Techniques in AQUATIC TOXICOLOGY

Volume 2

Edited by

Gary K. Ostrander

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Techniques in Aquatic Toxicology Volume II

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Dedication

Books such as this are usually dedicated to family members, colleagues, collaborators, or mentors who have played a meaningful role in the author's life and career. Sometimes, however, some of the most significant people in our lives are outside the circle of our family or our discipline.

I dedicate this volume to the folks at Johnson Orchards in Yakima, Washington, and in particular to Roy Johnson, Jr. (''Mister Johnson''), Donna Johnson, and Eric Johnson. I gratefully acknowledge that for many years I always had employment, whether it be for a summer or for a few hours over a weekend home from college. More importantly, I am most appreciative for their always believing in me and supporting me, no matter what objective I was pursuing. This has been true for nearly 30 years.

Preface

The initial volume in this series was published in 1996 and was well received by the scientific community. In fact, it has been most gratifying to observe the volume entering a second printing and the positive feedback I continue to receive from colleagues around the world. While the original intent was to follow with additional volumes every 2 to 3 years (wishful thinking!), other commitments have dictated a more modest approach. Nonetheless, herein I am pleased to present 39 additional techniques chapters.

As with *Volume 1*, this volume contains a blend of established and recently developed techniques that have the potential to significantly impact the expansive field of aquatic toxicology. I have divided the chapters into four broad sections to include techniques for assessment of toxicity in whole organisms, cellular and subcellular toxicity, identification and assessment of contaminants in aquatic ecosystems, and I conclude with a general techniques section, techniques for aquatic toxicologists, which contains chapters that could be of value for anyone working in this field.

Each of the individual chapters covers a specific procedure in detail. A brief *Introduction* serves to highlight the technique and the *Materials* section provides a very detailed list of what is needed to conduct the procedure(s). The *Procedures* sections are written so that the procedure can be easily followed and reproduced by a technician, graduate student, or someone with a basic knowledge of the field. In the *Results and Discussion* section, I have asked the contributors to provide and describe typical results, as well as anomalous results, false positives, artifacts, etc. In some instances, the contributors have provided data from their recently published work. Alternatively, some contributors have provided the data and discussed the previously unpublished experiments. Each chapter concludes with a list of pertinent *References* and a few also include appendices as necessary.

In addition to my review, all the chapters in this volume were reviewed by at least one individual with appropriate subject matter expertise. I am most grateful to the following scientists for providing thoughtful commentaries on one or more (usually more!) of the manuscripts contained herein: Gary Atchison, Keith Cheng, Dominic M. Di Toro, Craig Downs, Damjana Drobne, Howard Fairbrother, Jeffrey P. Fisher, Douglas J. Fort, Marc M. Greenberg, Mary Haasch, William E. Hawkins, James P. Hickey, James N. Huckins, David Janz, J. McHugh Law, Lawrence LeBlanc, Paul McCauley, Mark S. Myers, Dana Peterson, Taylor Reynolds, Colleen S. Sinclair, Terry W. Snell, Larry G. Talent, and Rebecca Van Beneden.

Finally, I express my sincere appreciation to the contributors for taking the time to distill their techniques into a consistent, easily accessible format. I believe that this consistency, more than anything else, has contributed to the success and the utility of this series.

About the Editor

Gary K. Ostrander received a B.S. degree in biology from Seattle University in 1980, an M.S. degree in biology from Illinois State University in 1982, and his Ph.D. degree in 1986 from the College of Ocean and Fisheries Sciences at the University of Washington, where he specialized in aquatic toxicology. He was an NIH postdoctoral fellow in the Department of Pathology, School of Medicine, at the University of Washington from 1986 to 1989 and also served as a staff scientist at the Pacific Northwest Research Foundation from 1986 to 1990.

In 1990, he joined the Department of Zoology at Oklahoma State University as an assistant professor and was tenured and promoted to the rank of associate professor in 1993. He assumed a dual role as director of the Environmental Institute and associate dean of the Graduate College in 1995.

Dr. Ostrander joined the faculty at Johns Hopkins University in 1996 and is currently the associate provost for research and chair of the Graduate Board at Johns Hopkins University. He holds his academic appointments in the Department of Biology in the School of Arts and Sciences and in the Department of Comparative Medicine in the School of Medicine.

Dr. Ostrander has authored over 80 technical papers and book chapters, edited 4 books, and has written a field guide. His primary research interest has been elucidating mechanisms of chemical carcinogenesis for which he employed aquatic, rodent, and human models. A second aspect of his work has been laboratory and field studies focused on understanding the mechanisms behind the worldwide decline of coral reef ecosystems.

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Techniques for assessment of toxicity in whole organisms

chapter one

Integrative measures of toxicant exposure in zebra fish (Danio rerio) *at different levels of biological organization*

Roel Smolders, Wim De Coen, and Ronny Blust
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Contents

Introduction

When aquatic organisms are exposed to pollutants, a cascade of biological events occurs if the exposure concentration is high enough and/or the exposure duration is long enough.[1–3] The general biochemical basis of stress responses was first described by Seleye[4] and is usually referred to as the general adaptation syndrome (GAS). This concept distinguishes three phases in response to stress: (1) primary alterations at the biochemical level, (2) secondary responses on a physiological level, and (3) tertiary effects on the whole organism level.

While a great deal of effort is directed towards eliciting the effects of pollutants on the first and second phases of the GAS, often referred to as biomarkers, there is still a profound need for a detailed description of effects at the whole organism level of biological organization.[1,5–7] Not only is the ecological relevance of effects at the whole organism level of biological organization, like condition, growth, or survival, often easier to establish than responses at lower levels, but also the integrative character of these

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measurements is one of the essential advantages of whole organism measurements.[8–10] These advantages promote the use of whole organism-based measurements, while a major difficulty with these measures is that there is often no identification of cause or mechanism of toxicity.

Though individual chemicals can have an impact on specific biochemical pathways, it is often difficult to extrapolate these effects to higher levels of biological organization, especially in the case of complex exposure scenarios, like field exposure or whole effluent toxicity.[10–12] Thus, although effects at the whole organism level in many cases lack the descriptive power to differentiate among the impact of specific toxicants, they give an integrative and holistic overview of the culminate effect of the disturbance of processes at lower levels of biological organization.

Moreover, effects on condition, growth, and survival, measured at the individual level, are an essential step to translate responses to higher levels of biological organization, like reproduction, population, and community effects.[9,13–16] There is, however, no ''right'' level of biological organization at which the effects of pollution should be studied and endpoints should be evaluated as part of a continuum of effects. Responses at a molecular or a cellular level of biological organization can provide detailed information on how chemicals interact with specific target sites and molecular pathways, but often provide little information concerning the ecological consequences of the effects. Studies on populations and communities do incorporate a high level of ecological relevance, since the well-being of populations and communities is the ultimate protection goal of ecotoxicology, but do not provide sufficient information on the eventual causes of the effects.[2,3,7,12,17]

What is required, is an integrated approach, where an understanding of the mechanistic basis of the stress responses in individuals is used to predict and interpret the penultimate effects at the population level. It should be obvious that the description of effects at the individual level is essential as a bridge between the mechanistic specificity of biochemical and physiological processes and the ecological relevance of population studies.

In order to obtain this holistic and integrative overview of how toxicants have an impact on organisms, the link should be made between different levels of biological organization and a ''currency'' has to be identified to extrapolate from one level to the next.[10,12,13,18,19] Energy budgets have been proposed as a very useful example of such a currency, not only because energy budgets can be determined at different levels of biological organization but also because they can provide a causal relationship between different levels, potentially relating cellular effects to growth or reproduction.[1,7,14,20]

Data that were previously presented by Smolders et al.[16,21] and unpublished data on the impact of different effluent concentrations on zebra fish (*Danio rerio*) will be used as an example on how endpoints describing cellular energy budgets, condition and reproduction are quantified, how data can be interpreted, and how these data can be combined to provide a holistic assessment of the impact at different levels of biological organization. All these data should be viewed within the framework of the metabolic cost hypothesis.[10,12,19,22] An active organism has a certain amount of energy available to it through its normal feeding, which will be used for storage in energy reserves, and will subsequently be used for investment in somatic growth, maintenance, or reproduction. If, however, a stressor impacts the fish, a shift in energy allocation will occur. Instead of the earlier balance between growth, maintenance, and reproduction, more energy will be allocated towards maintenance, leaving less energy available for growth and reproduction (Figure 1.1). Hence, from a theoretical point of view, stressed fish will show lower energy reserves, poorer condition, reduced growth, and impaired reproduction due to a decreased availability of energy.

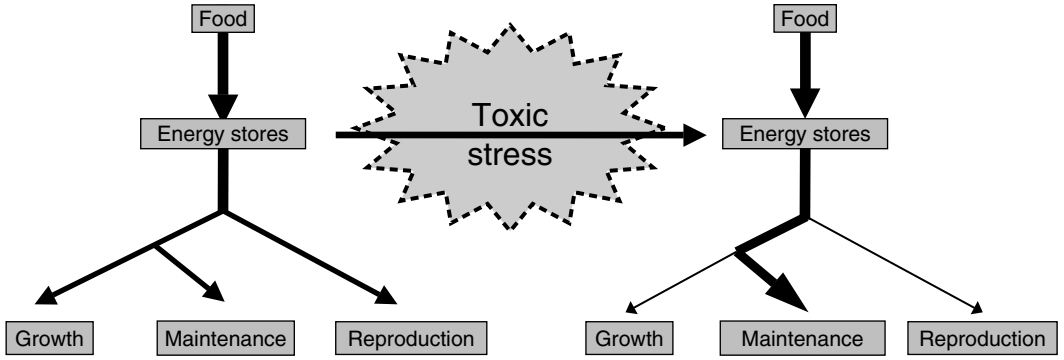

Figure 1.1 Outline of the metabolic cost hypothesis; in the presence of a toxic stressor, an increased amount of energy will be directed towards the basal maintenance of exposed organisms, leaving less energy available for growth and reproduction.

Also, indirect effects of pollutant exposure can be taken into account using this metabolic cost hypothesis. Even when organisms are not directly impacted by pollutant exposure, there may be other trophic levels that are impacted, leading to changes in food availability and trophic relations. Through a trophic cascade, this reduced food availability or change in food quality will eventually also have an impact on the energy availability of target organisms.[12,22–25]

In this chapter, we will present a number of different measurements, all focussed on determining the integrative effect of pollutant exposure at different levels of biological organization, illustrating the metabolic cost hypothesis. Briefly, methods will be presented that measure the energy reserves at a cellular level of biological organization, condition at the whole organism level, and reproduction as a final, ecologically relevant endpoint. These endpoints can be linked by one single currency, energy budgets, and we will try to illustrate how effects are interrelated.

Procedures and materials required

General zebra fish maintenance

Zebra fish *Danio rerio* (Hamilton) is recommended as test species in a number of ecotoxicological test protocols, e.g., different Organization for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO) guidelines. The advantages of zebra fish as bioassay organism include its small size, robustness, short life cycle, and the fact that under laboratory conditions it can be induced to breed all year round. Development from the fertilized egg to full reproductive maturity takes only 3–4 months. Additionally, this relatively short generation time makes zebra fish suitable for partial and full life cycle tests to evaluate the effects of chemicals on growth, development, and reproduction of fishes.[26–29]

Zebra fish are available at most pet stores and with commercial fish suppliers throughout the world. For general maintenance procedures, please refer to ''The Zebrafish Book. A Guide for the Laboratory Use of Zebra fish (*Danio rerio*)''* by

* This book is available online through the Zebrafish Information Network at http://zfin.org/zf_info/zfbook/zfbk.html.

Westerfield.[26] This standard work deals with most of the aspects of feeding, rearing, and breeding of zebra fish and provides detailed information on general test procedures.

For the experiments described in this chapter, we started the test with adult zebra fish with an average length of 37.7 mm (range 33–42 mm) and an average weight of 0.52 g (range 0.31–0.81 g). The test was performed following the OECD guideline 204 for fish toxicity tests and the USEPA guidelines for chronic toxicity testing.[27,28] Fish were fed commercial fish feed at a ratio of 2% of the maximum average body weight, set at 0.93 g. For optimal spawning conditions, water temperature should be around 25°C. The test lasted for 28 days and data were gathered weekly.

Cellular energy reserves

Toxicants can have an effect on the feeding rate, biomass conversion efficiency, and energy requirements of aquatic organisms.[1,30,31] All these different aspects will have an effect on the energy budget at a cellular level of biological organization. Carbohydrates, lipids, and proteins are the major energy reserves present in aquatic organisms, and thus according to the metabolic cost hypothesis, these reserves will be impacted by toxicant exposure. Methods to determine glycogen (as a main source of carbohydrate), lipid, and protein reserves have been developed and are easily performed using basic laboratory equipment.

Glycogen reserves: The analysis is based on the method by Roe and Dailey.[32]

- Pipette 200 µl of homogenate into an 1.5-ml Eppendorf tube and add 50 µl (1 *N*) perchloric acid (PCA).
- As preliminary measurements, determine the proper dilution of homogenate so that the measurements are within the range of the calibration curve (see the following).
- Vortex for 30 s and subsequently incubate on ice for 30 min.
- After incubation, centrifuge for 3 min at 10,000*g*.
- Transfer 200 µl of the supernatant into 14-ml test tubes (make dilutions if necessary) and add 1 ml of Anthrone reagent.
- For Anthrone reagent, measure 100 ml of concentrated sulfuric acid and place in a 250-ml beaker under a fume hood. Add 0.2 g reagent grade Anthrone, mix by stirring and cool on ice for 1–2 h.
- Make a calibration curve of at least five concentrations between 0 and 1000 µg/ml glycogen dissolved in 0.2 *N* PCA. Also add 200 µl of the calibration standards to 14-ml test tubes and treat equal to samples.
- Transfer 200 µl of the supernatant and calibration standards to 14-ml test tubes (make dilutions if necessary) and add 1 ml of Anthrone reagent.
- Incubate the test tubes at 95°C (in a water bath) for 30 min.
- Allow to cool down, transfer 200 µl to microtiter plates (perform measurements in triplicate) and read in microtiter plate reader at 620 nm.

Lipid reserves: The determination of lipids is based on the chloroform–methanol method described by Bligh and Dyer,[33] adapted for microplate reader. The extraction procedure can be performed in 1.5-ml test tubes and measurements are done in a microtiter plate reader.

- Take 250 μl of homogenate. Again, the proper dilution of homogenate needs to be determined first for the measurements to be within the range of the calibration curve.
- Add 500 μl of chloroform (reagent grade) and vortex for 5 s.
- Add 500 μl methanol and 250 μl water and vortex for 5 s.
- Centrifuge the test tubes for 3 min at 3000*g* at 4°C.
- After centrifugation, there will be three distinct fractions in the test tubes. The upper part is the methanol–water fraction, then there is a small layer containing the tissue homogenate residue, and the lower part is the chloroform fraction that contains the dissolved lipids.
- Remove the two first layers (pipette the methanol–water fraction and discard, remove the tissue residue layer with a pipette-tip), and transfer a 100-μl subsample of the third, chloroform fraction to a glass test tube.
- Make a calibration curve of at least five concentrations between 0 and 3000 μg/ml tripalmitin dissolved in chloroform. Also add 100 μl of the calibration curve to glass test tubes and treat equal to samples.
- Add 500 μl sulfuric acid to the 100 μl chloroform–lipid sample in the glass test tubes and incubate for 30 min in an oven at 200°C. Be careful to use only test tube racks and test tubes that can resist 200°C.
- Be extremely careful when taking the samples out of the oven because of the concentrated sulfuric acid at 200°C. Always use protective gloves and safety goggles.
- Let the samples cool down, first in air, later on ice. When samples have cooled down, add 500 μl of water. Again be careful because adding water to concentrated sulfuric acid may cause splashes. As always, make sure you wear proper protection. If sample absorption is higher than the range of the calibration curve, add more water.
- Transfer 200 μl of sample (in triplicate) to a 96-well microtiter plate, also incorporating the calibration curve in triplicate. Read in a microtiter plate reader at 340 nm.
- Recalculate lipid content of samples based on the known concentrations of the calibration curve.

Protein reserves: The procedure is based on the Bradford method.[34] It is a very simple quantification procedure that uses Coomassie brilliant blue dye:

- Add 50 μl of sample to a 1.5-ml test tube and add 950 μl of 1 *M* NaOH.
- Vortex for 5 s and incubate for 30 min at 60°C.
- After incubation, cool on ice, vortex again and transfer 200 μl of sample (in triplicate) to a 96-well microtiter plate.
- Make a calibration curve of at least five concentrations between 0 and 1000 μg/ml bovine serum albumin (BSA) dissolved in 0.2 *N* NaOH. Also add 200 μl of all concentrations of the calibration curve to microtiter plates and treat equal to samples.
- Subsequently add 50 μl of Coomassie brilliant blue dye reagent.
- Absorption measurements are done in a microtiter plate reader at a wavelength of 595 nm.
- Protein content is recalculated to micrograms per milliliter by means of the calibration curve.

Note: *It is essential that for every measurement of energy reserves, the sample preparation is accompanied by a calibration curve*. This calibration curve is needed to recalculate from the absorption data to the energy reserve concentrations (in μg/ml). Since calibration curves may slightly vary due to small differences in measurement procedures, the use of an average calibration curve is not advisable.

Calculation of energy budgets

Changes in body composition were expressed as changes in energy budget (EB_x) and were calculated using the following formula:[18,19,21]

$$EB_x = [((T_x - T_{x-1})(Y_x - Y_{x-1})/2) + (T_x - T_{x-1})(Y_{x-1} - Y_0)]/T_x \quad (1.1)$$

where T_x is the exposure time x, Y_x the composition (glycogen, lipid, and protein content) at time x, and $x - 1$ is the previous measurement time. Data on energy reserves and condition for Y_0 and T_0 need to be measured in the same batch of zebra fish before the start of the exposure. This approach allows the quantification of changes in the energy budget in zebra fish between different exposure regimes and periods. Since energy budgets reflect all physiological changes the exposed organisms had to make to survive during the entire exposure period, they are more relevant than the mere absolute levels of energy reserves. Whole body energy budgets can be calculated by summing the energetic values for the different reserves, using an enthalpy of combustion of 17 kJ/g for glycogen, 39.5 kJ/g for lipids, and 24 kJ/g for proteins.[21,35]

Condition indices

Since condition indices and growth describe key processes in individual aquatic organisms, these indices are a good basis for bridging the gap between cellular and population levels of biological organization. The most common type of condition indices is ratios between morphological features of fish.[36–38] Because length and weight are often routinely measured, measures of growth and condition can provide basic information about the general well-being of organisms. Length and weight should be determined for a relatively large number of fish (20–50 individuals) because the more data are available, the more accurate the condition determination will be. In the experiment presented here, length was measured for 50 fish per aquarium on a plastic covered sheet of millimeter paper, which was kept moist. Total length was determined up to 1 mm. Weight was determined up to 0.01 g using a simple analytical balance. In a review paper, Bolger and Connolly[39] identified as many as eight forms of condition index that have been used in fisheries research. However, only two frequently used indices describing condition in fish are described and illustrated:

Fulton's condition factor (FCF):

$$FCF = WL^{-3} \quad (1.2)$$

where W is the total body weight (in g) and L is the total length (in mm). Sometimes it may be more interesting to use the standard length (length of the fish from head to the base of the tail fin). This can be more accurate since the shape of the tail fin may vary significantly, thus skewing results. The main problem associated with FCF is that the formula

assumes isometric growth (growth with unchanged body proportions), an assumption often violated when adult fish are used.

Relative condition factor (RCF):

$$\mathrm{RCF} = W/(aL^b) \tag{1.3}$$

The parameters a and b are determined from a control (unstressed) population and are determined by $\log W = \log a + b \log W$ (i.e., a is the regression intercept and b is the regression slope). This procedure automatically leads to the result that the condition of the control population is 1 and the RCF of the exposed populations is a fraction of this value (hence, relative condition factor). A problem encountered with the RCF is that there needs to be a clear reference population to determine a and b. For laboratory experiments, this is usually not a problem, but for field experiments the choice is much less straightforward.

Other condition indices have been proposed, some using additional parameters like height of the fish.[38] Though these indices are reported to be less variable and more accurate, the need to measure both length and height will cause additional stress to the fish. Handling has been reported to cause severe stress in fish, including reduced growth, increased sensitivity to infections,[40,41] and delayed reproduction[42,43] and should therefore be minimized.

With the recent developments in image analysis and the declining cost of digital cameras, the analysis of digital pictures of exposed fish provides a new and very sensitive opportunity to measure subtle changes in growth, condition, and more complex aspects of development, including truss analysis[44] and fluctuating asymmetry.[45]

Reproduction

There are a number of methods to determine the reproductive potential of zebra fish. A method that has proven very efficient in our experiments is the marbling of small cages.[16,26] Briefly, four males (longer, slimmer, and more yellow especially on the belly) and two females (plumper and more silvery) are transferred from the exposure aquaria to smaller breeding containers. The bottom of every spawning container is covered with marbles, so any eggs spawned are protected from predation from the parents. Apart from the marbling technique, many varieties of mesh mating cages with pull-out dividers have been devised. Though these cages usually are custom-made, they should be large enough to enable the fish to swim freely and to prevent the eggs from being eaten by the parents. Zebra fish breed photoperiodically and normally produce eggs in the morning, shortly after sunrise.[26,46,47] The parent fish are left in the cages overnight, they are removed from the spawning containers and the marbles are carefully removed and cleaned. If spawning occurred, eggs will be visible at the bottom of the cage, and the number of eggs can be counted to quantify egg production (expressed as average number of eggs per female).

Endpoints that should be measured if a proper and accurate evaluation of effects of toxicants on reproduction is desired, should include the following:

- *Number of female fish spawning*: By individually caging females, this endpoint can be quantified. As we will illustrate later, the number of female fish spawning is related to energy budget of fish. However, there is a relatively high natural variability in zebra fish spawning, so the use of controls, replicates, and possibly repetition of experiments must be encouraged.

- *Number of eggs spawned per fish*: When parent fish are removed from the small spawning containers and marbles or cages are removed, eggs can be observed by eyes without the aid of any optical devices at the bottom of the containers as small spheres of about 0.5–1 mm diameter.
- *Hatching*: If the eggs are left in the small spawning tanks after quantification of spawning, the percentage hatching can also be quantified. Hatchlings can be observed after 3–4 days as little black juveniles (1–1.5 mm long).
- Since juvenile life stages are generally considered to be the most sensitive in ecotoxicological testing, juvenile growth, development rate, occurrence of malformations, and survival may be the very sensitive additional endpoints, which are beyond the scope of this chapter; details on rearing and maintaining juvenile zebra fish have extensively been discussed by Westerfield.[26]

Results and discussion

The data that are used here to illustrate the methods outlined in the previous section are extracted from Smolders et al.[16,21] and unpublished data. These data describe the impact of different concentrations of an industrial effluent on the energy budget, condition, and reproduction of zebra fish. As an example, we will compare responses of exposure to the control and 100% effluent aquarium.

Figure 1.2 illustrates how the total energy budget of zebra fish depends on both effluent concentration and exposure duration. The effects of effluent-exposure on the cellular energetics are expressed as energy budgets, instead of simply comparing energy content. As already indicated before, the presentation of energetics through energy budgets represents an integration over time. Because pollutant exposure is a continuous process, and the aim of our experiment is to correlate the cellular energy data with other integrative measures like condition, growth, or reproduction, the integrated approach is a

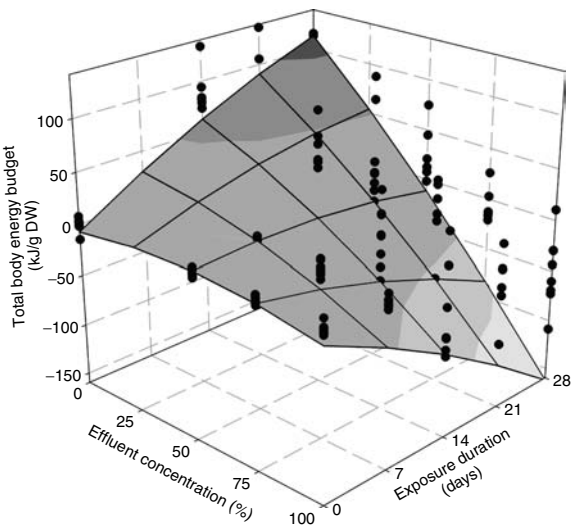

Figure 1.2 The effect of effluent concentration and exposure duration on the total body energy budget of effluent-exposed zebra fish (quadratic smoothening, $n = 156$; $R^2 = 0.531$).

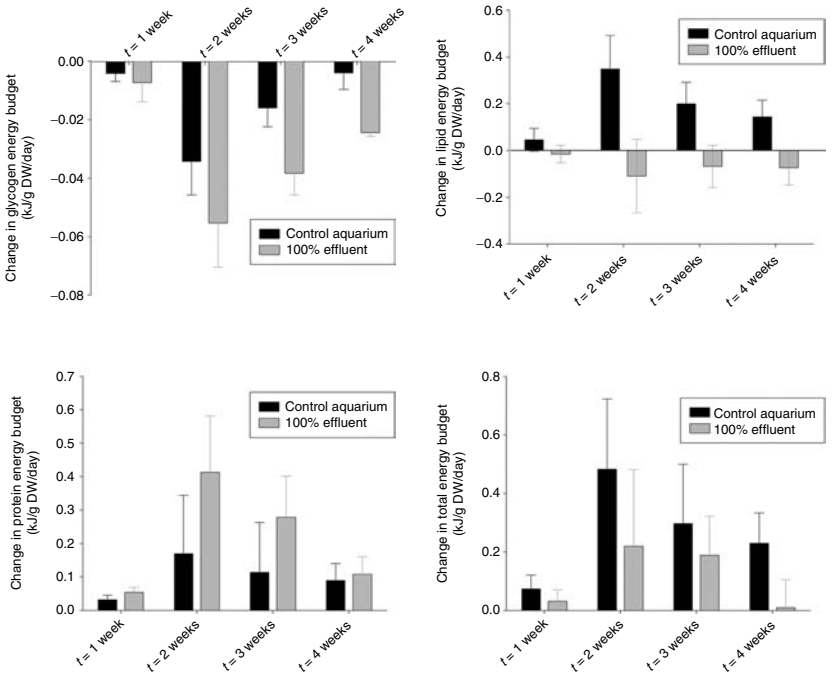

Figure 1.3 Overview of changes in energy budgets in control and exposed zebra fish ($n = 8$, mean $\pm$ SD). Complete data and discussion are presented in Smolders et al. (2003).[21]

good reflection of the continuous stress that is posed upon the fish throughout the experiment.

Figure 1.3 represents the total energy budget of effluent-exposed fish through time, and is the sum of the three major energetic components in fish, i.e., carbohydrates, lipids, and proteins. Though especially the total energy budget is relevant to extrapolate effects at a cellular level to effects at higher levels of biological organization, it also pays to look at the individual components. Our data showed that especially lipid budgets were related to effluent-exposure in a dose-dependent manner. Effluent-exposure caused a rapid and dose-dependent depletion of lipid energy budgets. The depletion of lipids by pollutant exposure has been documented for, among others, carp fingerlings,[48] rainbow trout,[49] and eel[50] chronically exposed to different pollutants. Also glycogen (as the primary source of carbohydrates) was significantly affected by effluent-exposure. Since glycogen is a rapidly available source of energy, it was not surprising, however, that this source of reserves was depleted by effluent-exposure. There are many examples in the literature where pollutant stress depleted glycogen levels in fish.[51,52]

Finally, protein levels were significantly different in fish exposed to effluents compared to the control. Even though protein is a prominent source of energy in fish, stress preferably causes depletion of glycogen and lipid reserves instead of protein.[1,51,53] We observed a significant increase in protein content in the second and third weeks of exposure to different effluent concentrations, but the levels restored to normal in the fourth week. An increase in protein content after pollutant exposure has also been observed by a number of other authors. For example, Wall and Crivello[54] found an increased microsomal protein content when starving juvenile winter flounder (*Pleuronectes americanus*) for 2 weeks, Brumley et al.[55] reported a 1.5-fold increase in

liver protein content when injecting sand flathead (*Platycephalus bassensis*) with up to 400 mg/kg Arochlor-1254, and a similar increase was observed in the liver of carp (*Cyprinus carpio*) injected with the herbicide 2,4-Diamin.[56] Smolders et al.[21] formulated the hypothesis that low to intermediate levels of pollution trigger increased protein synthesis (e.g., for detoxification processes and other defense mechanisms) when other sources of readily available energy like glycogen and lipids are still sufficiently present. On a more theoretical basis, Chapman[57] discussed this phenomenon using a hormetic concentration–response curve, where hormesis consists of a stimulatory response on a given endpoint (in our case whole body protein content) of 30–60% above the controls and comprises a general biological phenomenon that may represent overcompensation to an alteration in homeostasis.

Figure 1.4 shows the difference in length–weight relationship between the control and 100% effluent aquarium after 28 days of exposure. For fish of the same length, fish in the control aquarium are significantly heavier than fish exposed to the undiluted effluent. Equation (1.3) gives the values for *a* and *b* for the fish population in the control aquarium, which were used to determine the RCF. The advantage of using condition indices over simply determining changes in length or weight can be illustrated by the results presented in Figure 1.4. There will always be differences in growth within distinct populations when a large number of fish are used for testing. Even though fish in the control aquarium are of the same age and have the same life history, there is an intrinsic natural variability in length and weight. While this variability is natural, it hampers finding significant differences in average length among exposure aquaria due to high standard deviations. If, however, data are being rescaled based on length, which basically is what condition indices represent, much more subtle effects can be detected. Furthermore, since condition indices are a population response, they can also be used for populations with an uneven age distribution. As already mentioned, a problem often faced when using RCF is

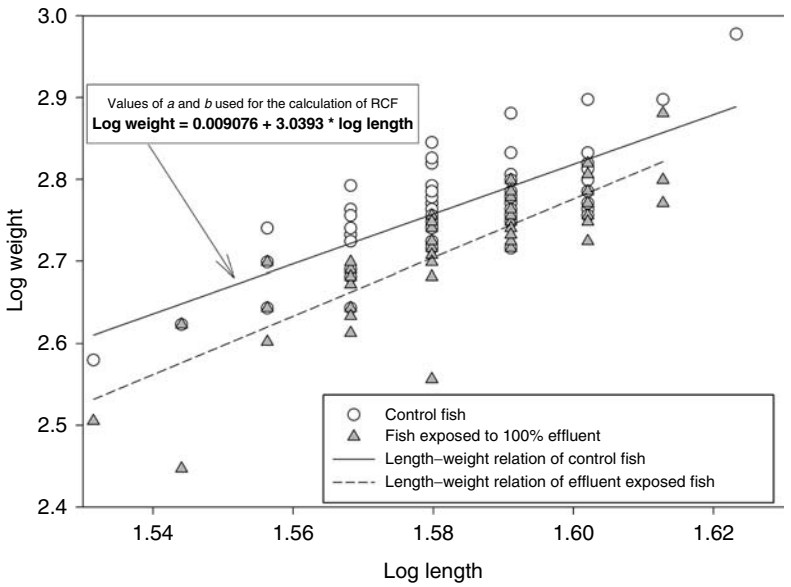

Figure 1.4 Length–weight for control and effluent-exposed zebra fish. The parameters of the length–weight relationship for the control fish are used to calculate the RCF.

the lack of a clearly defined reference population. While this is not a problem for laboratory experiments, it may be an important issue for field studies. Among different locations, food availability may not be comparable, thus making comparison among different locations difficult. Since in many rivers, pollution and eutrophication are linked, increased nutrient levels may be an important factor determining the growth performance of different food sources (e.g., macroinvertebrates and algae), masking the pollutant-related effects. Increased growth and/or condition of fish downstream point sources of pollution has been reported earlier, downstream bleached kraft mill effluents in particular. For example, Hodson et al.[58] reported elevated lipid levels and condition in white sucker (*Catostomus commeroni*) downstream BKME, and linked this with increased nutrient levels and richer food sources. Also Adams et al.[13] and Gibbons et al.[24] found increased energy storage, growth, and condition in redbreast sunfish (*Lepomis auritus*) and spoonhead sculpin (*Cottus ricei*) caught downstream bleached kraft pulp mills and contributed this improved performance to eutrophication and increased food sources associated with the mill effluent. In a comparison of 51 mills in Quebec, Langlois and Dubud[59] reported that if significant differences in the condition of fish were observed downstream pulp and paper mill effluents, in nine out of ten cases, fish exposed at the effluent discharge show a significantly better condition than fish at an unperturbed reference site. Food availability might have been one of the confounding factors, since they also reported that effluent discharge areas had a significantly higher abundance of macroinvertebrates, a very important food source for most fish species, compared to reference sites.

Also Fulton's condition factor (FCF) can be used to express the effects of effluent-exposure on the well-being of the fish. The interpretation of the FCF is not without danger; however, since the FCF requires isometric growth, an assumption that is often violated. However, RCF does not allow making a distinction between improved condition in the control aquarium and reduced condition in the 100% effluent aquarium. Figure 1.5 clearly indicates that the differences in condition among treatments are mainly due to an increased condition of fish in the control aquarium. FCF increases as the exposure period

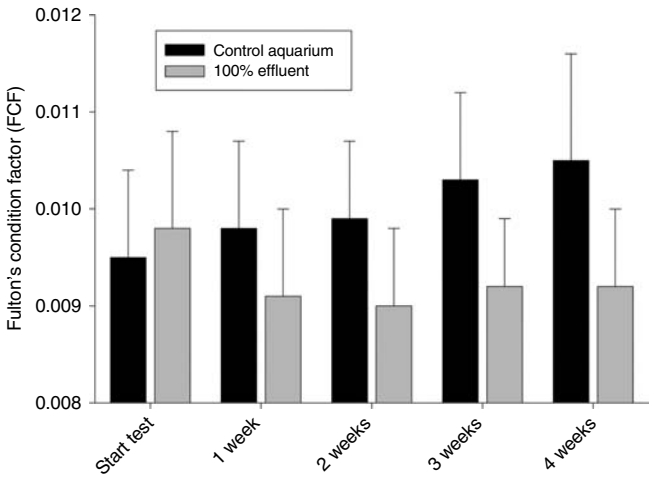

Figure 1.5 The effect of effluent-exposure on Fulton's condition factor (FCF; mean $\pm$ SD, $n = 50$). Note that this index requires isometric growth, an assumption frequently violated for adult fish.

increases, while fish in the 100% effluent aquarium have a reduced FCF compared to that at the start of the test, but no further decrease afterwards.

The ultimate level of concern in ecotoxicology is the stability and persistence of populations and communities of organisms. Therefore, spawning (the number of eggs produced) and hatching (the number of eggs leading to juveniles after fertilization) as endpoints of reproduction should be the penultimate result of all toxicological investigations. In addition, since the fecundity of fish often seems to be limited by the energetic cost of reproduction, spawning and hatching are key concepts within the bioenergetics approach.[60] Though spawning and hatching in zebra fish are relatively easy to quantify, the interpretation of the data is less straightforward. One of the main disadvantages of spawning and hatching as toxicological endpoints is that reproduction provides an ecologically relevant description of the effects of stress but does not, by itself, provide information on potential causes.

In the experiment presented here, there was a significant reduction of spawning and hatching in effluent-exposed fish (Table 1.1). However, this reduction was not caused by a reduction of the average number of eggs spawned per female, but by a reduction of spawning females. In other words, if fish in the 100% effluent aquarium spawned, they produced the same number of eggs as fish in the control aquarium, but not as many female fish in the 100% effluent aquarium actually made it to spawning. Again, this can be explained within the framework of bioenergetics. Fish will relocate energy towards maintenance, growth, and reproduction based on energy availability. If under toxic stress, however, female fish will not be able to relocate the necessary amount of energy towards reproduction, spawning frequency will reduce and the length of the reproductive cycle will increase. Within our effluent toxicity example, this was illustrated by the significant correlation between RCF of the mother fish and number of eggs spawned ($R^2 = 0.778$, $n = 20$, $p < 0.001$). Also multiple regressions describing the relationship between glycogen, protein, and lipid budgets and reproduction was significant ($R^2 = 0.410$, $n = 20$, $p < 0.034$),

Table 1.1 Effects of effluent-exposure on spawning and hatching of zebra fish (*D. rerio*)

Aquarium	Time (weeks)	No. cages	Spawning (% of cages)	Spawning (total No. eggs)	No. eggs/spawning female	Percentage of eggs hatching	No. juveniles/cage
Control	1	3	67	165	82.5	32.5	65
	2	4	75	485	161.7	55.5	304
	3	5	80	663	165.8	25.9	169
	4	5	60	589	196.3	73.8	371
50% effluent	1	3	67	328	164	26.9	70
	2	4	75	573	191	21.7	179
	3	5	40	275	137.5	32.7	72
	4	5	20	296	296	58.5	173
75% effluent	1	3	67	249	124.5	44.4	170
	2	5	40	173	86.5	32.3	66
	3	5	40	291	145.5	38.2	112
	4	5	60	601	200	33.3	210
100% effluent	1	3	33	51	51	58.8	30
	2	5	40	219	109.5	37.9	121
	3	5	40	105	52.5	47.9	75
	4	5	20	12	12	33.3	4

Table 1.2 Multiple linear regression describing the relationship between changes in energy budgets and integrated condition indices in effluent-exposed zebra fish (*D. rerio*). Data are given as average ($\pm$ SD), $n = 20$

	Intercept	Glycogen	Lipids	Protein	R^2	p-level
FCF ($\times$1000)	9.596 (0.091)	0.000066 (0.00029)	0.00016 (0.00003)	−0.000028 (0.000057)	0.643	$p < 0.001$
	$p < 0.001$	$p = 0.822$	$p < 0.001$	$p = 0.634$		
RCF ($\times$1000)	993.12 (8.20)	0.091 (0.026)	0.0105 (0.0029)	−0.0013 (0.0051)	0.795	$p < 0.001$
	$p < 0.001$	$p = 0.003$	$p = 0.002$	$p = 0.803$		
Reproduction	100.42 (13.89)	0.034 (0.044)	0.0113 (0.0049)	−0.0049 (0.0087)	0.410	$p < 0.0339$
	$p < 0.001$	$p = 0.455$	$p = 0.035$	$p = 0.579$		

indicating that indeed a reduced energy budget was causing a reduction in reproduction (Table 1.2). Based on this information, we concluded that, in this case, the effect of effluent-exposure on reproduction was not caused by a direct toxic effect but by an indirect reduction in energy availability for reproduction. The observation that there was no significant reduction in hatching also indicated that there was no direct toxic effect of the effluent on the reproductive process. There are, however, several examples where reduced spawning and hatching in aquatic organisms is caused by direct toxic action of chemicals, so caution in the interpretation of data is necessary. Many toxicants are reported to directly influence spawning and hatching, often through a delay of spawning and a reduction of hatching percentage. Though we did not quantify these endpoints in the example presented earlier, the time until hatching appears to be a very sensitive endpoint when exposing zebra fish[61–64] or Japanese medaka (*Oryzias latipes*)[65,66] to a wide variety of pollutants.

The aim of this chapter was to illustrate how relatively simple measures of energy budgets, condition indices, and reproduction can be combined within the framework of bioenergetics. The methods presented do not require high-tech equipment, are relatively easy and fast to perform but give a good overview of the potential effects of stress on fish. The main idea is that combining cellular energy budgets (fast and sensitive yet ecologically questionable endpoints) with reproduction through individual endpoints like growth and condition will increase the ecological relevance of toxicity testing and provide an integrative and holistic overview of effects. The results presented show that the use of different levels of biological organization within one test and one test species are not only useful to quantify effects but can also show how disturbance at one level of biological organization can influence other levels of organization.

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chapter two

Use of disease challenge assay to assess immunotoxicity of xenobiotics in fish

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Contents

Introduction

A properly functioning immune system is critical in maintaining the fitness or health of an organism. The immune system is a complex network that involves regulation by both the nervous and endocrine systems, which allows an individual to respond to or fight against an invading parasite or foreign material (antigens). An improperly functioning immune system may respond to ''self'' as foreign causing autoimmune disease or hypersensitivity or, the opposite can occur, leading to immunosuppression. Foreign chemicals, or ''xenobiotics'' have the potential of deregulating a healthy immune system. Xenobiotics can shift the system from operating at a healthy homeostatic level to being either

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hyper- or hyposensitive. Both results may have drastic consequences on the individual organism and at the population level as well.

An important tool for examining the status of the immune system is disease challenge studies, also referred to as host resistance challenge studies. In our target species, juvenile chinook salmon (*Oncorhynchus tshawytscha*), this technique provides an opportunity to determine if xenobiotic exposure can alter the ability of fish to respond immunologically to bacteria known to be harmful or pathogenic to the species in its natural environment. We will present a study that has been published in part[1,2] whereby we examined the effects of various xenobiotics on the disease susceptibility of juvenile chinook salmon. We will discuss the number of variables that need to be considered when planning a disease challenge experiment involving xenobiotics and how to address them.

Materials required

Equipment:

- Low temperature incubator: model 815 (Precision)
- Digital oscillating orbital shaker (Thermolyne)
- UV/Vis spectrophotometer: model Du 530 (Beckman)
- Tissue culture enclosure
- Biological safety cabinet
- Ultrapure water system
- Autoclave
- Magnetic stir plate
- pH meter
- Analytical balance
- Cryogenic can
- Adjustable micropipettes (10, 200, 1000 μl)
- Vortex mixer
- Compound microscope with oil immersion magnification

Supplies:

- 15-ml graduated tubes with screw caps and conical bottoms
- Sterile serological pipettes (10, 5, 1 ml)
- Micropipette tips (10, 200, 1000 μl)
- Latex gloves
- 1-cc tuberculin syringes
- 100 × 15 mm sterile Petri plates
- 1-μl sterile disposable inoculating loops
- Erlenmeyer culture flasks
- Weighing boats
- Cheese cloth
- Necropsy instruments
- Alcohol burner
- Frosted microscope slides
- Paper towels
- Spray bottle

- Ethanol
- Novobiocin sensitivity discs
- Vibriostatic sensitivity discs 0/129 (2,4-diamino-6,7-diisopropyl pterdine phosphate)
- Necropsy cutting board
- Metomidate hydrochloride (Wildlife Laboratories)
- Bacteriological media
  Tryptic soy agar (TSA)
  Tryptic soy broth (TSB)
  Sodium chloride (NaCl)
- *Vibrio anguillarum* media (VAM)
  Sorbitol
  Yeast extract
  Bile salts
  Cresol red
  Bromthymol blue
  Bacteriological agar
  Ampicillin
- Bacteriological diagnostic test kits
  Oxidase Test kit (bioMérieux Vitek)
  Rapid agglutination test: Mono-Va 50 Tests (Bionor)
  Gram stain kit (Sigma)
- Aquaculture supplies
  450-l plus 2400-1 circular aquaculture tanks
  Fishnets
  1.5-in.^{2} air diffuser for each tank
  Fish food (BioOregon)
  Aquaculture disinfectant: I-O-Safe
  Buckets

Xenobiotics:

- Polycyclic aromatic hydrocarbon (PAH) model mixture (analytical grade, Sigma)
  Fluoranthene
  Pyrene
  Benz[*a*]anthracene
  Chrysene
  Benzo[*b*]fluoranthene
  Benzo[*k*]fluoranthene
  Benzo[*a*]pyrene
  Indeno[1,2,3-*cd*]pyrene
  Dibenz[*a*,*h*]anthracene
  Benzo[*g*,*h*,*i*]perylene
- Hexachlorobutadiene (HCBD; Sigma)
- Polychlorinated biphenyl (PCB) mixture: Aroclor 1254 (AccuStandard)
- 7,12-Dimethylbenz[*a*]anthracene (DMBA; Sigma)
- Chlorinated-enriched Hylebos Waterway sediment extract (CHWSE)

Procedure

Contaminants

We are interested in examining the effect of contaminants that are representative of the type of chemicals found in a contaminated Puget Sound estuary, on the health of juvenile salmon. Accordingly, the following five contaminant solutions were chosen and prepared for the pathogen challenge study: (1) a mixture of organic contaminants extracted from contaminated sediment collected from the Hylebos Waterway (CHWSE) located in Puget Sound, WA. We used an extraction method that enriched for chlorinated butadiene-compounds inclusive of HCBD; (2) a solution of HCBD, which we determined to be a marker chemical for the Hylebos Waterway[3]; (3) a model mixture of PAHs prepared to represent high molecular weight PAHs (3–5 rings) found in the Hylebos Waterway sediment; (4) a PAH compound, DMBA, shown previously to suppress immune responses in juvenile chinook salmon[4]; (5) a commercially acquired PCB mixture (Aroclor 1254), similar to the mixture of PCBs found in the Duwamish and Hylebos waterways and shown previously to suppress immune responses in juvenile chinook salmon.[4] The sum concentrations and compositions of the chemicals and chemical mixtures are listed in a previous study.[2]

Caution must always be used when handling chemicals and contaminated sediments. This paper describes procedures that may involve hazardous materials but does not purport to address all of the safety issues involved. Collection and use of sediments with unknown chemical contamination and preparation of chemical test mixtures may involve substantial risk to personal safety and health. It is highly recommended that appropriate precautions be taken to minimize contact with test chemicals and sediments. Laboratory personnel should consult their facility's Chemical Hygiene Plan for specific policies and emergency procedures. In addition, current Material Safety Data Sheets (MSDS) should be consulted for all known chemicals used and/or suspected contaminants. Further information can be obtained from the U.S. Occupational Safety and Health Administration (OSHA)[5] and the Environmental Protection Agency (EPA).[6,7]

Contaminants in field-collected samples may include mutagens, carcinogens, and other potentially toxic compounds. When working with sediments with unknown contaminants, and/or with known chemical hazards, it is essential to minimize worker contact by employing appropriate safety equipment and procedures. This includes the use of appropriate gloves, laboratory coats or protective suits, safety goggles, face shields, and respirators where needed. Samples and chemical test mixtures should be handled and prepared in a ventilated safety hood, and used materials disposed of in an appropriate manner. Laboratory personnel should be trained in proper practices for handling, using, and disposing of all chemicals used in the procedures described here. It is the laboratory's responsibility to comply with federal, state, and local regulations governing waste management and hazardous materials disposal. Further information is available in ''The Waste Management Manual for Laboratory Personnel''[8] and from EPA.

Fish

Fish must be ''healthy'' so that confounding factors, such as a pre-existing disease, are not present. Inspections performed by state fish health inspectors and observations by the hatchery managers revealed no evidence or signs of the principal salmonid diseases in salmon (9–12 g) used in these studies. A fish health inspection[9] examines for the following salmonid pathogens: *Listonella anguillarum*, *Renibacterium salmoninarum*, *Yersinia ruckeri*,

infectious hematopoietic necrosis virus, infectious pancreatic necrosis virus, and viral hemorrhagic septicemia virus. If these pathogens are identified in the fish, the investigator may consider using a different source of fish for the experiment.

Juvenile chinook salmon were collected and transported to the laboratory for use in generating the lethal dose (LD)–response curve relationship for DMBA, Aroclor 1254, CHWSE, and HCBD solutions. Care must be taken so that undue stress does not occur during transport. To this end, the salmon were placed in 95-l coolers equipped with air stones to maintain proper oxygen levels during transport. Upon arrival, the fish were immediately transferred to 2400-l circular fiberglass tanks with fresh dechlorinated water. The juvenile salmon were slowly acclimated from freshwater to seawater over a 5-day period. Juvenile salmon were allowed to acclimate for a minimum 2-week period in seawater (9–12°C, 30–32 ppt salinity) prior to the beginning of the LD tests. The juvenile salmon were fed 3% of their total body weight per day with Biodiet Grower Peletized Feed (2.5 mm, BioOregon Incorporated, Warrenton, OR).

Generation of the LD–response curves for DMBA, Aroclor 1254, CHWSE, and HCBD solutions

''Measurement of lethality is precise, quantal, and unequivocal and is, therefore, useful in its own right if only to suggest the level and magnitude of the potency of the substance.''[10] Prior to examining an effect of a chemical on the immune response of fish, it is important to determine its LD–response curve with the species of interest. The contaminant may be delivered in a number of ways (i.e., diet, injection, or contaminated sediment using a MESOCOM approach for exposure). However, for initial studies, we recommend using injection delivery of the contaminant. This ensures that each fish receives a specific amount of chemical, and that you are not introducing other variables that come into play when using a diet or MESOCOM approach to contaminant delivery. For example, in a diet study, each fish may not eat equivalent amounts of diet thereby creating a range of contaminant exposure in the fish. Also, it may be unclear how a diet or sediment exposure may translate to contaminant body burden in the fish.

Our LD–response curves were determined by exposing 10 juvenile chinook salmon to each of the doses that consisted of: 50, 200, 400, 600, and 800 g sediment equivalent per kilogram of fish for CHWSE; 0.1, 1, 5, 30, 60, 100, 500, and 1000 mg kg^{-1} of fish for HCBD; 1, 10, 50, 100, 250, and 500 mg kg^{-1} of fish for Aroclor 1254; and 15, 25, 35, 50, 65, 75, and 88 mg kg^{-1} of fish for DMBA. Control fish were also exposed to the acetone/emulphor carrier solution. The fish were anesthetized with 1.5 mg of metomidate hydrochloride per liter (Wildlife Laboratories, Fort Collins, CO), weighed, measured, and injected intraperitoneally (i.p.) with 1 μl g^{-1} body weight of the contaminant. The salmon were held in 450-l tanks with running seawater and were observed for mortality over a 96-h period. This information generated for the PAH compound, DMBA, was also used to determine the dose of the PAH model mixture to be used in the disease challenge study.

Contaminant exposure and pathogen challenge

In Trial 1, fish were exposed by i.p. injection to sublethal doses of either CHWSE, HCBD or the PAH model mixture. In Trial 2, fish were injected with one dose of either Aroclor 1254 or DMBA. Doses of the contaminants for the *L. anguillarum* challenge were chosen based on their LD–response curve, targeting doses that would allow for a substantial but

sublethal dose. In both trials, control fish were injected with the acetone/emulphor carrier solution. One week after contaminant injection, fish were exposed to either *L. anguillarum* or to seawater diluent alone (see the following). In Trial 1, the juvenile salmon were anesthetized with 1.5 mg l^{-1} of metomidate hydrochloride, individually weighed, and then injected i.p. with 2 μl of inoculum per gram body weight of fish. Fish were injected with either HCBD, the PAH model mixture, CHWSE, or acetone/emulphor carrier solution. Concentrations of the test solutions were 10% of the LD_{50} of HCBD (20 mg of HCBD per kilogram of fish), 10% of the LD_{50} of the PAH model mixture (6.3 mg of the model PAH mixture per kilogram of fish), or 41% of the LD_{30} of CHWSE (307 g sediment equivalent per kilogram of fish). Since we were only able to achieve 30% mortality in the fish exposed to a very high concentration of CHWSE, we determined the LD_{30} for this contaminant instead of the LD_{50} (see the section ''Results and discussion''). The groups of fish injected with each contaminant were kept separate in individual tanks. Thirty fish were randomly assigned to six tanks for each of the four treatments. Three tanks of each treatment were challenged with *L. anguillarum*. The remaining three tanks in the treatment received only the TSB diluent. This latter unexposed group provided an estimate of background mortality.

In Trial 2, juvenile salmon were anesthetized as described earlier for Trial l, weighed and injected i.p. with 2 μl of inoculum per gram body weight of fish. Fish were injected with DMBA, Aroclor 1254, or acetone/emulphor carrier solution. Concentrations of the DMBA and Aroclor 1254 solutions were 20% of the LD_{50}. Therefore, the concentration of DMBA used was 13 mg of the PAH per kilogram of fish. The concentration of Aroclor 1254 used was 54 mg of the PCB mixture per kilogram of fish. The groups of fish injected with each contaminant were kept separate in individual tanks. Fifteen fish were randomly assigned to six tanks for each of the three treatments. Three tanks of each treatment were challenged with *L. anguillarum*. The remaining three tanks in the treatment received only the TSB diluent. This latter unexposed group provided an estimate of background mortality.

Infection of salmon with L. anguillarum

A number of factors need to be addressed when deciding which pathogen to use in the disease challenge. For example: Is the fish likely to come into contact with the pathogen in its natural environment? Is the pathogen easily grown in culture? Are there techniques for identification of the pathogen in exposed fish? For our disease challenges, we have chosen a Gram-negative bacteria *L. anguillarum*. Relative to some pathogenic bacterial species a lot is known about the epizootiology of this predominately marine pathogen.[11] *L. anguillarum* causes vibriosis that can occur in preacute, acute, and chronic forms in salmonids[12] and has been reported to have a major effect on over 48 species of marine fish. As discussed in the following, this pathogen can be grown to log phase within 24 h and both presumptive and confirmatory tests are available for identification of the pathogen. All of the above qualities make this pathogen an invaluable tool in disease challenges.

A lyophilized preparation of *L. anguillarum* (strain 1575), a gift from Biomed, Bellevue, WA, was kept at −70°C until use. The bacteria were rehydrated with 2 ml of TSB supplemented with 0.5% NaCl,[13] then the purity of the culture was determined by Gram stain, cell morphology, and agglutination with the rapid agglutination test (Bionar, Norway). To ensure further that the rehydrated preparation was free of contaminating bacteria, an aliquot was cultured onto a TSA plate supplemented with

0.5% NaCl for 24 h at 25°C. At the same time, 2 ml of the rehydrated preparation was placed into 100 ml of TSB supplemented with 0.5% NaCl for production of a stock culture. After 18 h of growth at 25°C on a shaker, the stock culture was mixed 1:1 (v/v) with a glycerol and saline buffer. The stock culture of *L. anguillarum* was aliquoted into cryovials (2 ml each) and stored at −70°C for future use.

A growth curve of *L. anguillarum* (strain 1575) at 25°C was determined to ensure that at the time of challenging the salmon with bacteria, the bacteria would be close to the peak of their exponential growth phase. In brief, 2 ml of the stock culture was placed into 500 ml TSB supplemented with 0.5% NaCl and placed on a shaker at 25°C. Two milliliters of aliquots were removed every hour from the 500 ml of bacterial suspension, and the turbidity of the culture determined with a UV–Vis recording spectrophotometer (Shimadzu Scientific Instrument, Columbia, MD) at 525 nm wavelength until just after the beginning of the stationary phase. During the exponential growth phase, the bacteria divide steadily at a constant rate and the population is uniform in terms of chemical composition of cells, metabolic activity, and other physiological characteristics.[14] The stationary phase of bacterial growth may be defined as the period of time after the exponential phase, in which the growth of the bacteria slows down or is no longer exponential either because a required nutrient is limiting or because the metabolic products become inhibitory to bacterial growth.[15]

Prior to conducting the disease challenge, a lethal concentration (LC) curve needs to be generated with the pathogen and the species of interest. Once the curve is generated, the low, medium, and high concentrations of the pathogen should be selected for testing host resistance.[16] If the contaminant is immunoenhancing, an increased resistance to the pathogen should be observed at the higher concentrations. If the chemical is immunosuppressive, an increase in disease susceptibility should be observed in the lower concentration. However, if the compound is immunosuppressive, care must be taken not to use too high or too low of a concentration of the pathogen. If the pathogen concentration is too low, contaminant-exposed fish may have the ability to fight the low number of bacteria in the same manner as the noncontaminant-exposed (control) fish. If the concentration of the pathogen is too high, it may overwhelm even the healthiest immune system and a difference in susceptibility will not be observed between the two treatment groups. Therefore, an LC–response curve for juvenile chinook salmon was determined with *L. anguillarum* (strain) 1575. Bacterial concentration was quantitated turbidimetrically at 525 nm, until an optical density of 1.7 was achieved, corresponding to the period near the end of the exponential growth phase. Seven log 10 dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-7}, and 0 ml bacterial culture per milliliter seawater) of the stock growth culture of *L. anguillarum* were used to determine the LC–response curve.

Bacterial plate counts of the stock culture were performed to determine the concentration of live *L. anguillarum*, to which the fish are exposed (Figure 2.1). To calculate the concentration of *L. anguillarum*, or colony forming units (cfu) per milliliter growing in a suspension culture, 10-fold serial dilutions were made of the bacteria and then plated onto TSA supplemented with 3% NaCl. Dilutions were made using eight sterile 15-ml graduated tubes with screw caps and conical bottoms filled with 9 ml of sterile TSB supplemented with 1.5% NaCl. With a sterile pipette, 1 ml of *L. anguillarum* suspension culture is transferred to the first tube, creating a dilution factor of 1:10. This dilution is mixed thoroughly by vortexing. With a sterile pipette 1 ml of the first dilution is transferred to the second tube creating a dilution factor of 1:100. This process was repeated until all successive serial dilutions are made. Each of the last three serial dilutions (10^{-6}, 10^{-7}, and 10^{-8}) are plated on three replicate Petri plates containing TSA supplemented

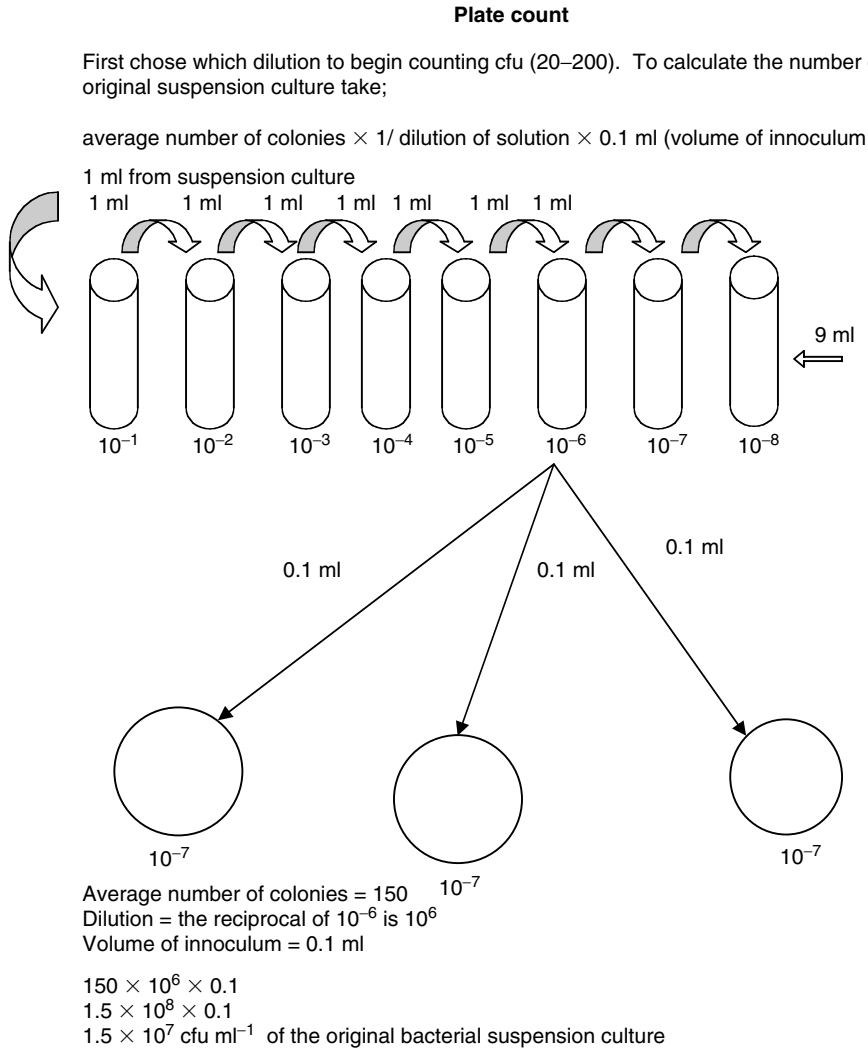

Figure 2.1 Plate count method for determining the number of colony forming units per milliliter of *L. anguillarum* in the stock solution.

with 3% NaCl. The center of the plates are inoculated with 0.1 ml of the dilution and a thin uniform layer of the inoculum is spread across the entire plate using a glass ''hockey stick'' dipped in 95% ethanol, flamed, and cooled on the edge of the agar plate. This procedure is repeated for each plate. The plates are labeled and incubated at 25°C for 48 h. Countable plates contain 20–200 colonies and the average numbers of colonies for the three plates of each dilution are used to calculate the bacterial concentration (cfu ml^{-1}) in the stock culture.

Twenty salmon, approximately 10 g each, were placed in 7.6-l buckets containing 4 l (1 l for every 50 g of fish) of the various concentrations of bacteria diluted in filtered seawater, or into a control bucket containing 4 l seawater. The fish were exposed to the bacteria under static conditions for 1 h with aeration. Following each challenge, the fish

were placed in 450-l tanks with flow-through sand-filtered and UV-treated seawater. Mortalities were collected and tabulated twice a day for a minimum of 7 days (168 h). The salmon were not fed during the experimental period.

Water treatment

When dealing with contaminants and pathogens, it is imperative to ensure that both the influent and effluent are contaminant and pathogen free. Our influent was sand-filtered and UV-treated seawater. Before the effluent was released into Yaquina Bay it was treated with charcoal and chlorine to prevent the introduction of chemicals and surviving bacteria, respectively.

Confirmation of pathogen-induced mortality

Necropsies should be performed on all mortalities to ensure that the dead fish have been infected with *L. anguillarum*. First, dead fish are sprayed with 75% ethanol. A small incision is made into the ventral abdomen with sterile scissors taking care not to damage any of the internal organs. With a sterile scalpel blade, the swim bladder and internal organs are pushed aside to expose the kidney. A sterile loop is inserted into the kidney and then aseptically struck onto a TSA plate supplemented with 3.0% NaCl. After 48 h of incubation at 25°C, colonies of *L. anguillarum* appear on the plate as shiny cream-colored raised and round mounds (0.5 × 1.5 μm; Figure 2.2).[11] Presumptive tests were initially performed on bacterial colony growth on the TSA plates. Presumptive identification of

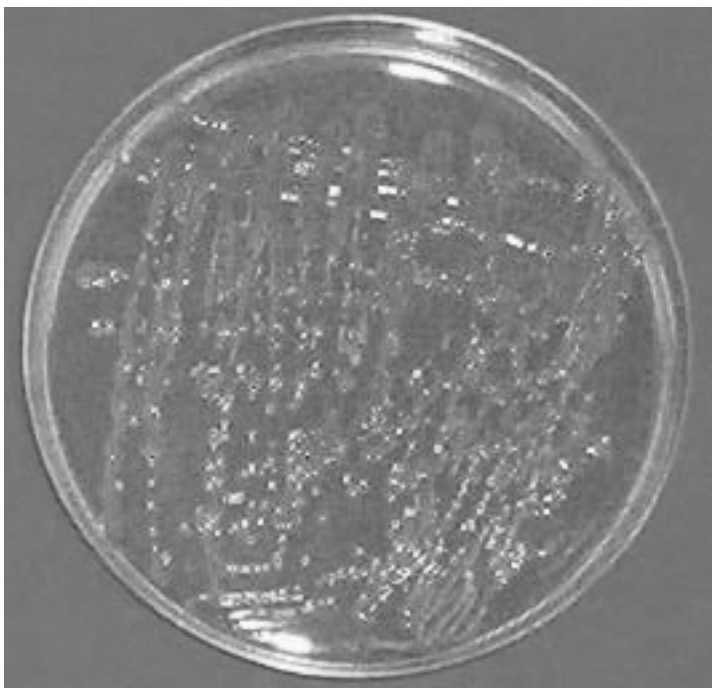

Figure 2.2 Colony morphology of *L. anguillarum* grown on TSA.

L. anguillarum infection was determined by inhibition of bacterial growth by novobiocin and the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropyl pterdine phosphate) on TSA (Figure 2.3A) and growth on VAM (Figure 2.3B). *V. anguillarum* media is a selective media for *Listonella* species and used to presumptively identify *L. anguillarum. L. anguillarum* on VAM produces a bright yellow, round, flat colony with a yellow halo after 48 h of incubation at 25°C.[17] Also as part of the presumptive identification of *L. anguillarum*, bacterial colonies were Gram stained and examined for the presence of cytochrome oxidase with a dry slide oxidase test (Figure 2.4). Bacteria presumptively identified as *L. anguillarum* were confirmed by a commercially available rapid agglutination test kit (Bionor; Figure 2.5).

Generalized linear models: Statistical analyses

Generalized linear modeling (GLM) was used to determine the LD_{30} for CHWSE and the LD_{50} for Aroclor 1254, HCBD, and DMBA solutions and the confidence limits for these values.[18] Statistical significance between treatments in the pathogen challenge studies was also assessed using GLM. We assumed that the number of survivors and mortalities in both assays follow a binomial distribution.[18,19] It has been determined that the most appropriate way to analyze the binomially distributed response data generated in toxicity tests is by using GLM.[18]

The analyses were performed with the GLMStat computer application.[20] To define a GLM it is important to identify the error structure and the link function that relates the linear predictor to the expected survival/mortality probabilities.[21] The logistic GLM was used for the analysis of data. For this model, the error structure is binomial and the linear predictor was related to the expected value of the datum by the logit link function.

Specifically for the pathogen challenge study, we used the logistic model to evaluate if survival/mortality of fish treated with a contaminant and fish treated only with the acetone/emulphor carrier solution were significantly different ($P_\alpha \leq 0.05$) beginning at 7 days post challenge. This analysis was conducted after correction for background mortalities. To correct for background deaths, the number of mortalities of a particular treatment group not treated with *L. anguillarum* were subtracted from the mortalities of that treatment group exposed to the bacteria. If required, analyses were conducted beyond day 7. Experiments were continued until mortalities began to level off at an asymptote in at least one treatment group.

Results and discussion

The cumulative 96-h LD curves of salmon given various doses of DMBA (Figure 2.6), Aroclor 1254 (Figure 2.7), CHWSE (Figure 2.8), or HCBD (Figure 2.9), were determined. The 96-h LD_{50} was determined for DMBA, Aroclor 1254, and HCBD to ensure the use of sublethal dosages of the test solutions for use in the pathogen challenge experiments. Since we were only able to achieve 30% mortality in the fish exposed to a very high concentration of CHWSE, we determined the LD_{30} for this contaminant. The LD_{50} determined for Aroclor 1254 was 270 mg kg^{-1} of salmon. The upper and lower 95% confidence limits were 500 and 100 mg kg^{-1}, respectively. The LD_{50} determined for DMBA was 63 mg kg^{-1} of salmon. The upper and lower 95% confidence limits were 73 and 56 mg kg^{-1},

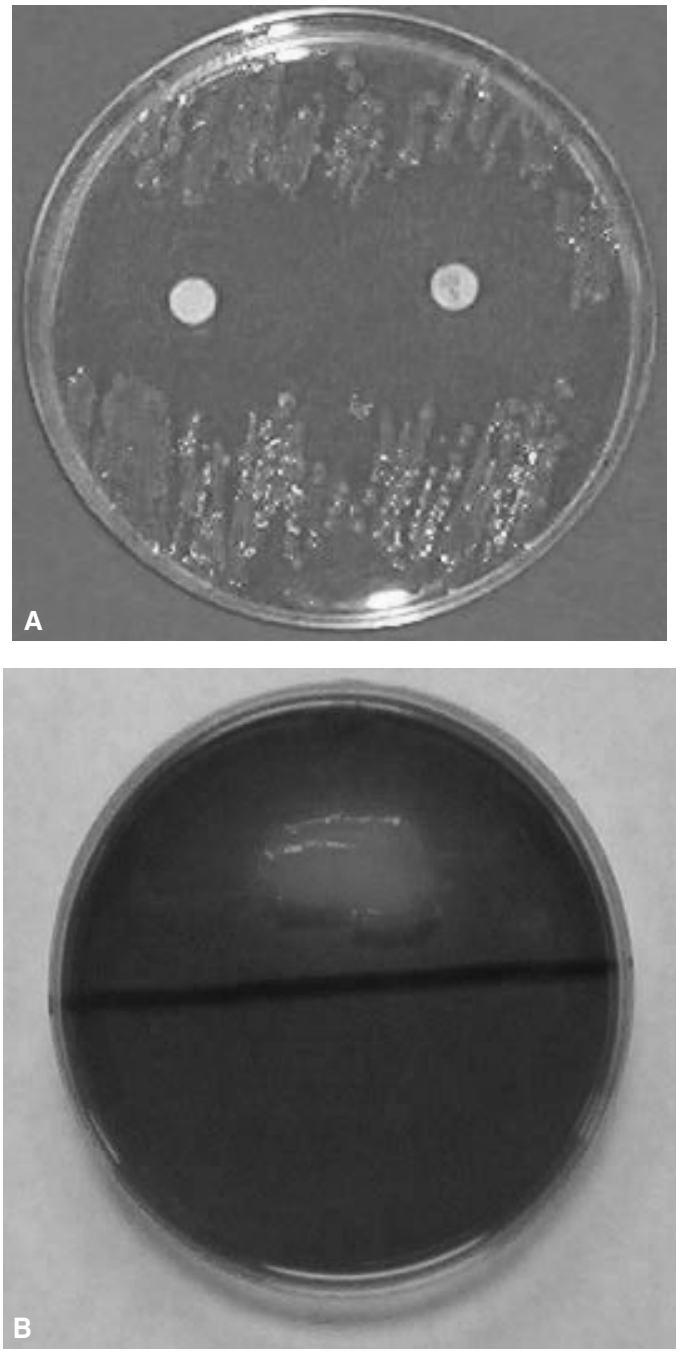

Figure 2.3 Two presumptive tests for *L. anguillarum*. (A) Zones of inhibition (or no bacterial growth) of *L. anguillarum* around the novobiocin and the vibriostatic agent 0/129 disks. The disks absorb water from the agar allowing the antibiotic to dissolve. The antibiotic migrates through the agar. There will be no growth in the areas where the antibiotic is at inhibitory concentrations for the bacteria on the plate.[21] (B) Growth of *L. anguillarum* on VAM. The media contains bile salts, a high concentration of NaCl and is at a high pH. These three factors select mostly for *Listonella* species. The color change (green to yellow) is due to the ability of *L. anguillarum* to ferment sorbitol.[17]

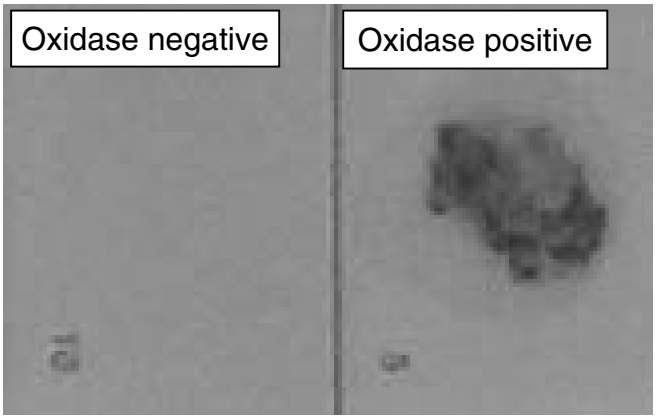

Figure 2.4 The presence or absence of cytochrome can be detected with an oxidase test that is commercially available. The enzyme is present if the bacteria are capable of oxidizing a reagent present on the test strip into a blue colored product. If a blue product forms, the bacteria is considered ''oxidase positive.'' *L. anguillarum* is oxidase positive.

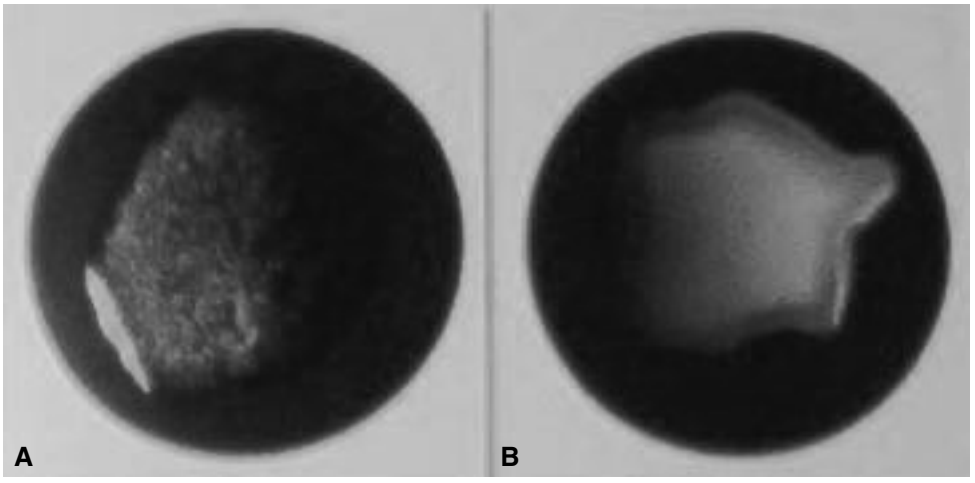

Figure 2.5 Agglutination of bacterial colonies from TSA with a monoclonal antibody to *L. anguillarum* is considered to be a confirmatory test for the identification of *L. anguillarum*. (A) A positive reaction or agglutination with the antibody. (B) The negative control shows no agglutination with the specific monoclonal antibody.

respectively. The LD_{50} for HCBD was 200 mg HCBD per kilogram of fish. The upper and lower 95% confidence limits were 450 and 130 HCBD per kilogram of fish, respectively. The LD_{30} determined for CHWSE was 741 g CHWSE equivalent per kilogram of fish. The upper and lower 95% confidence limits were 1500 and 540 g CHWSE equivalent per kilogram of fish, respectively.

The peak of the exponential growth phase of *L. anguillarum* grown in 500 ml of TSB supplemented with 1.5% NaCl and slowly agitated at 25°C occurred at approximately 15 h with an optical density of 1.9 (Figure 2.10). In subsequent challenge experiments,

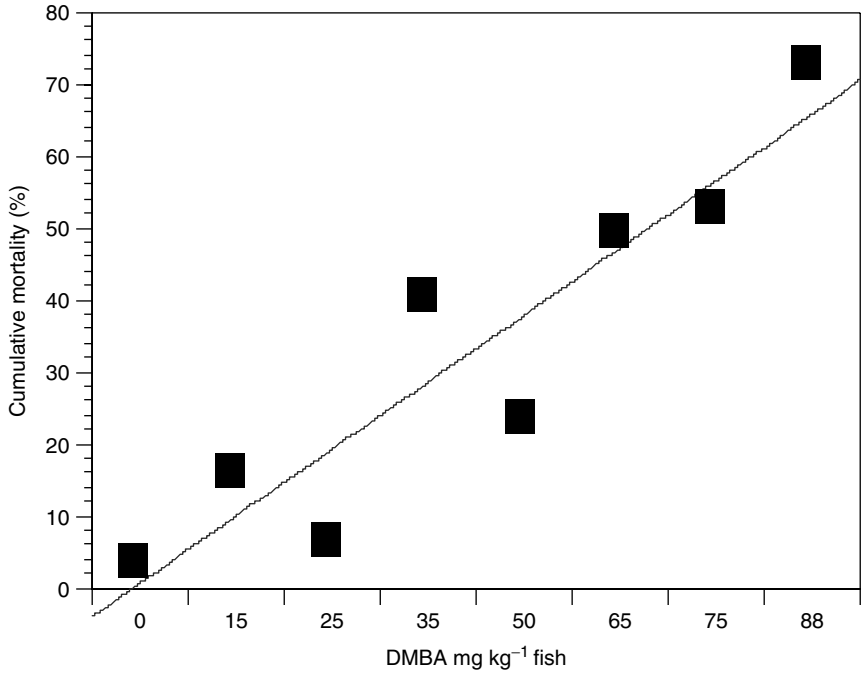

Figure 2.6 The cumulative 96 mortality of salmon given various doses of DMBA.[4] (From Arkoosh, M.R., Clemons, E., Huffman, P., Kagley, A., Casillas, E., Adams, N., Sanborn, H.R., Collier, T.K. and Stein, J., *J. Aquat. Anim. Health*, 13, 257–268, 2001. With permission.)

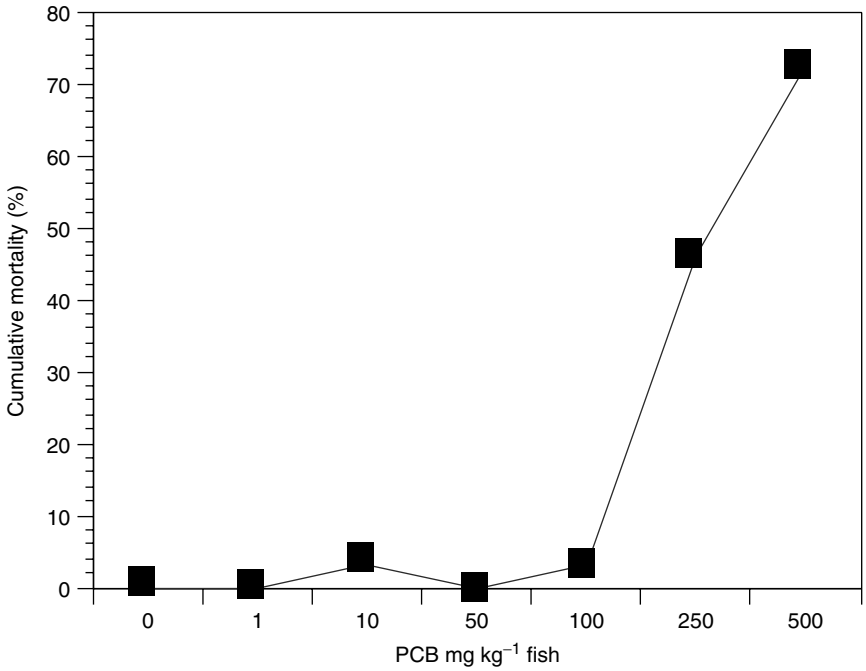

Figure 2.7 The cumulative 96 mortality of salmon given various doses of Aroclor 1254.[4] (From Arkoosh, M.R., Clemons, E., Huffman, P., Kagley, A., Casillas, E., Adams, N., Sanborn, H.R., Collier, T.K. and Stein, J., *J. Aquat. Anim. Health*, 13, 257–268, 2001. With permission.)

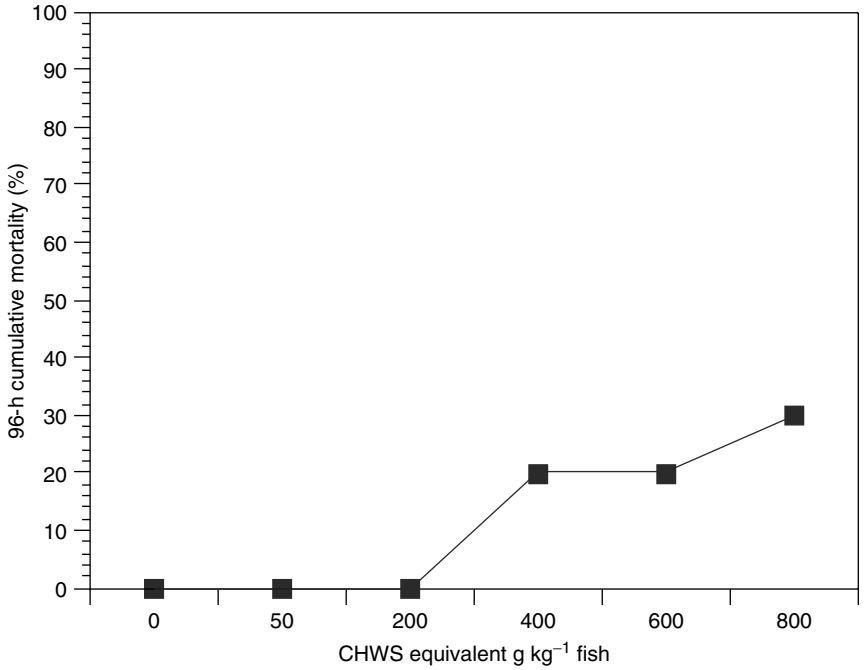

Figure 2.8 The cumulative 96 mortality of salmon given various doses of CHWSE.[2] (From Arkoosh, M.R., Clemons, E., Huffman, P., Kagley, A., Casillas, E., Adams, N., Sanborn, H.R., Collier, T.K. and Stein, J., *J. Aquat. Anim. Health*, 13, 257–268, 2001. With permission.)

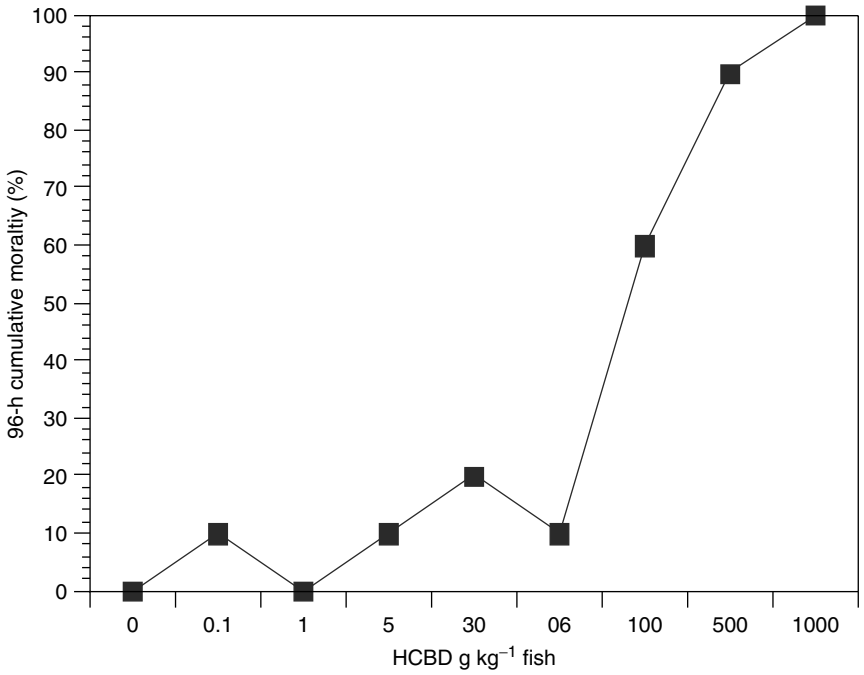

Figure 2.9 The cumulative 96 mortality of salmon given various doses of HCBD.[2] (From Arkoosh, M.R., Clemons, E., Huffman, P., Kagley, A., Casillas, E., Adams, N., Sanborn, H.R., Collier, T.K. and Stein, J., *J. Aquat. Anim. Health*, 13, 257–268, 2001. With permission.)

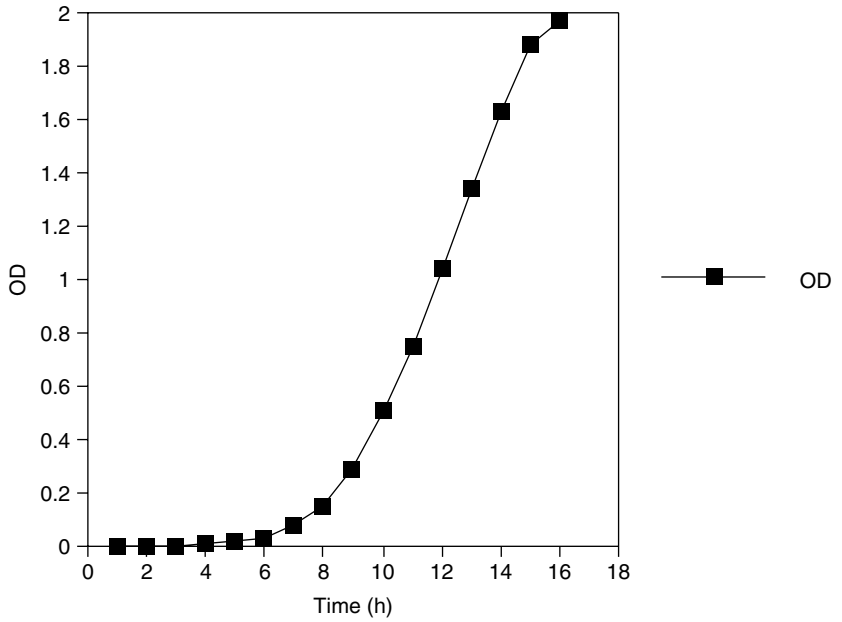

Figure 2.10 Growth curve of *L. anguillarum* measured as optical density (OD) at 525 nm (λ). The peak of the exponential growth phase of the bacteria occurs after 15 h.[1] (From Arkoosh, M.R., Casillas, E., Huffman, P., Clemons, E., Evered, J., Stein, J.E. and Varanasi, U., *Trans. Am. Fish. Soc.*, 127, 360–374, 1998. With permission.)

L. anguillarum cultures were grown to an optical density of approximately 1.7 prior to preparation of bacterial culture dilutions used for juvenile salmon exposures. The 168-h (7-day) LD–response curve for juvenile salmon exposed to *L. anguillarum* was determined by a logit regression analysis (Figure 2.11).

The percent cumulative mortality in Trials 1 and 2 of the different groups of juvenile chinook salmon exposed to *L. anguillarum* is shown in Figures 2.12 and 2.13, respectively. These figures represent the net cumulative mortality attributed to exposure to the bacteria after subtracting background mortality observed in juvenile chinook salmon that received chemical contaminants but were not exposed to bacteria. Background mortality at the end of the experiments was very low. Specifically, background mortality in Trial 1 at day 7 for the various treatments was the following: acetone/emulphor carrier solution (1.4%), CHWSE (1.2%), HCBD (2.7%), and the model mixture of PAH (0%). Background mortality in Trial 2 at day 9 for the various treatments was the following: acetone/emulphor carrier solution (6.6%), Aroclor 1254 (2.3%), and DMBA (6.7%). Statistical testing was performed beginning with 7 days post exposure data to determine treatment differences.[1] If statistical differences were not noted at 7 days post exposure, data obtained on the following days were also examined.

In Trial 1, the net cumulative mortality of juvenile chinook salmon exposed to *L. anguillarum* after receiving either CHWSE, HCBD, or the model mixture of PAHs ranged from 28% to 31% compared to the 16% observed in the acetone/emulphor control group at 7 days post bacterial challenge (Figure 2.12). Although in Trial 2, a significant difference in the net cumulative mortality between juvenile chinook salmon

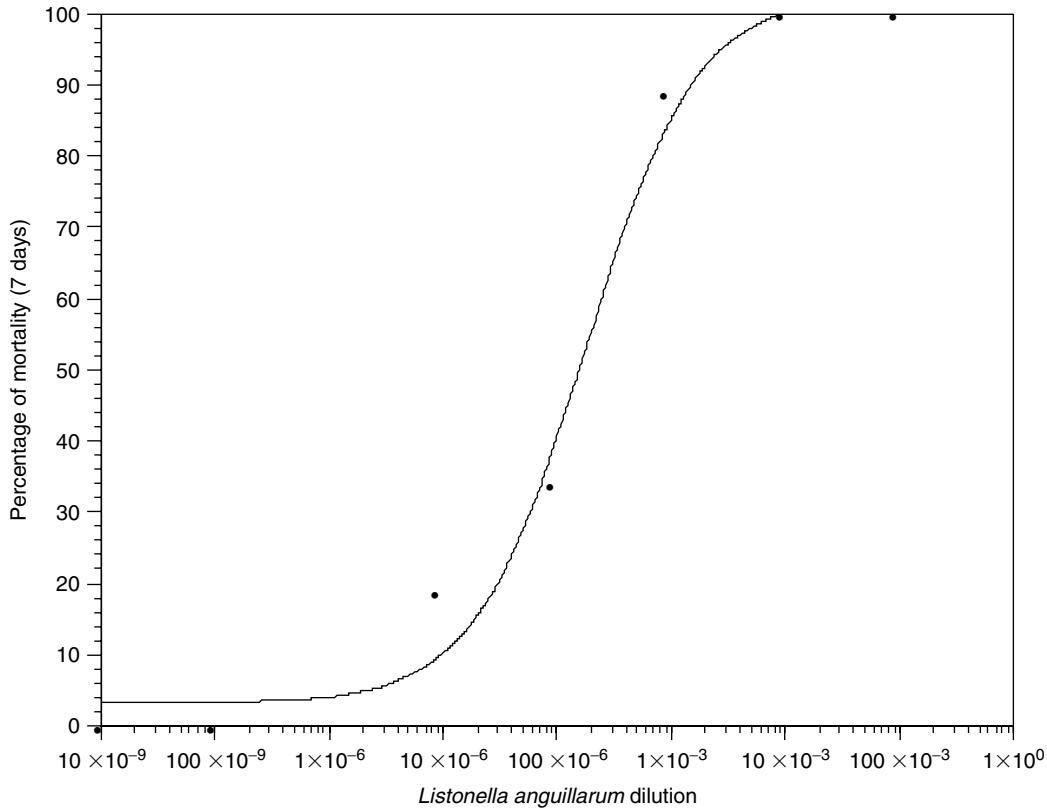

Figure 2.11 LD–response curve of juvenile chinook salmon at 7 days (168 h) post exposure to *L. anguillarum*.[1] (From Arkoosh, M.R., Casillas, E., Huffman, P., Clemons, E., Evered, J., Stein, J.E. and Varanasi, U., *Trans. Am. Fish. Soc.*, 127, 360–374, 1998. With permission.)

exposed to Aroclor 1254 and acetone/emulphor was detected on day 8, a difference between DMBA and acetone/emulphor was not detected until day 9 after challenge. The net cumulative mortality of juvenile chinook salmon exposed to the bacteria after receiving either DMBA or Aroclor 1254 ranged from 46% to 49% compared to the 25% observed in the acetone/emulphor control group at 9 days post challenge (Figure 2.13). The cumulative mortality was significantly higher in fish exposed to CHWSE, DMBA, Aroclor 1254, the PAH model mixture, or HCBD relative to fish receiving only the acetone/emulphor carrier.

Therefore, juvenile chinook salmon exposed to contaminants associated with urban estuaries in Puget Sound, such as the Hylebos and Duwamish waterways, exhibited a higher susceptibility to mortality induced by the marine pathogen *L. anguillarum*, than did the pathogen exposed juvenile chinook salmon treated only with the carrier acetone/emulphor. The contaminants tested represent specific subsets of predominant estuarine chemical pollutants. The chlorinated hydrocarbons tested were characterized by HCBD, PCBs, and CHWSE (which is composed primarily of HCBD-like compounds) and the aromatic hydrocarbons were characterized by the model mixture of PAHs and DMBA. Results obtained using the disease challenge assay described here, together with our previous studies,[4,22] support the hypothesis that chemical contaminant exposure

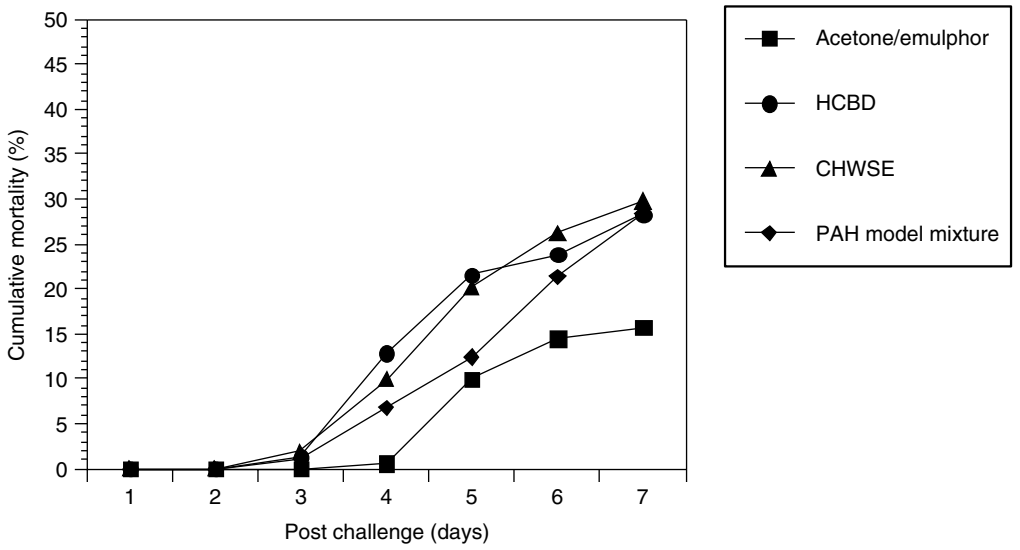

Figure 2.12 Percent cumulative mortality of juvenile chinook salmon injected with either HCBD, a model mixture of PAHs, a chlorinated-enriched sediment extract form the Hylebos Waterway, or the carrier control (acetone/emulphor) after exposure to 6×10^{-5} ml bacterial solution per milliliter seawater. The cumulative mortalities are corrected for mortalities observed in juvenile chinook salmon injected with either HCBD, a model mixture of PAHs, a chlorinated-enriched sediment extract form the Hylebos Waterway, or the carrier control (acetone/emulphor) but not exposed to *L. anguillarum*.[1] (From Arkoosh, M.R., Casillas, E., Huffman, P., Clemons, E., Evered, J., Stein, J.E. and Varanasi, U., *Trans. Am. Fish. Soc.*, 127, 360–374, 1998. With permission.)

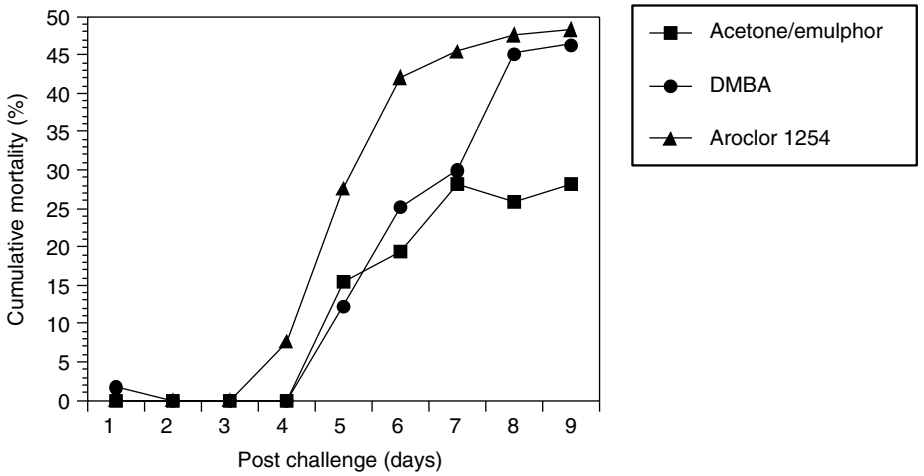

Figure 2.13 Percent cumulative mortality of juvenile chinook salmon injected with either DMBA, Aroclor 1254, or the carrier control (acetone/emulphor) after exposure to 6×10^{-5} ml bacterial solution per milliliter seawater. The cumulative mortalities are corrected for mortalities observed in juvenile chinook salmon injected with either DMBA, Aroclor 1254, or the carrier control (acetone/emulphor) but not exposed to *L. anguillarum*.[1] (From Arkoosh, M.R., Casillas, E., Huffman, P., Clemons, E., Evered, J., Stein, J.E. and Varanasi, U., *Trans. Am. Fish. Soc.*, 127, 360–374, 1998. With permission.)

of juvenile salmon in contaminated waterways can influence their ability to produce a protective immune response such that their survival potential may be significantly reduced.

Observations spanning the entire temporal scale of the experimental period in the present study provide useful data in evaluating differences in disease susceptibility of juvenile chinook salmon exposed to contaminants. This is especially apparent in Trial 2. Differences between DMBA groups and the control acetone/emulphor-injected groups were not significant until 9 days after exposure, whereas we were able to determine a difference between Aroclor 1254-injected fish and the control acetone/emulphor-injected fish after 8 days of exposure. Therefore, it is important to monitor the experiment to determine if it should be extend beyond the classical 7-day period in order to observe differences.

In conclusion, disease challenge assays in fish are useful in determining the effects of xenobiotics on their immune response. A number of variables need to be considered prior to performing the assay. They include but are not limited to the following:

- Which xenobiotic to expose the fish to, why, and what dose to expose the fish at? What method of exposure to use? How long after exposure to the contaminant prior to the disease challenge?
- Which pathogen to use in the challenge, how to challenge the fish with the pathogen (bath or injection) and what concentration to use. What confirmatory and presumptive tests are available?
- The temporal scale of the disease challenge assay needs to be determined.
- The fish need to be kept at a constant temperature, salinity, and flow since these variables have the ability to influence disease progression.
- Appropriate controls need to be used for quality assurance. For example, in our study, we used two types of controls. One control included juvenile chinook salmon, which did not receive bacteria, and the second control included juvenile chinook salmon, which received only the carrier acetone/emulphor. This way it is possible to determine how many fish might be dying from the procedure itself and not from *L. anguillarum* exposure.

If the xenobiotics under study are found to impair the ability of the fish to fight the pathogen, more specific tests can be used to determine which functional aspect (i.e., adaptive and/or innate immunity), or cell types (i.e., B cell, T cell, and/or macrophage) of the immune system is targeted by the xenobiotic.

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chapter three

Enhanced frog embryo teratogenesis assay: Xenopus *model using* Xenopus tropicalis

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Contents

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Introduction

The expense and time-consuming nature of conventional testing has warranted the development, validation, and eventual widespread use of alternative, nonmammalian model systems. Frog embryo teratogenesis assay—*Xenopus* (FETAX), a 4-day, nonmammalian, whole embryo bioassay designed to evaluate potential teratogenic hazard by direct chemical compounds and complex environmental mixture screening, and used as a tool to evaluate toxicological mechanisms of action, was developed to evaluate organ system malformation during early embryo and larval development.[1–3] In its original format, FETAX validation studies have been performed using the model as a screening assay using both pure chemical compounds and complex environmental mixtures (wastewater effluent, surface water, sediments, and groundwater).[4–12] Because *Xenopus* lack many metabolic enzyme systems, including the mixed-function oxidase (MFO) system, through the first 96 h of development, an exogenous metabolic activation system (MAS) was developed[13] and evaluated[14–21] using both Aroclor 1254- and isoniazid-induced rat liver microsomes. Currently, no standardized and well-validated alternative models exist for screening in teratogenesis. Today, only the limb micromass culture and the rodent whole embryo culture are used as an *in vitro* test of developmental toxicity.

An *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in 1994 by National Institute of Environmental Health Sciences (NIEHS) to develop a report recommending criteria and processes for validation and regulatory acceptance of toxicological testing methods that would be useful to federal agencies and the scientific community. In late 1998, ICCVAM initiated an evaluation of FETAX.[22] The ultimate objective of the ICCVAM evaluation was to determine applications in which FETAX may be successfully used and to determine areas of use that require further validation. Ultimately, the outcome of the ICCVAM evaluation prioritized further research needs and validation efforts to maximize understanding of how FETAX can be most effectively used as an alternative test method. From the ICCVAM, one overwhelming recommendation was brought forth.[22] This suggestion was to strongly consider replacing the currently used *Xenopus laevis* with a species with greater potential, *Xenopus tropicalis*.

The comparative advantages of using *X. tropicalis* over *X. laevis* are summarized in Table 3.1. FETAX can effectively be conducted in *ca*. 2-days-using of *X. tropicalis* as opposed to 4-days-using with *X. laevis*.[23] The increased rate of development of *X. tropicalis* is presently being evaluated, however, as it may have an impact on the sensitivity of the test. Many species that develop at cooler temperatures and, thus, develop more slowly than those at higher temperatures, are more sensitive, presumably due to longer developmental windows (i.e., larger exposure targets). Since FETAX uses an exogenous MAS consisting of rat liver microsomes, the increased temperature may increase the effectiveness and efficiency of the MAS. *Xenopus tropicalis*, being diploid, also represents a cytogenetic genetic advantage over *X. laevis*, which is oligotetraploid.

The smaller size of *X. tropicalis* compared to *X. laevis* represents practical advantages for the assay. Less test material is required for the smaller organism. More organisms can

Table 3.1 Comparison of *X. laevis* and *X. tropicalis* attributes as a test species

Consideration	*X. laevis*	*X. tropicalis*
Rate of development (time to complete FETAX test)	4-days	2-days[a]
Culture temperature	23.5 ± 0.5°C	26.5 ± 0.5°C[a]
Ploidy	Oligotetraploid	Diploid[a]
Egg/larvae size (culture density)	Larger	Smaller[a]
Waste production (NH_3)	Moderate	Negligible[a]
Clutch size	1500 per female	2500 per female[a]
Transgenic capacity	Moderate to good	Good[a]
Time to sexual maturity	1.5–2 years	4–6 months[a]
Consistency in long-term developmental kinetics	Moderate	Relatively tight[a]
Capacity to establish test battery with longer-term assay (including reproductive and life cycle assessments)	Low to moderate	Relatively high[a]
Chromosomes/genome size	$36/3.1 \times 10^9$ bp	$20/1.7 \times 10^9$ bp[a]
Disease susceptibility	Low to moderate[a]	Moderate
Capacity to develop inbred lines	Little	Great[a]
Literature available	Large database[a]	Emerging database

[a] Represent a significant advantage as a test species.

be accommodated in each replicated treatment, thus offering a density advantage over *X. laevis*. *Xenopus laevis* produce a greater quantity of waste products during the assay than *X. tropicalis* (*ca*. 1.5 mg/l NH_3-N versus <0.5 mg/l NH_3-N).[24] Increased clutch size also creates a significant production advantage for the FETAX model. One of the most exciting advantages is the capacity to create transgenic lines of *X. tropicalis* and the use of cDNA microarray technology. In addition to the aforementioned anticipated advantages of *X. tropicalis*, the shorter life cycle allows more expedient and practicable reproductive toxicity studies and endocrine evaluation of the thyroid axis during metamorphosis. It is also possible to create inbred strains of *X. tropicalis*, which will likely decrease the genetic variability observed in *X. laevis*. Developing inbred lines of *X. laevis* is difficult at best. In the present chapter, we describe a modified FETAX method using *X. tropicalis* and present results of studies comparing the sensitivity and practicability of using *X. tropicalis* in this developmental toxicity model system.

Materials and methods

Equipment and supplies

The equipment, chemicals, and consumable supplies required for performing FETAX are provided in Table 3.2.

Reagents

The following solutions were used to perform FETAX. Reagents that were used in solutions are presented in Table 3.2.

1. *FETAX solution*: A reconstituted water medium[4] was used as a negative control and as a diluent for preparing aqueous solutions of test chemicals by dissolving

Table 3.2 Equipment, chemicals, and supplies used to perform FETAX

Item	Model/CAS number	Source	Catalog number	Chemical purity (%)
Incubator, 23 ± 0.5°C	307C	Fisher	11-679-25C	—
Ohaus balance	E10640	Fisher	02-112-4	—
Zoom microscope	Stereomaster	Fisher	12-562-1	—
Denver pH meter	9340.1	Fisher	02-226-83	—
YSI DO meter	52	Fisher	13-298-00	—
Fume hood	47	Fisher	16-500-10	—
Corning stirrer/hot plate	PC-620	Fisher	11-497-8A	—
Nikon digital camera	Coolpix 5000	B&H Photo	NICP5000J	—
Rena submersible heater	RH100	Aquatic Eco	RH100	—
Sodium chloride	7647-14-5	Sigma	S9888	99.0
Sodium bicarbonate	144-55-8	Sigma	S8875	99.0
Magnesium sulfate	7487-88-9	Sigma	M7506	99.0
Calcium sulfate dihydrate	10101-41-4	Sigma	C3771	99.0
Potassium chloride	7447-40-7	Sigma	P3911	99.0
Calcium chloride dihydrate	10035-04-8	Sigma	C5080	96.0
Chorionic gonadotropin	9002-61-3	Sigma	CG5	—
L-Cysteine	52-90-4	Sigma	C7352	98.0
Streptomycin sulfate	3810-74-0	Sigma	S6501	98.0
Penicillin-G	69-57-8	Sigma	PENNA	—
Cyclophosphamide	6055-19-2	Sigma	C0768	98.0
Acetic hydrazide	1068-57-1	Sigma	A3031	90.0
6-AN	329-89-5	Sigma	A0630	99.0
NADP, B-	1184-16-3	Sigma	N0505	99.0
NADPH, B-	2646-71-1	Sigma	N1630	95.0
Glucose-6-phosphate monopotassium salt	103192-55-8	Sigma	G6526	99.0
Glucose-6-phosphate dehydrogenase	9001-40-5	Sigma	G7878	—
DMSO	67-68-5	Sigma	154938	99.9
3-Aminobenzoic acid ethyl ester (MS-222)	886-86-2	Sigma	A5040	—
Sodium hydrosulfite	7775-14-6	Sigma	S1256	85.3
Formaldehyde solution, 37% (w/w)	50-00-0	Sigma	F1635	95.0
Petri dishes, 60 mm	—	Fisher	08-757-13A	—
Petri dishes, 100 mm	—	Fisher	08-757-12	—
Transfer pipettes, 3 ml	—	Fisher	13-711-7	—
Tuberculin syringes, 1 ml w/26 g $\frac{3}{4}$ needles	—	Fisher	14-823-2E	—
Syringes, 5 ml	—	Fisher	14-823-35	—
Needles, 18 g $1\frac{1}{2}$	—	Fisher	14-826-5D	—

0.625 g NaCl, 0.096 g $NaHCO_3$, 0.075 g $MgSO_4$, 0.06 g $CaSO_4 \cdot 2\ H_2O$, 0.03 g KCl, and 0.02 g $CaCl_2 \cdot 2\ H_2O$ per liter deionized (DI) water.

2. *1% Dimethyl sulfoxide* (DMSO) (v/v) *in FETAX solution* water was used as a negative control and as a diluent for preparing aqueous solutions of test chem-

Table 3.3 FETAX culturing conditions for *X. tropicalis*

Parameter	Condition
Test type	Static renewal
Temperature	26.5 ± 0.5°C
Light quality	Ambient laboratory illumination
Light intensity	10–20 uE/m^2/s (50–100 ft-c)
Photoperiod	12 h light; 12 h dark
Test chamber size	25 ml
Test solution volume	10 ml per replicate
Renewal of test solutions	Daily
Age of test organisms	Small cell blastulae (stages 8 and 9)
Replicates per treatment	4 per negative control; 2 per positive control (reference toxicants); 2 per test substance
Test organisms per chamber	20
Test organisms per treatment	80 per negative control; 40 per positive control (reference toxicants); 40 per test substance
Test vessel randomization	Randomization of tray placement in incubator
Feeding regime	None, prefeeding stage
Cleaning	Siphoned daily, prior to treatment renewal
Aeration	None
Dilution water	FETAX solution prepared using E-PURE® deionized water and reagent grade chemicals
Test duration	Developmental stage 46 (*ca.* 48 h)
Endpoints	Survival, malformation, growth (length)
Test acceptability	Control mortality/malformation rates ≤10%

icals that are not readily dissolvable in water. Ten milliliters of DMSO were added to FETAX solution water and diluted to 1 l.

3. *6-Aminonicotinamide* (6-AN)[2] reference toxicant stock solution (at 2500 mg/l) was used as a positive control to induce mortality. The stock was prepared by dissolving 2.5 g 6-AN per liter FETAX solution water and storing at room temperature to prevent the formation of crystals.
4. *6-Aminonicotinamide* (6-AN)[2] reference toxicant stock solution (at 5.5 mg/l) was used as a positive control to induce effect (malformations). The stock was prepared by adding 2.2 ml of 2500 mg/l 6-AN stock solution to FETAX solution water to make 1 l of stock solution. The solution was stored at 4.0°C.
5. *Human chorionic gonadotropin* (hCG) *stock A solution* (1000 units/ml) was prepared by injecting 5.0 ml of 0.9% saline solution into a 5000 unit vial of hCG, using a 10-cc syringe and shaking until dissolved. The hCG stock A was used to artificially induce maturation and amplexus.
6. *Human chorionic gonadotropin* (hCG) *stock B solution* (150 units/ml) was prepared by adding 0.15 ml of hCG stock A solution to 0.85 ml of 0.9% saline solution using a 1.0-cc syringe and shaking until mixed. The hCG stock B was used to artificially induce maturation and amplexus.
7. L-*Cysteine solution* (2.0%) was prepared by dissolving 2.0 g of the chemical per 100 ml of FETAX solution water and used to remove jelly coat from fertilized eggs.
8. *3-Aminobenzoic acid ethyl ester* (MS-222) stock solution (at 100 mg/l) was prepared by dissolving 0.1 g MS-222 in FETAX solution water and diluted to 1 l. MS-222 was used to anesthetize test specimens at test takedown.

9. *Formalin solution* (3.0%) was used to preserve test specimens indefinitely. Formalin solution was prepared by mixing 40.5 ml of formaldehyde solution (37.0%) in 900 ml of FETAX solution water. The pH was adjusted to 7.0 using 0.1 *N* hydrochloric acid (HCl) solution. FETAX solution water was used to bring the reagent volume to 1 l. The formalin reagent was stored at room temperature.
10. *Penicillin–streptomycin stock solution* was used as an antibiotic to prevent bacterial growth and is prepared by mixing 10,000 units/ml penicillin G and 10,000 units/ml streptomycin sulfate into 100 ml FETAX solution water. Solution was stored at 4.0°C.
11. *FETAX antibiotics* (FAB) *solution* water was used in place of FETAX solution water in activated (MAS) FETAX. Ten milliliters of penicillin and streptomycin stock solution was added to FETAX solution water and diluted to 1 l. The solution was stored at 4.0°C. In the event a carrier solvent (1% v/v DMSO) was used as a diluent of the test material, a FAB and 1% v/v DMSO should be prepared and stored at 4.0°C.
12. *Acetic hydrazide* (AH) *stock solution* (at 3000 mg/l) was used as a reference toxicant by dissolving 3.0 g AH in FAB solution water and diluting to 1 l. The AH solution was stored at 4.0°C.
13. *Cyclophosphamide* (CP) *stock solution* (at 4000 mg/l) was used as a reference toxicant by dissolving 4.0 g CP in FAB solution water and diluting to 1 l. The stock solution was stored at 4.0°C.
14. *Metabolic activation system* (MAS): Rat liver microsomes. Microsomes were stored in freezer.
15. *MAS NADPH generator*: Dissolved 16.8 mg NADPH, 264 mg NADP, and 3.7 g glucose-6-phosphate in 33.6 ml FAB solution water and aliquots were separated into three scintillation vials. The generator was labeled and stored in freezer.
16. *MAS NADPH dehydrogenase*: Injected 5.0 ml of FAB solution water into a 500-unit vial of glucose-6-phosphate dehydrogenase, swirling to dissolve. The MAS enzyme was transferred to a scintillation vial, labeled, and stored in freezer.

Test system

Animal husbandry, breeding, and embryo collection were performed as described in American Society for Testing and Materials (ASTM) E1439-98.[2] *Xenopus tropicalis* embryos were harvested from adult breeding stock originally obtained from *Xenopus* I (Dexter, MI). Guidelines provided by Nieuwkoop and Faber[25] were used to stage the embryos. Both Nieuwkoop and Faber's[25] guidelines and the *Atlas of Abnormalities*[3] were used to help determine normal embryo and cleavage patterns.

Adult husbandry

Sexually mature *X. tropicalis* were separated by sex and housed in 50-l plastic tubs (6–8 animals per tub) containing 20 l of aged (dechlorinated) tap water. Temperature in each tub was maintained at 26 $\pm$ 1°C with the use of submersible heaters. The frogs were fed Salmon Starter (#4 pellet) from Zeigler Brothers (Gardners, PA), three times per week (Monday, Wednesday, and Friday), 3–4 h prior to cleaning the tubs. Feeding amounts varied between tubs based on number, size, and sex of the frogs. It was estimated that females received 40–50 mg and males received 25–30 mg per animal per feeding event.

Adult breeding

Adult female and male frogs were chosen based on their readiness for breeding. This was determined by physical appearances. Indicators used to determine female readiness were the appearance of full ovaries (sides of the abdomen appeared swollen from dorsal view) and protruding cloaca (small tail-like papillae located at the posterior end). Male frogs chosen for breeding possessed darkened nuptial pads on the palms of their forelimbs and/or light mating strips on the underside of their forelimbs.

Each male and female frog chosen for breeding was separated and administered a priming dose of 0.1 ml of hCG stock B (solution 6) subcutaneously in the dorsal lymph sac. Approximately 48 h later, immediately prior to introduction to the breeding chambers, each frog was given a booster injection of 0.1 ml of hCG stock A (solution 5). Each male and female was then paired and placed in 5 l breeding chambers containing approximately 3 l of FETAX solution (solution 1). Aquarium pumps were attached to each chamber to aerate the water. Each chamber was then covered and placed in a quiet, dark location overnight.

Embryo collection

Xenopus tropicalis embryos were collected by carefully pouring off as much water from the breeding chamber as possible, without losing eggs, and adding 500 ml of 2% (w/v) L-cysteine solution (pH 8.1) (solution 7). The eggs were gently swirled in the solution for 2–3 min to remove the jelly coat. The cysteine solution was then poured off and the remaining eggs were immediately rinsed with FETAX solution water until there was no cysteine odor (5–6 times). The de-jellied embryos were then divided into 100 mm Petri dishes containing FETAX solution water using 3-ml transfer pipettes. Unfertilized and necrotic eggs were discarded, leaving only normal-appearing fertilized embryos.[25] When developing embryos reached mid-to-late blastula stage (stages 8 and 9), they were transferred to 60-mm Petri dishes containing the test material.

Test substances

FETAX is designed to identify toxicity in environmental samples, both aqueous and solid phase, and aqueous solutions of chemical compounds.[2] Before initiating FETAX, environmental samples were analytically characterized by measuring pH, dissolved oxygen, conductivity, hardness, alkalinity, ammonia-nitrogen, and residual chlorine (residual oxidants). Only dissolved oxygen was adjusted by aeration if below 4.0 mg/l. Stock solutions made from chemical compounds were analyzed for pH and dissolved oxygen and adjusted if pH was outside the 6.0–9.0 range and dissolved oxygen was below 4.0 mg/l. Temperature, pH, and dissolved oxygen were monitored daily throughout the test.

Test design

The culture conditions used in the FETAX adapted for *X. tropicalis* are described in Table 3.3.

FETAX overview

The basic experimental designs may be considered to be the screen, the range finding or limit test, and the definitive concentration response assay. The FETAX screen was used to test material at 100% concentration, such as environmental samples. The FETAX range test was used to estimate the 96-h LC_{50} and EC_{50} and help define the definitive test concentrations. The FETAX definitive test was used to test materials at several concentrations to more precisely determine an LC_{50} and an EC_{50}. These materials included environmental samples and/or chemical compounds. The definitive assay was also modified to evaluate developmental toxicity for human health hazard assessment by incorporating an exogenous MAS. The MAS was composed of microsomes induced from rat livers and a nicotinamide adenine dinucleotide generator system, which simulated mammalian metabolism.

Microsome preparation

Rat liver microsomes were prepared using post-isolation mixes of phenobarbital-, β-naphthoflavone-, and isoniazid-induced preparations.[13] Microsomal P-450 activity was inferred by measuring the *N*-demethylation of aminopyrine (phenobarbital and β-naphthoflavone preparations) or *N*-nitrosodimethylamine (isoniazid preparation) to formaldehyde.[26] Post-isolation mixed microsome sets were prepared by mixing equal activities of each of the three differently induced microsomes. Post-isolation mixed MAS preparations have performed the best in FETAX validation studies and represent a broad spectrum of MFO activity.[27,28] Protein was determined by the BioRad method (BioRad, Richmond, CA).[29] Specific aliquots of the mixed microsomes were pretreated with carbon monoxide (CO) to inhibit MFO activity. Inhibition with CO was achieved by chemically reducing designated aliquots with dithionite and subsequent bubbling of CO gas through the reconstituted microsomes for 3 min.[13]

FETAX screen procedure for aqueous solutions

Sixty-millimeter Petri dishes were set up to test each treatment in duplicate (additional replicates may be tested, if desired). Four replicates of FETAX solution (solution 1) were used as the control. Also, four additional control replicates of 1% (v/v) DMSO in FETAX solution (solution 2) were used if test treatments were insoluble in water. Two replicates each of 5.5 mg/l and 2500 mg/l of 6-AN (solutions 3 and 4) were set as reference toxicants. Blastula stage embryos (*ca*. stages 8 and 9) were then added to each test dish containing treatments and controls. Loaded Petri dishes were then placed in an incubator with temperature maintained at 26.5 $\pm$ 0.5°C. The test was renewed daily and mortality and stage data were collected. When at least 90% of the control larvae reached stage 46, the test was terminated by anesthetizing the specimens in 100 mg/l MS-222 (solution 8) and fixing in 3% (v/v) formalin (solution 9). Data endpoints collected from the FETAX screen were percent mortality, percent malformation, and statistical differences in growth compared to the control.

FETAX range and definitive procedures for aqueous solutions

The FETAX range and definitive tests were performed in a manner similar to that performed for the screen, with only the treatment setup being different. Instead of screening different samples at concentrations of 100%, treatments in the range and definitive tests consisted of one sample or chemical tested at several concentrations. Data collected from the FETAX range test were used to better define concentrations selected for the FETAX definitive test. Data endpoints collected from the FETAX definitive test were percent mortality, percent malformation, LC_{50}, EC_{50}, and minimum concentration to inhibit growth (MCIG).

FETAX definitive procedure with MAS for aqueous solutions

The FETAX definitive MAS test consisted of two current definitive tests, one without MAS (unactivated) and one with MAS (activated). Enough 60-mm Petri dishes and stages 8 and 9 embryos were set up to test each of the following two sets:

1. Unactivated (without MAS) set:
   - Four replicates of FETAX solution as a control (solution 1)
   - Four replicates of FAB solution water without MAS (solution 11)
   - Four replicates of 1% (v/v) DMSO in FAB solution water (if required) (solution 11)
   - Two replicates of 6-AN solution at 5.5 mg/l concentration (solution 3)
   - Two replicates of 6-AN solution at 2500 mg/l concentration (solution 4)
   - Two or more replicates of each sample concentration to be tested
2. Activated (with MAS) set:
   - Four replicates of FAB solution water with MAS or 1% (v/v) DMSO in FAB solution water with MAS (if required) (solutions 11, 14–16)
   - Two replicates of 3000 mg/l AH with the CO-treated MAS and the MAS generator system (solutions 12, 14–16)
   - Two replicates of 4000 mg/l CP with the CO-treated MAS and the MAS generator system (solutions 13–16)
   - Two replicates of 3000 mg/l AH with the MAS and NADPH generator system (solutions 12, 14–16)
   - Two replicates of 4000 mg/l CP with the MAS and NADPH generator system (solutions 13–16)
   - Two or more replicates of each sample concentration to be tested

Twenty milliliters (10 ml per replicate) of each test treatment was placed in its own Erlenmeyer flask. Forty milliliters of reagent was used for the controls with four replicates. Microsomes (0.2 ml per 20 ml of solution) (solution 14) were added to each flask of the activated (MAS) set only, with the exception of unactivated AH and CP. Before adding microsomes to AH and CP, the microsomes inactivated were first treated by adding 5–6 granules of sodium hydrosulfite to *ca*. 0.5 ml microsomes and then bubbling CO gas through the microsomes for 3 min at a rate of one bubble every 1–2 s. MAS NADPH generator (192 μl) (solution 15) and 63 μl of MAS NADPH dehydrogenase (solution 16) were added to each activated (MAS) flask and the contents mixed. Ten

milliliters aliquots of solution from each flask were transferred to appropriately labeled Petri dishes. Blastula stage embryos (*ca*. stages 8 and 9) were then added to each test dish and dishes were placed in an incubator with temperature maintained at 26.5 $\pm$ 0.5°C.

FETAX scoring

After FETAX test takedown, larval specimens were anesthetized using a 100-mg/l solution of MS-222 (solution 8) and then preserved in 3% (v/v) formalin (solution 9). Specimens were then observed using a dissecting microscope and scored. Scoring data included total number of specimens per treatment, number of survivors per treatment, number of survivors per treatment with malformations, and a specific description of the malformations induced.

FETAX digitizing

After preservation, the larvae in each Petri dish were photographed using a digital camera and then electronically digitized using a personal computer and SigmaScan® Pro digitizing software (SPSS, Chicago, IL). Whole body length measurements, from snout to tail, were made of each specimen from each treatment.

Statistical analysis

Trimmed Spearman–Karber analysis was used to determine the median lethal concentration (LC_{50}), the EC_{50} (the concentration inducing gross terata in 50% of the surviving larvae), and their respective 95% fiducial intervals. A teratogenic index (TI) value was calculated by dividing the LC_{50} by the EC_{50} (malformation). The TI value represents the separation in the lethal and malformation-inducing concentration–response curves. Increasing TI values typically indicate increasing teratogenic potential.[5,21] Head-to-tail length was measured as an indicator of embryo growth using SigmaScan® Pro digitizing software (SPSS, Corte Madera, CA) and a personal computer. Concentrations inducing growth inhibition (MCIG) were calculated by ANOVA or ranked ANOVA [Bonferroni *t*-test, $P < 0.05$ (parametric data sets) or Dunn's test, $P < 0.05$ (nonparametric data sets)] using SPSS.

Results

Results of concurrent FETAX tests using *X. laevis* and *X. tropicalis* are presented in Table 3.4.

Control results

In 24 separate tests performed with *X. tropicalis*, the control mortality and malformation was 3.2 $\pm$ 0.8% and 4.8 $\pm$ 0.7%, respectively (present chapter and Reference 24). In either species, the incidences of mortality and malformation in the 5.5 mg/l 6-AN positive control treatment ranged from 0% to 15% and 47.5% to 82.7%, respectively. In either species at both culture temperatures, the incidences of mortality and malformation for the 2500 mg/l 6-AN positive control treatment ranged from 50% to 75% and 100%,

Table 3.4 Comparative sensitivity of *X. laevis* and *X. tropicalis* in FETAX model

Test substance [CAS No.]	Species	Temperature (°C) (± 0.5)	Exogenous MAS[a]	Unit	LC_{50}[b] (CI)	EC_{50}[c] (CI)	TI[d] (CI)	MCIG[e] (%LC_{50})	Reference
Acetylhydrazide [1068-57-1]	*X. laevis*	23.5	No	mg/l	9850.0 (9800.0–10150.0)	53.5 (48.2–58.8)	184.1 (164.0–204.2)	50.0 (50.0)	
	X. tropicalis	26.5	No	mg/l	8325.0 (8150.0–8500.0)	71.1 (64.1–78.2)	117.1 (102.0–132.2)	0.1 (9.1)	
	X. laevis	23.5	Yes	mg/l	8325.0 (8000.0–8650.0)	72.5 (64.0–81.0)	115.2 (102.1–128.3)	40.0 (0.5)	
	X. tropicalis	26.5	Yes	mg/l	4050.0 (3850.0–4250.0)	84.7 (80.5–88.9)	47.8 (33.5–56.5)	35.0 (0.9)	
6-AN [329-89-5]	*X. laevis*	23.5	No	mg/l	2264.6 (2249.1–2285.1)	4.7 (6.0–7.5)	481.8	100.0 (4.4)	24
	X. tropicalis	26.5	No	mg/l	2315.6 (2205.5–2420.7)	8.4 (8.1–8.7)	275.7	100.0 (4.3)	
Atrazine [1912-24-9]	*X. laevis*	23.5	No	mg/l	24.5 (19.3–31.1)	4.1 (3.0–5.7)	6.0	5.0 (20.4)	24
	X. tropicalis	26.5	No	mg/l	31.8 (29.8–33.8)	7.2 (5.2–9.2)	4.4	5.0 (15.7)	
Copper [7758-99-8]	*X. laevis*	23.5	No	mg/l	0.4 (0.3–0.4)	0.3 (0.2–0.5)	1.3	0.2 (50.0)	24
	X. tropicalis	26.5	No	mg/l	0.3 (0.2–0.3)	0.1 (0.1–0.2)	3.0	0.1 (33.3)	
Cyclophosphamide [6055-19-2]	*X. laevis*	23.5	No	mg/l	8150.0 (7500.0–8800.0)	6325.0 (6000.0–6650.0)	1.3 (1.0–1.6)	10,000.0 (>100.0)	
	X. tropicalis	26.5	No	mg/l	6350.0 (6100.0–6600.0)	5150.0 (5200.0–6450.0)	1.0 (0.9–1.1)	8000.0 (>100.0)	
	X. laevis	23.5	Yes	mg/l	1.1 (0.9–1.4)	0.4 (0.2–0.5)	2.8 (2.5–3.1)	50.0 (0.6)	
	X. tropicalis	26.5	Yes	mg/l	0.2 (0.1–0.3)	0.04 (0.02–0.06)	5.0 (4.0–6.0)	0.01 (5.0)	
Ethanol [64-17-5]	*X. laevis*	23.5	No	% (w/v)	1.0 (0.8–1.1)	0.7 (0.6–0.8)	1.4	1.0 (100.0)	24
	X. tropicalis	26.5	No	% (w/v)	1.6 (1.5–1.7)	1.0 (0.9–1.1)	1.6	1.3 (81.3)	
Methotrexate [60388-53-6]	*X. laevis*	23.5	No	mg/l	508.0 (475.0–543.0)	22.0 (21.0–24.0)	23.1 (20.1–26.1)	10.0 (2.0)	
	X. tropicalis	26.5	No	mg/l	322.5 (298.5–346.5)	39.3 (30.1–48.4) 8.2 (7.1–9.3) 5.0 (1.6)			
Semicarbazide [563-41-7]	*X. laevis*	23.5	No	mg/l	6956.0 (6642.0–7270.0)	9.5 (8.2–11.0)	734.2	7.5 (0.1)	24
	X. tropicalis	26.5	No	mg/l	7386.0 (7000.4–7768.6)	12.5 (11.4–13.8)	590.9	20.0 (0.3)	
Complex environmental mixture (Bermuda pond water-BARP)[7]	*X. laevis*	23.5	No	% (v/v)	32.4 (28.0–36.0)	10.2 (9.0–11.5)	3.2 (2.8–3.6)	6.1 (18.8)	
	X. tropicalis	26.5	No	% (v/v)	27.2 (23.1–32.3)	13.9 (10.3–17.5)	2.0 (1.5–25.)	12.5 (46.0)	

[a] Rat liver metabolic activation system.

[b] Median lethal concentration with respective 95% fiducial interval in parenthesis.

[c] Median teratogenic concentration with respective 95% fiducial interval in parenthesis.

[d] Teratogenic Index = LC_{50}/EC_{50}.

[e] Minimum concentration inhibiting growth (% of LC_{50} = MCIG/LC_{50}).

respectively. The performance results met the requirements established in ASTM E1439-98 and compared favorably to historical *X. laevis* tests.[2]

6-Aminonicotinamide

The stage 46 LC_{50}, EC_{50} (malformation), and TI values for 6-AN are presented in Table 3.4. The 6-AN[24] induced gut and cardiac miscoiling and abdominal edema in *X. tropicalis* at concentrations $\geq$10 mg/l. 6-AN induced notochord lesions, craniofacial mal-development, mouth abnormalities, rupture of the pigmented retina, and microcephaly at concentrations $\geq$10 mg/l in *X. tropicalis*. 6-AN induced gut and cardiac miscoiling, abnormal mouth development, and pericardial edema in *X. laevis* at concentrations $\geq$10 mg/l. 6-AN induced notochord lesions, craniofacial mal-development, abnormal pigmented retina formation, and microcephaly at concentrations $\geq$10 mg/l in *X. laevis*.

Ethanol

The stage 46 LC_{50}, EC_{50} (malformation), and TI values for ethanol are presented in Table 3.4. Ethanol[24] induced craniofacial dysmorphology, microphthalmia, and abnormal mouth development in *X. tropicalis* at concentrations $\geq$0.5% (w/v). Ethanol induced miscoiling of the gut, visceral hemorrhage and edema, and microcephaly at concentrations $\geq$1% in *X. tropicalis*. Ethanol induced abnormal gut development, craniofacial dysmorphogenesis, and mouth malformations in *X. laevis* at concentrations $\geq$0.5% (w/v). Ethanol induced abnormal myotome development, microphthalmia, microcephaly, and visceral hemorrhage and edema at concentrations $\geq$1% (w/v) in *X. laevis*.

Semicarbazide

The stage 46 LC_{50}, EC_{50} (malformation), and TI values for semicarbazide are presented in Table 3.4. Semicarbazide[26] induced notochord lesions, gut miscoiling, craniofacial and mouth mal-development, and microphthalmia at concentrations $\geq$10 mg/l in *X. tropicalis*. Semicarbazide induced other eye malformations, including rupture of the pigmented retina, at concentrations $\geq$100 mg/l in *X. tropicalis*. Semicarbazide induced gut malformations, craniofacial and mouth dysmorphogenesis, and notochord lesions in *X. laevis* at concentrations $\geq$5 mg/l. Semicarbazide induced eye malformations (ruptured pigmented retina and microphthalmia) and microcephaly at concentrations $\geq$100 mg/l in *X. laevis*.

Copper

The stage 46 LC_{50}, EC_{50} (malformation), and TI values for copper are presented in Table 3.4. Copper[24] induced gut miscoiling, craniofacial dysmorphogenesis, microcephaly, and mouth defects in *X. tropicalis* at concentrations $\geq$0.05 mg/l. Notochord lesions, abnormal coiling of the heart, cardiovascular malformations, microphthalmia, rupture of the pigmented retina, and abdominal edema at concentrations $\geq$0.1 mg/l in *X. tropicalis*. Copper induced gut miscoiling, abdominal and ophthalmic edema, craniofacial dysmorphogenesis, microphthalmia, rupture of the pigmented retina, and hypognathia in *X. laevis* at concentrations $\geq$0.05 mg/l. Microcephaly, hydrocephalus, and cardiovascular malformations were induced at concentrations $\geq$0.25 mg/l in *X. laevis*.

Atrazine

The stage 46 LC_{50}, EC_{50} (malformation), and TI values for atrazine are presented in Table 3.4. Atrazine[24] induced abnormal development of the mouth and craniofacial region in *X. tropicalis* at concentrations $\geq$1 mg/l. Atrazine induced notochord lesions, tail flexure, microphthalmia, rupture of the pigmented retina, and microcephaly at concentrations $\geq$10 mg/l in *X. tropicalis*. Atrazine induced notochord lesions, tail flexure, craniofacial defects, microphthalmia, lens and pigmented retina malformations, and abnormal development of the mouth in *X. laevis* at concentrations $\geq$5 mg/l. Microcephaly, visceral hemorrhage, and cardiovascular malformation were noted at concentrations $\geq$25 mg/l in *X. laevis*.

Methotrexate

The LC_{50}, EC_{50} (malformation), TI, and MCIG values for tests of methotrexate are presented in Table 3.4. Methotrexate induced gut and cardiac miscoiling and abdominal edema in *X. tropicalis* at concentrations $\geq$10 mg/l. Methotrexate induced notochord lesions, craniofacial mal-development, microophthalmia, and microcephaly at concentrations $\geq$10 mg/l in both species.

Cyclophosphamide

The LC_{50}, EC_{50} (malformation), TI, and MCIG values for tests of cyclophosphamide are presented in Table 3.4. Microophthalmia, mouth malformations, and gut miscoiling were induced in *X. tropicalis* as the result of exposure to $\geq$0.02 mg/l activated cyclophosphamide. In addition, concentrations $\geq$0.05 mg/l also induced rupturing of the pigmented retina, abnormal development of the lens, and microcephaly. These abnormalities were observed in markedly greater severity with increasing concentration of activated cyclophosphamide and were consistent with malformations induced in *X. laevis*.

Acetylhydrazide

The LC_{50}, EC_{50} (malformation), TI, and MCIG values for tests of acetylhydrazide are presented in Table 3.4. Gut miscoiling and notochord lesions were induced in *X. tropicalis* as the results of exposure to $\geq$20 mg/l acetylhydrazide. In addition, concentrations $\geq$150 mg/l produced abnormal development of the mouth, eye malformations, visceral edema, craniofacial defects, and abnormal heart development. These abnormalities dramatically increased in severity with increasing test concentration and were consistent with malformations induced in *X. laevis*.

Bermuda pond sediment extract (complex mixture)

The LC_{50}, EC_{50} (malformation), TI, and MCIG values for tests of pond sediment extracts from Bermuda are presented in Table 3.4. Microophthalmia, mouth malformations (including hypognathia), craniofacial defects, and cephalic malformations were induced in *X. tropicalis* as the results of exposure to these samples. These abnormalities dramatically increased in severity with increasing test concentration and were consistent with malformations induced in *X. laevis*.

Discussion

Results from these studies indicated that *X. laevis* and *X. tropicalis* generally responded similarly to the test compounds evaluated based on embryo lethality, the gross malformations observed, and embryo–larval growth. From a quantitative standpoint, some variability in the developmental endpoints was observed when comparing results for *X. tropicalis* to *X. laevis* results. These results were not necessarily unexpected. Since *X. tropicalis* is typically cultured at greater temperatures ($26.5 \pm 0.5^{\circ}C$) than *X. laevis* ($23 \pm 0.5^{\circ}C$), the rate of embryonic development is dependent on temperature, lethality in short-term alternative developmental toxicity test systems is influenced by exposure length, and malformation is specifically affected by exposure at critical periods of development, some endpoint variability was not unexpected. However, marked differences between the results in *X. tropicalis* and *X. laevis* were not found in this study. The magnitude of difference found is likely to be test compound dependent.

The teratogenic potential of chemicals assayed with FETAX has been routinely assessed based on TI values, growth endpoints, and types and severity of induced terata. In general, TI values <1.5 indicate low teratogenic potential as little or no separation exists between the concentrations that induce malformation without embryo lethality and those concentrations causing lethal effects. Thus, greater TI values represent a greater separation in malformation and lethal response curves and, thus, a greater potential for embryos to be malformed in the absence of lethality. Other endpoints, including types and severity of abnormalities and growth inhibition, are also considered in evaluating teratogenic hazard. Each of the compounds tested was determined to have teratogenic potential with varying degrees of potency. Regardless of species, the teratogenic potential (separation between lethal- and malformation-inducing concentration ranges) of the test materials evaluated in the present study was relatively similar: The teratogenic potency (concentration at which malformations are observed) and growth inhibiting potential (concentration that inhibit growth relative to the lethal concentration) were also similar regardless of species. The growth inhibiting potential tracked directly with the trends in teratogenic potential.[24] The same similarity in trends was identified during the validation of FETAX with *X. laevis*.[4–21,27,28,30,31,32]

In addition to the aforementioned anticipated advantages of *X. tropicalis*, the shorter life cycle allows more expedient and practicable reproductive toxicity studies and endocrine evaluation of the thyroid axis during metamorphosis. It is also possible to create inbred strains of *X. tropicalis*, which will likely decrease the genetic variability observed in *X. laevis*. Developing inbred lines of *X. laevis* is difficult at best. The only negative aspect of using *X. tropicalis* at this point is anecdotal evidence that this species may be more prone to diseases than *X. laevis*. More work will be required to determine the significance of the disease sensitivity issue.

Conclusions

In summary, results from the previous studies[23,24] suggested that *X. tropicalis* is an acceptable alternative species for *X. laevis* in FETAX. The revised alternative methods provided in this manuscript should enable interested investigators to conduct FETAX using *X. tropicalis*.

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chapter four

A short-term mummichog (Fundulus heteroclitus) *bioassay to assess endocrine responses to hormone-active compounds and mixtures*

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Introduction

There has been intense international interest in the development, validation, and standardization of laboratory fish tests to examine the responses of fish to endocrine disrupting substances (EDSs) and hormone-active effluents.[1–4] It is doubtful that a single surrogate species can be used to extrapolate results from laboratory testing to effects in wild species, as fish exposed to the same effluent source can vary in their endocrine and reproductive fitness responses.[5,6] Development of partial and full life cycle fish tests for EDS testing has primarily focussed on fathead minnow, *Pimephales promelas*[3,4] and other freshwater species, e.g., Japanese medaka (*Oryzias latipes*).[7] Less effort has been applied to estuarine and marine species, although efforts in sheepshead minnow[8] and eelpout[9] have yielded viable protocols. The application of full life cycle methodology for EDS testing can be costly and occurs in a timeframe not suited to testing purposes.[4] Therefore, there is a need to validate and characterize short-term, whole organism laboratory bioassays to identify responses suggestive of whole organism responses in the wild.

Extensive effort has been placed on the development of physiological tools, e.g., receptor,[10] vitellogenin,[11] plasma steroid,[12,13] and gonadal *in vitro*[14] assays. Additional advances in gene array[15] and molecular biology[15,16] techniques have also helped elucidate mechanisms of action, determine species differences, and define exposures. In total, studies measuring a variety of endpoints on numerous species have shown that although endocrine systems during vertebrate evolution have been relatively conserved,[17] significant species differences in receptor binding,[18] hormone-mediated responses,[6] and reproductive status[5] exist even among fish exposed to EDSs or hormone-active effluents.

To predict whole organism or population-level adverse health effects, exposure protocols must be designed to supply mechanism-specific information that provides opportunities to extrapolate across species and to use laboratory species as surrogates for wild fish. Here, we describe a short-term gonadal recrudescence bioassay for the estuarine killifish or mummichog (*Fundulus heteroclitus*), as well as the techniques used to assess changes in reproductive and endocrine status (including organ size, fecundity, plasma steroid and vitellogenin levels, and *in vitro* gonadal steroid production). The protocol has been validated with exposures to model compounds, representative of major routes by which EDSs exert their effects (anti/estrogens and anti/androgens).[2,19] The protocol has also been used to investigate causality at a Canadian pulp mill by determining the waste stream source and thereafter identifying hormone-active contaminants.[20–22] The mummichog is a good

candidate for bioassay development as it is the numerically dominant fish species in salt marshes along the eastern coast of North America, and much is known about its reproductive biology in the wild[23,24] and the laboratory.[25,26] In addition, mummichog were chosen for bioassay development as their natural range, size, and adaptability make them suitable for laboratory, artificial stream, and field studies, thus allowing extrapolation across study designs and enhancing the application of results to ''real world'' contaminant effects.

Materials required

Most of the chemicals, materials, and equipment can be purchased from any scientific or aquatic systems suppliers. Specific catalog numbers are included where relevant to specific techniques. Hagen® materials can be purchased from most local pet stores. Where helpful, sample recipes for relevant volumes are included. Animal care protocols should be approved by institutional animal care committees prior to beginning.

Mummichog field collection

Equipment required:

- Fishing license
- Seine net (approximately 6 m × 2 m, 0.6 cm mesh; e.g., Aquatic Eco-Systems, Apopka, FL, USA, catalog #HDS4)
- Chest waders
- Life jackets
- Buckets (approximately 20 l capacity)
- Aquarium dip nets
- Battery powered aquarium aerators (e.g., Aquatic Eco-Systems DC5)
- Air tubing
- Air stones
- Large insulated transport containers [approximately 150 gal (700 l) capacity; e.g., Aquatic Eco-Systems B2300]
- Plastic or galvanized minnow traps (43 cm L × 23 cm W; e.g., Aquatic Eco-Systems MT2)
- Floating rope
- Dog food (generic, approximately 1.5 cm diameter; cat food is too small and turns to mush quickly)
- Film canisters (optional)

Animal husbandry

Equipment required:

- Dechlorinated fresh water
- Salt water (true salt water or artificial, e.g., Coralife Instant Ocean™)
- Fish food (standard commercial trout pellets; crushed variety)
- Hagen® Nutrafin™ floating pellets for cichlids (Rolf C. Hagen Inc. International, Montreal, QC, Canada)
- Standard glass aquaria [e.g., 50 gal (190 l) minimum], larger stock tanks or water tables as available

- Multiparameter environmental meter capable of measuring dissolved oxygen (DO), temperature, pH, conductivity, and salinity (e.g., YSI 556 Multi-Probe System, Yellow Springs Instruments, Yellow Springs, OH, USA)
- Aquarium-size mechanical, chemical, and biological filtration filters (e.g., Hagen® AquaClear 500 System) for static systems
- Aquarium dip nets
- Air tubing
- Air stones
- Air supply (battery powered, standard electrical aquarium air pumps, or in-building system)
- Water quality test kits (e.g., Hagen® test kits)
- Water conditioner for biological systems (e.g., Hagen® Cycle™)

Artificial regression and recrudescence for year-round supply of fish

Equipment required:

- Standard glass aquaria [30–50 gal (114–190 l) capacity], larger stock tanks or water tables as available
- Filters (e.g., Hagen® AquaClear 200 suitable for 150 l aquaria)
- Flow-through or recirculation water chilling system for fresh and salt water suitable for holding tanks at 4°C or cold room set at 4°C for static system

Note: Chillers, pumps, and filters for chilling and/or recirculation systems are based on flow rates and volumes; please contact a supplier, such as Aquatic Eco-systems, to determine products suitable for your needs.

- Water quality test kits (e.g., Hagen® test kits)
- Multiparameter environmental meter
- Lights on timer device; either for whole room or in sectioned off area of room containing aquaria (various models are available at local hardware stores or from dealers, e.g., Intermatic, Energy Federation Incorporated, Westborough, MA, USA)
- Fish food, as described for animal husbandry

Short-term bioassay

Equipment required:

- Multiple glass aquaria [9 gal (34 l) minimum capacity]
- Dechlorinated fresh water
- Salt water
- Submersible fish tank filters (e.g., Hagen® Marina Jet Flow Corner Filter 10894)
- Filter media (e.g., Hagen® Poly Filter Wool A1031 and Living World Filter Box Carbon A1332)
- Large standard plastic garbage cans
- Air tubing
- Air stones
- Air supply (aerators or in-building)
- Dip nets

- Buckets [4–5 gal (16–20 l) capacity]
- Fish food (standard commercial trout pellets; crushed variety)
- Multiparameter environmental meter

Fish sampling

Equipment required:

- Bucket [approximately 2.5 gal (10 l) capacity]
- Air tubing
- Air stones
- Air supply (aerators or in-building)
- Latex or vinyl gloves
- Safety goggles
- Lab coat
- Aquarium dip nets
- Paper towel (cut into sizes approximately 2 to 3 $\times$ size of fish; 1-$\frac{1}{2}$ times the number of fish to be sampled)
- Needles (e.g., Beckton-Dickinson 26G3/8 or 25G5/8)
- Syringes (e.g., Beckton-Dickinson 1-cc syringe)
- Microfuge tubes (polypropylene, 1.5 ml)
- Microfuge tube racks (commercially purchased, or can be made easily by making suitable-size holes in sheets of Styrofoam)
- Borosilicate glass test tubes (12 mm $\times$ 75 mm)
- Test tube racks for 12 mm $\times$ 75 mm tubes
- Waterproof markers for labeling
- Ice
- Ice tray [dishpan or kitty litter tray or equivalent (46 $\times$ 76 cm)]
- Sharps container
- Refrigerated centrifuge (capable of at least 2500*g* and holding 1.5 ml Microfuge tubes; e.g., IEC Centra GP8R, with 316 rotor and 5827/5862 adaptor for 1.5 ml Microfuge tubes, Fisher Scientific, Nepean, ON, Canada, catalog #05-112-120 plus accessories; or IEC 3592 microcentrifuge, Fisher Scientific 05-112-114D)
- Freezer (-80°C)
- Electronic balance(s) ($\pm$ 0.001 g sensitivity)
- Dissection kit (scalpel, forceps, ruler)
- pH meter
- Magnetic stir plate
- Stir bars

Reagents required:

- Anaesthetic: tricaine methanesulfonate (TMS)–water
  - TMS (Syndel International, Vancouver, BC, Canada, catalog #18323)
  - Salt water
  - Dechlorinated fresh water

Dissolve TMS in combined salt and fresh water at 0.05–0.1 g/l. *Note*: TMS in fresh water should be buffered but does not need to be if used in salt or partial salt water as described

here. Please check the Material Safety Data Sheet (MSDS) for TMS, a potential carcinogen in humans.

- Heparin solution
  - 10 mg heparin sodium salt (Sigma-Aldrich H-0777, Sigma-Aldrich Corp., St. Louis, MO, USA)
  - 10 ml double-distilled water (ddH_2O)

Dissolve heparin in ddH_2O to obtain a concentration of 1 mg/ml; can be stored at 4°C for 2–3 days.

- NaCl solution (150 m*M*)
  - 0.876 g NaCl (Sigma-Aldrich A-9625)
  - 100 ml ddH_2O

Combine NaCl and ddH_2O; can be stored at 4°C for approximately 10–12 months.

- Aprotinin solution (1 KIU/μl)
  - NaCl solution (150 m*M*)
  - 1 mg aprotinin (Sigma-Aldrich A-1153; 4.3 TIU/vial where 1300 KIU = 1 TIU)

Add 1 mg aprotinin to 559 μl NaCl solution (150 m*M*) to produce a solution of 10 KIU aprotinin/μl. Store at 4°C. This solution is diluted further NaCl using (150 m*M*) to obtain an appropriate volume of 1 KIU aprotinin/μl. Store at 4°C.

- 1 m*M* hydrochloric acid (HCl) solution
- 1 m*M* sodium hydroxide (NaOH) solution
- Medium 199 buffer (M199)
- One bottle (final concentration 11.0 g/l) of M199 containing Hank's salts without bicarbonate (Sigma-Aldrich M-0393)
  - 6.0 g HEPES sodium salt (Sigma-Aldrich H-3784)
  - 0.35 g $NaHCO_3$
  - 0.1 g streptomycin sulfate (Sigma-Aldrich S-9137)
  - 1.0 g bovine serum albumin (BSA; Sigma-Aldrich A-7888)
  - 1l ddH_2O

Dissolve powdered M199 in 900 ml ddH_2O and stir gently with magnetic stir plate and stir bars until dissolved. Rinse original package with small amount of ddH_2O to remove all traces of powder. Add HEPES, $NaHCO_3$, and streptomycin and stir. Determine pH with a pH meter; adjust pH to 7.4 using either 1 m*M* HCl or 1 m*M* NaOH. Make up final volume to 1 l using ddH_2O. Add BSA when ready to use. M199 must be used within 24 h following addition of BSA. It can be stored for a limited time without BSA added (<1 week).

In vitro *incubations*

Equipment required:

- Glass Petri dishes (100 mm × 10 mm)
- Ice

- Ice tray [dishpan or kitty litter tray or equivalent (46 × 76 cm)]
- Scalpel
- Magnetic stir plate
- Stir bars
- Electronic balance (± 0.001 g sensitivity)
- 24-well culture plate (e.g., tissue culture treated non-pyrogenic polystyrene, Fisher Scientific C5003473) and/or 12 mm × 75 mm borosilicate glass test tubes
- Racks for 12 mm × 75 mm test tubes (if used)
- Waterproof markers for labeling
- Refrigerator (4°C)
- Repeater pipette (capable of dispensing 1000 μl
- 1000 μl pipette with disposable tips
- Incubator capable of holding at 18°C
- Scintillation vials (glass, 7 ml)
- Freezer (–20°C)

Reagents required:

- M199 buffer
- M199 + IBMX solution
  - 1 l M199 buffer
  - 0.222 g IBMX (3-isobutyl-1-methylxanthine; Sigma-Aldrich I-5879)

Add IBMX to M199 buffer and stir to dissolve using magnetic stir plate and stir bars.

- hCG stock solution
  - hCG (chorionic gonadotropin lyophilized powder; Sigma-Aldrich C-0434)
  - Ethanol (EtOH; Sigma-Aldrich E-7023)

Add hCG to EtOH to produce a 20 IU hCG per 5 μl EtOH solution.

Classification of follicle stage

Equipment required:

- Standard dissecting scope
- Dissecting forceps, blunt-pointed
- Glass Petri dishes (100 mm × 10 mm)

Reagents required:

- M199 or Bouin's solution (Sigma-Aldrich HT101128)

Bouin's contains picric acid; please check the MSDS.

- Plastic scintillation vials (20 ml, Fisher Scientific, 03 337 11B)

Plasma steroid extractions

Equipment required:

- Fume hood
- Borosilicate test tubes (16 mm × 150 mm)
- Racks for 16 mm × 150 mm test tubes
- Waterproof markers for labeling
- Glass scintillation vials (7 ml)
- Metal pan (approximately 20 cm wide × 30 cm long × 5 cm deep)
- pH meter
- Magnetic stir plate/hot plate combination
- Stir bars
- Pipette (50–200 μl tips)
- Repeater pipette capable of dispensing 500 μl
- Vortex mixer
- Freezer (–20°C)
- Thermos for liquid nitrogen (if this method of freezing is chosen)

Reagents required:

- Acetone (99.5% ACS reagent)
- Dry ice (approximately 1 kg)

Alternately, liquid nitrogen may be used instead of an acetone/dry ice bath.

- Diethyl ether
- ddH_2O
- 1 m*M* HCl
- 1 m*M* NaOH
- Phosgel buffer
  - 5.75 g Na_2HPO_4 (Sigma-Aldrich S9763)
  - 1.28 g $NaH_2PO_4{\cdot}H_2O$ (Sigma-Aldrich S9638)
  - 1.0 g gelatin (Sigma-Aldrich G2500)
  - 0.1 g thimerosal (Sigma-Aldrich T5125)
  - 1 l ddH_2O

Dissolve Na_2HPO_4, $NaH_2PO_4{\cdot}H_2O$, gelatin, and thimerosal in ddH_2O and heat to 45–50°C to dissolve the gelatin. Adjust pH to 7.6 (using 1 m*M* HCl or 1 m*M* NaOH) if required. Store at 4°C for up to 1 week.

Radioimmunoassay

Equipment required:

- Facilities licensed for radioactive use
- Borosilicate test tubes (12 mm × 75 mm) = assay tubes
- Assay tube rack for assay tubes (Fisher Scientific 14-809-22)

- Borosilicate test tubes (16 mm × 150 mm)
- Racks for 16 mm × 150 mm test tubes
- Waterproof markers for labeling
- Microfuge tubes (polypropylene, 1.5 ml)
- Test tube rack
- Pipette(s) and tips capable of 100, 200, and 1000 μl
- Vortex mixer (for test tube volumes)
- Refrigerated centrifuge (capable of holding at 2500*g* with buckets for multiple 12 mm × 75 mm glass test tubes; e.g., IEC Centra GP8R, with 316 rotor and 37-place 5737 adaptor; Fisher Scientific 05-112-120 plus accessories)
- Incubator capable of holding at 18°C
- Graduated cylinder (100 ml)
- Plastic disposable beakers with lids, 250 ml or 8 oz (e.g., Fisher Scientific 02-544-125)
- Fume hood
- Repeater pipette (5–200 μl)
- Paper towels or Kimwipes® (Fisher Scientific S47299)
- Plastic scintillation vials (7 ml, e.g., HDPE scintillation vials, Fisher Scientific 03-337-20)
- Scintillation counter (e.g., LS 6500, Beckman Coulter, Mississauga, ON, Canada) with counting racks for 7 ml scintillation vials
- pH meter
- Magnetic stir plate/hot plate combination
- Stir bars
- Electronic balance (± 0.001 g sensitivity)

Reagents required:

- Phosgel buffer (as described in the section ''Plasma steroid extractions'')
- Steroid standards
  - Estradiol (E_2; Sigma-Aldrich E8875)
  - Testosterone (T; Sigma-Aldrich T1500)
  - Ethanol (100%)

Both hormones are stored at a working concentration of 1000 ng/ml in ethanol (EtOH) at −20°C. Other hormones can be measured depending on commercial availability of standards, antibodies (Ab), and tracers.

- Steroid Ab (e.g., Medicorp, Montreal, QC, Canada or Steraloids, Newport, RI, USA)
  - E_2-Ab
  - T-Ab

Our Ab is received from Medicorp in 1 ml aliquots. Each aliquot is diluted in approximately 9.2 ml of phosgel (= stock dilution). This stock is then separated into 0.5 ml aliquots in 1.5 ml Microfuge tubes and stored at −20°C. The dilution required to provide 50% binding (= working dilution) is established by serial dilution of the stock followed by radioimmunoassay (RIA). The working dilution is specific to each shipment of Ab and must always be titered and assessed for binding prior to doing RIAs with

samples. For 11-ketotestosterone, a steroid synthesized in male fish, Ab cannot be purchased from commercial suppliers. Details for Ab production are too detailed to describe here.

- Steroid tracers (Amersham International, Buckinghamshire, UK)
  - 2,4,6,7-^{3}H-17β-estradiol (E_2*) [product TRK322, specific activity (s.a.) = 3.22 TBq/mmol, 87 Ci/mmol]
  - 1,2,6,7-^{3}H-testosterone (T*) (product TRK921, s.a. = 3.52 TBq/mmol, 95 Ci/mmol)
  - Ethanol (100%)

Stock steroid tracers are diluted (100 μl of tracer in 10 ml of ethanol) and stored in glass scintillation vials at −20°C until used in the assays.

- Inter-assay (IA) samples

Samples of a known concentration of steroid (pooled plasma or standard) must be run concurrently with each assay. Pooled plasma is obtained by bleeding a number of fish, centrifuging blood, combining resultant plasma, and redistributing into aliquots for freezing at −20°C. Alternately, as described for production of standards, a specific concentration of steroid can be made in phosgel, aliquoted, and frozen at −20°C. It is important to make up a large number of IA samples (in the 100s) to make them available over a large number of assays.

- Scintillation cocktail (e.g., ScintiSafe Econo 1 Sigma-Aldrich SX20-5)
- Charcoal solution
  - 0.5 g activated charcoal (Sigma-Aldrich C4386)
  - 0.05 g dextran T70 (Sigma-Aldrich D1537)
  - 100 ml phosgel

Add charcoal and dextran to phosgel and stir continually on magnetic stir plate. Store at 4°C for 2–3 days.

- Ice
- Ice tray (as previously described)

Vitellogenin assay

Equipment required:

- 1 l bottles with lids (e.g., Fisher Scientific 06-414-1D)
- Amber bottle (at least 500 ml) (e.g., Fisher Scientific 06-423-2C)
- Graduated cylinders (10, 100, 1000 ml)
- 12 mm × 100 mm test tubes
- Electronic balance (± 0.001 g sensitivity)
- Repeater pipette
- 8-Channel attachment for repeater pipette and appropriate tips (25–125 μl)
- Solution reservoirs for multi-tip pipette

- 96-well EIA/RIA polystyrene assay plates (e.g., Corning 3590, Fisher Scientific 07-200-35)
- 100 ml glass beakers
- Microfuge tubes (polypropylene, 1.5 ml)
- Pipette for 2–20 μl and disposable tips
- Incubator capable of maintaining 37°C
- Plastic wrap (e.g., Saranwrap®)
- Shaker plate capable of 0–500 rpm (e.g., Gyrotory Shaker, Model GZ; New Brunswick Scientific Co. Edison, NJ, USA)
- Vortex mixer
- Spectrophotometer capable of reading 96-well plates at 490 nm
- Magnetic stir plate
- Stir bars

Reagents required:

- ddH_2O
- 5 *M* H_2SO_4
- HCl (1 m*M*)
- NaOH (1 m*M*)
- $NaHCO_3$ (baking soda is acceptable)
- Sodium bicarbonate buffer (SBB) solution
  - 4.20 g $NaHCO_3$
  - 5.0 mg gentamycin sulfate (Fisher Scientific BP918-1)
  - 1 l ddH_2O

Stir on magnetic stirrer until all reagents are dissolved. Adjust pH to 9.6 with HCl or NaOH; may be stored up to 1 year at 4°C.

- Tris buffered saline — Tween (TBS-T) 10× solution
- 12.1 g Tris–HCl (Tris[hydroxymethyl]aminomethane hydrochloride; Sigma-Aldrich T-3253)
- 87.7 g NaCl
- 10 ml Tween 20 (1%) (enzyme grade polyoxyethylene 20-sorbitan monolaurate; Fisher Scientific BP337-500)
- 50 mg gentamycin sulfate (Fisher Scientific BP918-1)
- 1 l ddH_2O

Stir on magnetic stirrer until all reagents are dissolved. Adjust pH to 7.5 (with HCl or NaOH); may be stored up to 1 year at 4°C.

- TBS-T working solution
  - 100 ml TBS-T 10 × solution
  - 900 ml ddH_2O

TBS-T 10× solution is diluted with ddH_2O to create TBS-T working solution. Stir on magnetic stirrer until all reagents are dissolved. Adjust pH to 7.5 as previously described; may be stored up to 1 year at 4°C.

- TBS-T-BSA solution
  - 100 ml TBS-T working solution
  - 0.5 g BSA 98% ELISA grade (Sigma-Aldrich A-7030)

Note: BSA must be ELISA grade. Stir on magnetic stirrer until all reagents are dissolved. Adjust pH to 7.5 and the solution may be stored up to 3 weeks at 4°C (watch for flocculents).

- Ammonium acetate solution
  - 0.385 g ammonium acetate 98% (Sigma-Aldrich A-7330)
  - 100 ml ddH_2O

Stir on magnetic stirrer until all reagents are dissolved. Combine ammonium acetate and ddH_2O.

- Citric acid solution
  - 0.525 g anhydrous citric acid (Sigma-Aldrich C-0759)
  - 50 ml ddH_2O

Stir on magnetic stirrer until all reagents are dissolved. Combine citric acid and ddH_2O.

- Ammonium acetate–citric acid (AACA) solution
  - 100 ml ammonium acetate solution
  - citric acid solution

Adjust the pH of the ammonium acetate solution to 5.0 with the citric acid solution, while stirring on magnetic stir plate. Store at 4°C for 4 months in amber bottle.

- *o*-1,2-phenylenediamine (OPD) solution
  - 16 ml of AACA solution
  - 8 μl of 30% hydrogen peroxide (Sigma-Aldrich H-1009)
  - 8 mg OPD (Sigma-Aldrich P-1526) powder

Mix well on magnetic stir plate in 100 ml beaker. This is enough for 1 plate. Prepare immediately before use. (*Do not* make it up beforehand and store it.)

- Vitellogenin stock solution

Vitellogenin protein for standards for our work was prepared by Nancy Denslow, University of Florida, FL, USA, according to the methodology described by Denslow et al.[11] Currently, there is no commercial supplier for mummichog vitellogenin standards. Nancy Denslow may be contracted to create vitellogenin standards through the Molecular Biomarkers Core Facility, associated with the Interdisciplinary Center for Biotechnology Research at the University of Florida. The facility may be accessed through: www.biotech.ufl.edu/MolecularBiomarkers. Store 50 μl aliquots at −80°C.

- Vitellogenin coating solution
  - Vitellogenin stock solution (concentration will vary with batch)
  - SBB

Vitellogenin must be added to SBB to coat the wall of the plate. We have found that 16.5 ng vitellogenin per well is a good concentration for this solution and 150 μl of vitellogenin coating solution is required per well. Therefore, a solution of 0.11 ng/μl vitellogenin is used. For a 96-well plate, 18.8 ml of vitellogenin coating solution is required. (For example, assuming 0.60 mg/ml vitellogenin stock solution, we need 3.45 μl of 0.60 mg/ml solution in 18.8 ml of SBB to create the coating solution for 1 plate.) Vortex vitellogenin aliquot prior to use. Mix well on a magnetic stir plate in a small beaker.

- Primary Ab specific for mummichog vitellogenin aliquot

Our primary Ab was developed by Dr. C. Rice at Clemson University as described by MacLatchy et al.[2] It is now possible to purchase monoclonal Ab specific for mummichog vitellogenin from Euromedex, a company based in France (http://www.euromedex.com/New_t.html#) and EnBio Tec Laboratories in Japan (http:/www.enbiotec.co.jp/en/product/index.html). Ab should be diluted with TBS-T-BSA to aliquots of 50 μl and stored at −80°C.

- Primary Ab solution
  - TBS-T-BSA
  - Primary Ab aliquot

Each batch of antiserum will contain different levels of Ab and must be diluted to a working concentration. A dilution factor will need to be determined for each individual batch of Ab. (For example, our laboratory uses a 4250× dilution factor. The Ab stock required for 1 plate is the total volume of Ab solution needed divided by the dilution factor, 2.36 μl of antiserum in 10.0 ml of TBS-T-BSA.) Vortex primary Ab aliquot prior to use. Mix solution on magnetic stir plate in a small beaker.

- Secondary Ab aliquot
  - Peroxidase conjugate — goat anti-mouse IgG (whole molecule, Sigma-Aldrich A-6154)
  - TBS-T-BSA

The Ab should be initially diluted to 5× with TBS-T-BSA and frozen at −80°C in aliquots of 100 μl until use.

- Secondary Ab solution
  - Secondary Ab aliquot
  - TBS-T-BSA

Each batch requires different dilutions; optimal standard curves have been achieved with dilutions in the range of 100 to 2000×. For 1 plate, 18 ml of secondary Ab solution is needed. For a 200× dilution, the secondary Ab aliquots (diluted 5× prior to storage) must be further diluted by 40×. For example, 450 μl of the secondary Ab aliquots are pipetted into 18 ml of TBS-T-BSA to achieve the desired dilution. Vortex secondary Ab aliquot prior to use. Mix solution on magnetic stir plate in a small beaker.

- Vitellogenin IA
  - Vitellogenin stock solution
  - TBS-T-BSA

Vitellogenin stock solution is added to TBS-T-BSA to create a known concentration of vitellogenin (e.g., 18.75 ng/50 μl), which is pipetted into aliquots of at least 100 μl and stored at −80°C.

Procedures and protocols

Mummichog field collection

The mummichog is a euryhaline estuarine fish species found along the Eastern North American coast from northern New Brunswick to northern Florida. They are most easily located in shallow areas within tidal creeks where the tide flows over eelgrass.[27] Mummichog complete their entire life cycle within the estuaries.[28,29] Reproductive males have yellow bellies and a dark spot towards the rear of the dorsal fin. Females are generally larger and rounder, with dark vertical stripes on their bodies.

Seine netting

1. Once an appropriate fishing location is identified, a fishing license must be obtained for the area. The time of day for collection should also be considered to optimize catch success: mummichog tend to move closer to shore during rising tide and away during falling tide. However, it is not uncommon to find mummichog at the shoreline throughout the entire tidal cycle.
2. An appropriate area is located within the estuary where it is safe to pull the seine net through the water with minimal obstructions. Mummichog prefer soft or muddy bottoms as opposed to rocky substrates.
3. The seine is stretched perpendicular to the shoreline by one person, while a second person holds the net close to shore (chest waders and life jackets are recommended). An adequate maximum depth at the furthest distance from the shoreline is approximately 1 m; however, mummichog can also be found in shallower waters.
4. The net is pulled through the water parallel to the shoreline. It is imperative that the lead line be kept along the substrate throughout the duration of the tow to avoid fish loss. Each person should move through the water at the same rate, while maintaining a ''U-shape'' in the net. An appropriate tow distance is approximately 20–30 m; however, this will vary depending on environment and fish abundance.
5. To complete the tow, the person closest to shore slows, while the other person swings toward the shoreline. The loop is closed and the net is dragged up onto the shore, pulling the fish within the net completely out of the water.
6. The fish are quickly removed from the net and placed in a bucket (either by hand or with a dip net). It may be necessary to have multiple buckets available depending on the catch size, as it is imperative not to overcrowd the fish. Battery-powered aerators should be used to prevent low levels of DO.

7. Fish are sorted by size and by species (by-catch should be released as soon as possible). Small mummichog of less than 60 mm standard length should be released as they are too small to be used in the bioassay. Suitable mummichog are transported to the laboratory in a large well-aerated transport container.

Minnow trapping

1. Individual minnow traps are attached to floating rope, and spaced by approximately 3 m. The user may attach any number of traps to reasonable lengths of rope. A single buoy should be attached to one end of the rope to aid in trap positioning and recovery.
2. Dog food may be used to draw mummichog to the minnow traps (food may be placed directly in each trap or enclosed within an empty film canister containing various small holes).
3. Traps are placed in the water at the desired sites at an approximate depth of 30–100 cm. Traps should be used during low tide to ensure that fluctuating tides do not result in traps being exposed to air. In addition, a length of rope should be attached to the shoreline (above the maximum tide level) to prevent loss of the traps and ease of retrieval.
4. Traps should remain undisturbed for an appropriate length of time relative to the abundance of fish in the area (1–24 h). *Note*: the minnow trapping method may be used at any time during the tide cycle; however, caution must be taken so that traps are not exposed to air with the changing tides.
5. The rope is pulled toward the shore to retrieve the traps. Each trap is removed from the water one at a time to minimize air exposure and the fish are emptied into an aerated bucket of water.
6. Fish are sorted and transported as previously described.

Animal husbandry

Mummichog are easily maintained in the laboratory. Fish may be held in large glass aquaria filtered with high-quality standard aquarium filters (static system), in large flow-through stock tanks or in a flow-through system in a water table. We have found that flow rates of about 200 ml/min provide adequate renewal of the water. It is recommended that filters also be installed on flow-through system to optimize fish health. Stocking densities should be as low as possible; we have found that a good benchmark for fish density is 20–30 g biomass/l of water, roughly 2–3 fish/l in a static system and 30–50 g biomass/l of water, roughly 3–5 fish/l in a flow-through system.

1. Fish are held at ambient light and temperature, 15–20 ppt salinity, and DO of greater than 85%. Water temperature, salinity, and DO should be monitored regularly using a multiparameter environmental meter. If any of the parameters are out of a normal range, a partial or complete water change should be done for static systems, or a partial water change with adjustment of the freshwater and saltwater flow rates for flow-through systems.
2. Filter components are regularly cleaned and/or replaced. When cleaning filters, changing water or establishing new tanks, a water conditioner for biological

systems should be used according to instructions provided. These practices will help maintain fish health.
3. Other water quality testing (ammonia, nitrates, nitrites, pH, etc.) should be carried out periodically using standard test kits. Abnormal levels in any of the parameters should be corrected with a partial water change and cleaning or replacement of the filter components.
4. Overall fish health should be observed daily and any symptoms of fish disease, such as abnormal behavior, changes in activity levels, presence of physical peculiarities (color, skin, and fin condition), or external parasites, should be noted. Any sick or dead fish should be removed immediately. Sick fish should be treated in an isolation tank with a treatment specific to their ailment and not returned to the stock tanks.
5. The fish should be fed daily with standard commercial crushed trout pellets at approximately 1–3% body weight, and this diet should be supplemented regularly (every 2–3 days) with cichlid pellets (1–3%). Food consumption should be monitored and the amount of food given should be adjusted accordingly. Uneaten food should be removed regularly along with any waste products.

Artificial regression and recrudescence for year-round supply of fish

It is well established that reproductive cycles in mummichog can be artificially manipulated to induce spawning.[2,26] During April–September, laboratory fish captured from the wild can be used as spawning cycles are maintained in laboratory on 2–4 week cycles. Bioassay exposures are optimal when fish are sampled a few days to a week prior to the full moon during recrudescence (period of gonadal maturation). Availability of pre-spawning fish year-round is essential if studies are to be done outside the natural spawning cycles. Fish can be artificially regressed and recrudesced by the following protocol.

1. Male and female mummichog are separated into tanks by sex. Separation of sexes during regression yields pre-spawning fish that have not initiated spawning behaviors and gamete release.
2. Fish can be kept in filtered aquaria with maximum density of 20–30 g biomass/l water. For 114-l aquaria, we use one or two AquaClear 200 filters. Filters should be cleaned as needed and water quality parameters measured as previously described in ''Animal husbandry.'' Alternately, fish can be kept in flow-through systems. Salinity should be maintained at 16 ppt and DO $> 85\%$.
3. Once acclimated (usually 1 week), photoperiod and water temperature are adjusted to 8-h L:16-h D and 4°C over a period of a week. Water temperature can be maintained by placing static aquaria in a cold room. Flow-through systems can be maintained at 4°C by using readily available in-line commercial chillers (contact Aquatic Eco-systems or other suppliers for examples suited to particular water flow needs).
4. Fish should be fed to satiation daily (approximately 1% body weight/day). *Note*: fish feeding rates decrease in cold temperatures and they can be fed every other day depending on consumption.

5. After 8 weeks of cold exposure fish should be completely regressed. A good external indicator of regression is the fading of male spawning colors (yellow ventral surface).
6. Following 8 weeks of cold exposure, day length and temperature should be increased gradually to 16-h L:8-h D and 18–20°C, respectively, over a period of a week.
7. Gonadosomatic index (GSI) should be significantly increased by 2 weeks; hormone levels should be equivalent to pre-spawning fish in the wild or laboratory by 4 weeks[2]). Noticeable yellowing of male ventral surfaces at 2 weeks identifies progressing gonadal recrudescence.
8. Once fish have recrudesced, they can be used in the short-term bioassay as per the following descriptions.

Short-term bioassay

The following protocol is a description of a static exposure with daily renewal. However, flow-through exposures can be done using diluter systems.[3] These systems are costly but ensure constant exposure concentrations and minimize exposure to metabolic by-products and wastes. Flow-through systems are recommended when available.

1. Glass aquaria are filled with 16 ppt water (equal parts of de-chlorinated fresh water and salt water). Aquaria are aerated and filtered using corner filters during the acclimation period (at least 1 week).
2. Mummichog with a minimum standard length of 70 mm are selected, weighed (0.01 g), and randomly allocated to each aquarium (6–8 fish/12–16 l of 16 ppt water). Fish smaller than 70 mm may not provide adequate volumes of blood for steroid and vitellogenin analysis. Sex ratio should be 1:1 in all tanks. Fish can be kept at ambient photoperiod in spring/summer or on 16-h L:8-h D.
3. Fish are fed approximately 3% body weight/day with standard crushed commercial trout pellets and maintained under static conditions with a natural spring/summer photoperiod throughout the acclimation period.
4. Water quality parameters should be measured daily with the multiparameter environmental meter. DO should be >75%, temperature between 10°C and 20°C, and salinity 16 ppt. Filter media may be changed as necessary during the acclimation period.
5. Following acclimation, filters are removed and aquaria are randomly allocated to treatment groups (each group consisting of a minimum of four separate tanks). Treatments may include whole effluents (e.g., pulp mill effluent), model steroid compounds (e.g., ethynyl estradiol), or other compounds of interest.
   (a) If the chemicals used for the exposure are mixed in a solvent, such as methanol or ethanol, equivalent amounts of the solvent must be added to the control groups. We have done trials that show small solvent volumes (35 μl in 16 l = 35×10^{-6} l/16 l = 2.2×10^{-4} % solvent) have no effect on the reproductive endpoints measured in this bioassay (unpublished data). An extra control group in which no solvents are administered may be added to the experimental design if it is necessary to determine the effects of the solvent.
6. Static water is completely replenished daily. Water should be allowed to come to ambient temperature in large plastic garbage cans prior to use. To safely perform

water changes, fish are removed with a dip net (one tank at a time, separate nets per treatment) and placed into a bucket containing the pre-determined volume of 16 ppt water (e.g., 16 l). Aquaria are then emptied, and fish are replaced in the aquaria with the new water.

7. Each tank receives the designated treatment following water renewal. It is recommended that the treatments be administered for each tank immediately following the water change in order to minimize the duration of time that the fish are unexposed. *Note*: when effluents are used in place of water during water changes (e.g., 100%, 50%, 1%, etc.), dilute and use as exchange water accordingly.
8. During the exposure period water quality measurements are taken (temperature, DO, conductivity, salinity), and fish are fed as described for acclimation period. Fish should be allowed at least 1 h for food consumption prior to water renewal.
9. Repeat steps 6–8 every 24 h for 7–15 days. Length of bioassay is dependent on potency of material being tested; we have found that 7-day exposures are adequate for demonstrating endocrine effects for many EDSs and pulp mill effluents.[2,19,21]
10. Throughout the exposure period, fish should be monitored daily for overall health status (e.g., presence or absence of parasites), abnormal behaviors, and physical appearance (color, skin, and fin condition). Any dead or sick fish should be removed immediately. Sick fish should be treated for any infections but must not be returned to the exposure tanks following removal.
11. On the final day, fish are sampled as described in the section ''Fish sampling''.

Fish sampling

1. Prior to sampling, needles and syringes must be heparinized (usually a day or more before sampling date). Heparin solution is drawn into and emptied from each assembled needle-and-syringe pair 2 to 3× (approximately 1 ml each draw). *Note*: no liquid is to be left in the syringe or needle, and the plunger should be used repeatedly to clear out any of the remaining liquid.
2. All required Microfuge tubes (for blood when sampling and later for plasma after centrifugation) should be prelabeled using a waterproof marker prior to the sampling day. These Microfuge tubes can be laid out in racks in order of sampling to enhance organization on the sampling day. As well, test tubes for holding gonads following dissections can be prelabeled and placed in racks.
3. Sampling with a group of people in an assembly line works best given the number of fish (e.g., 8 fish × 4 replicate tanks × 5 treatments = 160 fish) to be sampled. Preprinted index cards with spaces for all necessary information (treatment group, fish number, sex, length, total body weight, liver weight, and gonad weight) can be used and kept with each fish during sampling to avoid confusion. We will generally have two bleeders (one for males, one for females), as well as one or two dissectors for each sex. An additional person acting as ''gopher'' to get fish and run other errands is helpful to have on hand. We usually line up the two teams across from each other on opposite sides of a long table, with enough balances, dissecting tools, etc., for each person or for easy sharing. In step 2, we will generally separate tubes for male and female fish to organize the assembly lines.

4. Fish are sampled one tank at a time. Fish are anesthetized by placement in bucket(s) of 8 l of aerated 16 ppt water containing TMS–water. Gloves must be worn by all people in contact with TMS–water or fish placed in TMS–water. Lab coats and safety goggles are also recommended (refer to MSDS or equivalent information provided by supplier).
5. To simplify sampling we find it easiest to separate fish by sex at this time by placing female and male fish in different buckets. Three to four fish can be anesthetized at one time.
6. Fish are anesthetized until they are no longer swimming but opercular movement is still evident. Fish are removed one at a time for sampling.
7. Anesthetized fish are bled while held in a piece of paper towel (allows a firm grip on the fish). The fish is held ventral side up and the needle is inserted posterior to the pelvic fins and anus, until it hits the vertebrae (caudal puncture). The needle is just slightly withdrawn away from the vertebrae. Suction is then applied on the syringe plunger until blood begins to enter the needle and syringe; usually 100–300 μl of blood can be collected. Blood samples are then ejected, without the needle attached to prevent hemolysis, into a 1.5-ml Microfuge tube. To the Microfuge tube, the aprotinin solution (a protease inhibitor) must be added at this time at a concentration of 10 KIU/ml blood (e.g., 2 μl KIU solution/200 μl blood). Syringes and needles are disposed of into a biohazard/sharps container. Microfuge tubes are kept on ice until centrifugation. If kept cold, blood can be held prior to centrifuging for a few hours until all fish are sampled.
8. Fish are measured (standard length; rostrum to peduncle; $\pm$ 1 mm) and weighed ($\pm$ 0.01 g).
9. Fish are euthanized by spinal severance with scissors or scalpel.
10. Fish are dissected; liver and gonadal tissue are removed and weighed separately ($\pm$ 0.001 g). If multiple balances are used among dissectors, balances need to be calibrated prior to sampling. Gonadal tissue is placed into the prelabeled borosilicate glass test tubes containing 1 ml medium 199 buffer and kept on ice for *in vitro* assays.
11. Fish carcasses are disposed of properly according to local regulations.
12. Plasma is centrifuged (15–20 min at 2400*g* at 4°C) to separate the plasma from the red blood cells. Plasma is then transferred to new, clean, prelabeled 1.5 ml Microfuge tubes and stored at −80°C until analysis. We separate plasma into two Microfuge tubes at this point, one for subsequent steroid analysis and one for subsequent vitellogenin analysis. For steroids, freeze >50 μl plasma. For vitellogenin, freeze 10–20 μl.
13. Transfer data from index cards to an electronic spreadsheet. Columns for fish number, treatment, sex, weight, length, gonad weight, liver weight, GSI (gonad weight/body weight × 100), liver-somatic index (liver weight/body weight × 100), condition factor (weight/length^3 × 100), and comments are typical values recorded and calculated at this point.

In vitro *incubations*

The following method is based on the protocol of McMaster et al.[14]; included here are the directions applicable to mummichog. However, McMaster et al.[14] is a detailed protocol,

and it is advised that it be reviewed for additional information regarding methodology for measuring *in vitro* gonadal steroid production in fish.

1. The protocol for gonadal *in vitro* incubations should be begun as soon as sampling is finished for all the fish, or even part-way through sampling if there are available people. Gondal tissue must be kept on ice until the incubations are started. Gonadal tissue is removed from one test tube at a time and placed into a glass Petri dish on ice containing M199. Tissue from each fish is individually processed. For testes, and ovaries with no distinct follicles easily separated by the naked eye, tissue is divided with a scalpel, and 18–25 mg of tissue is placed into individual wells each containing 1 ml M199 in a 24-well cell culture plate. Alternately, borosilicate glass tubes (12 mm × 75 mm) may be used for tissue incubations. If ovaries have prematurational follicles (<1.25 μm in diameter), the follicles should be separated and counted, and 10–15 follicles (consistent within experiment) placed in each well (24-well culture plates should be used if follicles are counted). Maturational follicles and mature eggs will probably also be present; however, steroid production of T and E_2 are generally low in these samples (unpublished data). The number of wells that can be processed per fish is dependent on the amount of gonadal tissue available, up to 6–8 wells/fish. The mass of the tissue (or number of follicles) and fish identification number must be recorded for each well. The tissue in each well should be in two pieces to maximize exposed surface area. Tissue should be kept on ice at all times. When the plate is full, store in a refrigerator at 4°C until step 2. Repeat for all gonadal samples.
2. When all gonadal tissue has been allocated, the media in each well is drawn off using a 1000-μl pipette. Each well then receives 1 ml of M199 + IBMX solution. We have shown that IBMX, a phosphodiesterase inhibitor that stimulates steroidogenesis by prolonging the presence of cAMP (3′,5′-cyclic adenosine monophosphate), enhances steroid production in mummichog incubations.[2] We use steroid production in the M199 + IBMX samples as our ''basal'' production levels. Within the 6–8 wells of gonadal tissue or follicles for each fish, half of the wells (3–4) are designated with this basal treatment label and require no further additions. The remaining wells are designated stimulated treatment and receive 5 μl hCG stock solution. HCG is an analog of gonadotropin hormone, and stimulates cAMP mobilization of cholesterol into the steroidogenic pathway. The basal and stimulated samples are then placed into an incubator at 18°C for 24 h. For additional precursor incubation options refer to the work of McMaster et al.[14]
3. After incubation, media is drawn off plates using a 1000-μl pipette using a separate tip for each well. Media from each well is then placed into appropriately labeled glass scintillation vials, capped, and frozen at −20°C until analyzed by RIA.

Classification of follicle stage

Follicles produce different levels of steroids at different stages (e.g., pre-vitellogenic, vitellogenic, maturational),[14] therefore effects on plasma steroids or *in vitro* steroid production due to treatment can be difficult to tease apart from stage-dependent changes. As in other fish, mummichog generally have high levels of circulating estradiol (and T) when

undergoing vitellogenesis.[26,30] Steroid levels, particularly estradiol, drop off during maturation; the maturation-inducing progesterones then become important.[31] Therefore, treatment effects causing changes in gonadal maturation can potentially be confounding when plasma steroid levels are looked at in isolation from developmental stage of the follicles.

It is, however, possible to classify follicular development stage and to determine if there are treatment effects. This can be done precisely by histology[32] or electron microscopy,[33] but the methodology is too detailed to relay here. It is also possible to detect treatment differences in fecundity and mature egg size in mummichog using image analysis.[34]

In a less precise fashion, one can separate and count under a dissecting scope different follicle classes. This can be done on sampling day, using follicles in M199 or at a later date with follicles preserved in Bouin's solution. Counting mature follicles and eggs also provides a fecundity estimate for each fish, i.e., an estimate of the number of mature eggs to be released during the next spawn. It is possible to count either all the (mature) follicles in the ovaries, or to count follicles in only part of the gonad (e.g., the left ovary).

1. On the sampling day, if follicles are to be sized immediately, place the ovary (or part thereof) of one fish in a glass Petri dish with a small amount of M199 to keep the ovary moist. If follicles are to be sized at a later date, store the ovary from each fish separately, in a plastic scintillation vial, with a generous amount of Bouin's solution to completely cover the ovary. At the time of staging for stored follicles, place the ovary of one fish in a glass Petri dish with a small amount of Bouin's solution.
2. Using two pairs of forceps, gently separate the follicles from each other and the connective tissue. Separate opaque and translucent (yellow) follicles. Maturational follicles and mature eggs are generally >1.3 mm in diameter (this can vary depending on fish stock[33]) and are translucent, pale yellow, and have lipid droplets at one pole. Mature eggs are generally loose and 1.7–1.8 mm in diameter.
3. Count the number of maturational follicles and mature eggs, as well as the opaque (previtellogenic and vitellogenic) follicles. The number of maturational follicles and mature eggs represents a fecundity estimate for each fish. The relative proportions of opaque follicles, translucent follicles, and mature eggs provide an estimate of staging or maturity for the gonad and may be useful for discriminating between differences in hormonal levels due to reproductive stage and differences due to treatment effects.

Plasma steroid extractions

Steroid hormones are bound to steroid binding proteins in the plasma, and they must be separated before they can be assayed using RIA. Ether extraction based on the methods of McMaster et al.[12] using an acetone dry ice bath (or liquid nitrogen) is a relatively quick, inexpensive, and simple method to achieve separation of the protein–hormone complexes.

Spiking samples (with cold or radiolabeled steroid) can be done to validate recovery and, therefore, the extraction procedure. The phosgel solution should be at 4°C and

should be made on Day 1 for use on Day 2. Following extraction, phosgel-reconstituted extracted steroids can be stored at −20°C for up to a year.

Day 1:

1. Plasma samples are thawed.
2. 16 mm × 150 mm test tubes are labeled as per the samples to be extracted.
3. Equal volumes (200, 100, 50 µl; dependent on the amount available in the samples) of plasma are pipetted into the test tubes.
4. Using a repeater pipette, 500 µl of ddH_2O are added to each tube.

Note: The following steps must be performed under the fume hood.

5. Ether (5 ml) is added to each of six tubes.
6. Each tube is vortexed for 20 s and allowed to settle.
7. Each tube is vortexed again and allowed to settle.
8. An acetone/dry ice bath is set up by placing 2–3 pieces (5–10 cm diameter, 3–4 cm thick) of dry ice in a metal pan and adding a volume of acetone to have a pool of approximately 4 cm depth. *Note*: it helps to tip the metal pan so as to be working only in one corner. Alternately, the tube may be immersed into a thermos of liquid nitrogen.
9. The tubes are slowly placed in acetone (liquid nitrogen) to freeze the aqueous fraction. Do all six (one or two at a time).
10. One tube at a time, the edges are thawed at the solid phase level (using your hands) to ensure all the solid phase goes to the bottom.
11. The tube just thawed is re-frozen.
12. The ether phase is decanted into respective, labeled glass scintillation vials.
13. The vials are left in the fume hood to evaporate the ether overnight. Alternatively, the ether can be evaporated under N_2 to shorten the evaporation time.

Day 2:

14. When the scintillation vials are dry, 1 ml of phosgel buffer is added.
15. The reconstituted samples are frozen at −20°C until use. The reconstituted samples should be left at room temperature for 3 h prior to freezing to ensure complete reconstitution of the steroids into the phosgel.

Radioimmunoassay

RIAs are used to measure the concentration of steroids in the blood (plasma protocol) or the biosynthetic capacity of the gonads (*in vitro* protocol). The assay is based on competitive binding by a fixed concentration of steroid tracer (E_2* or T*) with unlabeled steroid (E_2 or T) in standards or in samples. Competition is for a limited and constant number of binding sites available on the steroid Ab (E2-Ab and T-Ab), such that only 50% of total steroid concentration will be bound by the Ab.

There are a number of quality controls required to ensure precision and accuracy within and between RIAs in a laboratory. These parameters must be established for each laboratory. The following is a summary of RIA performance characteristics described in detail by McMaster et al.[12] In the initial stages of establishing an RIA, parallelism

(presence of interfering compounds) should be examined by analyzing serial dilutions of media or plasma; details of linearity should be provided. Similar to parallelism, specificity (cross-reactivity of the Ab) must be examined by comparing the ability of related hormones to bind the specific Ab. Accuracy is simply determined by spiking samples with known concentrations of unlabeled steroid and doing regression analysis to determine the slope of the line when steroid added is plotted against steroid measured. A slope different than 1 indicates the presence of interfering compounds and the RIA must be reassessed. Precision (inter- and intra-assay variabilities) must be considered by running a sample from a common pool of plasma or media in each assay (IA) and by replicating this IA several times within the same assay (intra-assay variability). An IA variability >15% is unacceptable, as is an intra-assay >10%. The IA calculation is the standard deviation divided by the mean of your IA samples from multiple assays. The values used in the similar intra-assay calculation come from 3–5 samples of the common pool run in the same assay. Upper and lower detection limits must be determined prior to interpreting a data set, and there is no universal means by which to establish these limits. The lower limit can be taken to be the 0 steroid standard value minus 2 standard deviations,[12] and this value should be reported with the data. The upper limit can be established as the steroid concentration that gives less than 10% of total binding. If a sample falls within this upper limit, it should be diluted to give approximately 50% binding and re-assayed.

The RIA described here has a number of modifications from the original assay described by McMaster et al.[12] Optimization was required for mummichog due to the small blood volumes and sometimes low steroid production levels obtained from these small-bodied fish. We increase the number of standard replicates (from 3 to 5) to allow for a more precise standard curve in the 0–12.5 ng/tube region of the curve. Binding of the ''0'' standard is set at 50% rather than the broader acceptable range of 35–50% described by McMaster et al.[12] Adding Ab prior to the addition of tracer gives increased opportunity for endogenous hormone to bind to the Ab. The volume of extracted sample used in the assay is always 200 μl and the incubation time is 24 h versus the less conservative parameters (50–200 μl and 3–24 h, respectively) described by McMaster et al.[12]

The phosgel and charcoal solutions should always be at 4°C. A little extra of each solution should be made to ensure there is always enough.

Day 1:

1. Samples (plasma or *in vitro*) and IA are thawed at room temperature.
2. Steroid standards are made up as follows:
   (a) Stock steroid (100 μl of 1000 ng/ml) is diluted with 24.9 ml of phosgel buffer (Table 4.1, row 1) in a plastic beaker to produce a final steroid concentration of 800 pg/tube.
   (b) Serial dilutions are performed in 16 mm × 150 mm test tubes to prepare subsequent standards: final steroid concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56 pg/tube (Table 4.1, rows 2–10). Each standard should be vortexed prior to serially diluting.
3. Assay tubes (12 × 75 mm) are placed in racks. Respective standards, samples and the IA and intra-assay sample(s), and phosgel are added as indicated in Table 4.2, using 200-μl pipettes and separate pipette tips for each different addition.
4. Aliquots of the desired Ab (E_2-Ab or T-Ab) are thawed and phosgel is added to make the working Ab dilutions. *Note*: Ab for all tubes = 200 μl × (No. standard tubes + No. samples + 2 IA).

Table 4.1 Steroid standard preparation by serial dilution for the RIA

Initial [steroid]	Serial dilution: volume of initial [steroid] to be combined with volume of phosgel to yield final [steroid]	Volume of phosgel to be added (ml)	Final [steroid] (pg/tube)	Standard No. (std #)
STOCK (1000 ng/ml)	100 µl	24.9	800	1
800 pg/tube	1 ml	1	400	2
400 pg/tube	1 ml	1	200	3
200 pg/tube	1 ml	1	100	4
100 pg/tube	1 ml	1	50	5
50 pg/tube	1 ml	1	25	6
25 pg/tube	1 ml	1	12.5	7
12.5 pg/tube	1 ml	1	6.25	8
6.25 pg/tube	1 ml	1	3.125	9
3.125 pg/tube	1 ml	1	1.56	10

Table 4.2 Assay tube contents for the RIA

Tube No.	Contents	Phosgel (µl)	Standard (µl)	Unknown (µl)
1,2,3	NSB[a]	400	—	—
4,5,6,7,8	0	200	—	—
9,10,11,12,13	1.56	—	200 (std #10)	—
14,15,16,17,18	3.125	—	200 (std #9)	—
19,20,21,22,23	6.25	—	200 (std #8)	—
24,25,26,27,28	12.5	—	200 (std #7)	—
29,30,31	25	—	200 (std #6)	—
32,33,34	50	—	200 (std #5)	—
35,36,37	100	—	200 (std #4)	—
38,39,40	200	—	200 (std #3)	—
41,42,43	400	—	200 (std #2)	—
44,45,46	800	—	200 (std #1)	—
47,48	Unknown 1	—	—	200
—	Unknown 2 to *x* (including some intra-assay controls)	—	—	200
145,146[b]	Unknown *x*	—	—	200
147,148	IA[c]	—	—	IA 200

[a] Non-specific binding (NSB).

[b] Unknown samples up to the maximum capacity of the centrifuge (minus spaces for the intra-assay controls).

[c] IA aliquots: always included as the last two tubes in each assay.

Note: Ensure all steps from this point forward are being performed in a radioactivity-licensed area.

5. The incubator is turned on and set to 18°C.
6. The working steroid tracer solution is made by adding an appropriate amount of phosgel to stock steroid tracer.
7. The tracer dilution is verified.

   (a) Two scintillation vials containing 200 μl of working steroid tracer solution + 600 μl phosgel + 5 ml scintillation cocktail are counted in the scintillation counter and the total counts should be approximately 5000 counts per minute (cpm).
8. Working Ab solution (200 μl) is added using a repeater pipette to every assay tube except tubes 1, 2, and 3 as these tubes are used to calculate non-specific binding (NSB).
   (a) NSB accounts for the presence of tracer not directly related to binding by the Ab (possible contamination or background counts). The mean value of the counts in these tubes is later subtracted from the counts per minute of the standards and tracer in subsequent calculations.
9. Steroid tracer (200 μl) is added to every assay tube using a repeater pipette.
10. Tubes are incubated at 18°C for 24 h.
11. Total counts reference (TCR) tubes are made by adding 200 μl of tracer and 600 μl of phosgel to each of three scintillation vials. These tubes are capped and incubated with the other tubes in the assay.

Day 2:

12. Centrifuge is turned on (4°C).
13. At the end of the incubation period, the tubes are placed in ice/ice water for exactly 10 min.
14. After 10 min in ice-cold water, 200 μl of charcoal is added to each tube (not the TCRs) using a repeater pipette. Time from the addition of charcoal to the last assay tube to the beginning of the spin (step 16) should be 10 min exactly. *Note*: the charcoal solution must be stirred with a magnetic stirrer at all times during additions so that the charcoal is suspended evenly in the solution.
15. Tubes are wiped with paper towels or Kimwipes®, vortexed, and loaded into the centrifuge.
16. Tubes are spun at 4°C for 12 min at 2400–2500*g*.
17. The liquid phase is decanted from the tubes into the scintillation vials, by quickly pouring the contents of the tubes into the vials, being careful not to disturb the charcoal pellet. Scintillation vials can be ordered in bulk to save money. It is recommended that in preliminary scintillation vial orders, the vials be ordered with the re-usable cardboard racks included to provide racks for holding of vials during decanting. Set up the vials for decanting and counting in the order of the assay: TCR, NSB, standards, samples, IA.
18. Scintillation cocktail (5 ml) is added to each scintillation tube, including the TCRs. A preset volume pump attached to the scintillation fluid container makes dispensing easier.
19. Scintillation tubes are capped and vortexed.
20. Scintillation tubes are loaded into the counter, using a counting program appropriate to the radiolabeled ^{3}H steroid isotope.
21. Waste materials are disposed of in a manner consistent with local radioactive garbage regulations.

Data analysis

The raw data (cpm) produced by the scintillation counter can be analyzed using commercially available programs or programs associated with the specific scintillation counter used.

Presently, we are using templates set-up using Microsoft Excel® and SPSS SigmaPlot® version 8.0 or higher. The pharmacology software in SigmaPlot (Simple Ligand Binding using the 4-parameter logistic curve fit) is used to define the parameters of the standard curve, and these parameters are then inserted into an Excel worksheet that is set up to calculate the concentration of steroid hormones in the original samples. The first step required is subtracting the NSB values from the standard curve counts and sample counts. The concentrations in the samples are then calculated as follows.

Example calculations

A typical standard curve data set might give the following parameters for the equation to the 4-par logistic curve:

$$y = D + \frac{A - D}{1 + 10^{(x - \log C)^B}} \tag{4.1}$$

$A = 1973.69, \quad B = 1.12, \quad C = 30.6300 (\log C = 1.4862), \quad D = 18.2951$

These values are calculated by SigmaPlot® using the standard curve data generated by the scintillation counts and the Simple Ligand Binding function. The 4-par logistic curve equation [Equation (4.1)] can solve for x (the sample steroid concentration) using the counts per minute of the sample (y) in the Microsoft Excel® spreadsheet. For example, if $y = 1750$ cpm and this value is entered into Equation (4.1) as solved for x, one would find $x = 4.93$ pg/tube. The units are pg/tube at this point because this value reflects the concentration of steroids in that particular scintillation vial of the RIA. To calculate back to the original sample one must factor in the volume of plasma used in the steroid extraction (typically 30–100 μl), the volume of phosgel used to reconstitute the steroids after the extraction (typically 1 ml) and the volume of sample used in the RIA (typically 200 μl). Therefore, if 100 μl of plasma was extracted and reconstituted in 1 ml of phosgel, then:

$$\begin{aligned}[\text{steroid}] &= \frac{\text{steroid content in tube}}{\text{volume of sample used}} \times \frac{\text{reconstituted plasma volume}}{\text{volume of plasma extracted}} \\ &= \frac{24.93\,\text{pg/tube}}{200\,\mu\text{l}} \times \frac{1000\,\mu\text{l}}{100\,\mu\text{l}} \\ &= 1.25\,\text{pg}/\mu\text{l}\,(= 1.25\,\text{ng/ml})\end{aligned} \tag{4.2}$$

The respective calculations for an *in vitro* RIA are slightly different. For example, assume a gonadal tissue mass of 23.6 mg used in the incubation, 1000 μl of M199 used in the incubation period of 24 h and a volume of incubation media used in the RIA of 200 μl/tube. If the steroid content of one tube in the assay is 57 pg/tube, the calculations are as follows:

$$\begin{aligned}&[\text{steroid}]\ (\text{produced by gonad } \textit{in vitro}) \\ &= \frac{\text{steroid content in tube}}{\text{volume of sample used}} \times \frac{\text{media volume in incubation}}{\text{gonadal tissue mass used in incubation}} \times \frac{1}{\text{time}} \\ &= \frac{123\,\text{pg/tube}}{200\,\mu\text{l/tube}} \times \frac{1000\,\mu\text{l}}{23.6\,\text{mg}} \times \frac{1}{24\,\text{h}} \\ &= 1.09\,\text{pg produced/mg testes/h}\end{aligned} \tag{4.3}$$

Vitellogenin assay

This assay measures vitellogenin levels in plasma. Vitellogenin is a precursor to the egg yolk protein and is synthesized in the liver under estrogenic control. While both males and females carry the gene for this protein, under normal circumstances male fish do not synthesize vitellogenin. However, when exposed to substances with estrogenic properties, males will begin to synthesize the protein. Vitellogenin induction has been explored as a biomarker for several fish species.[11]

The assay to detect vitellogenin works through a chain of reactions. The plates are precoated with vitellogenin. The plasma samples and a primary Ab are then added; this Ab binds to the vitellogenin in the samples and to the vitellogenin that is coating the walls. A secondary Ab is subsequently added, which binds to the primary Ab. The secondary Ab is conjugated to an enzyme, which will act upon a substrate (OPD) to produce a characteristic color. The more enzyme present, the more color that will be developed. If there is a low level of vitellogenin in the plasma, relatively more Ab will bind to the vitellogenin on the side of the wall of the plate and more color will be produced.

1. Ensure there is enough of each of the buffer solutions and that all pH values are correct. Also ensure that the solutions are not past their expiry dates.

Day 1:

2. Vitellogenin coating solution (150 μl) is added to each well with a repeater pipette. The plate is covered tightly with plastic wrap and incubated for 3 h at 37°C.
3. The standards, samples, IA, and primary Ab are prepared during this incubation.
   (a) Vitellogenin standards are made up by diluting with TBS-T-BSA stock vitellogenin to a final concentration of 75 ng/50 μl in a test tube. Serial dilutions are performed to prepare subsequent standards with final concentrations of 37.5, 18.75, 9.4, 4.69, 2.34, 1.17, 0.59, 0.29, and 0.15 ng/50 μl. Each standard should be vortexed prior to the subsequent dilution. The initial dilution will vary according to the concentration of the stock solution. [For example, 5 μl vitellogenin stock solution (assuming 0.60 mg/ml) is pipetted into 2000 μl of TBS-T-BSA to create the required 75 ng/50 μl.] Subsequent dilutions are 1:1 volumes of previous standard and TBS-T-BSA as with the RIA standard dilutions.
   (b) Our laboratory has found that laboratory-held mummichog plasma must be diluted further in order to have vitellogenin levels low enough to compare to the standard curve. We have found that a 1001× dilution works best for these fish. This dilution may be accomplished by pipetting 2 μl of plasma into 2000 μl TBS-T-BSA. For field samples, we have found the best dilution to be much lower, about 501×, which may be achieved by pipetting 4 μl into 2000 μl of TBS-T-BSA. The dilution that results in the most plasma vitellogenin samples within the limits of the standard curve is dependent upon the type of plasma samples that are being analyzed and should be tested for the best dilution each time samples are to be analyzed from a specific experiment.

(i) IA: Thaw aliquot
(ii) Ab: Thaw and prepare as described in reagents section

4. Plates are removed from the incubator and excess vitellogenin solution is shaken off into the sink.
5. The plate is washed by adding 300 µl of TBS-T going up and down the plate with the multi-tip repeater pipette (three runs of 100 µl) followed by shaking the solution off into the sink. This is repeated five times.
6. TBS-T-BSA (200 µl) is added to every well.
7. The plate is incubated for 30 min at 37°C under plastic wrap. This time is crucial, so that the BSA builds up but does not coat over the vitellogenin. The solution is discarded afterwards, and the plate is not washed.
8. Primary Ab, standards, samples, and IA are added to the appropriate wells as described in Table 4.3.
9. The plate is covered with plastic wrap and incubated on a desktop overnight.

Day 2:

10. The plate is rinsed five times with TBS-T as in step 5.
11. Secondary Ab solution (150 µl) is added to every well.
12. The plate is incubated under plastic wrap for 2 h at 37°C.
13. OPD solution is prepared when the 2-h incubation period is nearly over.
14. The plate is removed from the incubator and rinsed five times with TBS-T (as in step 5).
15. OPD solution (150 µl) is added to each well with a repeater pipette, and the plate is covered with plastic wrap. The plate is allowed to incubate at room temperature in a dark drawer for 30 min.
16. The spectrophotometer is turned on and set at 490 nm.
17. The plate is removed from the drawer. Some of the wells should have yellow coloration.
18. H_2SO_4 (50 µl) is added to each well to denature proteins and stop the enzymatic production of color.
19. The plate is placed on the shaker for 10 min at 100 rpm.

Table 4.3 Suggested assignment of wells within plate for vitellogenin assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	Buf	Buf	Buf	Buf	9.4	9.4	9.4	9.4	S9	S9	S17	S17
B	Ab	Ab	Ab	Ab	18.75	18.75	18.75	18.75	S10	S10	S18	S18
C	0.15	0.15	0.15	0.15	37.5	37.5	37.5	37.5	S11	S11	S19	S19
D	0.29	0.29	0.29	0.29	75	75	75	75	S12	S12	S20	S20
E	0.59	0.59	0.59	0.59	S1	S1	S5	S5	S13	S13	S21	S21
F	1.17	1.17	1.17	1.17	S2	S2	S6	S6	S14	S14	S22	S22
G	2.34	2.34	2.34	2.34	S3	S3	S7	S7	S15	S15	S23	S23
H	4.69	4.69	4.69	4.69	S4	S4	S8	S8	S16	S16	IA	IA

Buf: Buffer only, 150 µl of TBS-T-BSA. Ab: Ab only, 50 µl of TBS-T-BSA, plus 100 µl of antiserum. 0.15–75: standards from serial dilution, add 50 µl plus 100 µl antiserum. S1–S23: samples, 50 µl of samples plus 100 µl antiserum. IA: inter-assay, 50 µl of IA plus 100 µl antiserum.

20. The plate is read on the spectrophotometer at 490 nm.
21. The acid is neutralized by shaking $NaHCO_3$ over the plate so that it enters each well. The plate is then discarded.

Data analysis

The data are analyzed by comparison to a standard curve. This curve is generated from the absorbance values ($A_{490\,nm}$) of the vitellogenin standards. The data are transformed prior to construction of the standard curve by taking the log of the concentrations of the vitellogenin standards, thus providing the *x*-values for the curve. Secondly, the $A_{490\,nm}$ values for each vitellogenin standard concentration are converted to a binding over origin binding (B/B_o) or relative maximum absorbance value. This is done by subtracting the mean absorbance values of the blank (or buffer-only) wells (which removes the amount of color production that is not a direct result of the OPD acting upon secondary Ab bound to the walls of the plate) and then setting the absorbance of the Ab-only wells (less the absorbance of the blank wells) to 100% or maximum absorbance, and determining the percentage of maximum absorbance for each standard concentration. These values become the *y*-values, and a standard curve such as Figure 4.1 may be plotted.

It is imperative that the standard curve produces a reliable regression, as it will be used to determine the concentration of vitellogenin in the plasma samples. Therefore, we recommend that an effective standard curve is one that has an r^2 of at least 0.95. When new reagents are acquired or the assay has not been used recently, the standard curve should be performed to ensure the assay is working correctly (i.e., there has been no

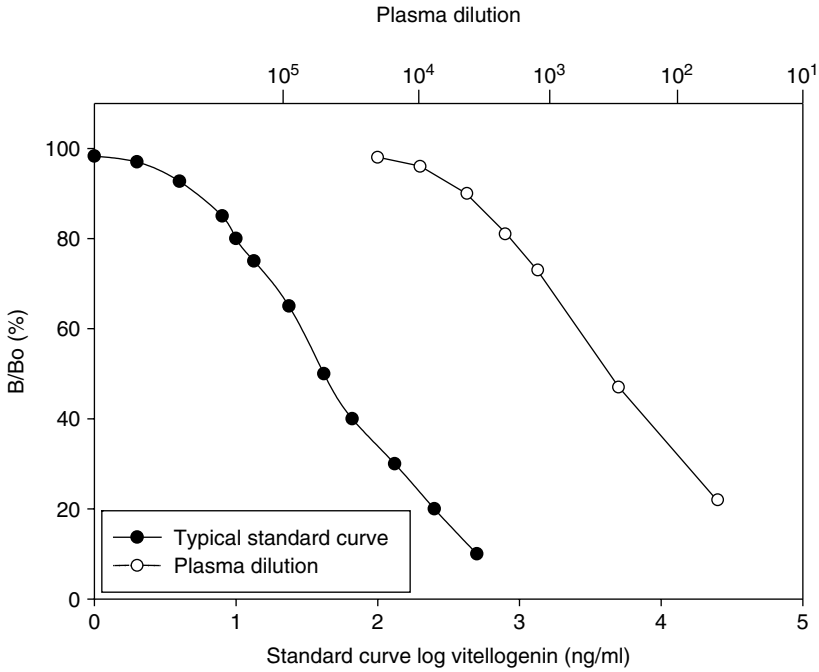

Figure 4.1 Lower *x*-axis: typical binding displacement curve (–●–) obtained with vitellogenin standards. Upper *x*-axis: Parallel binding displacement curve (–○–) obtained with serial dilution of plasma from a male mummichog exposed to waterborne ethynyl estradiol.

degradation of Ab or other reagents). In addition, parallelism of multiple standard curves should be confirmed using analysis of covariance (ANCOVA) to ensure that there is no significant difference among the slopes of multiple standard curves.

It is important that the diluted plasma samples fall within the standard curve of purified vitellogenin. It is recommended that a serial dilution of a plasma sample be tested prior to running samples, in order to show linearity of the plasma dilution (Figure 4.1), as well as to determine the dilution of the plasma that lies within the vertical slope of the standard curve. IA variability should be assessed with every assay by including an IA sample as described earlier. Please refer to the work of Denslow et al.[11] for further details on assessing assay quality.

Once the regression on the standard curve values has been determined to be acceptable, the sample concentrations can be calculated. Two replicate $A_{490\,nm}$ values for each plasma sample are averaged, and the relative absorbance for each sample as a percentage of the maximum absorbance is determined as described earlier. If this value is >95% of the maximum absorbance, the concentration of vitellogenin is considered to be below detectable limits. If this value is below 95% of the maximum absorbance, then the log concentration of vitellogenin in the sample is calculated from the parameters of the regression equation. This value must then be inverse-log transformed to give the concentration of vitellogenin (ng/50 μl as per the standard curve) within the well. To convert this concentration (ng/50 μl) to the actual concentration of vitellogenin in the sample, this must be multiplied by the plasma dilution factor. This will give a concentration in nanograms per milliliter that may be converted to more appropriate units if necessary (e.g., mg/ml). We have used both Corel QuattroPro® and Microsoft Excel® to create templates to extrapolate the vitellogenin concentrations for the samples from the standard curve.

Statistics

Differences in mean gonad and mean liver size among treatments can be tested with an ANCOVA with total fish weight as the co-variate of organ weight. Condition factor can be tested in a similar way, with weight as the dependent variable and standard length as the co-variate. When the *p*-value of the interaction term is >0.1, treatment and covariate are considered as the only possible sources of variation in subsequent analyses.[35] Scatterplots, with regression, of log covariate (*y*-axis) against the log dependent value (*x*-axis) can be given; however, it is often easier to indicate differences by listing in a table the morphological variables as the index or calculated value (Table 4.4).

Statistical test(s) involved in the data analysis (e.g., steroid hormones and vitellogenin) are dependent on outliers, normality, and variability. Before performing any tests of significance, the data can be examined for outliers using a test such as the Dixon test for outliers.[36] Given a normal distribution, a nested analysis of variance (ANOVA) can be performed with fish as the units of replication to determine presence or absence of tank effects and to test for treatment differences. If the ANOVA identifies treatment differences, Tukey's, Dunn's, or Dunnett's *post-hoc* tests can be used to determine where these treatment differences exist. A decision should be made *a priori* if there is an interest in comparing all treatments (Tukey's or Dunn's multiple comparison test) or in comparing each treatment to the control (Dunnett's multiple comparison test). For the former, Tukey's test is used if sample sizes are equal, Dunn's test if they are not.[37]

Table 4.4 Effects of 7 days of exposure of female (F) and male (M) mummichog to final effluent (0–100%) collected from a Canadian paper mill. Values are means (standard errors of the means). Differing superscript letters indicate groups that are different

Sex	Effluent concentration	Weight (g)	Gonadosomatic index, GSI (%)	Liver-somatic index, LSI (%)	*In vitro* Testosterone (T) production (pg/mg tissue/h)	Maturational follicles and mature eggs (*N*)	Plasma vitellogenin (mg/ml)
F	0	13.0 (0.662)	10.4 (1.35)^{a}	5.75 (0.244)		156.5 (20.3)^{a}	3.14 (0.189)^{a}
	1	12.6 (0.332)	9.58 (0.758)^{a}	5.58 (0.202)		143.7 (11.4)^{a}	2.12 (0.231)^{ab}
	5	12.9 (0.281)	8.38 (1.01)^{a}	5.18 (0.376)		117 (13.3)^{a}	2.54 (0.456)^{ac}
	15	12.6 (0.624)	7.86 (0.888)^{a}	6.088 (0.290)		91.0 (12.0)^{b}	2.78 (0.329)^{a}
	30	11.5 (0.721)	6.89 (0.864)^{a}	5.04 (0.313)		103.3 (13.0)^{a}	1.56 (0.274)^{bc}
	50	12.6 (0.625)	6.48 (0.749)^{a}	5.32 (0.473)		82.9 (10.4)^{b}	1.47 (0.129)^{bc}
	100	13.6 (0.682)	6.07 (0.800)^{b}	5.118 (0.285)		68.9 (8.64)^{b}	1.24 (0.165)^{b}
	p-value	0.067	0.011	0.222		<0.001	<0.001
M	0	15.4 (0.581)	1.65 (0.226)^{a}	2.52 (0.262)	1.25 (0.115)^{a}		1.51 (0.134)
	1	13.8 (0.644)	1.39 (0.118)^{a}	2.46 (0.226)	1.06 (0.0900)^{ab}		1.71 (0.540)
	5	15.3 (0.501)	1.35 (0.0808)^{a}	2.43 (0.138)	0.978 (0.136)^{ab}		1.43 (0.157)
	15	15.3 (0.373)	1.25 (0.105)^{a}	2.27 (0.142)	0.943 (0.160)^{b}		1.58 (0.482)
	30	14.3 (0.499)	1.21 (0.0115)^{a}	2.45 (0.145)	0.767 (0.110)^{b}		1.69 (0.132)
	50	14.5 (0.563)	1.23 (0.0843)^{a}	2.98 (0.137)	0.570 (0.0496)^{c}		1.56 (0.142)
	100	14.7 (0.291)	0.981 (0.0706)^{b}	2.62 (0.141)	0.436 (0.0675)^{c}		1.39 (0.278)
	p-value	0.257	0.022	0.265	<0.001		0.988

If tank effects are present as determined by the nested ANOVA, the unit of replication becomes the tank rather than the individual fish (with an associated decrease in statistical power). RIA data are often non-normally distributed. Performing a log-transformation will typically permit use of parametric tests; however, if the transformation is not effective or desirable, the equivalent non-parametric test (e.g., Kruskal–Wallis ANOVA on ranks) can be performed.[37]

Any statistical software program with these various tests can be used. Examples of programs we have used include SAS/STAT (SAS, Cary, NC, USA) and Systat (Systat Software, Richmond, CA, USA) and SPSS (SPSS, Chicago, IL, USA).

Results and discussion

As an example of the type of date resulting from the mummichog bioassay, mummichog were exposed to final effluent from a Canadian paper mill to determine fish responses at environmentally relevant and greater effluent concentrations. Pre-spawning fish were exposed in static systems for 7 days in February 2001 following use of the artificial regression-recrudescence protocol. In brief, 3 male and 3 female mummichog were weighed prior to allocation into 32.4 l filtered aquaria containing 16 l of 16 ppt water at 18–20°C and >85% DO. Fish were acclimated for 1 week in the exposure aquaria. Beginning on Day 1, fish were exposed in replicates of four aquaria to the following concentrations of paper mill effluent: 0%, 1%, 5%, 15%, 30%, 50%, and 100%. The receiving environment normally receives 1–5% dilutions of the effluent. Water and effluent changes were made daily. Following exposure, fish were sampled as previously described. *In vitro* incubations were done with testes tissue only. The number of

maturational follicles and mature eggs were counted; opaque (pre-maturational) follicles made up only 5% of the total follicles counted.

Female ($p < 0.11$) and male ($p < 0.022$) GSIs were significantly reduced at 100% effluent (Table 4.4). There was no effect on liver-somatic index (LSI) (Table 4.4) or condition factor (data not shown) in either males or females. Female plasma T was depressed at 30–100% ($p < 0.001$), while male T was depressed at 100% ($p < 0.001$) (Figure 4.2). Responses for

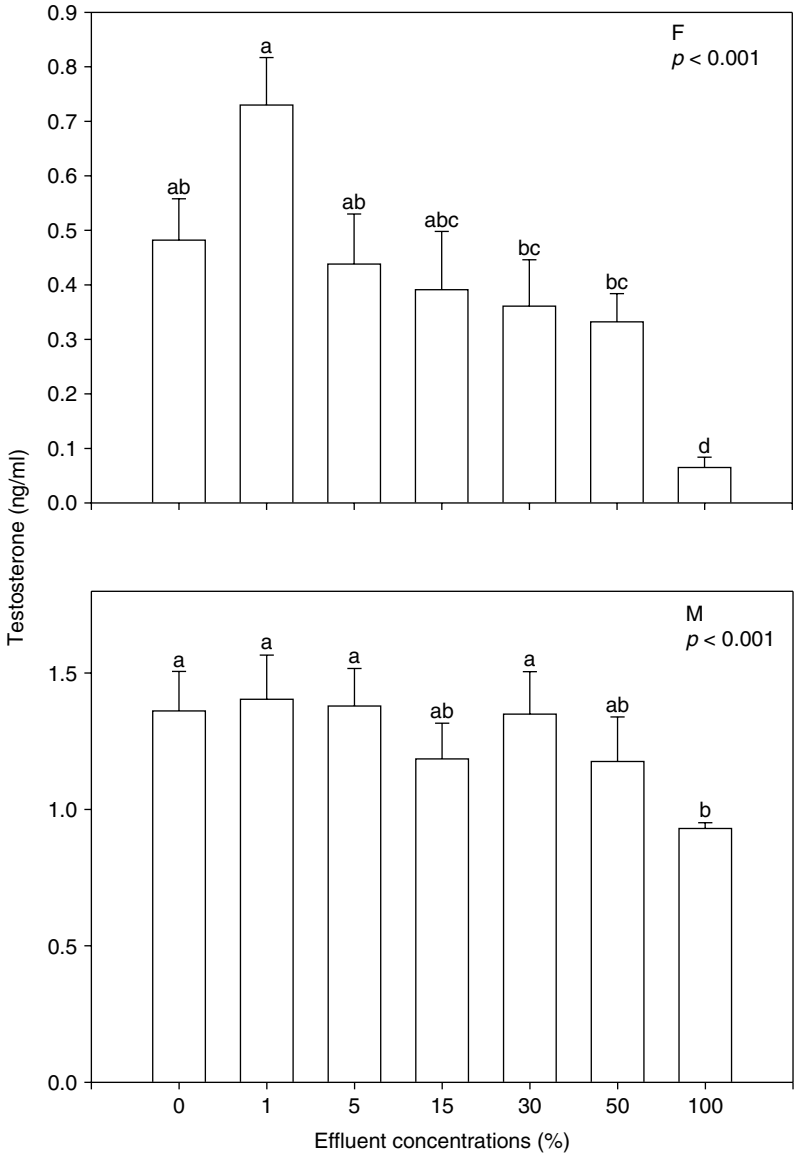

Figure 4.2 Plasma testosterone (T) levels of female (F; top) and male (M; bottom) mummichog exposed to effluent from a Canadian paper mill for 7 days in the bioassay. Values are mean (+standard error of the mean). Significant differences are indicated by differing letters. Plasma hormone levels are routinely presented in graphical form. Figures are especially suitable for clearly representing dose–response data.

plasma estradiol (females) and 11-ketotestosterone (males) paralleled those of plasma T (data not shown). *In vitro* incubations of testes pieces were decreased at 50% and 100% ($p < 0.001$) (Table 4.4).

These results are typical of those obtained when using the bioassay to assess effects of hormone-active effluents (or EDSs). Depressions in T production in males have been observed in males exposed to environmentally relevant concentrations of anti/androgens,[19] high concentrations of anti/estrogens,[2] and pulp mill effluents.[20,21] In males, *in vitro* depressions in T production were affected at lower effluent concentrations that plasma T (Table 4.4, Figure 4.2). Observed treatment differences in gonadal steroid production are often correlated with changes in circulating steroid levels. Gonadal production is often altered at lower exposure concentrations than plasma steroid levels.[2,19] Pulp mill effluent effects, and effects of other EDSs, are often manifested at the level of the gonad[38] through effects on steroid production.[39,40] The *in vitro* incubation assay is a relatively simple method of determining effects at the gonadal level. Additional methodologies using precursor additions can be used to identify enzymes within the steroidogenic pathway affected by exposure.[14]

The number of maturing eggs (maturational follicles plus mature eggs) was decreased starting at 15–30% ($p < 0.001$; Table 4.4). This is the first time in our bioassay in which we have detected significant changes in follicle development during the 7-day bioassay. At 100% effluent, male testes size was also reduced. In females, high endocrine toxicity at 15–30% was observed; the lack of follicular development correlates with reduced plasma steroid levels. The number of mature follicles/eggs is in the same range as those previously found in field studies,[33,34] as well as our own fecundity values for pair-breeding laboratory stocks (R. Ibey and D. MacLatchy, unpublished). Whether follicular development was delayed by direct effects of the effluent on follicular cell growth and differentiation, or indirectly by effects on endocrine parameters, is unknown. Mummichog follicular development is under hypothalamo-pituitary control,[41] and pulp mill effluents are known to interfere with the pituitary–gonadal axis (decreased gonadotropin level, decreased gonadotropin response at gonads) in fish.[42] Estradiol is also required in fish to direct ovarian growth and differentiation[43]; depressions in estradiol production may have caused changes in paracrine or autocrine control. Further work is required to fully understand the control and function of endocrine systems in fish. Without such understanding, our ability to understand EDS effects is limited.

No effects on plasma vitellogenin levels were found in males (Table 4.4). Induction of vitellogenin production in (male) fish is a commonly measured indicator of exposure to an estrogenic compound or effluent.[44] Pulp and paper mill effluents are not normally associated with estrogenic activity[45] although constituents of pulp mill effluent have been shown to be estrogenic.[46] This is because final effluent chemistry can be quite distinct from the chemistry of the component waste streams that make up the final effluent. It has previously been established that mummichog do induce vitellogenin when exposed to estrogen mimics (ethynyl estradiol[2]) and sewage.[6] Females in this study had depressed vitellogenin levels (Table 4.4), most likely due to depressed estradiol levels (data not shown). We have obtained similar results in female mummichog exposed to methyl T.[19] In juvenile fathead minnow, high concentrations (>75 μg/l) of a pharmaceutical antiestrogen (ZM 189,154) caused depressions in whole-body homogenate concentrations of vitellogenin.[47] It is important to note that many EDSs are able to interact with both estrogenic and androgenic receptors,[48] and a vitellogenic response to EDSs indicates interaction with just one receptor-mediated pathway.

In sum, these results are typical of those observed when mummichog are exposed to hormone-active compounds or effluents. Taken together, the endpoints are able to identify whole organism responses (plasma steroids and vitellogenin) and a population-level effect (reduced fecundity). As well, one mechanism by which the effluent manifests its effects is decreased steroidogenesis. The effluent does not appear to be estrogenic (vitellogenin data). This paper mill data reflects pulp mill effluent studies using this bioassay in that the effects resemble responses found in fish exposed to model anti/androgenic compounds rather than model estrogenic compounds.[45]

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chapter five

*Conducting dose–response feeding studies with salmonids: Growth as an endpoint**

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Contents

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Introduction

This chapter provides details on the procedures for conducting toxicant dose–response feeding studies with salmonids. The main focus in our laboratory is to assess the effects of various toxicants that juvenile salmonids are exposed to as they migrate through urban estuaries. Many of the procedures outlined here are general and may be applied to other fish species. This chapter will primarily discuss how growth is evaluated; however, once the fish are dosed for a specific time period, most other desired biological response, such as immunochallenge, physiological function, behavioral assays, or reproductive abnormalities may also be assessed.

Feeding toxicants to fish is an ideal way to mimic environmentally realistic exposure. Injecting toxicants in large doses can overwhelm physiological systems producing results that may never be observed in fish from contaminated sites. Information on contaminant concentrations in stomach contents and prey species can be used to design dose–response studies where single toxicants or mixtures can be applied to food and fed for a specified period of time. Although it is difficult to accurately mimic the exposure fish experience in the field, the results from controlled laboratory studies with defined doses can add greatly to ecological risk assessments, critical body residue determinations, and bioaccumulation evaluations. These types of studies are also valuable for the researcher interested in physiological mechanisms of response from field-like exposures.

Materials

Water system

Fiberglass tanks for holding fish and conducting experiments (e.g., 1.3 and 2 m diameter), polyvinyl chloride (PVC) water supply lines, valves, flexible tubing, and PVC drain lines; biofilter (surface area determined by biomass loading), nitrifying bacteria to seed biofilter, biosolids filter (capacity determined by biomass loading), charcoal filter (size determined by flow rate), submersible pumps, water chiller (size determined by flow rate and degrees cooling), water heater (size determined by flow rate and degrees heating), and ultraviolet (UV) sterilizer (wattage determined by flow rate); airstones, Tygon tubing, and air blower (oil free).

Fish husbandry/experimental

Egg transfer containers, coolers, egg incubation trays, automatic fish feeders, fish nets (large and small), iodophor for the eggs, disinfectant for fish nets and floor mats, and

several clean 20-l buckets; balances (e.g., basic toploader for weighing food and a high capacity balance for weighing fish in water); water chemistry test kits (e.g., ammonia, nitrite, alkalinity), oxygen meter, flow meters, pH meter, salinometer, water level sensor, and thermometer (continuous/recording).

Fish food and dosing

Fish food (commercial food for the early life stages, low fat pellets for the exposure phase, and food with antibiotics for disease outbreaks); pipettes, covering the range of 10–100 μl, 100 μl to 1 ml, 1–10 ml, Erlenmeyer flasks with glass stopper, and a large spoon; balance (high precision for weighing test chemicals); tricaine (MS-222), dichloromethane ($MeCl_2$), fume hood, stainless steel bowls, trays/tubs (e.g., polycarbonate, stainless steel), aluminum foil, and polyethylene tubs for food storage.

Procedures

A. Source of fish

Fish for experimentation can be obtained from many sources and usually at any desired life stage. They can be obtained from hatcheries as eggs and raised in the laboratory to the desired life stage for testing or collected from the field. We recommend obtaining eggs (as gametes or at the eyed stage) and culturing fish to ensure high quality juveniles for testing and total control over all stages. We routinely raise 6000 juveniles from eggs, which is not difficult, if water quality parameters are kept within prescribed limits (see Appendix I). We obtain our eggs from the University of Washington hatchery and in the past have acquired fish in the smolting stage from the Soos Creek Hatchery in Auburn, WA. Salmonids at different stages of development can be purchased from various sources (e.g., Trout Lodge, P.O. Box 1290, Sumner, WA. 98390, 253.863.0446; AquaSeed, 2301 NE Blakeley Street, Suite 102, Seattle, WA 98105, 206.527.6696, info@aquaseed.com).

B. Raising fish from eggs

1. Obtain the desired number of high quality eggs from a reputable source that can certify the eggs are disease free. If the eggs are to be transferred to another location, a permit may be required from the appropriate agency (e.g., State Department of Fisheries).
2. The transportation of eggs requires egg containers. In our laboratory, we use sections of 4-in. PVC pipe with screens attached to one end, a cooler, and clean cotton towels. For transport, eggs are placed in the containers, which are then stacked in a cooler. Wet towels are placed on all sides of the egg containers. Eggs are then transported to the laboratory and placed in an incubation rack.
3. The total number of eggs received can be estimated by the following method. The number of eggs in a small volume is determined (e.g., 100-ml beaker). The total volume is then determined with a suitable container (e.g., 1-l glass Pyrex beaker). This total volume and the actual count of eggs/ml are multiplied to determine the total number of eggs in culture. Estimating the total number of eggs can also be accomplished by weighing; however, determination by volume is more convenient for dispensing eggs to the incubation trays.

4. The eggs are then placed in an incubation chamber that has been disinfected with an appropriate disinfectant (e.g., iodophor). The incubation tray should be mounted on supports over a water reservoir tank (e.g., 2-m-diameter fiberglass tank). When additional water (makeup water) is needed to keep a constant volume, it should be passed through an activated charcoal filter to remove contaminants and chlorine and a UV sterilizer to reduce pathogen exposure. Water in the reservoir tank is recirculated through a chiller or heater, biofilter, and UV sterilizer. This water is then pumped to the top of the incubator by submersible pump and allowed to flow over all trays holding eggs. Two pumps are used for redundancy and should be plugged into separate electrical circuits. A recirculating water system is desirable for this phase to control water quality parameters; however, freshwater can be supplied from diverted natural sources (e.g., lake or stream) or from a municipal system and treated.
5. The density of eggs in the hatching trays should be low enough to allow a complete flow of water over the eggs, as well as sufficient access to remove dead eggs. A flow of 1 l/min should be more than sufficient for a large number of eggs. It is important that the oxygen content of the water exiting the incubation trays is maintained at 100% saturation. If less than 100%, the flow rate will have to be increased. The front and back of the incubator are covered with black plastic to protect the eggs from exposure to light. The temperature of the water should be optimized for this life stage. This is approximately 10°C for chinook salmon (*Oncorhynchus tshawytscha*).
6. Environmental parameters (see Appendix I).
7. The incubation chamber should be checked daily for dead eggs or larvae, which must be removed to reduce fungal infection.
8. Alevins (newly hatched salmon with yolk sac attached) should remain in the incubator until the yolk sac is almost completely absorbed or when larvae are swimming freely around the trays. Fish can then be gently transferred to a holding tank (e.g., the 2-m water reservoir tank).

C. *Raising salmon fry to presmolt juveniles*

1. A loading density not to exceed 20–30 larvae per liter of water is recommended. The characteristics of the water source will have to be taken into consideration for the loading density. A flow-thorough system will support more fish than a recirculating system.
2. Environmental parameters (see Appendix I).
3. Feeding the larvae is initiated at an appropriate time post hatch. For chinook salmon, this occurs when fish begin spending more time in the water column instead of the tank bottom. Commercially prepared fish food specially formulated for salmon larvae is recommended. This food should be introduced every 0.5–1 h via automatic feeders, at the rate and pellet size recommended by the feed manufacturer. A 2-m diameter fiberglass tank with autofeeder is shown in Figure 5.1.
4. When fish reach an average size of approximately 1–2 g, they may be fed 3–4 times/day by hand. The size of the fish food should be increased according to the schedule presented in Table 5.1, which is approximate for chinook salmon held at 9–11°C. Additional details on pellet sizes and feeding rates can be obtained from

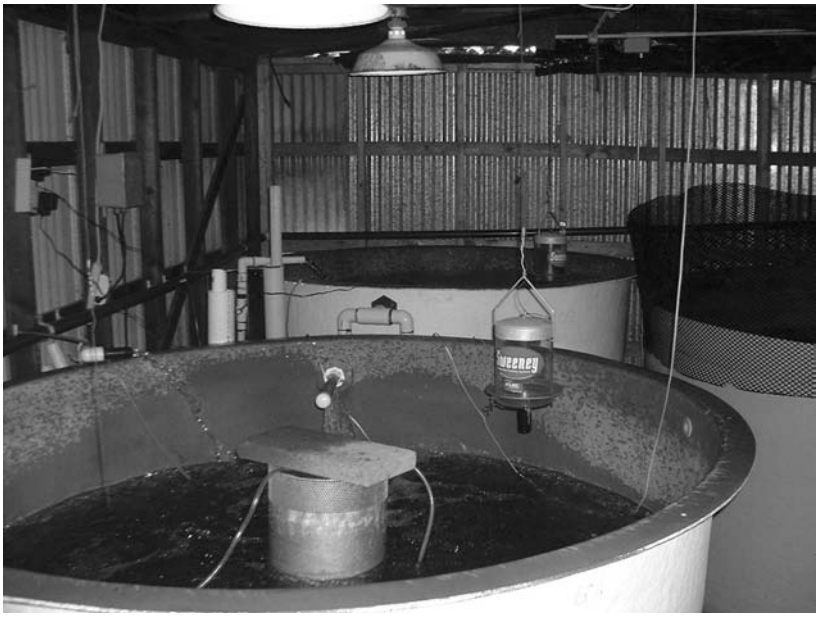

Figure 5.1 Large fiberglass tanks (2 m diameter) used for rearing juvenile chinook. Automatic feeder shown above each tank.

Table 5.1 Example of feeding schedule for juvenile chinook salmon

Stage	Size of fish	Size of pellet	Percentage of bw/day
1	Larvae to 4 g	Micro pellet	2.8–2.0 as fish increase
2	4–6 g	1.2 mm	1.7
3	6–25 g	1.5 mm	1.6–1.8
4	25 to 80+ g	2.0 mm	1.4–1.5

manufacturers (e.g., EWOS Canada, www.EWOS.ca). The schedule in Table 5.1 showing pellet size and rate of feeding may be different for other species of fish in culture and should be adjusted according to feed manufacturer specifications.

5. As soon as the fish weigh approximately 1 g, a subsample of 100 fish should be weighed individually to determine their mean weight. After this, all juvenile fish can be weighed in large batches to determine the total biomass (see Appendix II). The total biomass in grams and the average weight of the sample will be used to determine the total number of fish in culture. This value can be used to determine feeding rates.
6. Once the total number of fish and biomass are established, the amount of food to be dispensed can be determined. For juvenile chinook salmon, we dispense food at a rate of 1.5–2.0 % body weight (bw)/day. An appropriate amount of food is weighed and can be given to the fish in 2 or 3 feedings within normal working hours (e.g., at 10 am and 4 pm).
7. A sample of 100 fish should be weighed once per week to determine the average weight of all fish in the population. This average weight is used to adjust the amount of food given to the entire population.

8. When juvenile fish weigh approximately 2 g, they are switched to a fish pellet that contains a total lipid content that mimics the prey for wild fish (see Appendix IV.A).

D. Introducing smolts to seawater

If experiments are conducted in seawater, the fish must be acclimated to high salinity water. Several parameters may be used to determine when smolts are ready for entry into seawater.[1] These include gill ATPase, endocrine hormones (e.g., thyroxin), body lipid, blood sodium, or skin coloration. The following procedures are described for juvenile chinook salmon. This protocol may need to be adjusted for other salmon species.

1. When the juveniles weigh approximately 2–4 g, they should be moved into larger tanks (e.g., 2-m diameter) at a density not to exceed approximately one fish per liter. At this time, the fish should remain in the freshwater system and allowed a few days after the transfer to acclimate. In our laboratory, we keep up to 2000 fish in 2-m-diameter tanks, which hold approximately 2000 l of water. For each tank, we maintain a flow of 40–50 l/min, which is well within the prescribed limits of 0.6 l/min/kg at 10°C.[2] It has been our experience that fish at a density of one fish per liter need relatively high flows, otherwise dissolved oxygen levels will decline rapidly.
2. Environmental parameters (see Appendix I).
3. For juvenile chinook salmon, it has been demonstrated that the physiological parameters for making the transition to seawater are optimized during the new moon.[3] When our young of the year chinook salmon are larger than 5 g and their skin coloration is silvery, we introduce them to seawater during the new moon in May, June, or July. During this time, the salinity is raised from 0 to 7 parts per thousand (ppt) on the first day. After this, the salinity is increased by 5 ppt/day until full strength seawater is attained. After achieving full strength seawater, the fish are given a minimum of 2 weeks to acclimate before handling.
4. Once in seawater, these fish are fed 1.5% of their body weight per day with the low fat fish food at rate of 5–6 times per week. The food is generally delivered in two or three equal batches over the day to total the 1.5% bw ration.

E. Distribution of fish to experimental tanks

1. Before fish are distributed, the experimental tanks should be thoroughly cleaned with acid and disinfectant. All fouling organisms need to be removed from the tanks, and all water delivery lines and drains need to be checked for obstructions. In our laboratory, we use a dilute solution (1 *M*) of muriatic or hydrochloric acid to remove fouling organisms from the tanks, which is applied twice. After this treatment, we apply a solution of quaternary ammonium for 15 min to disinfect the tanks. This disinfectant comes with directions for concentration and time. These cleaners can be purchased from vendors specializing in aquaculture supplies. After cleaning the tanks, the entire system should be operated for at least 24 h to flush out any remaining

acid and disinfectant. After this, the tanks should be drained, refilled, and allowed to operate normally for at least 48 h before fish are added.

2. Within 1 week prior to distribution of fish to experimental tanks, a subsample of fish (e.g., 100 fish) should be selected at random from the common pool of fish to determine the mean and standard deviation (SD) of their weight. This information will be used to select the size range of fish to be included in the experiment. For growth studies, it is ideal to keep the SD of the mean weight for each experimental tank relatively low and uniform across tanks.
3. For growth studies, a size range of fish that produces a relatively low SD should be selected before hand. In our experiments, we strive for a coefficient of variation (CV = SD/mean * 100) of approximately 10–15%. This range in weights is subject to change to meet experimental needs and open to interpretation at the time of distribution. One major consideration is the number of fish available and the expected rejection rate. For example, in one of our experiments, we selected a weight range of 8–10 g, which produced a mean fish weight for each tank of approximately 9 g with SD = 0.5–0.6 (= CV of 7%). In our experience, a CV of 10–15% requires twice as many fish as needed to run the experiment due to a rejection rate of approximately 50%. Another strategy is to distribute all fish randomly to the experimental tanks; however, this will likely lead to greater difficulty in data interpretation because of the larger variance for mean weight.
4. Fish are weighed individually to determine their weight (Appendix II). If a weight range has been selected as described in step 3, each fish will be accepted or rejected for the experiment based on its weight. Two clean buckets with approximately 15 l of clean water from the system should be close by. Fish that will be used in the experiment go into one bucket and rejected fish into the other. Each bucket will need an airstone to keep the oxygen saturation level between 70% and 100%, and the temperature should not rise more than 1°C from the system temperature.
5. The number of fish added to each experimental tank will vary depending on the species selected, tank volume, and flow rate of water. In our experiments, we place approximately 75 fish in each 1.3-m-diameter tank. This has proven to be an optimal number of fish for these tanks based on consideration of biomass/liter, behavioral interactions, and water quality. The density for salmonids of this size is approximately 1.5 g of fish/l. Figure 5.2 shows an array of 1.3-m-diameter fiberglass tanks. This facility has 32 experimental tanks.
6. The number of experimental tanks for each treatment should be determined by a power analysis. See Appendix III for statistical methods. The order of filling of the tanks with fish will be determined with a random number generator. Because selecting fish from the common pool of fish is often not random, fish need to be added to the experimental tank in batches. When dip-netting fish from the common pool, often the smaller and slower fish are caught more easily and these should be randomly distributed among the tanks (see Appendix III.B). Fish are usually added in three equal batches. If 75 are to be added, approximately 25 fish will be added to each tank based on the order determined with the random number generator. After this, the next 25 should be added in the same order as the first round of additions.
7. Once all the fish are distributed to the experimental tanks, an analysis of variance (ANOVA) and *post-hoc* test is performed to determine if the mean

Figure 5.2 Picture shows an array of 1.3-m-diameter (500 l) fiberglass tanks. Total array is 32 tanks. Note the air supply lines, drain stand pipes, spray bars for water delivery (inside the tanks), overhead lighting, and the tank with mesh cover.

fish weight in any tank is significantly different from that in any other tank. If a mean fish weight of a tank is significantly different, fish may be removed or new fish added until no significant differences are observed. This can also be accomplished in an iterative fashion by examining the ANOVA for each round of 25 fish that are added to tanks. If any of the tanks exhibit deviation at any stage, corrective measures (e.g., adding heavier or lighter fish within the selected range) can be taken to ensure that a non-significant mean weight will be obtained. It is also important that the variance in fish weights among tanks is uniform. A test to examine the homogeneity of variance (e.g., Bartlett's) should be performed.

8. After all fish are distributed to the experimental tanks and the mean weights for each tank are deemed non-significant, then a treatment designation for each tank will be chosen with a random number generator such that replicates from a given treatment are scattered through the complex of tanks. This procedure will minimize the effects of any potential environmental gradients that may occur in the physical space containing the experimental tanks (see Appendix III.B).
9. Any fish that dies between the time of distribution and the start of the experiment should be replaced. Because dead fish will assume the same osmotic pressure as their environment and dehydrate or superhydrate, depending on the salinity, they should placed in a physiologically isotonic solution (0.17 *M* or approximately 1% salt solution) for 2 h, removed, blotted of excess water, and weighed. A fish of comparable weight should then be removed from the database for that tank and the weight of the replacement fish inserted in its place.
10. After distribution to the experimental tanks, the fish should be allowed to acclimate for 7–10 days before feeding with experimental food. During this

period, the fish are fed unadulterated fish pellets that will be used in the experiment.

11. Tank covers are highly recommended to keep fish from jumping out and to exclude predators. We use a soft fiber mesh material (white) attached to two curved pieces of flexible 1-in. tubing that allows easy access to the fish and the ability to add food without lifting the cover (Figure 5.2). The covers should also be disinfected and dried thoroughly before fish are added to the tanks.

F. Conducting the experiment

1. After distribution of the fish to experimental tanks is completed and an acceptable mortality has been achieved (e.g., <0.2%/day), fish will be fed contaminated fish pellets at rate to be determined (e.g., 2.0% bw/day). The total amount fed per tank is based on this rate and the total biomass for that particular tank. See Appendix IV.B for information on preparing toxicants-dosed fish pellets. Fish are fed 5 or 6 days/week. Appendix I.D contains details on feeding schedule and increasing the food ration.
2. In our laboratory, juvenile chinook salmon are usually fed 2.0% bw/day to achieve a sufficient rate of growth for testing the hypothesis of toxicant effects on growth. A feeding rate of 1% bw/day for juvenile salmonids at their optimum temperature (approximately 10–11°C) is very close to a maintenance dose that would produce very little growth. For juvenile chinook salmon in our system under our usual conditions, the growth efficiency is approximately 20%, i.e., 1 g of dry food produces approximately 1 g of fish biomass based on wet weight (≈ 0.2 g of fish dry weight).
3. Environmental parameters (see Appendix I).
4. The fish in each tank are fed contaminated pellets that have been dosed with toxicants for a period of time appropriate for the study. Considerations for the dosing period include the growth rate and life cycle of the fish, the amount of dosed food that can be made in one batch, and the types of biological responses measured. In our laboratory, we commonly feed dosed food for 45 days and unadulterated food for several months after the dosing period.
5. After a predetermined time period (e.g., 45 days), the fish in each tank are weighed. Each individual fish in a tank is weighed by one of the methods described in Appendix II. The data are generally recorded and saved in an Excel spreadsheet then exported to a statistical program for analysis (see Appendix III). If the experiment continues, the total biomass obtained for each tank should be used to adjust the amount of food given for the next feeding cycle. Each tank is then given a unique amount of food based on the total biomass obtained from the weighing. See Appendix I.D for information on increasing the weekly food ration.
6. After a predetermined time (e.g., another 45 days or day 90 of the experiment), the fish in each tank can be weighed again in the manner described in Appendix II and at regular time intervals, if so desired.
7. This experimental design also allows several other chemical and biological responses to be quantified. If sufficient fish are available from each tank, samples for whole-body chemistry, blood for physiological parameters,

internal organs for biomarkers or morphological indices (e.g., hepatosomatic index) can be taken. A select number of dosed fish may also be removed for separate experiments, such as a disease challenge or behavioral tests.

8. At a minimum, the fish food should be analyzed for the added toxicants to determine the actual exposure concentrations for experimental fish. If resources permit, a broad analytical scan of several potential contaminants [e.g., polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), phthalates, and metals] should be conducted for one or two samples of the control food to determine background contamination.
9. For some experimental designs, it may be desirable to determine the toxicant concentrations in fish tissue (e.g., whole-body, liver, gill) or metabolites in bile. Our laboratory routinely analyzes whole-body fish to determine bioaccumulation of toxicants from fish food and for correlation analysis with biological responses. Fish are starved for 24 h before whole-body or bile samples are taken. Stomach contents and the alimentary canal should be removed during sampling if correlations are to be generated for fish responses, or they may be left in place if the total concentration is desired for assessing exposure to those species that prey on salmon.

G. Quality control/assurance

The details of a quality assurance program are to be decided by the laboratory conducting the experiments and the intended use of the data. Examples of quality assurance plans and guides can be found on various government websites.[4] It is highly recommended that a database program (e.g., Filemaker Pro™ or MS Access™) be used to keep track of feeding regimes, mortalities, and fish removed for various samples or other experiments. Chain of custody numbers and forms are desirable for every sample, even if this research is not conducted under the auspices of any environmental statute. For these types of studies, standard practice for quality control includes calibration of balances and measuring instruments (e.g., thermometers, oxygen meters), feed quality (low contaminants, correct nutrient mix), and associated controls for analytical chemistry. Periodic checks on food allocation should also be made to ensure correct dosage amounts.

All results for water quality measurements, adjusted water quality parameters, fish weights, feeding schedules and amounts, mortalities, abnormalities, corrective actions taken, and any observational notes are recorded in a laboratory notebook by the scientist in charge of fish husbandry. These notes will be useful for assessing problems and should be reported with the results of each experiment.

Discussion/considerations

The preceding has been a discussion on how to set up and conduct growth studies with juvenile salmonids exposed to toxicants in their food. Some of these techniques and analytical tools will be appropriate for other species of fish; however, several aspects of the species life history will need to be considered.

Growth is just one biological response that can be assessed with this design. Once the fish are dosed, they can be used in a multitude of other studies designed to assay for effects. If growth is the main concern, the researcher may want to delve into the

mechanism responsible for the response. For example, decreased growth may be due to a physiological response, and even though the treatment fish are eating just as much as the control fish, their metabolism may be altered producing a negative effect on growth. A reduction in growth may also be caused by the fish becoming lethargic and not ingesting the same amount of food as the control fish or rejecting food because of reduced palatability. These should all be part of the routine suite of behavioral observations for this type of experiment.

In our experiments, we consider fish weight to be a more important variable than fish length for assessing growth impacts due to toxicant exposure. This is supported by a few studies noting that fish weight was more affected than fish length for salmonids exposed to toxicants.[5,6] Measuring length is important, however, for determining the condition factor (*K*) (see Appendix III.C), which can provide general information about the health of the fish. In general, when fish are stressed, the condition factor usually declines indicating that a fish will be lighter for a given length.

There are several excellent books on fish aquaculture, systems engineering, and fish diseases that should be consulted for more details and specific situations.[7–11]

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Appendix I Environmental conditions

Freshwater or seawater water can be supplied to fish with a recirculating or flow-through system. This delivery system should have a charcoal filter in line to remove contaminants and sufficient UV sterilizers to keep the water pathogen free. For both flow-through and recirculating water systems, a biosolids filter is highly recommended to remove wastes and excess food. A biofilter is highly recommended for recirculating systems to provide sufficient surface area for nitrifying bacteria to keep ammonia and nitrite levels low. It is highly recommended to seed the biofilter with a bottle of nitrifying bacteria, which can be purchased from any aquarium or pond store. A flow-through system is recommended for the toxicant exposure phase because recirculating systems are difficult to maintain and it is a challenge to keep fish in different treatments from being cross-contaminated. Figure 5.3 shows the biosolids filter, air blower, and UV sterilizers for the experimental facility in Figure 5.2.

I.A Water quality parameters

Water quality (pH, ammonia, nitrite, temperature, hardness, oxygen content, and alkalinity) should be checked frequently. Recirculating systems will require more frequent analysis of water quality parameters than flow-through systems. Additionally, some parameters are more variable in freshwater systems (e.g., pH, hardness, alkalinity) than in seawater systems. Parameters that are likely to change quickly and can cause acute stress in fish (e.g., pH, temperature, ammonia, nitrite) should be checked several times per week and other

Figure 5.3 Picture shows a drum filter to remove solid material from the water line, two UV light sterilizers (mounted on wall), and an air blower for supplying air to each tank. The large UV unit contains 8 tubes (40 W each) and the small unit under the plywood contains two tubes (200 W each). These components are for the tank array in Figure 5.2.

parameters (alkalinity and hardness) may be checked less frequently (e.g., once or twice per week). All values should be recorded in a notebook or computer spreadsheet. Water temperature should be maintained at an appropriate level for the species of choice and it should be checked several times per day, preferably on a continuous basis.

Many of these parameters can be determined with electrodes (pH, oxygen content) or colorimetric test kits (hardness, ammonia, nitrite, alkalinity). Corrective measures must be taken if any of the parameters are outside the accepted range. The values in Table 5.2 are for juvenile chinook salmon, different values for each parameter may be required for other species. Any corrections made to water quality should always be done in a cautious manner to minimize any large changes in the parameter being optimized. The parameter being changed should be measured within 1 h after corrective action is taken and every hour thereafter until the parameter value has stabilized. Oxygen content should be monitored continuously until optimized. If a flow-through seawater system is being used for delivery, it is the discretion of the researcher to determine how to optimize the parameter (e.g., increase flow if oxygen content is low, addition of a charcoal filter if ammonia is high, use chillers to reduce temperature).

I.B Water flow rate

The flow of water will depend on the biomass in the tank. For the incubation trays, a flow sufficient to keep the eggs at the proper temperature should be maintained. For the small salmonids (<10 g), Heen et al.[7] recommend 0.3–1.0 l/min/kg fish, for temperatures found in Table 5.2. In our laboratory, we have found that a flow rate of approximately 2–4 l/min for the 1.3-m-diameter tanks (500 l) with 75 fish is adequate to maintain proper water

Table 5.2 Example of water quality parameters for juvenile chinook salmon in a freshwater recirculating system

Parameter	Minimum limit	Maximum limit	Optimum value
Ammonia	0 ppm	0.5 ppm	<0.1 ppm
Nitrite	0 ppm	0.5 ppm	<0.1 ppm
pH	6.5	8.5	7.8
Hardness	50 ppm	200 ppm	125–150 ppm
Alkalinity	50 ppm	150 ppm	75–100 ppm
Temperature	8°C	12°C	10–11°C
Oxygen (saturation)	70%	100%	95–100%

quality. For our larger 2-m tanks (2000 l) with up to 2000 juveniles (4–10 g), we maintain a flow rate of at least 40 l/min to maintain the oxygen content above 85–90% saturation. In each of our tanks, we have a ''spray bar'' connected to the inflow hose. This is a section of PVC pipe (2 cm diameter by 30–50 cm length) with approximately 8–10 holes (0.6–1 cm) drilled in a line. Water exits the spray bar at a high rate into the tank, which helps aerate the water (Figure 5.2). The jets of water are set at about 45° to the water surface and help to create a current in the tank that the fish orient to and swim against. As a fail safe measure, a sufficient number of airstones should be placed in each tank to ensure that the oxygen content does not fall below 70% saturation at any time, especially in the event that the recirculating or flow-through delivery system fails (Figure 5.2). This should be tested periodically by shutting off the water flow to a tank with fish and recording the change in oxygen content. Corrective measures may be needed (e.g., more air stones or a more powerful blower).

I.C Light

If the tanks are in a building, the light will be artificial. It is important to have adequate light in the building and bulbs that reflect the natural spectrum as close as possible. The daily light schedule should match that of ambient daylight. Ideally, the light fixtures will be on timers and rheostats that will gradually increase and decrease the light level.

I.D Feeding fish and increasing food ration

Fish in culture and those in the experiment are fed 5–6 times/week. Generally, an appropriate amount of food will be weighed and given to the fish in 2–3 feedings over the day. Personnel feeding the fish should take all the necessary precautions to avoid contact with the food containing toxicants. The amount of food placed in each tank is usually the same for a 1-week period and is based on the total biomass and the selected feeding rate (e.g., 2.0% bw/day). At the beginning of the second week, the amount of food distributed to each tank is increased to reflect an increase in fish biomass. The rate fed will be the same 2.0% bw/day, and the new total biomass is based on a growth curve projection from previous studies or actual data. The actual data can be obtained from total biomass weights that are determined from a separate ''growth control'' tank of fish that is treated identically as the experimental tanks but fed control food (solvent treated). Weekly or daily growth increments and increased ration amounts may also be calculated

with equations similar to those used in financial applications to determine compound growth.

I.E General husbandry

Each tank should be inspected daily for dead fish and checked for properly working spray bars, airstones, and drains. If any abnormalities are observed, they should be corrected immediately. Dead fish are removed daily and recorded for each tank and the data used in the statistical analysis. Once per week, excess food and fish waste should be removed from the bottom of each tank. This can be accomplished by gently breaking the waste loose for suction down the drain or by a separate device that will siphon or suck the material out of the tank. If the fish appear lethargic or mortalities increase independent of toxicant treatment, a fish health specialist should be consulted immediately. The specialist may recommend fish food with antibiotics, which can be purchased from feed manufacturers, or possibly a formalin dip if external parasites are evident. There are several references (e.g., Stoskopf[8]) with additional information on treatments for fish diseases. It is also recommended to have floor mats (specially designed) with disinfectant at the entrance to the fish holding/experiment area to reduce the transfer of pathogens. In addition, materials that come in contact with the fish (e.g., buckets and nets) should also be disinfected after each use.

The size of the experimental tank is also an important consideration. We have found the 1.3-m-diameter circular tank to be ideal because of its volume and the ability to allow a circular current for the fish to swim against. In the past, we conducted experiments in 0.7-m square tanks (approximately 200 l) that were shown in a paired comparison test (unpublished) to cause increased mortality in juvenile chinook salmon. This increased mortality was likely due to increased stress caused by crowding and the lack of a circular current.

I.F Miscellaneous

The building that houses the tanks should be secured such that predators and pests (e.g., cats, otters, rats) cannot enter. If there is evidence of such animals in the building, they should be trapped and removed. Sensors to detect system malfunctions are highly recommended. In our laboratory, temperature probes are connected to a computer that can notify personnel by auto dialer and pager if values occur outside a preset range. We also have flow meters that connect to our security system, which is monitored continuously. Any interruptions in flow are detected by the security monitoring company, which can initiate a call to the appropriate personnel.

Appendix II Method of weighing fish

Fish can be weighed as a group for determining total biomass, as individuals when distributing to experimental tanks, or when assessing the frequency distribution of their individual weight. Weighing individual fish can be done by two different methods. One method is to narcotize the fish and weigh in a tared plastic container on a top-loading balance. Another method of weighing is by water displacement, which is less stressful on the fish and minimizes handling.

For the first method, a narcotizing agent, such as tricaine methanesulfonate (also known as MS-222), is used to immobilize the fish. For juvenile chinook salmon, we have used a solution of 125 mg/l, which takes approximately 5 min to affect the fish. This narcotizing agent is difficult to work with because it suppresses the respiratory system and fish can die if exposed too long. Research has also shown that MS-222 may adversely affect physiological parameters[12] and possibly the toxicological response of the fish.

For the water displacement method, we use a Sartorius 4000 balance that weighs items up to 50 kg with a 0.1-g accuracy. A 20-l bucket with approximately 15 l of water is placed on the balance and tared to 0.0 weight. Fish are netted and placed in the bucket as a group or individually to obtain their weight. The net with fish is allowed to drip for a short period of time (e.g., 3–5 s) such that very few, if any, drips fall from the net. This will ensure relatively accurate weight of each fish being added to the container of water. At the beginning of each day when fish are weighed, each scientist weighs one fish 3 times to assess variability in their technique. This information can be used as a quality control guide for fish weighing.

For all weighing methods that hold fish in buckets, airstones must be present to keep oxygen content between 70% and 100% saturation and fish biomass kept below 20 g/l. Oxygen content should be checked frequently with an oxygen meter to ensure adequate levels (70–100% saturation) for fish respiration. Fish are generally held in the buckets for no longer than 20 min. When this time limit is reached, the bucket with fish is gently poured into the holding tank from where they originated or a suitably prepared tank for long-term holding or experimentation.

Appendix III Statistical design and analysis

III.A Determining the number of experimental tanks

The first step is to determine how many treatment concentrations are to be tested. This usually requires some previous information regarding the expected response for a given exposure concentration, such as previous studies in your laboratory or literature values. If this type of information is not available, a range-finding assay can be conducted to provide a crude estimate of the concentration needed to produce an adverse effect. This can be accomplished with one or two replicates for each order of magnitude increase in exposure concentration (e.g., 0.1, 1, 10, 100 μg/g of food). This design will likely produce results with no response for the lower concentrations and a 100% response for one or more of the higher treatment concentrations. Once an adverse exposure concentration range is determined, a more refined experiment can be designed with smaller differences in exposure concentrations (e.g., 2- or 3-fold increases) that will likely to provide partial responses for one or two of the exposure concentrations (e.g., 25% reduction in growth). Statistically, it is advantageous to use the same factor difference between exposure concentrations, the same number of replicates per treatment, and to have partial responses. The number of fish (sampling unit) per replicate (experimental unit) will depend on various parameters for the test species, such as accepted biomass per liter, behavioral interactions, the number of desired samples, and long-term goals.

To determine how many replicates are needed for each treatment, a discussion regarding the ''power of the test'' is relevant. It should be noted that independent replicates are required for hypothesis testing and inferential statistics. It is often tempting to have only one or two tanks (experimental units) and measure some response in several individuals and call these ''replicates'' (i.e., the sample size (n) becomes all measured

values). Using data from several individuals from one tank would be pseudoreplication because they are not truly independent.[13] For testing the hypothesis that there are no differences among treatment means ($\mu_1 = \mu_2 = \mu_3 = \mu_n$), each tank would be a replicate and one data-point (e.g., mean weight) from that tank would be used for the analysis.

Power is the ability to avoid false negatives or type II errors. A false negative is equivalent to finding no effect when in fact one actually exists. Also, a false negative is accepting the null hypothesis (H_0: $\mu_1 = \mu_2 = \mu_3 \cdots$,) of equal treatment means when you should reject the null hypothesis and is determined by β, the type II error. In many experiments, β is high, meaning there is a high probability that the null hypothesis will not be rejected. A power analysis is highly dependent on the experimental variance, which may be higher or lower than estimated for a proposed experiment. The variance that determines power is the variance among tank means within treatments. The actual power analysis cannot be conducted until after the experiment is performed. Because the variability between replicates cannot be known before the experiment is conducted, maximizing the number of replicates in the experimental design is advantageous to assure sufficient power of the test. An example of the expected power for a typical experiment follows.

Example:
Treatments (k) = 9, replicates (n) = 5, total tanks (N) = 45, $\alpha = 0.05$, $v_1 = 8$ degrees of freedom (df) = $k - 1$, $v_2 = 36$ df = $k(n - 1)$, σ^2 (mean square error) is estimated by $s^2 = 1.4\,\mathrm{g}^2$ (from previous experiment); δ = minimum detectable difference = 3 g (a value based on previous work to maximize the power of the test):

$$\phi = \sqrt{\frac{n\delta^2}{2ks^2}} = 1.33.$$

Once the quantity ϕ (phi) is calculated, the power of the test is then computed for a specific difference. From a statistical table (e.g., Figure B.1h in Reference 14), this ϕ value equates a power of 0.7. This is equivalent to saying that there will be a 30% chance of committing a type II error in this analysis. Compared to other biological experiments, this power is acceptable.[15] If only 4 replicates were used, the power would drop to 0.67 and it would increase to 0.72 for 6 replicates.

To determine the number of replicates for each treatment, the following equation for independent samples can be used[16];

$$N = \frac{(Z_\alpha + Z_\beta)^2 2\sigma^2}{\delta^2}$$

Z_α and Z_β are the probability associated with one tail of the normal distribution. For a two-tailed probability, use $Z_{\alpha/2}$. For example, using a type I α error rate of 0.05 and a type II β error rate of 0.2 (20%; which equals a power of 80%), $Z_{0.05/2} = 1.96$ and $Z_{0.2} = 0.85$ for a two-tailed test. As suggested by Steel and Torrie,[16] because the variance is often not known, it would be advantageous to define the minimum detectable difference in terms of the SD. If the SD is set to 1, $2\sigma^2/\delta^2 = 2$, our result for a two-tailed test would be $N = (1.96 + 0.85)^2 * 2$ or a sample size of 15.8 (round up to 16). For most experiments, this is far too many replicates for the available resources. By adjusting the minimum detectable difference, type I or type II error rate, or the experimental variance, the number of replicates needed for avoiding type II errors can be reduced.

There is nothing sacred about using $\alpha = 0.05$ for determining statistical significance. Because α and β are inversely related for a given sample size, choosing an α of 0.10 would decrease β and increase the power of the test. For many situations, a higher α value is desirable to assure environmental protection (see an in-depth discussion by Peterman[15]).

By weighing several fish from each tank, we may have much more information that may be used to determine the power of the test; however, a statistician should be consulted if this approach is used. By taking multiple samples per tank (e.g., 15 fish), we may be able to consider the power analysis in terms of composite sampling. Recent research has provided formulae for calculating power when composites are used.[17] According to the method provided by Edland and van Belle,[17] the power for our example above would be approximately 0.88, or there would be a 12% chance of making a type II error. This would be for a smaller minimum detectable difference (= 0.6 g). Predictions for power using the same design but with 4 replicates would yield a power of 0.80. Six replicates would boost the power to 0.95.

III.B Randomization of fish and treatments

It is crucial that each replicate in the experiment is treated identically. Because gradients exist, a simple way to minimize the impact of all uncontrolled factors on the dependent variable is to randomize individuals over treatments and experimental units. When designing an experiment, there are many physicochemical and biological factors, such as light, temperature, salinity, food ration, behavior, and organism interaction that must be considered. These factors are either controlled, i.e., included in the experimental design, or their influence is minimized.

The experimental units in the study are the tanks, and these can affect the dependent variable in a variety of ways. The most obvious sources of tank effect are location and the order that the tanks are filled with fish. For example, tanks closest to an exterior wall may experience greater temperature fluctuations, light levels, or disturbance than the interior tanks. Also, the first tanks filled may contain fish that are less robust than fish placed later in the tanks because the ''weaker'' ones may be captured more easily from the larger pool of fish. To avoid confounding these effects with treatment effects, the order that the tanks are filled should be done randomly with respect to location.

As described in step 6 of the section ''Distribution of fish to experimental tanks,'' we add fish in three groups of 25 to the experiment tanks. A recent study that examined the statistics of various random allocation schemes of organisms to experimental chambers (6 treatments, 4 replicates, 10 fish per tank) found that the best scheme was to randomly add each individual to tanks over all treatments.[18] The authors also concluded that a scheme that randomly allocates all fish or half of the total to each tank over all treatments also produces acceptable statistical results. Our scheme for allocation is similar to the latter one recommended by the authors and is more appropriate for experiments with large numbers of fish.

After fish allocation is completed, the next step is to randomize treatments (and replicates) among the experimental units (tanks). The treatments must be randomized in regard to the order the tanks were filled because of the potential for interactive effects of the toxicant with some factor related to the order of tank filling. Each tank is assigned to a treatment in a random fashion until all treatments and replicates are determined.

The order by which fish and treatments are randomized is accomplished with a random number generator. These can be obtained from statistical tables (e.g., statistical tables of Rohlf and Sokal[19]) or from a spreadsheet (e.g., Microsoft Excel).

III.C Analyzing the results

While it might be tempting to weigh only a subsample of all fish in a tank, there is usually such high variability among fish weights that a relatively large proportion of the total would be required for sufficient representation. This, in addition to occasional non-random sampling, argues for weighing the entire population of each tank.

While we do not measure length in the beginning of the experiment, it is useful to determine length at the end. Using length and weight, a condition factor (K) can be calculated that will provide information on the general health of the fish. Comparing K values among treatments can be an important endpoint for assessing toxicant impacts.

$$\text{condition factor } (K) = \frac{\text{weight}}{\text{length}^3} * 100$$

Weight is in grams and length is in centimeters. For most salmonids, a value of 1.0 is considered healthy.[20] See Reference 20 for a discussion on condition factor for other fish species.

Several types of analyses would be appropriate for analyzing the results of a growth study. The two basic approaches are ANOVA and regression analysis.

ANOVA

The first step is to test the assumptions of the ANOVA. (That is: Do these data come from a normal distribution and are the variances homogeneous?) References on basic statistical analysis[14,16] will have information on how to determine if the data are suitable for ANOVA and, if not, how data transformations may help. If the fish are weighed just once, a univariate ANOVA consisting of treatment concentrations (independent variable) and fish weight (dependent variable) will suffice. A repeated measures ANOVA would be statistically appropriate if fish weights are taken at several time points from the same fish. *Post hoc* tests, such as Scheffe's or Student–Newman–Keuls, can be run to determine which pairs of treatments are significantly different from each other. Dunnett's test, which generates p-valves for comparisons involving the control to each treatment, may be a more appropriate *post hoc* test.

The ANOVA can be run on the tank weight means or growth rate constants, if several weights are obtained at several time points. Growth rate can be determined with the equation

$$W_t = W_0 * e^{k_g t}$$

where W_t and W_0 are tissue weights at time t and time 0, k_g is the growth rate constant in units of d^{-1}, and t is the specified time interval.

Regression analysis

Many different types of growth curves are possible[20]. For many, standard regression analysis can be performed and their slopes tested for significant differences. All intercepts will be the same because the experiment will have started with no significant differences in fish weight.

Growth and mortality data can be analyzed by generalized linear models (GLM) to produce LCp, ECp, LRp, and ERp values. LCp and ECp statistics are point estimates that define the lethal and sublethal (i.e., growth) exposure concentration for a given proportion of the population. LRp and ERp values are also point estimates for lethal and sublethal responses that are based on tissue concentrations (residue). The point estimate for the degree of growth inhibition (e.g., EC10, ER25) can be determined with equations provided by Bailer and Oris.[21] Growth, which can be exponential, would be modeled as:

$$\text{net growth } (g) = e^{(a+bx)}$$

where x is the fish food or whole-body tissue concentration, and a and b are coefficients determined by GLM using a log link function and a gamma error distribution. The confidence intervals for these growth ECp and ERp values can be determined with the delta method.[22] If mortality occurs, GLM equations can also be used to generate point estimates[23] (e.g., LC10, LR50), which are more accurate than standard probit methods.

Appendix IV Fish food

IV.A Fish food for experimentation

It is desirable to keep the total fat content of food given to experimental fish low and close to that found in wild fish and their prey. A diet that is artificially high in fat will produce fish with excess body fat[24] that may lead to physiological abnormalities. This is also important for toxicity evaluation because body fat can affect the toxic response for hydrophobic contaminants.[25,26] These studies identify an inverse correlation between the toxicity response and lipid content (higher lipid reduces the response), implying that studies with laboratory fish fed high fat commercial diets may underestimate the toxic response. This is especially important for studies that attempt to mimic the toxic response in juvenile salmonids in an estuary. As these fish transit from freshwater to seawater (smoltification) their whole-body lipid content falls to low levels (5–10% dry weight).[27–29]

Juvenile salmonids generally prey on invertebrates[30] that contain variable amounts of lipids. The lipid content for polychaetes and molluscs are generally in the range of 5–10% dry weight[30,31]; however arthropods (insects and crustaceans) can be more variable and substantially higher for some species.[30] Commercially prepared fish food for salmonids is usually very high in total fat content (15–25%), which causes the lipid content of juvenile fish to increase beyond that found in wild fish.[24,29] Additionally, commercially prepared feeds can contain relatively high levels of contaminants, such as PCBs, DDTs, and PAHs.[32,33] In order to mimic as closely as possible the food that a wild fish would be obtaining, it may be necessary to formulate your own fish pellets or contract a laboratory or wholesaler to make it to your specifications. For studies using juvenile salmonids, a lipid content of approximately 7–10% dry weight is desirable to mimic natural prey.

Prepared fish food can be purchased from one of several manufacturers. The basic ingredients (fish meal, vitamins, and binder) can also be obtained from some of these manufacturers, and pellets can be made in the laboratory with the appropriate machinery. We prefer to use cod liver oil purchased from a health food store because we have found it to be very low in contaminants, such as PCBs. We have found that by making our own fish food, the total fat content can be minimized (e.g., 7–10% of dry weight) to mimic natural prey levels.

When juvenile fish weigh approximately 2 g, they should be switched to low fat pellets to allow their body lipid composition to reflect their diet before they are fed dosed food. This will continue until the experiment begins, at which time fish will be fed with the same low fat food that has toxicants added. It would be advantageous to sample a few fish occasionally from the common pool of fish and conduct a proximate analysis[27,30] to specifically determine lipid content. Ideally, experimental fish that are in the presmolting or smolting phase should have whole-body lipid levels in the 5–10% (dry weight) range. Lipid content in the fish pellets should be maintained above 6.5% (dry weight) to assure adequate energy and growth for juvenile chinook salmon.[30] If the experiment is conducted past the smolt phase, the lipid content of the feed should be increased to match the life stage and trophic level at which the fish would be naturally feeding.[30]

IV.B Dosing the fish pellets

It is crucial that all preparations of fish food are treated identically to avoid confounding factors. The only differences among treatments should be the amount of toxicant being tested. Ideally, all food should be dosed in one batch to avoid batch-to-batch variation. Testing the dosed food before the experiment starts is required. A simple test with several fish is required to ensure there are no problems with pellet size or palatability of the food, especially at the high doses. Depending on the toxicant of interest, fish pellets with high concentrations may be rejected by fish.

The main consideration for dosing fish pellets is how to get the toxicants on the food. Toxic elements (metals) may be dissolved in acidic water and added to the dry ingredients during formulation of the fish pellets. One important aspect for testing the toxicity of an element is the species (form) that is added to food (e.g., inorganic versus alkylated, metal valence, redox state). For organic toxicants, non-polar solvents are often required to dissolve the compound to assure uniform amounts on the pellets. Some of the solvents used by researchers to add organic toxicants to fish food include fish oil, dichloromethane (methylene chloride, $MeCl_2$), petroleum ether, acetone, and hexane.

Methylene chloride is a popular solvent because it is non-polar. It is advantageous over a more polar solvent, such as acetone, which may soften the fish pellets and cause them to disintegrate. Disadvantages of using $MeCl_2$ include its toxicity to humans and emissions as it volatilizes. Other solvents may be used, depending on the toxicant of interest. Fish oil may be preferred for some contaminants; however, non-polar solvents may be necessary for the more hydrophobic test compounds. We have found that $MeCl_2$ works very well for PAHs and PCBs because the more hydrophobic congeners in these groups are difficult to get into solution. At least one study has looked at the effects on fish growth from treating fish pellets with $MeCl_2$.[34] These authors found no statistical difference in fish growth when comparing $MeCl_2$ treated pellets with unadulterated pellets.

In our laboratory, we have noticed that the moisture content of the fish pellet can change during the dosing procedure, which may be important for comparing growth between treatments and controls (solvent control and regular control, if used). Because the amount fed per day is based on a percentage body weight of fish and a consistent dry weight to wet weight ratio for the fish pellets, each batch of pellets should be checked for moisture content. If differences are found, adjustments in the amount fed should be made so a consistent amount of food (based on dry weight) is given to each group of fish.

The first step in preparing dosed fish pellets is to make a concentrated stock solution of the selected toxicant. For example, if dosing fish pellets with Aroclor 1254 (a mixture of PCB congeners), a stock solution of 4000 μg/ml would be reasonable. Based on the density of Aroclor 1254 (1.5 g/ml) it would take 0.133 ml in 50 ml of an appropriate solvent, such as $MeCl_2$ to make a stock solution of 4000 μg/ml. The stock solution is kept in an appropriate sized Erlenmeyer flask (with glass stopper) in the dark. The same approach can be taken with toxicants that occur as powders, which are weighed out on a balance and added to the $MeCl_2$ solution.

One approach for dosing the fish pellets with organic toxicants is to add an appropriate amount of food to a stainless steel bowl. In our studies, we routinely dose a batch of 3500 g of fish food, which is enough to feed 150 fish (10 g each) at 2% bw/day for 45 days. After the food is weighed out and placed into the bowl, an appropriate amount of stock solution is added to a 4-l bottle of $MeCl_2$. For example, to generate a PCB dose of 1.5 μg/g on the fish pellets, one would pipette 1.31 ml of the stock solution (see earlier) into the 4-l bottle. The solution is shaken, allowed to equilibrate for at least 10 min, and then the entire bottle is poured onto the pellets in the stainless bowl. The size of the bowl should be large enough to hold the entire batch of food but allow the solution to completely cover the pellets.

This mixture is stirred with a large metal spoon for 1 min initially, for 1 min after a 1-h period, and then for 1 min on the following day. Progressing from low to high doses and rinsing the spoon with $MeCl_2$ between bowls will reduce cross-contamination. All bowls are left under a fume hood and the solution is allowed to evaporate for approximately 1 week, which is usually sufficient for dryness. After this time, the fish pellets are placed into a large plastic or stainless steel tub lined with aluminum foil and allowed to dry for another 24 h. Once dried, the pellets are placed into a plastic tub with a tight-fitting lid and kept in a freezer at −20°C until needed to feed fish. A comparison of our nominal concentrations for this procedure to the analytical results for fish food and tissue concentrations have shown a high degree of success for this method.

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chapter six

Field experiments with caged bivalves to assess chronic exposure and toxicity

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Applied Biomonitoring

Contents

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Introduction

This chapter reviews the use of field experiments with caged bivalves as an evolving technique in aquatic toxicology to assess chronic exposure and toxicity. The caged bivalve approach has become more feasible, practical, and routine as a result of experience and expertise gained in a number of developing paradigms, including (1) ecological risk assessment, (2) tissue residue effects approaches, and (3) bivalve biomarkers. Ecological risk assessment has helped focus a more equal emphasis on the importance of characterizing exposure and effects, tissue residue effects approaches have facilitated the utility of tissue chemistry data from laboratory and field studies, and bivalve biomarkers have helped characterize and understand the biochemical processes linking exposure and effects.[1] While there are no ''ideal'' indicator organisms, bivalves have many attributes necessary for effective sentinels of exposure and effects in environmental monitoring and assessment programs in aquatic toxicology.[2]

Nearly all clinical measurements (e.g., histological, biochemical, and physiological) used in laboratory tests are applicable to field testing, but their limited application in the field is due to a lack of understanding of the utility and knowledge in the flexibility of these diagnostic tools.[3] We believe that the same is true for caged bivalves. While caged and indigenous bivalves have been used extensively in ''Mussel Watch'' programs to quantify chemical exposure, they have been used less frequently in effects-based monitoring programs. Nevertheless, caged bivalves are equally suited to characterizing exposure and effects. Caging facilitates exposure and effects measurements. Caging also facilitates the utility of mussels as monitoring and assessment tools for several reasons. These include a well-defined exposure period, comparisons between beginning- and end-of-test measurements, and experimental control of size range, exposure history, and genetic makeup of the test animals. Therefore, field experiments using caged bivalves utilize many characteristics of traditional laboratory bioassays in terms of experimental control and many characteristics of field monitoring in terms of environmental realism.[4] Strategic deployment of caged bivalves along suspected chemical gradients can help identify the source of chemicals in the water column and the bioavailability of sediment-associated chemicals. Transplant studies have been used successfully to identify the fine vertical structure of bioaccumulation and associated bioeffects in the water column when the cages were separated by as little as 2 m vertical distance.[5–8]

McCarty[9] proposed developing a single bioassay methodology that includes an integration of tissue chemistry and effects measurements. Bivalves are well suited for this approach because they integrate external chemical exposure as they filter the water for food. With respect to the methods described here, the most important attribute may be their ability to be easily collected, measured, caged, and transplanted.[4] Since the gill is both a respiratory and food-collecting organ, chemical exposure in the field includes both waterborne and dietary exposure pathways, an element often missing from laboratory exposures. Parrish et al.[10] suggest that the real variable in risk assessment is exposure rather than toxicity, and that shifting from laboratory tests to field testing could reduce the uncertainty in the assessment. We believe that the variability in laboratory measurements of exposure and effects are inexorably linked, and that field experiments are necessary to bridge the gap and establish links with more traditional assessment methods.

The inclusion of more sophisticated measurements, such as biomarkers, in field experiments further increases the ability to bridge gaps between exposure and effects.

Many biochemical methods developed for marine, estuarine, and freshwater bivalves can be performed quickly and inexpensively. Some of those developed for reproduction (e.g., the vitellin assay) give an useful estimate of reproductive condition in a relatively short period of time.[11–13] These biochemical measurements be supplemented with other observations (e.g., sex reversal which is a more direct method of demonstrating possible endocrine disruption) to produce more conclusive information on effects.[14]

Aquatic toxicology would be enhanced by using a more ecological risk assessment-based approach that includes field experiments. The methods described here are important to aquatic toxicology because they provide a standardized field approach to implement long-term, chronic testing.[4] We believe that most toxicity and bioaccumulation tests conducted in the laboratory[15–18] can be conducted in the field, and that field experiments are necessary to increase the utility of the data in terms of predicting effects and establishing causality. The purpose of this chapter is to advance the knowledge of practitioners, and promote field experiments with caged bivalves as a useful, cost-effective, and practical diagnostic tool in aquatic toxicology.

Test organisms and materials required

Recommended test organisms

Recommended test species include (but are not restricted to) those shown in Table 6.1. These species have been used more than any other bivalve species but because of the similarity in bivalve shape and structure, almost any bivalve is suited to caging and transplantation.

Source of test organisms

Most marine bivalves are bottom-dwellers that can be easily collected in shallow water by wading or by divers or dredging in deeper water. Some marine bivalves are routinely cultured, and culturing facilities can generally provide test animals of uniform size. Obtaining bivalves from a culturing facility eliminates uncertainties associated with age, genetics, and previous exposure history. Many freshwater bivalves are becoming more difficult to collect in large numbers because of their threatened or endangered status. Regardless of whether marine or freshwater bivalves are being collected, a permit will be necessary for collection and transplantation because of concerns regarding introduction of exotic species.[4]

Table 6.1 Species most commonly used in field testing

Marine bivalves		Freshwater bivalves
(1) Family Mytilidae	(3) Family Ostreidae	(1) Family Unionidae
Mytilus californianus	*Crassostrea gigas*	*Elliptio complanata*
Mytilus edulis	*Crassostrea virginica*	*Pyganodon grandis*
Mytilus galloprovincialis	*Ostrea edulis*	(2) Family Corbiculidae
Mytilus trossulus	(4) Family Tellinidae	*Corbicula fluminea*
Perna perna	*Macoma balthica*	(3) Family Sphaeridae
Perna viridis	*Macoma nasuta*	*Sphaerium simile*
(2) Family Pectinidae	(5) Family Veneridae	(4) Family Dreissinidae
	Mercenaria mercenaria	*Dreissena polymorpha*

It is recommended to sample a number of surrogates at the beginning of the test to establish their condition and to quantify the concentration of chemicals in their tissues to assess their chemical exposure history. It is usually preferable to transplant bivalves to test areas as soon as possible after collection because laboratory holding can also induce stress to test animals. The spawning period should be avoided because of potential effects on bioaccumulation and growth. If it is necessary to hold bivalves for extended periods in the laboratory, they must be fed, even if unfiltered water is used in the holding tanks.[4]

Materials required

The supplies and materials shown in Table 6.2 may be required to conduct a field experiment with caged bivalves. The quantity of each item depends on the number of bivalves used and the study design.

Table 6.2 Materials and supplies commonly used for field testing with caged bivalves

Field supplies	*Decontamination supplies*
Aluminumfoil	Brush, scrub
Buckets, 1-gal	Biodegradable cleaning solution
Buckets, 5-gal	Distilled water
Cable ties, 14-in.	Weigh pans
Cable ties, 4-in.	*Data sheets and forms*
Cable ties, 8-in.	Chain of custody forms
Calipers, field-plastic	Electronic data sheets
Clip boards	Hard copy data sheets
Compartmentalized trays	Extra hard copy data sheets
Cutting boards (plastic/glass)	*Sample jars*
Deployment line	Sample jars, supplied by analytical lab
Distribution rack	Sample jar labels
Extension cords	Tamper-proof tape
Forceps, plastic	*Electronic gear*
Gloves, surgical	Balance, electronic
Ice chests	Calipers, electronic
Labels for mesh bags	Computer, portable
Mesh bags	Data transfer hardware
Trays, flat w/grid	*In-situ* temperature monitoring devices
Paper towels	Surge protectors
Pliers, cross-cut (small and large)	Power strips
Predator mesh	*Miscellaneous*
Scalpels/knives (stainless/ceramic)	Tables
Scissors	Chairs
Tarp	Bottle to stretch bags
Trash bags	Identification tags for cages
Tubs, bus	Tape, duct
Weights/anchors, cinder blocks	Tape, electrical
Ziploc® polybags	Marking pens, fine and thick point

Procedures

Data set

Controlled experiments with caged bivalves under field conditions facilitate the simultaneous collection of data to help characterize chemical exposure and associated biological effects in the same organism and at the same time. The procedures outlined here are designed for collecting measurements of bivalve shell lengths, whole animal wet weights, tissues, and shell weights. These field measurements are used to characterize effects due to exposure at the deployment site. The tissue chemistry data, reported by an analytical laboratory, are used to characterize exposure conditions. If *in-situ* temperature monitors are used, these data can be used to help characterize other factors that may be affecting bivalve growth and response.

Exposure chambers

Although it is possible to mark individuals, cages with individual compartments are generally recommended for field studies with caged bivalves to minimize potential interferences, standardize the procedures, and minimize the time associated with handling individuals. For long-term exposures to characterize sediment, compartments may not be appropriate. The basic concept behind the cage design is to maximize water flow to the test animals, while containing test animals within the cage and ensuring that each individual has the same water exposure. Cages can be flexible material with compartments attached to a rigid frame (i.e., mesh bags attached to a PVC frame or heavy plastic mesh), rigid with fixed compartments (i.e., plastic trays or wire baskets with internal divisions), or a benthic cage where test animals are held in contained sediment without compartments (Figure 6.1). The separation of test animals into individual compartments allows equal exposure to each bivalve. Compartmentalization also facilitates tracking individuals throughout the test and eliminates the need to mark or notch individuals.[4] Although cages without compartments are not recommended for most water column exposures because of the potential for clumping of individuals and uneven exposure conditions, a ''bucket'' or benthic cage is appropriate for long-term exposures where the bivalves must have the ability to migrate within the surficial sediments.[19,20]

Figure 6.1 Cage examples: (A) compartmentalized flexible mesh; (B) compartmentalized rigid; (C) non-compartmentalized benthic.

Flexible mesh bags and rigid PVC cage construction

The dimensions of the flexible mesh bags and the PVC frames depend on the species, size of the individual test organisms at the start of the test, expected growth, and the number of organisms per cage. Oyster culch netting, similar to that used in bivalve aquaculture, is recommended to make the mesh bags. This netting is available in many diameters and mesh sizes, comes in tubular form, and can be cut to length as needed. An approximate 6-in. diameter mesh material is recommended for smaller smoothed-shelled species like mussels and clams because there is less mesh at the point of constriction. For larger bivalves with rough shells and irregular shapes, such as oysters, a tubing of larger diameter should be used. Individual compartments are created by separating the bivalves within the mesh bags with a plastic cable tie or other restricting device. Sufficient space should be provided in each compartment to allow test animals to grow during the exposure period. The mesh bag should be long enough to accommodate the desired number of bivalves per bag plus sufficient material to allow secure attachment to the PVC frame. Approximately 30 cm of mesh netting on either end of the bag is generally sufficient for attachment to a PVC frame constructed from 1.9 cm diameter material.[4]

For most applications, schedule 40 PVC pipe (3/4 in diameter) is sufficient for frame construction (Figure 6.2). Practical frame sizes range from 30 cm × 30 cm to about 20 cm × 100 cm. To minimize crowding and possible damage to bivalve shells, the PVC frame should be approximately 5 cm longer and wider than the space occupied by the bivalves once placed in the mesh bag. To remove any water soluble and/or volatile chemicals, the frames should be soaked, preferably in flowing fresh or seawater, for at least 24 hours after construction. Number each cage with an indelible marker to facilitate attachment of mesh bags.

Negative buoyancy can be achieved by drilling the frame approximately every 25 cm. with a hole between 1/8 and 3/16 in. to allow water to enter the pipe and remove trapped air. The corners of the frame should not be drilled to eliminate the potential for weakening the overall structure of the frame. Frames that are not drilled may tend to float.

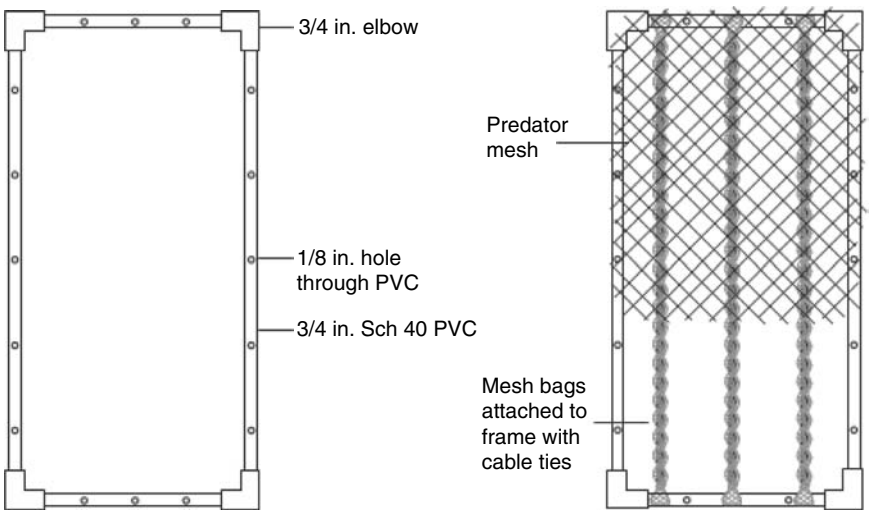

Figure 6.2 Supporting frame with and without predator mesh.

Because each PVC frame will hold one or more mesh bags containing bivalves, each mesh bag must be labeled with an identification tag. Durable plastic or other inert material is recommended. For consistency and ease in identifying the beginning of a bag, it is recommended that the bag be knotted approximately 23 cm from the end and the tag be attached near the knot with a plastic cable tie. Tags should include both the cage number and the bag number, i.e., a label of 9–3 indicates that this is the third bag assigned to Cage #9.

Benthic cage

The overall design of the benthic cage (Figure 6.3) includes a solid outer container and an inner mesh chamber held in place with plastic cable ties.[19,20] Mussels are confined by this mesh insert; there are no individual compartments. The bottom of the mesh chamber is lined with biodegradable newspaper, and the mesh chamber is then filled with clean sand. The newspaper helps retain the clean sand during set up and deployment. Mussels are distributed evenly on top of the clean sand in the benthic chamber before deployment. The benthic cage is buried in sediment with only the top 15 cm exposed. Sediment trapped by the cage will be retained by the solid outer walls.

Conducting the tests

Pre-sort

To minimize the potential for size differences among cages at the start of the test and to achieve a more even size distribution, the bivalves should be pre-sorted into size groups, with each size group in its own container. Sorting can be based on either shell length, as determined with vernier calipers with a measurement accuracy of 0.1 mm, or whole-animal wet-weight, as determined with an analytical balance with a measurement accuracy of 0.01 g. For most species, the pre-sort is based on shell length, with size groups in 1-mm increments (e.g., 50, 51, 52 mm, etc.). Measure the shell length and place the individual in the appropriately labeled bucket or holding container. The bivalves should be kept moist and cool by using wet ice and moist paper towels as necessary. They should

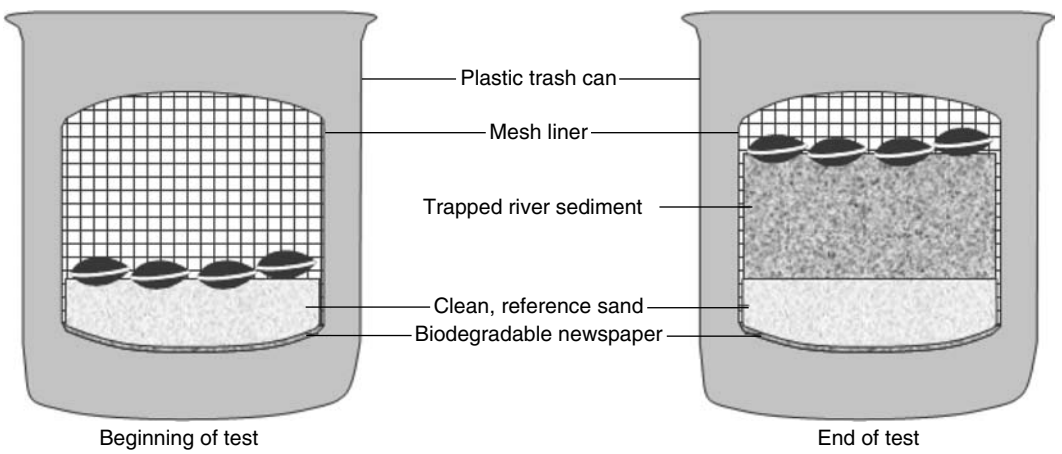

Figure 6.3 Detailed diagram of benthic cage.

be handled as carefully, gently, and quickly as possible so that animals are not unnecessarily stressed. Water should not be placed in the holding containers during the pre-sort because it could lead to oxygen-deficient conditions. The bivalves should be kept out of direct sunlight to eliminate heat stress. The bivalves can be held in an ice chest with wet ice if air temperatures are excessively warm.[4]

After the pre-sort, count the number of bivalves in each size category. To achieve the goal of starting the test with bivalves of a similar size, identify the smallest series that contains the number of bivalves required to initiate the test. The overall target is a 5- to 10-mm range for the size of bivalves used (i.e., 20–25, 33–38, 36–46 mm), except for very large freshwater bivalves where it may be very difficult to obtain a sufficient number in a narrow size ranges. Once the test specimens have been identified, wet ice and paper toweling can be used to keep them wet and cool. The bivalves can be placed in a flow-through system, or other aerated holding device, if measurement and distribution will not occur until a later time.

Set-up for distribution

A PVC distribution rack (Figure 6.4) is used to hold the mesh bags to facilitate distribution of measured mussels. Stretch the mesh material with your hand or an object approximately so that the bivalve will easily fall within the bag during the distribution process. To ensure an even distribution of mussels in each size group across all stations,[4] attach all bags with the ''–1'' designation to the distribution rack (i.e., for

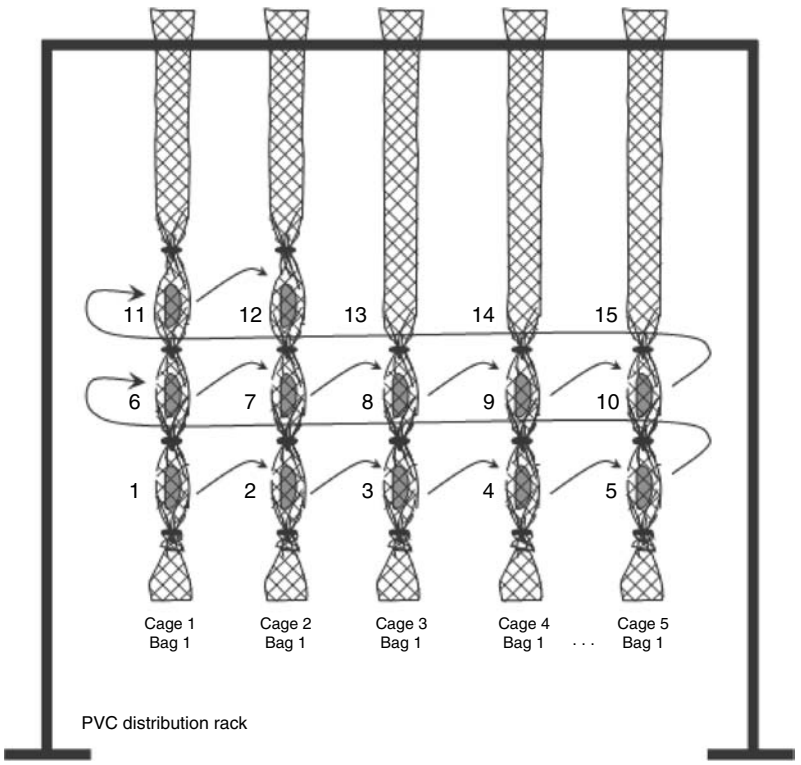

Figure 6.4 Bivalve distribution system.

the first round of distribution, attach bags that are labeled 1-1, 2-1, 3-1, 4-1, 5-1, etc.). The bags should be hung from the distribution rack in cage number sequence. Once the ''-1'' bags are filled, the second round of distribution will utilize all bags with the ''-2'' designation.[4]

Measurement and distribution

Starting with either the smallest or largest size group identified in the pre-sort, place all bivalves within the size group into a tray or tub containing water. The bivalves need to be held in water before measurement to eliminate air between the valves. Bivalves must be completely submerged and flat on the bottom prior to measurement. Bivalves that float on the surface or sit upright on the bottom may have air trapped between their valves. These individuals should not be used because the air will bias the whole-animal wet-weights, because air weighs less than water. Transfer these suspect individuals to a separate container containing water. If left undisturbed for a brief period (e.g., 5–10 min), they will likely purge the trapped air. Water temperature should be maintained as close as possible (approximately $\pm$ 5°C) to temperatures at the deployment site or lower. Plastic bags containing wet ice can be placed in the tub with the bivalves to maintain desired water temperature. A rapid change in water temperature could induce spawning in adult organisms that have ripe gametes.

Shortly after placement in the tub with water, the bivalves will begin to respire, and their shells will be slightly agape (approximately 2–3 mm). Most species will tightly close their shells upon light physical stimulation. Bivalves that do not completely close their shells upon movement or light physical stimulation (i.e., agitation of the water around the bivalves or lightly tapping the shell) should not be used. Prior to making the final measurements, examine the exterior of the shell. Individuals that have broken shells or holes in their shells should not be used.

Randomly select one specimen from the holding tray. Using a paper towel, blot excess water from exterior of the shell, measure its shell length with a caliper, and weigh with an analytical balance. The data can be recorded electronically and/or manually. Once the specimen is measured and weighed, place it into the first mesh bag on the distribution rack.

Affix a 4-in. plastic cable tie around the mesh material above this individual. The cable tie should be adjusted so that it is tight enough to prevent the animal from passing through, but loose enough so that the cable tie can be moved if necessary. Do not constrict the mesh to the point that it might restrict valves from opening. There should be enough slack in the mesh to allow movement and growth of the individual during the test. Randomly select another specimen from the tray and make desired measurements. Place this individual into the second mesh bag on the distribution rack, affixing a cable tie as previously described. Repeat this process until one individual has been placed into each mesh bag. Continue adding bivalves, one at a time to the mesh bags, completing one row before another is started, until each mesh bag contains the desired number of individuals. When all of the bags on the distribution rack have been filled, remove the bags, knot or cable tie the open end, leaving a tail length of approximately 25 mm. Place the completed bags into a cooler lined with ice and moist paper towels. Repeat the above process until all the mesh bags are filled.[4]

This distribution process is also recommended for bivalves to be deployed in the benthic cage even though the bivalves will be removed from the mesh bags before

deployment. It is not necessary to separate bivalves with plastic cable ties during the distribution process if the benthic cages are used. The mesh bags facilitate transportation to the deployment sites, and identification of individuals assigned to a given benthic cage.

Once the bivalves have been distributed to all mesh bags, sort the bags by cage number (i.e., the first number on the label). Either attach the bags to the PVC frames, as described below, or place them in uncontaminated flowing water (either laboratory or field) until ready to attach bags to the PVC cages or distribute the mussels in the benthic cage.

Measuring and distributing bivalves to be used for baseline (beginning-of-test) tissue chemistry

Bivalves to be used for baseline tissue chemistry should be identified during the measurement and distribution of test specimens to ensure similar sizes. This can be accomplished by randomly assigning a cage number for each baseline tissue chemistry sample. However, instead of distributing the bivalves for baseline tissue chemistry to mesh bags, they can be distributed to rigid, compartmentalized boxes, which eliminates the need to remove individuals from the mesh bags once the distribution process is completed. The individuals should be placed into the compartmentalized boxes in order (i.e., with the first individual measured placed into compartment #1, the second into compartment #2, etc.). At the end of the distribution process, the tissues are removed for chemical analysis.

Although it is possible to measure chemicals in individual bivalves and this approach could provide more data on individual variability, the cost of chemical analysis of individuals is usually prohibitive. Furthermore, pooling provides a more integrated sample that reduces individual variability. The methods described here assume that tissues will be pooled to create composites for chemical analyses. The number of individuals necessary for the composite will depend on the (1) the size of the soft tissue mass and (2) the amount of tissue required by the analytical laboratory for the desired analyte(s).

Attachment of mesh bags to PVC frame

A set of mesh bags attached to a PVC frame constitutes a cage (Figure 6.2). The mesh bags can be attached to the PVC frame by (1) knotting the tail ends of the mesh directly to the PVC, or (2) using cable ties to firmly attach mesh to the PVC frame. There should be sufficient slack in the mesh bag after it is attached so that the bivalves are able to open their shells and filter, but not so much slack that the bivalves hang more than about 5 cm below the plane of the cage. If the mesh is stretched too tightly, it will restrict valve opening, filtration, and growth. If the bag is too loose, it will tend to get tangled in the deployment array. If necessary, slide the cable ties to increase or decrease the space between individuals without compromising the space available for each individual (i.e., do not decrease the space between animals so that there is insufficient space for them to open their valves during respiration). A temperature-recording device can be attached to the frame at this time. If desired the cage can be enclosed within a heavy duty plastic mesh envelope, with a mesh size appropriate to exclude predators (e.g., approximately 1.25–2.5 cm).[4]

Water column deployment

For water column deployments, cages can be either suspended from a fixed mooring, such as a floating pier or piling, or they can be suspended within the water column by attaching them to a line that has an anchor on one end and a surface or subsurface buoy attached to the other end. Factors that should be considered during the deployment of cages for surface water assessments include change in tidal height (i.e., to ensure the cages are at the desired depth during both low and high tides), slope of bottom material (i.e., to ensure the cages do not slide down a steep slope during the exposure period), and boating and recreational activities in the vicinity of the cages (i.e., to avoid cages being removed by or tangled within propellers). Water column deployments are primarily used to assess chemicals in the water column, but this approach can also be used to assess chemicals associated with sediments if the cage is suspended so that it is close to the bottom (e.g., ≈ 1 m).

Fixed bottom deployment

PVC cages can be deployed directly on top of the sediments or set at a fixed distance from the bottom by attaching legs to the cage and pushing the legs into the sediments to hold the cage in place. Depending on the species of bivalve used, bivalves in cages deployed directly on top of the sediments can be used to assess chemicals associated with both sediments and the water column. If the goal is to assess subsurface sediments, it is recommended to place the cages directly on top of the sediments. The bivalves gain exposure to chemicals in the sediments as the sediments infiltrate the mesh material or as the foot rakes the sediment surface. Attach sufficient weights or anchors to ensure the cages remain at the desired position. Rebar can also be bent into a ''U'' and pushed over the cage into the sediment to secure the cage in position. Iron rebar should be coated with appropriate rubberized coatings or covered by plastic bags to prevent potential metal exposure.[4]

Benthic cage deployments

Prior to deploying the benthic cage, remove bivalves from their mesh bags and place them on the clean sand. Affix the flexible mesh top to the inner mesh with cable ties, so the bivalves cannot escape during transport to the bottom. Benthic cage deployment is best accomplished with divers. The diver will need to dig a hole in the bottom substrate, set the benthic cage in the hole, place sediment around the outside of the benthic cage, and secure the cage to the bottom with either weights or rebar.[19,20]

Deployment period

The deployment period is a function of the type of chemicals being accumulated. A minimum 30-day deployment period is recommended; a preferred deployment period is 60–90 days. A period of less than 30 days is not recommended unless the chemicals of concern are low molecular weight organic compounds, such as some PAHs. Equilibrium for most other chemicals, such as metals and high molecular weight organic compounds, is generally achieved in marine and freshwater bivalves within a period of approximately 60–90 days. A test period of 60–90 days helps ensure reaching chemical equilibrium and provides sufficient time for adverse effects to manifest themselves. The benthic cage is recommended for long-term exposures where more subtle effects, such as endocrine disruption and sex reversal, are the measurement endpoints.[19,20]

Retrieval and end-of-test measurements, collection and preparation of bivalve tissues for chemical analysis

The same care in maintaining moisture and water temperatures should be followed at the end of the experiment as in the beginning. It is critical to retain the order of bivalves during the end-of-test measurements, so the measurements can be paired with beginning of test values. Compartmentalized trays are used to hold the bivalves during the end-of-test measurements. Otherwise, procedures are virtually identical to those employed at the beginning except that tissues must be removed from all the animals exposed in the field. Additional details on these procedures can be found elsewhere.[4] Specific procedures for collection, preparation, and preservation of bivalve tissues are often provided by project requirements or the analytical laboratory but additional guidance is also provided.[4]

Results and discussion

Three case studies are presented to demonstrate that the caged bivalve methodology can identify the fine structure of exposure and effects along suspected chemical gradients. The rationale is, if the methodology is useful for identifying differences over these small spatial and temporal scales, it will also be useful over larger scales. These studies also show that the methodology is versatile and robust. The San Diego Bay study is important because it was the first study, in which these specific methods were used to demonstrate spatial differences in exposure and effects over a small vertical scale (3 m). It represents the initial development and field testing of the method and application of a long-term record (3 years) at several different sites that facilitated development of tissue residue effects relationships. The Port Valdez study is important because it represents the deepest transplant (70 m) we conducted at that time. It was used to demonstrate near-bottom gradients in PAH tissue burdens horizontally (kilometer scale) and vertically (2 m). The Port Alice study is important because it represents near-surface horizontal (kilometer scale) and vertical (2 m) gradients in growth rates. Mussel survival was high in all three tests with exposure periods from 56 to 84 days.

Regarding the relevance to ecological risk assessment, exposure and effects were measured in all three studies and the San Diego Bay results were used as part of the U.S. Navy's ecological risk assessment for utilization of TBT antifouling coatings. Since the San Diego Bay study provided data over a series of years, it was most applicable for determining tissue residue effects, and these relationships have been discussed elsewhere.[6] Biomarkers were also measured in subsequent San Diego Bay studies that established links with the growth endpoint.[21,22] Biomarkers and tissue residue effects approaches will not be emphasized in this chapter because they have been addressed extensively elsewhere. It should be remembered, however, that in its simplest form the caged bivalve methodology could be considered as a platform for almost any clinical measurement. This combination of caged bivalves, tissue residue effects approaches, and biomarkers is what makes the approach so potentially powerful.

In each study, exposure was characterized by measuring bioaccumulation of chemicals of concern and effects by measuring survival and growth. Three different species of marine mussels in the genus *Mytilus* were used (*Mytilus galloprovincialis*, *M. trossulus*, and *M. edulis*), and in each study, bioaccumulation and growth were used to assess exposure and effects over relatively small spatial scales of either 2 or 3 m vertical distance in areas

suspected of physical and chemical stratification. All cages were deployed with floats to maintain a fixed position relative to the surface of the water column or bottom sediment, unlike natural populations that are attached to fixed substrates where water exposures would change with tidal cycle. A general description of each of these studies is provided in Table 6.3.

San Diego Bay, California

The San Diego Bay study is important because it shows the ability to quantify exposure and effects over a small spatial scale (3 m). These relationships were quantified over an extended temporal scale (3 years).[5] Concentrations of TBT in seawater were also measured to complete the exposure-dose–response relationships. These San Diego Bay data led to our development of the exposure-dose–response paradigm.[6] These data were also important from the ecological risk assessment perspective because they showed that ship hulls floating on the surface were the source of TBT in mussel tissues and not the contaminated sediment.[23]

Statistically significant differences were found in seawater TBT concentrations, tissue TBT concentrations, and mussel growth rates when the site 1 m below the surface was compared with the site 1 m above the bottom (sites were only 3 m apart) (Figure 6.5). The data show a precipitous decline in seawater TBT concentrations (exposure) that coincides with restrictions on the use of TBT antifouling coatings (Figure 6.5A). This decline was also associated with a general decrease in TBT concentrations in mussel tissue (Figure 6.5B), although confounding factors, such as adverse effects, of weekly measurements in the first four tests made data interpretation more difficult. Excessive handling during these measurements reduced mussel growth rates and probably TBT accumulation as well. When length and weight measurements were made every other week, both growth rates and TBT accumulation increased. The important lesson here is that measurement stress can affect both laboratory and field experiments. Another apparent anomaly appears in the growth rate data where growth rates generally increase at both sites as TBT in water and tissues decreases. In Test 8, however, growth rates decrease significantly, and this was attributed to lower water temperatures near 14°C. Water temperatures above 20°C also reduced mussel growth rates. This corroborated results from other studies suggesting that 20°C was near optimum for mussel growth.[6,23]

Port Valdez, Alaska

The Port Valdez study is important because it shows the ability to quantify total PAHs in mussel tissues (dose) over a small vertical spatial scale (2 m) and a larger horizontal spatial scale (1 km). The main purpose of this test was to demonstrate if the caged mussels would survive, grow, and accumulate chemicals of concern; the test was considered successful from that perspective. Differences were found in PAH bioaccumulation and mussel growth rates among sites. At the time, this caged mussel study was the best example of how a gradient design could be used to demonstrate that the source of PAHs in mussel tissues was the Ballast Water Treatment Facility diffuser, which discharged at a depth of 70 m. This is shown by the decreasing gradient of PAHs in mussel tissues (Figure 6.6) with distance away from the diffuser.[7]

The gradients shown for each of the three monitoring depths (i.e., 5, 7, 9 m above the bottom) are very similar, except for the concentration of total PAHs at 1200 m. At 9 m above the bottom, the cage was lost and no data are available, at 7 m above the

Table 6.3 Summary of case study details

Year	Location	*Mytilus* species	Size range (mm)	Exposure duration (days)	Number of mussels	Percent survival	Depths/ distances	Type of gradient
1987–1990	San Diego Bay, CA	*M. galloprovincialis*	10–12	84	300	96	1 m below surface, 1-m above bottom	Vertical
1997	Port Valdez, AK	*M. trossulus*	30–36	56	2100	97	5, 7, and 9 m above bottom, 200 m intervals from diffuser	Vertical and horizontal
1997	Port Alice, B.C., Canada	*M. edulis*	14–21	68	1620	95	2, 4, and 6 m below surface, 0.3, 3, and 10 km from diffuser	Vertical and horizontal

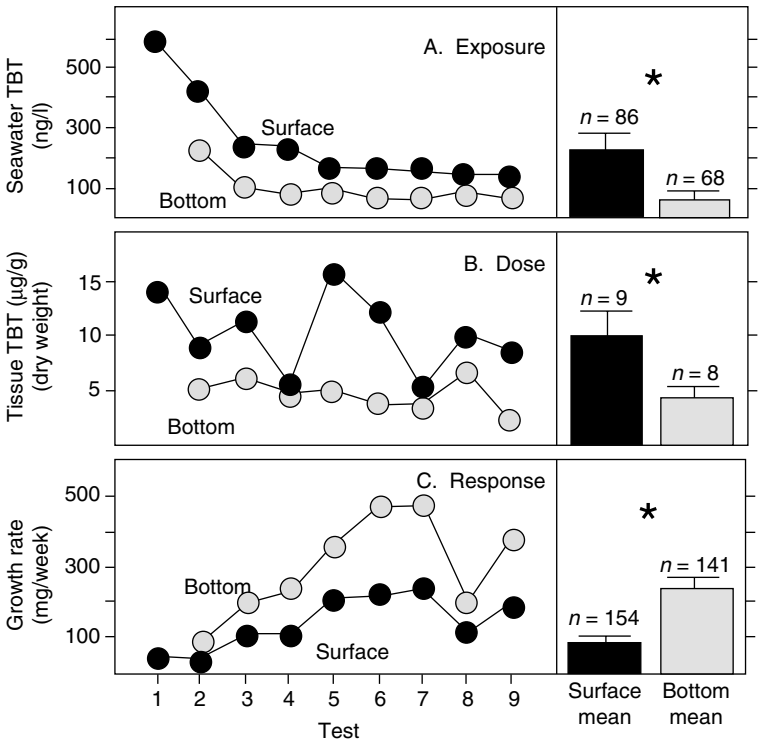

Figure 6.5 Examples of fine structure in (A) chemical exposure, (B) internal dose, and (C) growth response at two sites separated by 3 m vertical distance.

bottom the data point fell along the regression line, and at 5 m above the bottom the value was higher than expected. These anomalies may preclude a more extensive interpretation. Without the data point for 9 m above the bottom, it was difficult to hypothesize whether the regression should be more like that for 5- or 7-m above the bottom, as well as the significance of the regression. When using all the data, the poorest relationship is found at 5 m above the bottom. For the 5-m depth, it is possible that the total PAH concentration in mussel tissues from the 1200 m site is either an outlier or representing another source. Chemical fingerprinting suggests that it is another source. If only the data between 200 and 1000 m are used, the significance of the regression is much better.[7]

Port Alice, B.C. Canada

The Port Alice study shows the ability to detect differences in effects among sites along a suspected chemical gradient even when the chemical of concern has not been identified.[8] Environment Canada has based its Environmental Effects Monitoring program on effects because they are the ultimate concern. In addition, scientists still have not identified the causal factor for those effects measured near pulp and paper mills over the last 25 years. While most of these effects have been measured in fish, we have recently demonstrated similar effects through elevated vitellin production that suggest effects on reproduction and endocrine disruption in freshwater bivalves. The main purpose of the Port Alice test was to quantify effects in caged mussels and determine if possible tracers or causative

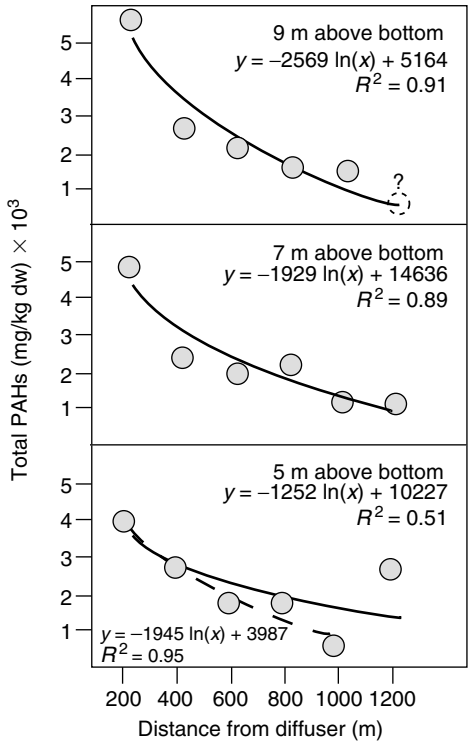

Figure 6.6 Examples of fine structure and decreasing chemical gradient with distance at 5-, 7-, and 9-m above the bottom in a depth of 70 m.

agents responsible for these effects could be identified. Correlations were found for growth effects and the plant sterol campesterol, but campesterol may not be the causative agent because it has not shown significant adverse effects in other studies. Effects were shown along the suspected chemical gradient, as well as differences among sites (Table 6.4).

Table 6.4 summarizes results of statistical comparisons for the three most important growth rate metrics at pooled Stations 1 and 2, 3 and 4, and 5 and 6 at distances of 0.3, 3, and 10 km from the diffuser, respectively. The lack of continuity in statistical results for these three metrics suggests that there is some uncertainty regarding the spatial influence of the pulp mill effluent. Statistical analyses of weight and length growth rate suggest that growth was reduced only at a distance of approximately 0.3 km from the mill. End-of-test tissue weights, however, suggest that growth was reduced up to a distance of 3 km from the mill. Growth rates were correlated with spent sulphite liquor, dissolved oxygen, temperature, and distance from the diffuser. The relationship between growth rate and distance from the diffuser and depth are shown in Figure 6.7. The best relationship between plant sterols and distance from the effluent diffuser was for campesterol. Not only was there a gradient of decreasing concentrations of campesterol with distance from the diffuser, but there was also a statistically significant relationship between the decreasing concentration of campesterol in mussel tissues and the increasing mussel growth rates. Although these relationships do not establish causality, the data suggest that campesterol could be a factor affecting mussel growth. Even if it does not adversely affect growth, it might be useful as a tracer for pulp and paper mill effluents.[8]

Table 6.4 Statistical comparisons among pooled stations at distances of 0.3, 3, and 10 km from the diffuser (statistically similar values are italicized)

	Pooled stations		
	1 and 2	3 and 4	5 and 6
Weight growth rate (mg/week)	218	*236*	*248*
Length growth rate (mm/week)	1.22	*1.28*	*1.32*
EOT tissue weight (g, wet)	*0.68*	*0.69*	0.78
EOT percent lipid (%)	*1.00*	*1.14*	1.48
EOT percent water (%)	*79.5*	*78.4*	*77.5*

As with the Port Valdez study, not all questions were answered, but the advantage of an experimental field approach is the ability to improve the experimental design to answer the questions being asked and develop new questions and new approaches. This is important to aquatic toxicology in the sense of hypothesis development and hypothesis testing and is the core of the caged mussel methodology.

Summary and conclusions

We believe that field experiments using caged mussels can enhance modeling and assessments in aquatic toxicology because of the ability to characterize exposure and effects over

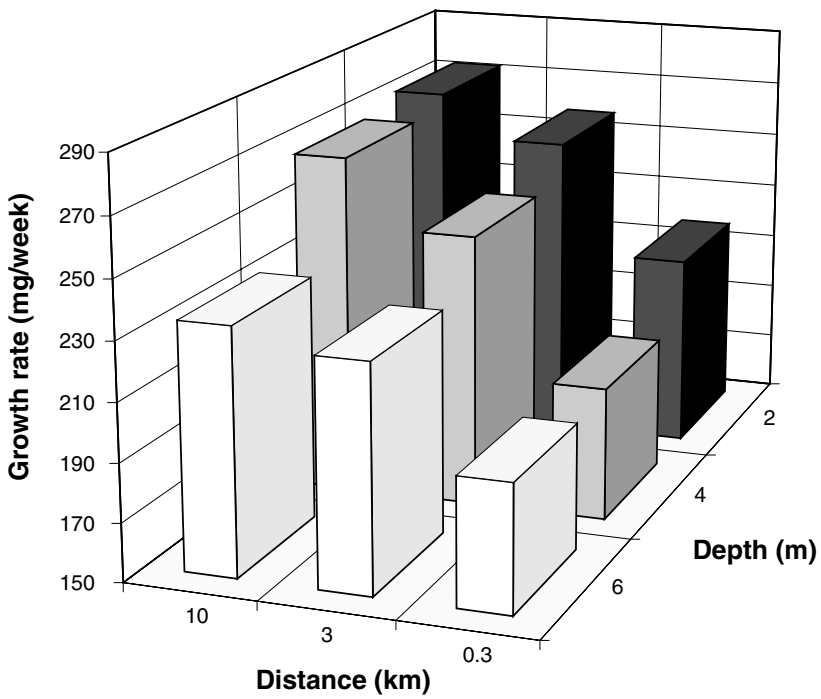

Figure 6.7 Examples of fine structure and increasing growth gradient with distance at 2-, 4-, and 6-m below the surface.

space and time. Caging facilitates the utility of both tissue residue effects approaches and bivalve biomarkers. The field experiments account for the importance of receiving water because the tests are conducted under environmentally realistic conditions that include all possible exposure pathways. It is this ability to characterize exposure and effects that makes these field experiments consistent with ecological risk assessment-based monitoring and a weight of evidence approach. The examples provided here demonstrate how caged bivalves can be used to characterize the fine structure of exposure and effects in horizontal and vertical chemical gradients. This approach can also help establish causal relationships between exposure, dose, and response, particularly when paired with tissue residue effects approaches and bivalve biomarkers. While there are no perfect monitoring and assessment tools, field experiments with caged bivalves possess many characteristics of a practical and useful methodology. In each of the examples provided here, field experiments using caged bivalves provided important information that could not have been obtained through traditional field observations or laboratory toxicity tests. More importantly, the experimental approach is consistent with a basic tenet of aquatic toxicology, which includes hypothesis testing and hypothesis development.

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chapter seven

Application of computer microscopy for histopathology in isopod toxicity studies

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Contents

Introduction

Histopathological changes are integrators of the cumulative effects of alterations in physiological and biochemical systems in an organism exposed to a natural or anthropogenic stress.[1,2] The primary advantage of histopathology is that it permits the visual localization of injury to cells and tissues in multiple organs as it existed just prior to sacrifice and fixation of the tissue. In contrast to tissue homogenates, this approach provides a window to understanding the functional architecture of cells, tissues, and organs. The use of histopathology as a research tool also permits characterization of the essential biological features of the animal being investigated for its sexual, histological, and reproductive status, all variables that may also influence the outcome of experimental manipulations. Furthermore, histopathological examination can be used as an adjunct method to diagnose certain infectious diseases that may be linked to environmental or anthropogenic stressors.

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In mammals and in fishes, histopathological examination is widely recognized as a reliable method for disease diagnosis and for assessing acute and chronic effects of exposure to toxicants at the cellular and organ levels. This is not the case in invertebrates. One reason for this lies in the fact that knowledge and experience of fundamental invertebrate biology, pathology, and toxicology is far behind that of mammals and fishes.

For an accurate histological interpretation of sections, the tissue must be properly fixed, processed, and stained, and the examiner must be familiar with the range of normal morphological variations due to sex, reproductive and nutritional status, age, and season. In recent years, histopathology has reached a breakthrough due to the availability of computer-assisted tools for microscopy.

The aim of computer microscopy is the accurate mapping and quantification of biological tissue whether it is of individual cells, groups of cells, or of entire histological regions.[3] In computer microscopy, the user selects those features of the tissue that are to be studied and then makes boundary contours and/or sequences of isolated points. The contours and points are coded for identification purposes. Line lengths and closed areas are measured by tracing and standard computer numerical integration techniques, respectively.

In our work, computer-assisted morphological mapping was employed for estimation of the histological characteristics of the digestive gland epithelium. We determined the morphometric parameters and lipid surface density on serial sections of digestive glands (hepatopancreas) of the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea). Isopods are among the most popular organisms in terrestrial, as well as aquatic, toxicology.[4] A variety of biomarkers have already been developed to be used in isopod toxicity studies. We established some standardized criteria for histopathological assessment of the state of the organism, and an example is given of a terrestrial isopod *P. scaber* exposed to metal- and pesticide-contaminated food.

Materials required

Tissue preparation

The terrestrial isopods, *P. scaber* (Latreille, 1809) (Isopoda, Crustacea), were collected under concrete blocks and pieces of decayed wood lying on earth in the garden. We followed the toxicity testing protocol as proposed by Drobne and Hopkin.[5] Before dissection, sex, moult stage, presence or absence of marsupium, and weight of each animal were determined. Sex was determined by the characteristic shape of the male's first and second pleopods, which differ from the other pleopods (Figure 7.1b). In females, all pleopods have a similar shape. Moult stage could be determined by milky white deposits on the ventral part of the pereon, indicating the premoult stage (Figure 7.1b).[6] The absence of deposits is a characteristic of the intermoult and postmoult animals. The postmoult stage is difficult to determine antimortem. One characteristic of this stage is an empty gut. In the toxicity studies, only the intermoult animals, as determined postmortem, were included.

After exposure to contaminated food, the animals were dissected and the digestive system was isolated (from 9 to 10.30 a.m.). The digestive system of *P. scaber* is composed of a short stomach, a gut, and four blind ending digestive gland tubes (Figure 7.1c). The animals were decapitated, and the last two pereonies were cut off (Figures 7.1a,b). The gut and four digestive gland tubes were pulled out by tweezers and immediately dipped into fixative. The hepatopancreas is best preserved if it is pulled out together with the stomach and the gut. In such a case, the digestive juices remain within the lumen of the tube, and the least mechanical damage is caused to the gland epithelium. If the digestive gland

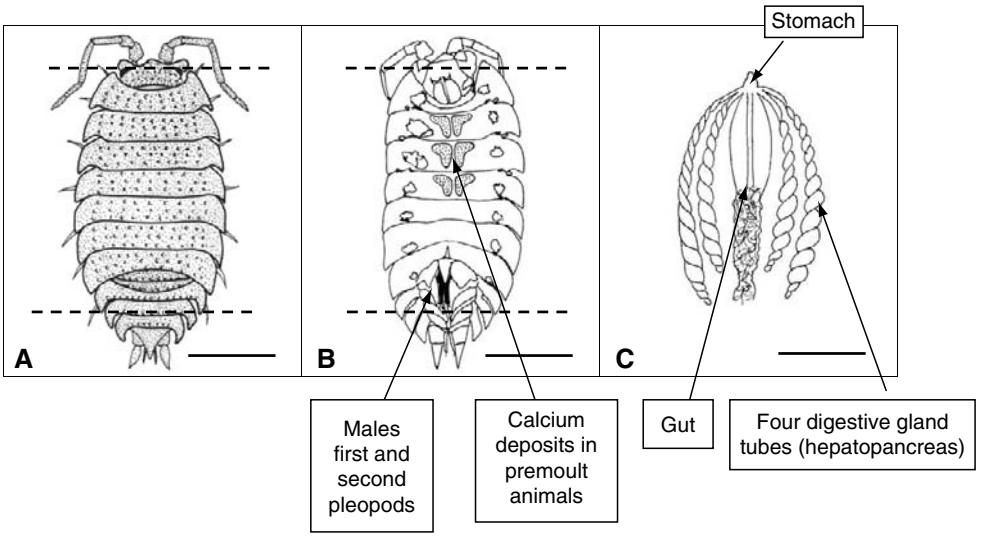


Figure 7.1 (a) Dorsal site of *P. scaber*. (b) Ventral site of *P. scaber*. Broken lines indicate parts of body that are cut off before the digestive system is isolated. (c) The isolated digestive system: stomach, gut, and four digestive gland tubes. Scale bar is 2.5 mm.

tubes are not pulled out together with the gut, it is not recommended to pull them out tube by tube, but to open the lateral parts of the body, in order to gently remove the ventral site and only then pull out the gland tubes. In such cases, the most anterior part of the tube is damaged, but the rest is satisfactorily preserved. If the gland tube is stretched during the isolation, it can be further processed; however, some artifacts are expected, for example, a changed luminal area. After isolation and before fixation, the color and the shape of the gland tubes are described, as well as the amount of digesta (Tables 7.1a and 7.2a).

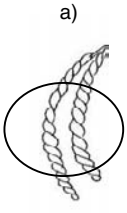
Procedures

The isolated digestive gland tubes were fixed in Carnoy-B fixative, a mixture of glacial acetic acid (10%), absolute ethanol (60%), and chloroform (30%) for 3 h at room temperature. Fixative was washed out with absolute ethanol for 2 h. Then the tissue was transferred to xylene (3 × 15 min) and subsequently embedded in the melted paraplast at 58°C. Samples were oriented parallel to the edges of the model and incubated in the melted paraplast for 12 h. The paraplast was allowed to harden for 2 days at room temperature. Then 8-μm sections (Reichert-Joung 2040 rotary microtome) of the entire tube were cut (approximately 600–700 sections per gland tube). All sections were stained with eosin for 30 min, then briefly rinsed in 70% alcohol (2 s) and in 96% alcohol (2 s), and dehydrated in 96% ethanol (3 × 2 min) and xylene (2 × 5–10 min). In staining only with eosin, no differentiation of the dye is needed and thus the procedure is highly repeatable. This is of significant importance for comparative, computer-based objective analyses of histological images.

Manual contouring of the inner and the outer epithelial surface of a serial section of one gland tube was performed to calculate the average epithelial area and epithelial thickness on a section (computer program for image analyses, KS-400 Kontron Electronic,

Table 7.1 Description of the digestive system of a control, intermoult *P. scaber*

a) Description of the digestive system of a control *P. scaber*

(a)	(b)	(c)
		
Full gut	Half-full gut	→ Moulting animal
a)	b)	
		
Medio-posterior part of gland tubesis NOT enlarged in diameter Creamy colour	Medio-posterior part of gland tubes is enlarged in diameter Yellow-brown or clear yellow color	

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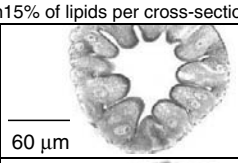
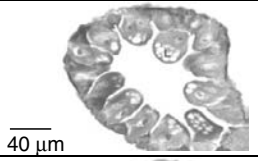
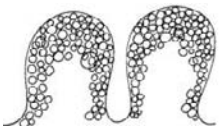
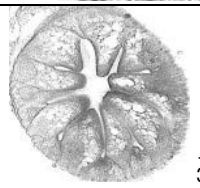
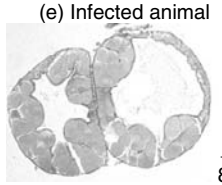
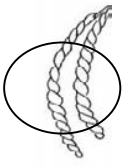
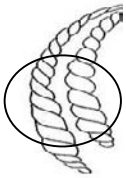
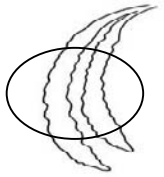
(b) Histological description of hepatopancreas of a control *P.scaber*			
Epithelial thickness: 40–60 µm			
70% of investigated glands have on average more than15% of lipids per cross-section			
(a) Up to 10% of lipids		 60 µm	
(b) More than 10% of lipids		 40 µm	
(c) More than 20% of lipids		 40 µm	
(d) More than 25% of lipids		 30 µm	(e) Infected animal  80 µm

Table 7.2 Description of the digestive system of a stressed *P. scaber*

(a) Description of the digestive system of stressed *P. scaber*.

(a)	(b)	(c)	
 Full gut	 Half-full gut		Moulting animal
(a)  Medio-posterior part of gland tubes is NOT enlarged in diameter Creamy color	(b)  Medio-posterior part of gland tubes is enlarged in diameter Yellow-brown or clear yellow color	(c)  The spiral shape of the tube is not observed Medio-posterior part of the gland tube is enlarged in diameter Creamy color or yellow-brown or clear yellow color	

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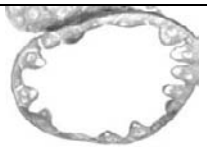
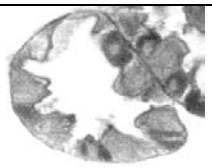
(b) Histological description of hepatopancreas of a stressed *P. scaber*		
Epithelial thickness: less than 40 μm With increasing stress the epithelial thickness decreases		
With increasing stress the lipid droplets in most cases significantly decrease on an average of 0–10% per cross-section		
(a) Approximately 10% of lipid droplets or less		 40 μm
(b) No lipid droplets	Occasionally observed stage of cell degradation	 40 μm
(c) No lipid droplets	Stress  Extreme stress 	 20 μm

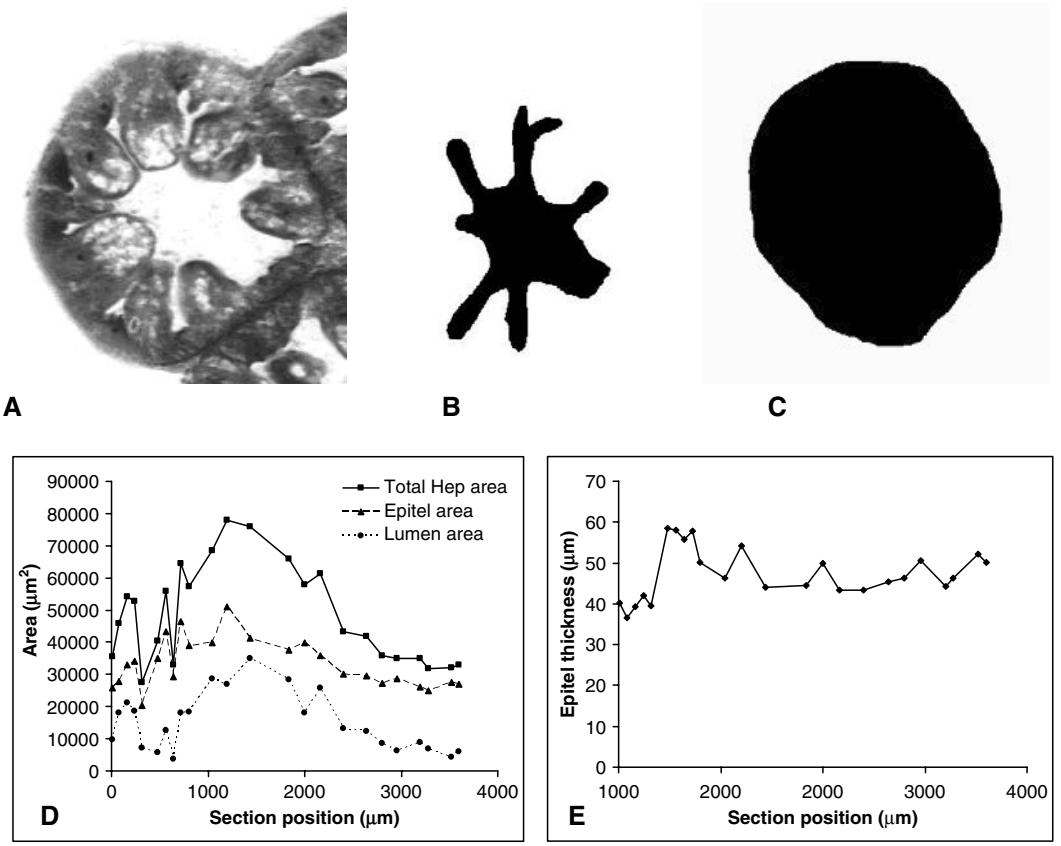


Figure 7.2 An example (Animal 387) of determining the lumen, epithelial and total tube area on cross-sections along a gland tube. See also http://www.fgg.uni-lj.si/~/sdrobne/DDrobne/CRC_Histopathology/. (a) The image of a histological section of hepatopancreas. (b) A contour of the inner epithelial area. (c) A contour of the outer epithelial area. (d) Lumen, epithelial, and total gland tube areas on serial sections along one gland tube. (e) Epithelial thickness on serial sections along the same gland tube.

Germany, Figure 7.2). Manual contouring of the inner surface was necessarily performed, since, using the computer program, the borders between the apical epithelial surfaces usually cannot be distinguished from the lumen that is filled with digestive juices. In some control animals, contouring of all serial sections was performed (http://www.fgg.uni-lj.si/~/sdrobne/DDrobne/CRC_Histopathology/). The morphological data of all serial sections were used to evaluate statistically the number of sections needed to be analyzed along the gland tube to obtain a consistent morphometric description of the tube. It was calculated that the image analyses of each 25th section along the gland tube provide the same morphometric data that cumulative analysis of all serial sections would yield.

On the selected section, the lipid surface density ($SD_{lipid} = S_{lipid}/S_{epithelium}$, SD, surface density; S, surface) was also determined. In this case, the surface is the epithelial area on a cross-section. The median lipid surface density along a tube was calculated. An

example of a report on lipid surface density determination (Animal 387) that was generated in ''Mathematica'' and ''Excel'' is shown in Table 7.3.

Results and discussion

Histological characteristics of unstressed, intermoult digestive glands of P. scaber

Intermoult *P. scaber* have either full or half-full guts. Those with half-full guts have brown-yellow or clear yellow glands with an increased diameter in the medio-posterior part. The glands of animals with full guts are of uniform diameter and are of white or white-yellow color. Microscopically, the digestive gland epithelium is composed of dome-shaped cells protruding into the lumen of a gland tube. They are filled with lipid droplets, but the quantity of lipid droplets varies among individuals. In the majority of animals, the cell cytoplasm is composed of up to 25–30% lipids (Table 7.3). In more than 300 animals investigated, none were found to possess digestive glands totally free of intracytoplasmic lipid droplets. The droplets are situated in the apical part of the cytoplasm or are distributed throughout the entire cytoplasm. The distribution of lipids in the cytoplasm along the entire gland tube does not vary significantly (Table 7.3).

Irrespective of the fullness of the gut, the color of the glands, and the diameter of the medio-posterior part of the gland, the average epithelial thickness along a gland tube is no less than 40 μm. This indicates that the epithelial thickness does not change as the diameter of the gland tube changes during the digestive cycle.[7] The epithelial thickness for that reason is a more reliable measure of stress than the epithelial area, expressed as a percentage of the cellular area on a cross-section. However, in severely stressed animals (long duration of exposure and higher doses of chemicals), the epithelial area as a fraction of tube area is also significantly reduced (Table 7.2b). This was previously shown by Odendaal and Reinecke.[8]

In a group of unstressed animals some have empty guts; these are probably post-moult animals and were therefore not included in our study. Also, in the unstressed group, some animals have larger and softer glands than expected. A microscopic investigation of these glands revealed that they are infected by intracellular bacteria.[9,10] In the infected individuals, the epithelial cell cytoplasm is filled with vacuoles of different sizes, which contain bacteria (Table 7.1b). The infected animals were also excluded from this investigation.

Macroscopic and histological characteristics of the digestive system of stressed P. scaber

As in unstressed *P. scaber*, the animals fed with metal- and pesticide-contaminated food have either full or half-full guts. The majority of glands have a similar macroscopic appearance to those in the unstressed group. However, some animals have large and soft glands, and the spiral shape of the tube is not seen.

In comparison to control animals, the average microscopic thickness of the digestive gland epithelium is less than 40 μm, and the cell cytoplasm usually contains less than 10% lipid droplets. With increasing stress, the percentage of cytoplasm occupied by lipid droplets drops to under the detectable limit. The epithelial cells have a pyramidal shape

Table 7.3 An example of a report on lipid droplets (Animal 387) generated in Mathematica and Excel

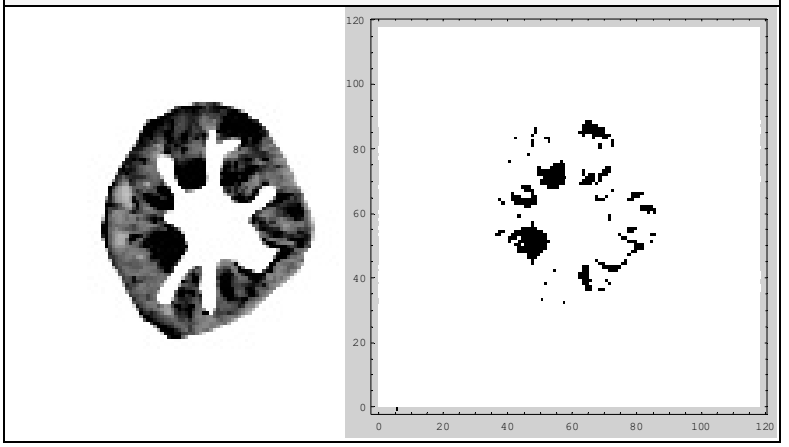
Relative statistics: evaluation of the quantity of lipid droplets

Animal:	387

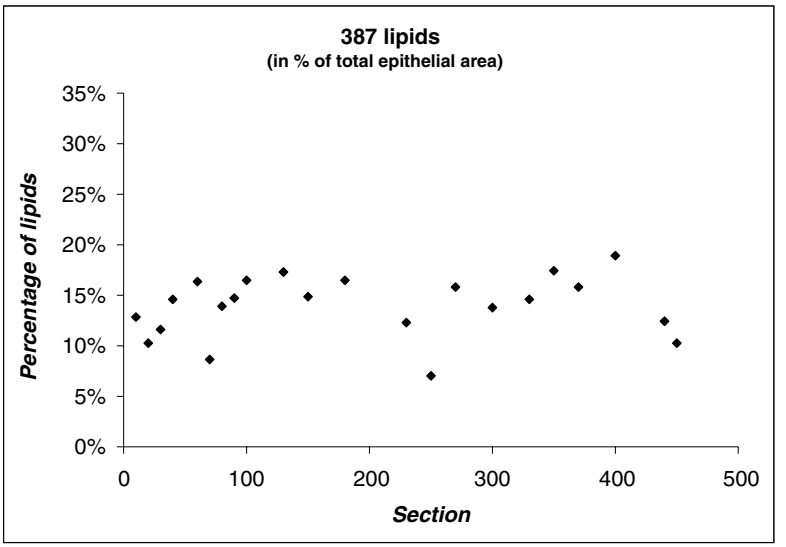
m =	558	mm =	475	d =	10	k =	0.90
n =	750	nn =	475	dout =	4	level =	1.35

	Lipids
N_total=	24
N_bad=	2
Avg =	13.9 %
StDev =	3.0 %
Min =	7.1 %
Max =	18.9 %
D1 =	10.2 %
D9 =	17.2 %

Section: 387-009-400



Section	Lipids
1	
10	0.127888
20	0.102136
30	0.115739
40	0.146259
60	0.163593
70	0.086154
80	0.138809
90	0.147927
100	0.165459
130	0.172546
150	0.148345
180	0.165293
230	0.123077
250	0.070901
270	0.158668
300	0.138303
330	0.145473
350	0.174803
370	0.158543
400	0.189398
410	
440	0.124360
450	0.102524



Note: `N_total` is the total number of the analyzed sections, `N_bad` is the number of bad (not analyzed) sections, `Avg` is the average of lipids in the analyzed sections, `StDev` is the standard deviation, Min and Max are the minimum and maximum of lipids, and `D1` and `D2` are the first and the ninth decile of values in the analyzed section. Parameters `m`, `n`, `mm`, `nn`, `d`, and `dout` are used in previous phases of preparation and alignment of section images. Parameter `k` defines the percentage of central pixels that were analyzed (sometimes there occur some errors in the margins of the outlined sections), and `level` is an empirically defined level of lipid droplets in the section.

(Table 7.1b). In some animals, degradation of epithelium was observed over regionally extensive areas all along the tube (Table 7.2b).[11] With increased stress, the flattened regions become larger and the pyramid-shaped cells are fewer. The overall change consisted of an absence of lipid droplets and the extreme attenuation of the digestive gland epithelium, which were correlated with the duration of exposure to the stressor and with the dose of the chemical.

Distinguishing histological characteristics of unstressed and stressed digestive glands of P. scaber

An adult intermoult *P. scaber* (40–60 mg wet weight) is fit if the average epithelial thickness of a gland tube is usually more than 40 μm, the cells are dome shaped and filled with lipids along the entire gland tube.

The histopathological characteristics of the digestive glands of an organism exposed to stress are as follows: the average epithelial thickness along a gland tube is less than 40 μm, up to 10% of the epithelial cell cytoplasm is composed of lipids in some cells only, there are large areas with pyramid-shaped cells, areas with flattened cells, and/or areas with degrading cells. The epithelial thinning and reduction of lipids in cells show a dose response.

It is difficult if not impossible to distinguish on the basis of histological parameters whether an organism is fit or slightly stressed. Characteristics of the ''borderline'' group include an epithelial thickness of less than or around 40 μm, regions in the tube where up to 25% of lipids are observed and also regions with no lipids in the cells, and flattened regions, as well as regions with degrading cells. However, dome-shaped cells prevail. We suggest that in animals exposed to low and moderate levels of stress, the judgment whether histological examination indicates the effect of a toxicant or not should be based on observations of more digestive glands of organisms exposed to the same stress. Based on our experiences, 5–8 organisms per experimental group would give reliable information on the effect of a stressor.

Histopathology in isopod toxicity studies

In the protocol suggested for histopathological studies on isopods, the two most serious limitations of histopathology are satisfactorily reduced. The tissue fixation, processing, and staining procedures are highly reproducible. A range of normal morphological variations due to sex, reproductive and nutritional status, and season were thoroughly examined before the histopathological criteria were selected.

An additional advantage of using the digestive glands of isopods in toxicity studies is that each gland tube can be used for a different type of analysis; for example, histopathology, analyses of biochemical biomarkers, analyses of energy reserves (lipids, proteins, glycogen), etc.

It is concluded that in analogy with mammalian and fish toxicology, the histopathology of invertebrates deserves a place in the toxicological toolbox (Table 7.4). Various computer-assisted approaches in pathology can equally be applied to the isopod tissue, which will in turn increase its value in environmental toxicity studies.

Table 7.4 Characteristics of histopathology in toxicity studies with isopods

Animals	Terrestrial and aquatic isopods
Equipment	Standard equipment for a histological laboratory and computer-assisted light microscopy
	Digestive gland histopathology
Characteristics	Applicability
Easy to perform	Moderately
Reliable	Yes, when a large data base on histological responses of selected species is established
Repeatable	Yes
Fast screening	Yes, using reference light micrographs for comparison
Sensitive	Moderately
Early warning	No
Ecologically relevant	When applied with other lower and higher level biomarkers
Laboratory validated	For Cd, Zn, Hg, organophosphorus pesticide diazinon

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chapter eight

Sperm cell and embryo toxicity tests using the sea urchin Paracentrotus lividus *(LmK)*

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Contents

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Introduction

A major requirement in environmental health assessment and monitoring of coastal marine environments is the availability of ''laboratory biological instruments'' allowing reliable evaluation of sublethal toxicity endpoints for single pollutants and environmental matrices. In this field, the sea urchin broadly meets the above-mentioned requirements, allowing evaluation and integration of a multiple set of sublethal endpoints in early life stages, and thus has great importance for population health, although conducted on a single-species basis. In fact, key-events evaluated in sea urchin bioassays include: (a) reproductive success, (b) offspring quality following gamete exposure, (c) larval development, and (d) cytogenetic anomalies. Any change in these events is evaluated by following exposure to single toxicant or polluted environmental matrix of sea urchin gametes or embryos, which are easy to obtain in a good state of health of adult organisms during their reproductive period.

There are many advantages with the utilization of sea urchin bioassays. They include: the availability of detailed basic information on echinoid biology, the year-round availability of animals, the possibility of using organisms at the top of evolutive scale of invertebrates, of having available multiple life stages together with short exposure times, sensitive responses and multiple test endpoints. As regard the test itself, it has low laboratory costs, it is highly reproducible, it has a cosmopolitan test system, and there is also a wealth literature available.[1]

This work describes the procedure developed in our laboratories for performing two toxicity bioassays: the first based on reproductive success as endpoint (sperm cell toxicity test) and the second on larval development (embryo toxicity test), using the sea urchin *Paracentrotus lividus* Lmk. The procedure, derived from the original protocol by Dinnel et al.,[2] was developed following the auto-ecological characteristics of *P. lividus* and in harmony with US Environmental Protection Agency standard procedures using the species *Arbacia punctulata* and *Strongylocentrotus purpuratus*.[3,4] *P. lividus* is the most abundant sea urchin species along northern Atlantic and Mediterranean coasts, with a long reproductive period, from October to June.[5]

Toxicity tests using the early life stages of *P. lividus* are demonstrated to be reliable and useful tools in assessing the toxicity of xenobiotic compounds, which are very dangerous for aquatic life. The contemporary use of these two toxicity tests combines very important toxicological information, for a predictive view of the effects that dangerous pollutants may have on populations in coastal marine environments.[6] Moreover, our research group proposes bioassays with *P. lividus* as methods for quality assessment and monitoring of marine coasts and transitional environments in particular, the Lagoon of Venice. The suitability of bioassays for assessing lagoonal sediments has been tested by their application at sites in the Lagoon of Venice typified by differing kinds and levels of pollution. Elutriates were chosen to assess the potential effects of pollutants that are made available in the water column as a consequence of sediment resuspension (dredging, fishing gear, etc.). Both tests were effective in discriminating several different pollution/bioavailability situations, although their combined use showed higher efficacy in discriminating between stations and also periods.[7]

A preliminary investigation with pore waters (by extraction with centrifugation) was also carried out. Preliminary results showed that both bioassays were able to highlight differences in the toxicity of sediment samples from varying contamination sites (unpublished data).

Materials required

Animal collection and culture

- PET vessel (30 l) for animal transfer preferably in an insulated transport case
- Sampling sack
- Portable aeration systems
- Glass aquarium (250 l) (thermostated, or in a climate-controlled chamber)
- Reconstituted seawater prepared with a commercial salt for aquaria
- Aeration system, using filtered compressed air and equipped with air lines, tubes, and air stones
- Filter systems equipped with filtering materials and blast holes
- Temperature (*T*), pH, and oxygen meters

Dilution water

- Analytical balance.
- Dilution water tank to prepare reconstituted water.
- Deionized water purified by the Milli-RO system/Milli-Q system (Millipore, Bedford, MA, USA).
- Reconstituted seawater prepared by adding sea salts reported in Table 8.1 following the ASTM formula[8] in due amounts and order list, to 890 ml of distilled water (Milli-RO) to reach a salinity value of 34%.

Reagents

- Reference toxicant: copper standard solution for atomic adsorption (1000 mg/l) in nitric acid 0.5 *M*/l (Baker, Deventer, Holland)
- KCl reagent grade

Table 8.1 Salts used for preparing reconstituted seawater following the formula ASTM[8]

Compound	Amount (mg)
NaF	3
$SrCl_2$ $6H_2O$	20
H_3BO_3	30
KBr	100
KCl	700
$CaCl_2$ $2H_2O$	1470
Na_2SO_4	4000
$MgCl_2$ $6H_2O$	10,780
NaCl	23,500
Na_2SiO_3 H_2O	20
$NaHCO_3$	200

- Formaldehyde (37%) buffered with sodium tetraborate
- Glacial acetic acid 10% reagent grade
- Kits for NH_3 and NO_3 analysis
- Chloride acid
- Acetone

Test apparatus and equipment

- Small syringe
- Parafilm
- Latex gloves
- Glass vessels
- Pasteur pipettes
- Eppendorf vessels
- Ice bucket
- Pipettes (1, 10 ml)
- 10-ml graduated cylinders
- Neubauer hemacytometer counting chamber
- Slides
- Count register (at least two places)
- Dissecting microscope, at 10× and at 40× for determination of number of eggs and sperm cells, respectively
- Fume hood
- Gauze or sieve (200 μm)
- Beakers (100, 500, 1000 ml)
- Flasks (100, 250, 500 ml)
- Sterile polystyrene 6-well micro-plates with lids (Iwaki Brand, Asahi Techno Glass Corporation, Tokyo, Japan) as test chambers
- Thermostatic bath (at least 45 × 45 cm) and/or thermostatic room
- Invert microscope for counting eggs and embryos (optional)
- pH meter.

Procedures

Animal collection and culture

Collection: Adult sea urchins are collected in marine coastal zones at depths from 1 to 4 m, far from sources of large-scale industrial and domestic pollution.

Collection procedure: A variable number of adult sea urchins (we suggest 30–70, at least 4 cm in diameter) are collected by divers during the natural reproductive period, in order to avoid any stress to the organisms. The adults should be handled carefully and gently, so we suggest that divers should be experienced.

Animal transfer: A 30-l PET vessel in an insulated, aerated transport case, partially filled with seawater from the sampling site, is used to transfer animals to the laboratory.

Animal culture: A maximum of 20–30 animals for each 100 l of water is stored in one 250-l glass aquarium containing aerated seawater from the sampling site, kept in a conditioned room (18 ± 0.5°C) with a natural photoperiod. Animals already used

for experiments are kept in an identical aquarium and then taken back to the sea. The temperature of seawater is gradually changed until the culture temperature was reached.

The aquaria must be provided with both aeration and filter systems; the later may be composed, for example, of a series of two filtering units, each of which is used to filter about 50 l of seawater. These units are made of 1-l PET pierced columns and filled with various filtering materials and blast holes. The first column is filled with a porous substrate that supplies bacteria of a suitable substratum for their growth; the second column contains granular activated carbon. Each column is kept topped up by a marine pH stabilizer. A small pump (e.g., Mod. Zippy 50-Ocean Fish, Prodac International, Cittadella, PD, Italy) is applied to each column, for continuous re-circulation and filtering of seawater.

Sea urchins are fed every 2–3 days with macroalgae (e.g., *Ulva* sp.) and molluscs (e.g., *Mytilus* sp.), collected at the same sampling site.

Sea urchins are kept at mean salinity 35 $\pm$ 1‰, pH 7.8–8.2, and oxygen at saturation level, respectively. Fecal pellets must be removed every 2 days and the seawater partly replaced by filtered artificial seawater (e.g., Ocean Fish, Prodac International).

Sea urchins can be used for tests after their gradual acclimatization to culturing conditions. Our experience suggests that 1 week of acclimatization is usually sufficient, when differences in water temperature between sampling site and culturing conditions were at most 8–10°C. Acclimatization must be longer, up to 3 weeks, when differences in temperature are higher (about 15°C).

Values of *T*, pH, DO, O_2, NH_3, and NO_3 are periodically checked, and any dead organisms are removed. If a culturing step is not possible, tests can also be performed with animals as soon as they are collected; but this approach, in our opinion, may not guarantee high data reproducibility (see the following). Some of our data also suggest that organisms are more sensitive as soon as they are collected with respect to cultured ones: EC_{50} data obtained with the reference toxicant (copper) showed values of 28 (26–30) μg/l and 41 (40–43) μg/l with animals just collected, but 46 (44–49) μg/l and 60 (58–62) μg/l, after 1 week of acclimatization, respectively.

Pre-test phases

The test procedure has been developed with reference to the protocol proposed by Dinnel et al.[2] according to the auto-ecological characteristics of *P. lividus*. It is strongly suggested to execute both toxicity tests at the same time in order to use the same pool of gametes. Step-by-step procedure is reported as follows:

Gamete emission: Sea urchins are induced to spawn by injecting 1 ml of 0.5–1 *M* KCl solution into the coelom through the peristome; the animals are then allowed to spawn for about 30 min in 50 ml of artificial seawater in a glass vessel. We suggest inducing spawning at least 9 animals, in order to obtain a minimum of 3 males and 3 females with a good emission. If no good emission is achieved, or there are not enough males and females, other animals must be induced to spawn.

Male gamete collection: The sperm cells (lactescent emission) obtained from a minimum of 3 males are put together in a seawater volume ranging from 50 to 100 ml, depending on the abundance of emission, washing the gonadic plate of each male with the 5-ml pipette. This wet collection is suitable if the test is performed within 1 h of collection. Otherwise, it

is advisable to collect the pool of sperm cells dry with a Pasteur pipette in an Eppendorf vessel, and keep it in a covered ice bucket or refrigerator (4°C). However, before cell density is determined, the dry pool needs to be diluted in 50 ml of artificial seawater in a glass vessel, for more reliable evaluation of the sperm cell concentration.

Determination of sperm cell density: The density (no. sperm cells/l) of this pre-diluted sperm suspension is determined by adding a 0.1-ml subsample to 1 ml of glacial acetic acid in a 10-ml graduated cylinder, brought to volume with artificial seawater (dilution factor, df = 100). After the mixing of the suspension by cylinder inversion and Pasteur pipette, one drop is added to each of the two counting places of the Neubauer hemacytometer counting chamber. The count is performed, after 15 min waiting, under a dissecting microscope at 40×. Sperm cell density is determined by applying the formula:

$$[\text{sperm/ml}] = [(\text{df})\ (\text{sperm counted})\ (\text{hemacytometric conversion factor})/\text{no. squares}]\ \text{mm}^3/\text{ml}$$

$$[\text{sperm/ml}] = [(100)\ (\text{sperm counted})\ (4000)/400] \times 1000$$

Sperm/egg ratio: The optimal value for *P. lividus* is 20000:1. In order to keep this ratio constant, sperm density must be adjusted suitably (4×10^7) by applying the formula:

$$\text{df} = [(\text{sperm/ml})/\text{no. sperm desired}] \times 0.1 = [(\text{sperm/ml})/4 \times 10^7] \times 0.1$$

where 0.1 is the sperm volume in ml to add to 10 ml test solution.

The results of a previous set of experiments, in which four different sperm/egg ratios were used (5000:1, 10000:1, 20000:1, 30000:1), showed that, at a sperm/egg ratio of 5000:1, fertilization success was low (39 ± 3.5%), whereas a high percentage of fertilized eggs resulted (88 ± 4.24%, 92 ± 3.46%, 95 ± 1.15%) at higher ratios, respectively. Consequently, a sperm/egg ratio of 20000:1 was chosen as the most suitable, to ensure higher constancy in the percentage of fertilization in controls (Figure 8.1).

Egg collection: The eggs (orange emission), obtained by washing the gonadic plates of minimum of 3 females with the 5-ml pipette, are filtered through a 200-μm gauze and put together in a large beaker (500 ml) containing artificial seawater, in order to obtain a pre-diluted egg suspension.

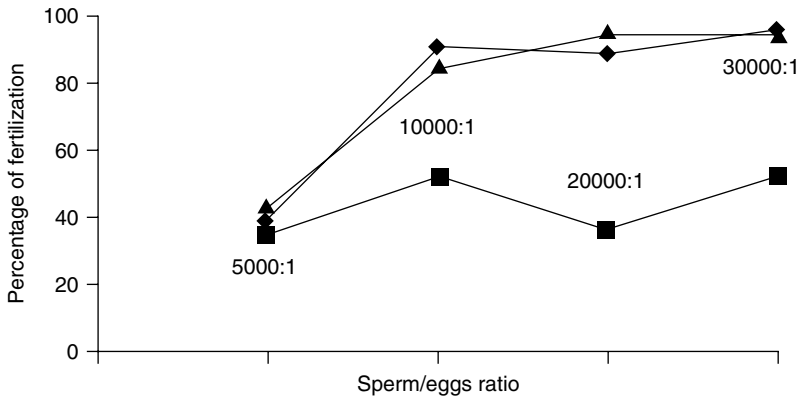

Figure 8.1 Results of sperm cell toxicity test conducted at different sperm/egg ratios (5000:1, 10000:1, 20000:1, and 30000:1). Execution conditions: gametes pool from 3 males; ◆, eggs from first female; ■, eggs from second female; ▲, eggs from third female.

Determination of egg density: Egg suspension density is determined by adding 0.1 ml of the pre-diluted suspension of eggs in a 10-ml graduated cylinder and counting 1 ml of this diluted suspension using a slide (a plankton counting camera with vertical bars facilitates counting), under a dissecting microscope at 10×.

In order to keep the sperm/egg ratio constant, egg density must be standardized to 2000/ml by applying the formula:

$$\text{df} = (\text{no. eggs}/\text{no. eggs desired}) \times 1 = (\text{no. eggs}/2000) \times 1$$

where 1 is the egg volume in ml to add to 10 ml test solution.

Some experiments showed that, using the eggs obtained from 3 females separately, the success of fertilization was closely linked to differences in egg quality, less evident when the eggs were put together in a single pool (Figure 8.1).

Sperm cell test procedure

Test procedure conditions: Temperature 18 ± 0.5°C; salinity 35 ± 2‰ $O_2 > 40\%$; pH 7.8–8.2.

Execution: 0.1 ml of adjusted sperm suspension is exposed to 10-ml aliquots of test solution in test chambers, and left to incubate in a thermostatic bath at 18°C for 60 min. It is recommended to shake gently and constantly the flask by hand during the transfer of the 0.1 ml of sperm in each well.

After 1 h of exposure, 1 ml of standardized egg suspension is added directly to the solution, in which the sperm cells are exposed. Like the sperm, the flask containing the egg suspension must be gently and constantly shaken by hand during the transfer of the 1 ml of eggs to each well. The number of eggs added to each well containing sperm cells exposed to toxicant solution is about 2000. A period of 20 min is allowed to pass, to ensure that fertilization has occurred.

End of test: The test is stopped adding 1 ml of concentrated buffered formalin, which also allows samples to be preserved for the test lecture from few days up to 1 month.

Endpoint: The endpoint considered in this test is fertilization success/failure, based on observation of the presence of a fertilization membrane surrounding the eggs (Figure 8.2).

Counting eggs: The percentage of fertilization in each treatment is determined by counting 200 eggs at 10× using a plankton counting camera. For correct evaluation, each suspension must be gently mixed by hand in order to re-suspend the eggs uniformly, in a subsample of 1 ml. When using an invert microscope, count directly by observing the whole well randomly.

Repeated counts of 50, 100, 200, and 300 eggs showed that determination of the fertilization percentage is more reliable when the number of counted eggs was at least 200. Counts of 50 and 100 eggs provided coefficients of variation, respectively, of 11.6%

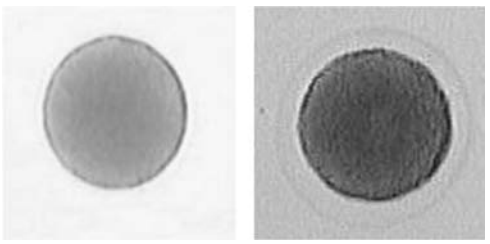

Figure 8.2 Fertilized and unfertilized eggs in sperm cell toxicity test (10×).

and 14%, whereas when the number of counted eggs was increased, the coefficient of variation decreased to 4% and 3% for 200 and 300 eggs, respectively (Figure 8.3).

Embryo toxicity test procedure

Test procedure conditions: Temperature 18 ± 0.5°C; salinity 35 ± 2‰ O_2 > 40%; pH 7.8–8.2.

Zygote achievement: Adjusted sperm and egg suspensions are put together at a sperm/egg ratio of 10:1. For example, prepare at least 100 ml of egg suspension in a beaker, add 10 ml of sperm suspension, and put in the thermostatic bath or room.

Wait for fertilization: A period of 20 min is allowed to pass, to ensure that fertilization has occurred.

Test procedure execution: The test is performed by adding 1 ml of fertilized egg suspension to 10-ml aliquots of test solution contained in test chambers, incubated in a dark thermostatic room or bath at 18°C for 72 h. The number of embryos in the 10-ml test solution is about 2000 (in each well). Normally, zygotes develop in embryos after 48 h, but the time chosen for the test guarantees that all zygotes reach the embryo stage in the negative control.

End of test: The test is stopped by adding 1 ml of concentrated buffered formalin, which also allows the embryos to be preserved for the test lecture from a few days up to 1 month.

Endpoint: The endpoint considered in this test is larval (pluteus) development success/failure. Test results may be evaluated following two different approaches, corresponding to different levels of in-depth examination of anomalies in development.

The first level is a simple, quick discrimination between the normal and anomalous development of each (pluteus) larva (considering all anomalies, including malformations, delays, and blockages at pre-larval stages together). This procedure, which is also currently used in standard U.S. EPA tests, gives the percentage of effect and allows calculating EC_{50} and NOEC. Most data reported in the section ''Sensitivity toward pure substances'' (and related tables) are of this kind.

The second level requires more accurate assessment of larval anomalies, which may be distinguished into at least four categories:

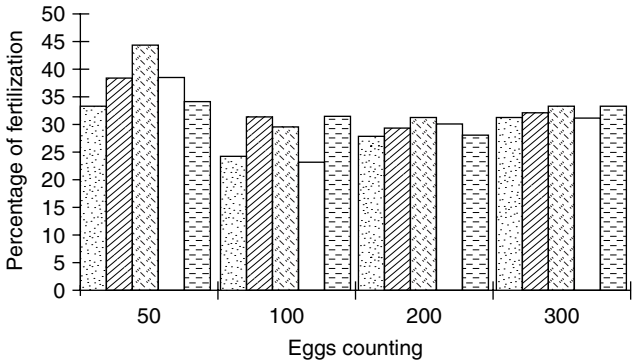

Figure 8.3 Results of repeated counts of 50, 100, 200, and 300 eggs for the sperm cell toxicity test. Data are referred to an experiment using the reference toxicant (60 μg/l test concentration, 1 replicate). Execution conditions: pools of gametes from 3 males and 3 females.

(1) ''Malformed plutei,'' including larvae that are developed but show some malformations (e.g., defects of skeleton and/or digestive apparatus) (Figure 8.4B).
(2) Prisms, including the stage that is normally reached at about the 40th h but abnormal after 72 h (Figure 8.4C).
(3) ''Retarded plutei,'' including larvae that are late, even after 72 h of development time (Figure 8.4D).
(4) Phases blocked before differentiation in larvae (i.e., pre-gastrula stages, gastrula) (Figure 8.4E).

Such careful examination of results after an experiment better discriminates only embryotoxic effects from teratogenic ones. A case study for heavy metals is reported at the end of the section ''Sensitivity toward pure substances.''

Counting: The percentage of plutei with normal development in each treatment is determined by counting 100 larvae. Each suspension must be gently mixed by hand in order to re-suspend larvae uniformly in a subsample of 1 ml. When using an invert microscope, count directly by observing the whole well randomly.

Repeated counts of 50, 100, 200, and 300 larvae showed more reliable counts when the number of plutei is at least 100: counts of 50 plutei provided larger coefficients of variation (6.9%) than counts of 100 or more larvae (2.6–2.8%) (Figure 8.5).

Preparation of test solutions

Preparation of solutions to perform tests with a pure toxicant (copper was chosen also for its use as a reference toxicant) and with elutriate from a contaminated sediment as a complex matrix, is briefly described here. Results are reported in the following.

The copper solution is prepared by adding 1 ml of the concentrated standard solution (1000 mg/l) to 100 ml of double-distilled water (Milli-Q system, Millipore, Bedford, MA, USA). The solution at concentration 10 mg/l is then diluted using artificial seawater (35‰, 18°C, pH 8) to prepare the toxicity test solutions. The number of reference toxicant

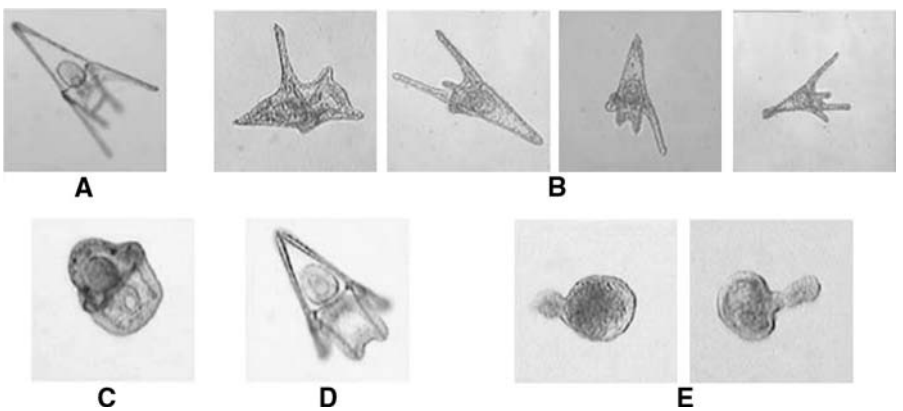

Figure 8.4 Anomalies in embryo toxicity test: (A) normal pluteus; (B) ''malformed plutei,'' including developed larvae that are developed but show some malformations (e.g., defects of skeleton and/or digestive apparatus); (C) prisms; (D) ''retarded plutei''; (E) phases blocked before differentiation in larvae (in this example exogastrulas) (10×).

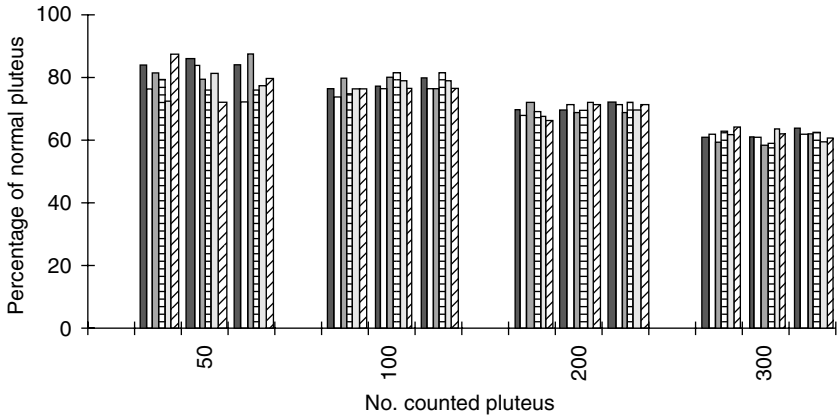

Figure 8.5 Results of repeated counts of 50, 100, 200, and 300 embryos for the embryo toxicity test. Data are referred to a negative control (3 replicates). Execution conditions: pool of gametes from 3 males and 3 females.

concentrations is usually at least 6, chosen in logarithmic scale (24, 36, 48, 60, 72, 84 μg/l) plus the negative control, and the test is performed with 3 replicates.

Elutriates are usually prepared performing dilutions at 6‰, 12%, 25%, 50%, 75%, and 100%, using artificial seawater. Three experimental replicates are used for each dilution and for negative controls.

Data analysis

Toxicity data obtained from a single concentration of pure toxicant or environmental sample may be expressed as the percentage of effect, taking into account the effect of the control, according to Abbott's formula[9]:

$$\text{percentage of effect} = \frac{100 \times \text{percentage of effect in sample} \; - \; \text{percentage of effect in control}}{100 - \text{percentage of effect in control}}$$

Percentage of effect obtained from all following dilutions can be used to calculate the EC_{50} and the NOEC values. Two statistical methods, Trimmed Spearman–Karber[10] and Probit,[9] are available to calculate the EC_{50} value with 95% confidence limits. Trimmed Spearman–Karber is a non-parametric test, the most used method; Probit is a test allowing to calculate also the slope of the concentration–response curve. In our opinion, the slopes as calculated by Probit statistical method are very important because they add important information on toxicity of a compound. The higher values correspond to steeper slopes meaning that small increases in exposure concentrations are associated with large increases in observed responses. In any case, we suggest using both methods, in order to verify statistical results better. If an experiment is programmed in order to investigate low concentrations producing effects, the NOEC value can be calculated using the Dunnett program.[11] In Table 8.2, an example with an inorganic toxicant (copper) is reported, with EC_{50} values calculated with the two statistical methods, and the NOEC. Table 8.3 gives an example of spermiotoxicity determination (as percentage of effect and EC_{50}) of environ-

Table 8.2 Example of determination of EC_{50} (with two statistical methods) and NOEC starting from experimental data of sperm cell toxicity test with a pure substance (copper). All data are expressed as μg/l

Copper (Cu^{2+})	Percentage of unfertilized eggs			
	1 replicate	2 replicates	3 replicates	Mean SD
Control	10	14	13	12
24	15	19	23	19
36	26	27	32	21
48	42	31	35	36
60	49	32	43	41
72	76	72	75	74
84	81	85	92	86
	EC_{50} trimmed Spearman–Karber method			62.42 (59.46–65.53) μg/l
	EC_{50} Probit method			64 (60–68) μg/l, slope = 7.73
	NOEC Dunnett program			24 μg/l

Table 8.3 Examples of spermiotoxicity determination (as percentage of effect and EC_{50}) of environmental samples (elutriates): sample n.1, sample with a low toxicity; sample n.2, sample with a high toxicity; n.c., not calculable

	Percentage of unfertilized eggs			
Sample n.1, percentage of sample	1 replicate	2 replicates	3 replicates	Mean
0 (control)	7	9	8	8
6	6	8	10	8
12	10	7	11	9
25	11	10	10	10
50	10	12	11	11
75	15	14	14	14
100	22	25	20	22
	Percentage of Effect (Abbott's formula)			15
	EC_{50}			n.c.
Sample n.2, percentage of sample	1 replicate	2 replicates	3 replicates	Mean
0 (control)	10	8	8	9
6	15	17	17	16
12	28	28	27	28
25	42	50	44	45
50	54	55	55	55
75	85	88	80	84
100	100	100	100	100
	Percentage of Effect (Abbott's formula)			100
	EC_{50} (Spearman-Karber)			33 (29–37)

mental samples (elutriates). Percentage of effect ranks less toxic samples whereas, EC_{50} allows to better discriminating most toxic ones.[7]

Quality assurance/quality control

A good quality assurance (QA)/quality control (QC) program in toxicological experiments requires various phases, including: (a) biological materials (adult quality used for obtaining gametes, negative and positive controls necessary to ascertain gamete quality and to accept or reject experimental data); (b) experimental conditions (constancy of conditions, cleaning of equipment); (c) toxicant characteristics (water solubility and possible used carrier, stability, volatility, ability to modify media, e.g., pH values); (d) abiotic matrices for testing (sampling methods ensuring representative samples of environmental media, sample storage, standardization of procedures to obtain test matrices). In this context, only some guidelines pertaining to points *a* and *b* are reported in the following because of the extent of topics in points *c* and *d*.

Adult quality

Adult quality can be periodically checked by morphometrical–physiological parameters, such as the gonadal index (G.I.) and percentage of maturity. These parameters are closely related to the maturity and health of the sea urchin population used for bioassays and can reveal possible reproductive anomalies. Our experience highlighted the importance of this check, also when the sampling site was changed, sacrificing some adult specimens for information on their morphometrical–physiological characteristics, in order to ascertain the state of the animals before conducting toxicity bioassays.

According to Fenaux[12] the G.I. for sea urchin is calculated as follows:

$$\text{G.I.} = \frac{\text{humid weight of gonads (ml)}}{\text{total humid weight of adult}} \times 100$$

Each adult organism is first weighed using a technical balance and the weight expressed as gram per liter. Through an incision in the peristomial membrane, all five gonads are gently extracted (using a spoon) and the sex recorded. In a 10-ml graduate cylinder containing 5 ml of seawater, the gonads are then added and the weight calculated in milliliter (the water volume displaced by the gonads). In any case the literature also reports other methods for calculating G.I.[5,13]

The percentage of maturity is calculated as the rate between the number of spawning animals during each bioassay and the total number of animals injected with KCl. For *P. lividus* at a stage of good maturation, the G.I. ranges from 5 to 9.[5] The percentage of maturity in males is never lower than 50% but is very variable in females, depending on seasons.[12]

Negative controls

Negative controls are necessary to ascertain gamete quality and to accept or reject experimental data. In all kinds of experiments, first of all artificial seawater must be used as a negative control. The acceptability of test results is fixed both at a fertilization rate and at a percentage of normal plutei of $\geq$70% in all negative controls.[8]

For experiments performed with environmental samples (e.g., elutriates and pore water extracted from sediment), an unpolluted natural or artificial sediment (control

sediment) is recommended, in order to take into account also the possible ''matrix effect.'' The experiment excludes a matrix effect when no statistically significant differences are found with tests using the control sediment.

Control chart with a reference toxicant (positive control)

In examining the intralaboratory precision of a method, a reference toxicant control chart must be generated, reporting EC_{50} values as they are obtained by new experiments.[14] This control chart includes both valid and not-valid EC_{50} data considered for the precision of the methods used and intralaboratory reproducibility calculations, and also reveals the sensitivity of the test organisms each time a new test (e.g., with environmental samples) is performed.

For both toxicity bioassays with *P. lividus*, the iterative use of the reference toxicant (copper) and the building up of the control chart yielded information on method precision (considering the minimum number of variables as one operator and one/few batch of organisms) and on intralaboratory reproducibility in several years, with different operators and a lot of batches of organisms from different sampling sites (spatial–temporal variability).[6,14] Copper was selected because it answers to most requisites for a good reference toxicant.[15]

Equipment cleaning

At the end of each test, equipment used to prepare and store water, and to prepare gamete dilution and positive control solutions are rinsed with water and left in 10% chloride acid overnight in separate tanks; this measure is strongly recommended in order to avoid any cross-contamination between the equipment used for biological materials and that used for the reference toxicant or other contaminated material. The materials are then rinsed at least six times with Milli-RO water and the same with Milli-Q water. The glass vessels in which environmental sample dilutions are prepared are washed with detergent, rinsed with water, washed with an organic solvent, such as acetone, and then the normal cleaning procedure is followed.

Results and discussion

Quality assurance/quality control

Adult quality

The G.I., calculated according to Fenaux,[12] found for *P. lividus* of the north Adriatic Sea during the period 1998–2001, revealed that the species generally shows good maturation during the period February–June. In 1999, the mean G.I. value was 7.1 ± 3 ($n = 48$, one sampling site), and in 2001, in three different sites of the Gulf of Venice, the mean G.I. values were 5.4 ± 2.2 ($n = 38$), 4.7 ± 2.3 ($n = 46$), and 5.0 ± 2.7 ($n = 35$) (unpublished data). Anomalous spawning was found in one site of the Gulf of Venice in 1997, with gametes very sensitive to the reference toxicant: the G.I. values were 2.2 ± 2 ($n = 150$) in January and 3.4 ± 2.4 ($n = 10$) in February, lower than those from a reference site (7.7 ± 1.8, $n = 115$ in January).

Negative controls

For each set of experiments (with pure substances or environmental samples), the data for negative controls are expressed by averaging all tests performed with diluted water. For example, in all experiments with organotin compounds, controls showed 95 ± 2% of fertilized eggs and 83 ± 5% of normal plutei,[6] in experiments with heavy metals, controls showed 88 ± 5% of fertilized eggs and 84 ± 3% of normal plutei.[16]

Control chart with copper as reference toxicant

The control charts of the embryo toxicity and sperm cell toxicity tests are reported in Figures 8.6(a) and (b), respectively. Detailed indications for the building up of the control chart with increasing numbers of variables are reported for the sperm cell toxicity test by Volpi Ghirardini and Arizzi Novelli.[14] After many years, and considering both the natural variability and the high number of introduced variables (i.e., sampling sites, operators, organism batches), the acceptability range considered for EC_{50} is 39–71 and 50–86 μg/l for the sperm cell and embryo toxicity tests, respectively. When EC_{50} values fall outside this range, all data produced using the same pool of gametes should be rejected or considered with due caution.

Intralaboratory variability obtained on the sperm cell toxicity test (CV = 16%, $n = 46$) was lower than the intralaboratory variability range reported for the Atlantic species *Arbacia punctulata* (23–48%)[17]; data for the embryo toxicity test were comparable with those reported by Phillips et al.[18] (CV = 12%, $n = 30$, for *P. lividus* versus CV = 20% for *S. purpuratus*) using the same reference toxicant and similar methodological procedures. Moreover, the NOEC values (copper) calculated for both sperm cell (years 1998–1999) and embryo toxicity tests (years 1997–1999) yielded means ± SD of 0.032 ± 0.008 mg/l ($n = 12$) and 0.037 ± 0.015 mg/l ($n = 9$), respectively. The mean $NOEC/EC_{50}$ ± SD ratios were, 0.60 ± 0.12 and 0.47 ± 0.29, in good agreement with those of 0.53 ± 0.13 and 0.46 ± 0.093 ($n = 6$, calculated) found for *S. purpuratus*[19] for the sperm cell and the embryo toxicity tests, respectively.

Sensitivity toward pure substances

In order to investigate the sensitivity and discriminatory ability of both tests, various compounds, such as anionic and non-ionic surfactants (only with the sperm cell toxicity test),[19] organotin compounds,[6] heavy metals,[16] and sulphides and ammonia[20,21] were studied in our laboratory.

As regards surfactants, the investigated compounds were aromatic and aliphatic surfactants of anionic (linear alkylbenzene sulfonates, LAS) and non-ionic (alcohol polyethoxylates, AE, nonylphenol polyethoxylates, NPE) types, and their aerobic biodegradation products, sulfophenylcarboxylates (SPC), polyethylene glycols (PEG), carboxylated polyethylene glycols (PEGC), carboxylated AE (AEC), and nonylphenol (NP). Results are reported in Table 8.4. The sperm cell toxicity test showed good discriminatory ability among all tested surfactants and their biodegradation products: EC_{50} values differed by about 4 orders of magnitude, ranging from 0.06 to >200 mg/l. This feature allows the test to record significantly different toxicity responses, even when differences in molecular structure are slight. The toxicity of anionic surfactants depends on the length of the alkyl chain, and that of non-ionic surfactants is due to their length and branching. Much lower toxicity was shown by aerobic biodegradation products, in comparison with that of their parent compounds, with the exception of NP. In this study, the sperm cell test

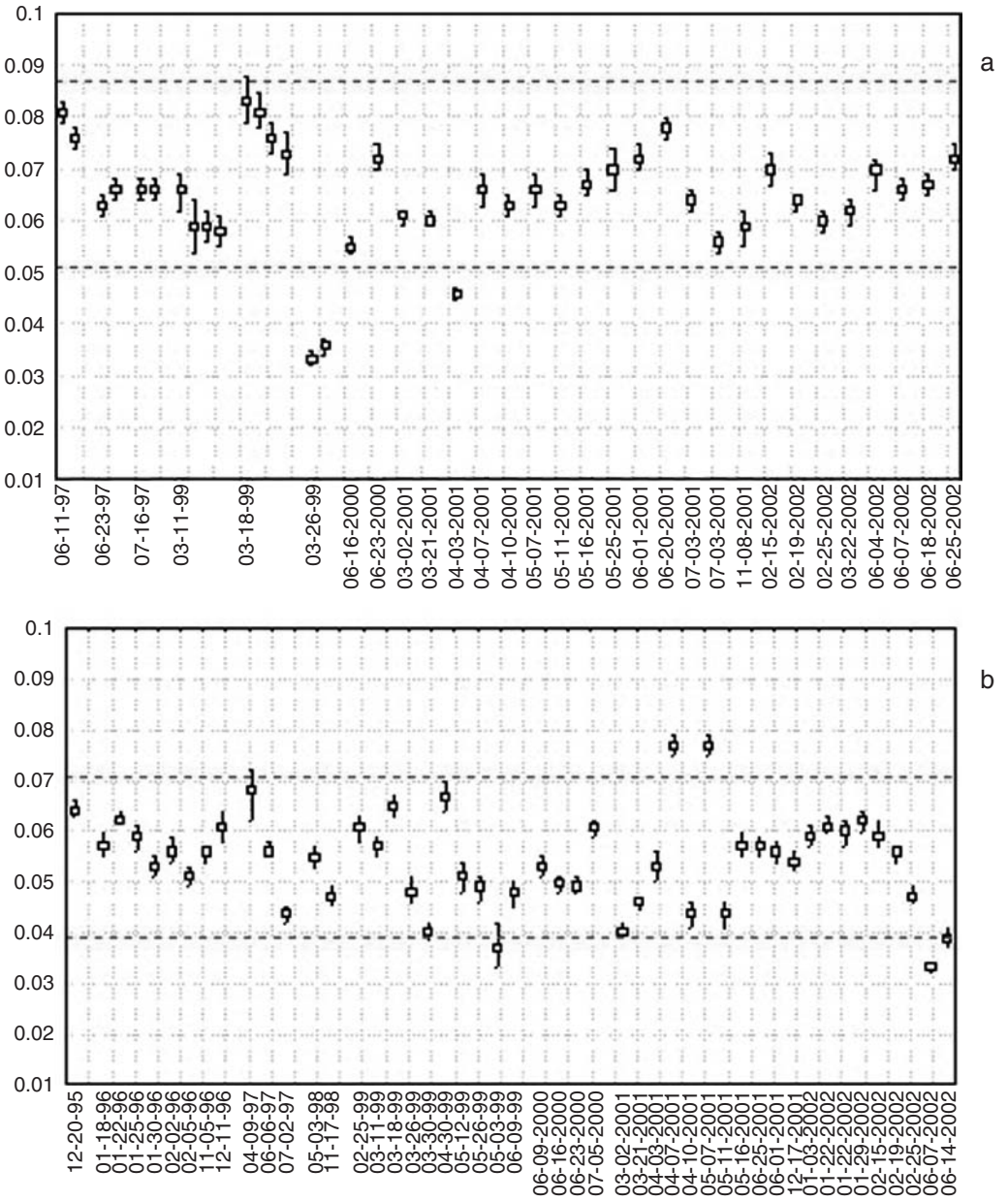

Figure 8.6 Control charts for sperm cell toxicity test (b) and for embryo toxicity test (a) obtained by repeated experiments with the reference toxicant (copper). Dotted lines: acceptability ranges. In ordinate the EC50 values in mg/L are reported.

demonstrated its predictive value in determining the toxicity of xenobiotic compounds with similar molecular structures.

As regards organotin compounds, EC_{50} and NOEC values are listed in Table 8.5. Comparisons between sperm cell and embryo assay results highlighted the fact that the latter has greater sensitivity toward triphenyl, trimethyl, and triethyl compounds, which were instead more toxic for embryos. Comparable sensitivity between sperm and

Table 8.4 Toxicity values (EC_{50} with 95% confidence limits) of anionic and nonionic surfactants for sperm cell toxicity test with *P. lividus*: euAV, average number of ethoxy units; acAV, average length of alkyl chain

Compound		Sperm cell toxicity test, EC_{50}
C_9-LAS	Nonylbenzene sulfonate	2.71 (2.64–2.77)
$C_{11.6}$-LAS	C_{10}–C_{13} alkilbenzene sulfonate mixture	1.12 (1.10–1.14)
C_4-SPC	2-sulfophenyl butirrate	>200
C_{11}-SPC	11-sulfophenyl undecanoate	21.3 (21.1–21.5)
NPE	Nonylphenol polyethoxylate with euAV = 10	1.94 (1.91–1.97)
NP	Nonylphenol	0.27 (0.27–0.28)
L-C_{12}AE	Dodecanol polyethoxylate with euAV = 9	0.94 (0.87–1.01)
L-$C_{13.7}$AE	Linear $C_{12,\ 14,\ 16,\ 18}$ AE blend with acAV = 13.7 and euAV = 10	0.062 (0.060–0.064)
O-$C_{13.6}$AE	Oxo-C12-C15 (46% linear, 54% mono-branched) blend with acAV = 13.6 and euAV = 7	0.12 (0.11–0.12)
B-C_{12}AE	Monobranched C12AE with euAV = 5	4.03 (4.01–4.05)
M-C_{13}AE	Multibranched AE blend with acAV = 13 and euAV = 8	0.92 (0.89–0.95)
PEGs 200, 400, 3000	PEG blend with euAV = 4.5, 9, 68	>200
E_4DC	Carboxylated PEG with euAV = 4	>200
AEC-3.5; AEC-5.5	Carboxylated AE blend with euAV = 3.5, 5.5	2.98 (2.87–3.10); 4.41 (4.33–4.50)

Table 8.5 Toxicity values (NOEC and EC_{50} with 95% confidence limits) of tributyltin and triphenyltin for sperm cell and embryo toxicity tests with *P. Lividus*. Data expressed in μg/l (n.c., not calculable)

Compounds	Sperm cell toxicity test		Embryo toxicity test	
	NOEC	EC_{50}	NOEC	EC_{50}
Tributhyltin chloride[a]	1.3	5.8 (5.5–6.2)	1.3	2.5 (2.4–2.6)
Tributhyltin oxide[a]	n.c.	3.0 (2.7–3.3)	1.8	2.6 (2.5–2.7)
Triphenyltin acetate[a]	n.c.	18.5 (17.8–19.3)	0.5	1.17 (1.11–1.24)
Triphenyltin hydroxide[a]	11	16.5 (15.8–17.2)	0.4	1.11 (1.04–1.18)
Trimethyltin chloride[b]	25,000	30,360 (29,040–31,730)	n.c.	139 (129.1–149.6)
Triethyltin bromide[b]	n.c.	728.7 (699.7–758.9)	n.c.	16.9 (15.8–18.1)

[a] Data from Reference 6.

[b] Unpublished data.

embryos was observed for tributyltin compounds. Investigation of larval anomalies highlighted the fact that damage caused by triorganotins is highly concentration-dependent, and that the most sensitive stages correspond to the crucial phases of differentiation (gastrula and prisma). Data for tributyltin compounds compared well with literature values for early life stages of other marine organisms. NOEC values for the sea urchin embryotoxicity test ranged from 0.4 to 1.8 μg/l and are thus not far from chronic toxicity data in the literature, in spite of very different exposure times (72 h versus several weeks).

Despite the high number of researches on the effects of tributyltin compounds, there is scarce comparable information about sea urchin early life stages sensitivity towards them. Our results concerning embryo toxicity are in agreement with those obtained for tributyltin oxide by Ozretic et al.[22] for *P. lividus*; our EC_{50} values are close to those reported by Ozretic et al. ($EC_{50} = 2\ \mu g/l$), although they used a different methodology. The proportional relationship between the damage rates occurring during differentiation with increasing toxicant concentration were confirmed by Ozretic et al.[22] and Marin et al.[23] Other authors investigated the toxicity of TBTO with other echinoid species, but it is very difficult to compare the results.[24,25] The only toxicity data concerning effects on sea urchin sperm cells are reported by Ringwood[26] for tributyltin chloride towards *Echinometra mathaei* (1-h $EC_{50} = 1.8\ \mu g/l$).

For improved information about the comparative sensitivity of sea urchin bioassays to heavy metals, which are one of the most important causes of contamination in the ecosystem of the Lagoon of Venice, the toxicity of As^{3+}, Cd^{2+}, Cr^{3+}, Ni^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+} was investigated. Toxicity values, expressed as EC_{50} and NOEC, are listed in Table 8.6, together with the EC_{50} literature range for other echinoids. Both tests were able to discriminate heavy metal toxicity, showing differences in EC_{50} values of 3 and 2 orders of magnitude for sperm and embryo toxicity tests, respectively. The latter test was particularly sensitive to Cd^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}, whereas both tests showed similar sensitivity toward Cu^{2+}, Cr^{3+}, and As^{3+}. Mercury was shown to be the most toxic of all the heavy metals examined: EC_{50} values were of the same order of magnitude, although the sperm cell test was about three times more sensitive. However, the general higher sensitivity of the embryo toxicity test with respect to the sperm cell test was also confirmed with these pollutants, and is still clearer when the NOEC values are examined. Moreover, for both heavy metals and organotin compounds, the quality and degree of defects in developmental stages depend on increasing concentration.

For all these compounds, toxicity tests using the early life stages of *P. lividus* were demonstrated to be reliable and useful tools to assess the toxicity of xenobiotic compounds, which are very dangerous for aquatic life. The combined use of these

Table 8.6 Toxicity values (NOEC and EC_{50} with 95% confidence limits) of heavy metals for sperm cell and embryo toxicity tests with *P. lividus*. EC_{50} range from literature are reported, regarding the following sea urchins species: *P. Lividus*,[27–30] *Arbacia punctulata*,[31–34] *Arbacia spatuligera*,[31] *Echinometra mathaei*,[26,31] *S. droebachiensis*,[31,35] *S. purpuratus*,[18,31,35] *S. franciscanus*,[31,35] *Dendraster excentricus*,[35] *Lytechinus variegatus*.[31] Data from Reference 16, expressed as mg/l

Metal	Sperm cell toxicity test			Embryo toxicity test		
	NOEC	EC_{50}	EC_{50} range from the literature	NOEC	EC_{50}	EC_{50} range from the literature
As^{3+}	1.9	3.9 (3.7–4.2)	No data as EC_{50}	0.1	1.9 (1.8–2.1)	No data as EC_{50}
Cd^{2+}	1.6	8.4 (7.3–9.7)	8–38[33–35]	0.01	0.2 (0.2–0.3)	0.5–11.24[29,32,35]
Cr^{3+}	0.7	3.2 (3.0–3.5)	20.7 and 341.8[31]	0.2	3.1 (2.4–3.8)	No data as EC_{50}
Ni^{2+}	0.5	5.1 (4.7–5.6)	No data as EC_{50}	0.05	0.32 (0.28–0.35)	No data as EC_{50}
Pb^{2+}	0.5	16.2 (15.6–16.8)	1.3–19[34,35]	0.003	0.07 (0.06–0.08)	0.04–0.54[26,28,34,36]
Cu^{2+}	0.03	0.07 (0.05–0.07)	0.01–0.06[26,31,34,35]	0.04	0.06 (0.05–0.07)	0.02–0.11[28–30,32]
Zn^{2+}	0.1	0.21 (0.20–0.23)	0.028–0.38[31,33–35]	0.01	0.049 (0.045–0.053)	0.02–0.58[17,30,35]
Hg^{2+}	0.01	0.017 (0.016–0.017)	0.0014[32]	0.01	0.05 (0.04–0.05)	0.008–0.044[27–29,32]

two toxicity tests supplies very important information, for a predictive view of some effects, which dangerous pollutants may have on populations in coastal marine environments.

Sulfide and ammonia are considered possible confounding factors when toxicity bioassays are used for monitoring purposes in environmental matrices (e.g., elutriates and pore water extracted from sediments). To improve the very scarce information on the sensitivity of both sea urchin methods to sulfide and ammonia, experiments with *P. lividus* were performed in the same aerobic conditions used for testing environmental samples. Toxicity data for sulfide and ammonia expressed as EC_{50} and NOEC are given in Table 8.7. Both bioassays are very sensitive toward sulfide, their EC_{50} values being comparable. In the sperm cell toxicity test, increasing sulfide concentrations corresponded to an increase in the toxic effect (i.e., an increase in unfertilized eggs). In the embryo toxicity test, although sulfide was almost completely oxidized during the 72-h period, increasing sulfide concentrations corresponded to an increase in the sum of anomalies, meaning that a sufficient sulfide concentration was present in the delicate phases of larval development to determine toxic effects. The embryotoxicity data are in agreement with previous literature toxicity data on other species of echinoids.[20]

As regards ammonia, results confirmed that for sea urchin embryos, the toxicity of ammonia is strictly pH dependent; the higher is the pH of the medium, the lower is the concentration of total ammonia required to reach 50% of embryotoxic effect (due to increased undissociated form). The sperm cell toxicity test was less sensitive to total ammonia than the embryo toxicity test by 1 order of magnitude.

Toxicity tests using the early life stages of *P. lividus* are demonstrated to be reliable, useful tools in assessing the toxicity of xenobiotic compounds, which are very dangerous for aquatic life. Both tests demonstrated high intralaboratory reproducibility, considering data obtained using the reference toxicant over many years of experimentation. Experiments with pure compounds confirmed the high sensitivity of both tests; the sperm cell test has sensitivity comparable to that of an acute sensitive test, whereas the embryo toxicity test shows sensitivity close to that shown in chronic toxicity tests. The combined use of these two toxicity tests yields very important toxicological information, for a predictive view of some effects, which hazardous pollutants may have on populations in coastal marine environments.

Table 8.7 Toxicity values (NOEC and EC_{50} with 95% confidence limits) of sulfide and ammonia for sperm cell and embryo toxicity tests with *P. lividus*. The only EC_{50} data available in literature regarded the sea urchins *S. purpuratus*.[36,37] Data from References 20 and 21, expressed as mg/l

mg/l	Sperm cell toxicity test		Embryo toxicity test		
	NOEC	EC_{50}	NOEC	EC_{50}	EC_{50} range from the literature
Total sulfide	0.1	1.2 (1.1–1.3)	0.1	0.4 (0.4–0.5)	0.19[32]
Total ammonia					
pH 7.7	—	—	1.0	5.70 (5.3–6.1)	7.2[33]
pH 8.0	—	20.7 (18.0–23.9)	0.5	4.24 (3.9–4.6)	2.98[33]
pH 8.3	—	—	0.1	3.10 (2.9–3.3)	1.38[33]

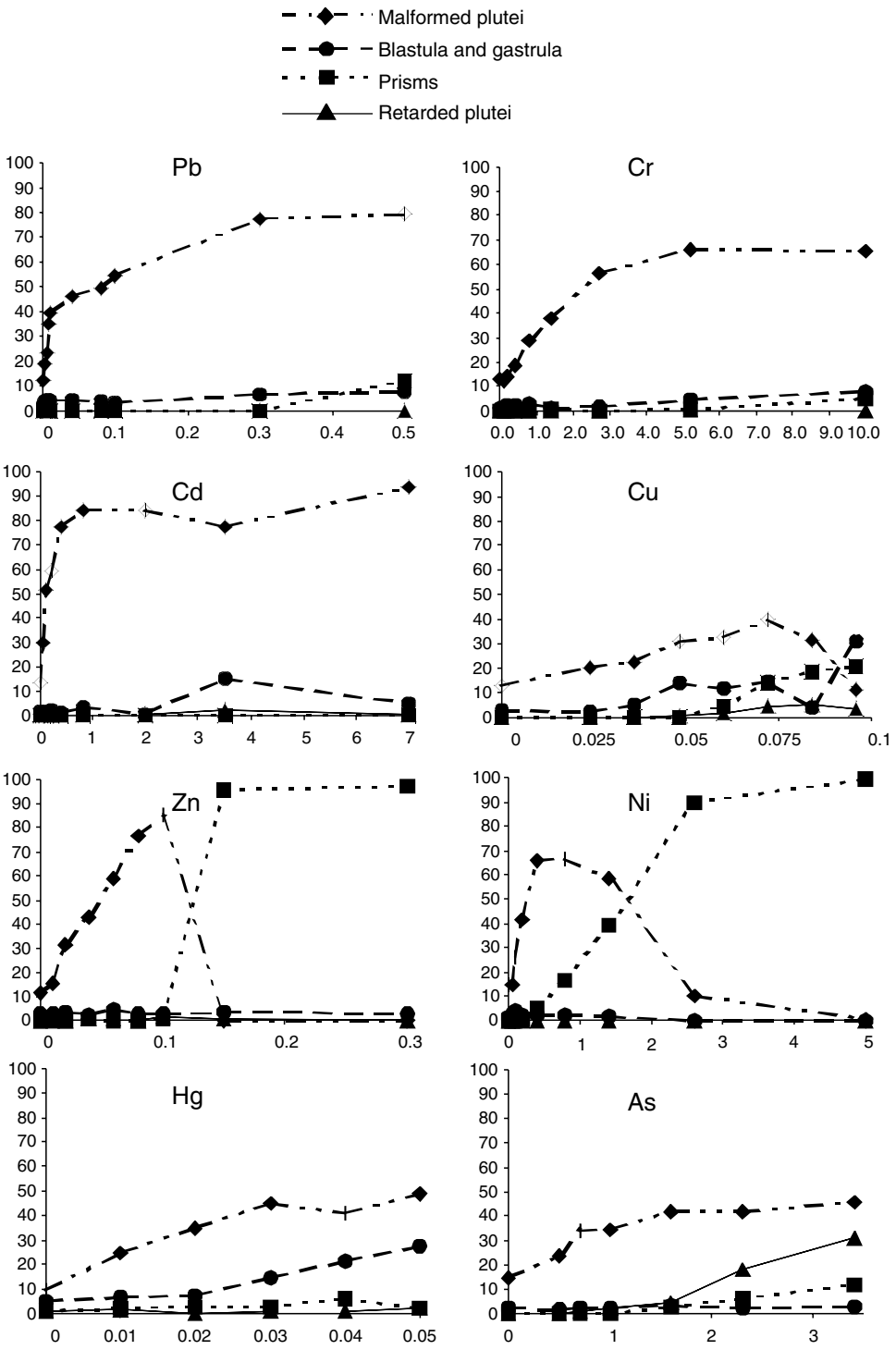

Figure 8.7 Quality and degree of defects in the developmental stages of embryos of *P. lividus* exposed to heavy metals. In abscissa concentrations in milligrams per liter are reported. In ordinate the percentage of effects is reported.

Determination of anomalies in embryo development

Careful examination of anomalies in embryo development after an experiment allows us to better discriminate the kinds of effects produced by each toxicant or environmental matrix. Determination of larval anomalies may yield many indications on the toxicity of pure substances and/or environmental samples tested. An example of the quality and degree of defects caused in developmental stages by pure substances is shown in Figure 8.7 and concerns the study carried out on heavy metals.[16] Generally, the increased concentrations of heavy metals mainly caused larval malformations, particularly evident for Pb^{2+}, Cr^{3+}, and Cd^{2+}, which showed similar trends. For Ni^{2+} and Zn^{2+}, increasing concentrations gave rise to irregular development, which led to an increase of prisms. Malformed prisms were found with 2.6–5 mg/l of Ni^{2+} and 0.15–0.3 mg/l of Zn^{2+}. For Hg^{2+} and As^{3+}, increasing concentrations caused a linear increase in malformed plutei, blastula, and gastrula stages from 0.02 mg/l for Hg^{2+} and in prisms and retarded plutei from 1.6 and 2.3 mg/l for As^{3+}. Cu^{2+} showed a different pattern in metal toxicity, with a decrease in malformed plutei and an increase in prisms, at about 0.072 mg/l and at the blastula and gastrula stages at 0.048 mg/l.

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chapter nine

Assessment of metal toxicity to sulfate-reducing bacteria through metal concentration methods

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Contents

Introduction

This is a method for the quantification of the toxic effects of a heavy metal on the activity of the sulfate-reducing bacteria (SRB). SRB have tremendous potential as remediating agents for the treatment of acid mine drainages or other metal-contaminated, sulfate-rich streams through the reactions shown in the following[1]:

1-56670-664-5/05/$0.00+$1.50

$$\text{organic matter} + SO_4^{2-} \rightarrow HS^- + HCO_3^- \quad (9.1)$$

$$Me^{2+}\ (\text{metal ion}) + HS^- \rightarrow MeS\ (\text{metal sulfide}) \downarrow + H^+ \quad (9.2)$$

The overall effect of the two reactions is the reduction in sulfate concentration, acidity, and dissolved metal concentrations. Heavy metals present in the waste streams can impact the bacterial community adversely through various mechanisms, including enzyme deactivation, protein denaturation, and exclusion of essential cations.[2,3] Quantification of the toxic effects is vital for the design and operation of an SRB-based treatment process for remediating these waste streams.[4] The technique presented in this chapter allows the determination of the toxic concentration — defined as the minimum metal concentration at which complete cessation of the sulfate reduction activity takes place, and an EC_{50} concentration — effective concentration 50, the initial metal concentration at which the sulfate reduction activity is reduced by 50% over that of a culture not exposed to the metal. The toxic concentration of the metal is determined by exposing the SRB culture to progressively higher concentrations of the metal under investigations and monitoring the activity of the culture. An active culture generates sulfide according to reaction (9.1), which then combines with the ferrous ion present in the system to form a black precipitate of ferrous sulfide. This blackening of the culture serves as the visual indicator of the activity. The concentration at which the characteristic blackening is not observed is the toxic concentration of the metal. If the metal concentration is lower than the toxic concentration, then the SRB culture retains its activity and sulfide generated due to the bacterial metabolism reduces the metal concentration to zero through precipitation as metal sulfide. Consequently, monitoring the metal concentration with time allows one to quantify the sulfate-reduction activity. The SRB culture is exposed to different concentration levels (lower than the toxic concentration) and the sulfate-reduction activities are determined as a function of the initial toxicant metal concentration. The activity of the control culture not exposed to the toxicant metal is monitored through the ferrous ion concentration measurements.

Materials required

SRB culture

Analytical instruments

Inductively coupled plasma (ICP) emission spectrometer, Perkin-Elmer Optima 3300 DV or equivalent
pH meter, Accumet AR25 (Fisher Scientific, catalog #13-636-25) or equivalent

General purpose laboratory equipment

Aluminum Seal Crimper/Decrimper, Fisher Scientific, catalog #03-375-24A/24AC
Anaerobic chamber system, Model 855 AC, Plas Labs, Lansing, Michigan
Analytical balance, Fisher Scientific, catalog #02-112-8
Autoclave, Barnstead/Thermolyne 215560
Drying oven, Fisher Isotemp* Model 506G Economy Lab Oven
General purpose refrigerator, Fisher Scientific, catalog #97-920-1
New Brunswick I2400 incubating shaker, Fisher Scientific, catalog #14-728-2
Magnetic stirrer, Barnstead/Thermolyne Fisher Scientific, catalog #11-496-30 to 32

Model 225 Benchtop centrifuge with rotor and tubes, Fisher Scientific, catalog #04-978-50, 05-111-10, 05-111-18
Muffle furnace, Thermolyne F114215

Reagents

Ammonium chloride, Fisher Scientific, catalog #A661-500
Argon gas, prepurified grade, for ICP analysis
Calcium chloride, Fisher Scientific, catalog #C79-500
Copper sulfate, Fisher Scientific, catalog #C495-500
Distilled deionized (DI) water
Ferrous sulfate, Fisher Scientific, catalog #I146-500
Hydrochloric acid, trace metal, Fisher Scientific, catalog #A508-500
Magnesium sulfate, Fisher Scientific, catalog #M63-500
Nitric acid, trace metal, Fisher Scientific, catalog #A509-500
Nitrogen, hydrogen, and carbon dioxide gases as needed for the anaerobic chamber
Potassium phosphate monobasic, Fisher Scientific, catalog #P285-500
Resazurin, Aldrich, catalog #19930-3
Sodium acetate, Fisher Scientific, catalog #S210-500
Sodium citrate, Fisher Scientific, catalog #S279-500
Sodium hydroxide, Fisher Scientific, catalog #S318-500
Sodium sulfate, Fisher Scientific, catalog #S421-500
Yeast extract, Fisher Scientific, catalog #BP1422-100
Zinc sulfate, Fisher Scientific, catalog #Z68-500

Glassware/miscellaneous supplies

0.2-μm Gelman Nylon Acrodisc Syringe Filter, Fisher Scientific, catalog #4550
Desiccator
Disposable needle, B-D 305194, Fisher Scientific
Disposable syringe, B-D 309603, Fisher Scientific
Falcon Blue Max Jr. graduated tubes (for ICP analysis), Fisher Scientific, catalog #14-959-49B
Fisherbrand Finnpipette pipetters, 100–1000 μl and 1–5 ml, Fisher Scientific, catalog #14-386-74 and 21-377-244
Fisherbrand Finntip pipet tips, Finntip 1000 and Finntip 5 ml
Fisherbrand graduated polypropylene tube (for sample storage), Fisher Scientific, catalog #14-375-150
Flexible tubing (for vacuum filtration, sparging)
Gas regulators, for gases used with anaerobic chamber and ICP
Glass beakers, from 50 ml to 4 l capacity
Glass fiber filter disks, Gelman type AE or Whatman grade 934AH
Gooch crucible and crucible holder
Gray butyl stoppers for serum bottles, Fisher Scientific, catalog #06-447G
Metal tubing (for gas connections)
Oxygen trap, Alltech Associate part #4831
Stir bars, Fisher Scientific, catalog #14-511-59
Suction flask
Volumetric flasks (for dilutions), 250 ml

Weighing dishes
Wheaton aluminum seals, Fisher Scientific, catalog #06-406-14B
Wheaton serum bottles, 125 ml, Fisher Scientific, catalog #06-406K
Wide-bore pipets, Kimble #37005 or equivalent

Procedures

Preparation of the nutrient medium

The high pH recommended for the most of the nutrient media[5] for the cultivation of SRB would result in the precipitation of the heavy metal as hydroxide. Therefore, it is recommended to adjust the pH to a lower value where the heavy metal would remain in the dissolved state. Further, the phosphate concentration also needs to be reduced to prevent metal-phosphate precipitation. The composition of the nutrient medium is shown in Table 9.1.

One liter of nutrient medium is prepared by starting with slightly less than 1 l (ca. 950 ml) of distilled, DI water in a 1.5- or 2-l glass beaker. Quantities of the components 1, 3, 4, 5, 6, and 9 shown in Table 9.1 are weighed using the analytical balance and added to the water while stirring. Components 2, 7, and 8 are added through concentrated stock solutions having the following concentrations: KH_2PO_4 5000 mg/l; $CaCl_2{\cdot}2H_2O$ 6000 mg/l; $MgSO_4{\cdot}7H_2O$ 6000 mg/l. The stock solution concentrations are tenfold higher than the component concentrations in the nutrient medium, and the desired component concentration is achieved by adding 10 ml of the stock solution to the beaker. The order of addition of the components has no effect on the medium; however, it is recommended that the component 9 (ferrous sulfate) be added at the end. One milliliter of 0.1% (w/v) indicator resazurin solution is also added per liter of the nutrient medium. The nutrient medium volume is adjusted to 1 l by adding the required amount of DI water after all the components are dissolved in water. The pH of the medium is adjusted to 6.6 ± 0.1, by addition of dilute hydrochloric acid or sodium hydroxide solutions. This nutrient medium is used for the cultivation of control cultures — cultures that are not exposed to the toxicant heavy metal. The media containing the toxicant heavy metal are obtained by spiking the control medium with varying amounts of stock solutions of the heavy metal sulfate solution. For example, spiking 200 ml of the control media with 0.1, 0.2,

Table 9.1 Nutrient medium composition

No.	Component	Concentration (mg/l)
1	Ammonium chloride (NH_4Cl)	1000
2	Potassium phosphate monobasic (KH_2PO_4)	50
3	Sodium acetate (CH_3COONa)	6000
4	Sodium sulfate (Na_2SO_4)	4500
5	Yeast extract	1000
6	Sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$)	300
7	Calcium chloride ($CaCl_2{\cdot}2H_2O$)	60
8	Magnesium sulfate ($MgSO_4{\cdot}7H_2O$)	60
9	Ferrous sulfate ($FeSO_4{\cdot}5H_2O$)	500

0.4, and 0.8 ml of 5000 mg/l of Zn^{2+} solution (as zinc sulfate) yields nutrient media containing 2.5, 5, 10, and 20 mg/l (ppm) of Zn^{2+}. The 200 ml of nutrient medium at each metal concentration and the control medium are divided into two aliquots of 100 ml each and dispensed into the 125 ml serum bottle. The serum bottles are stoppered with the butyl rubber stoppers and sealed using the aluminum crimp seals. These are then autoclaved at 121°C for 15 min for sterilization. The medium shown in the table is specific to acetate-utilizing SRB, as the organic substrate (electron donor) in the medium is acetate. The media for other SRB can be similarly designed to prevent precipitation of the heavy metal studied. The high concentration of the ferrous ion in the nutrient medium serves to trap the biogenic sulfide and thus is essential for measuring the SRB activity.

Removal of dissolved sulfide from SRB culture

It is presumed that an active SRB culture is available for toxicity assessment. The culture may be a pure strain procured from an organization, such as ATCC (American-type culture collection), and activated according to the procedure suggested, or it may be a mixed culture obtained from a bioreactor. The dissolved sulfide (hydrogen sulfide) associated with an active culture needs to be removed from the system as it will cause metal precipitation and confound results. The SRB culture obtained above is subjected to centrifugation at 2500*g* for 10 min to form a pellet. The clear supernate containing nutrient salts and sulfide is discarded. The pellet is resuspended in a volume of deaerated DI water equal to the original culture volume and again subjected to centrifugation at 2500*g* for 10 min. The deaerated DI water is obtained by purging oxygen-free nitrogen (nitrogen stripped off the residual oxygen by passing it through the oxygen trap) gas through it for 30 min prior to use. The clear supernate left after the settling of the SRB culture is discarded. This wash procedure is repeated twice to remove all the traces of dissolved sulfide. The washed culture is resuspended in a smaller quantity of deaerated DI water to obtain a concentrated suspension and used for the inoculation of the nutrient media. The culture should be washed preferably just prior to the inoculation and used without delay. However, it can be stored in the refrigerator at 4°C for several hours if needed.

The SRB concentration in the toxicity experiments is approximately 100 mg/l on a dry basis. Thus ca. 100 mg SRB culture is needed per liter of the nutrient medium prepared as described earlier. The volume of the SRB culture activated or withdrawn from the bioreactor must contain at least as much SRB biomass on a dry basis. For example, if the bioreactor SRB concentration is 2000 mg/l, at least 50 ml need to be withdrawn for the experiment. The actual volume withdrawn should be preferably between 150 and 200 ml to account for any losses, as well as the determination of biomass concentration as described in the following. The final concentrated suspension after the removal of dissolved sulfide should have the biomass concentration ca. 10,000 mg/l, so that the addition of 1 ml of this suspension to 100 ml nutrient medium results in the SRB concentration of 100 mg/l without any appreciable change in volume.

Determination of biomass concentration

The suspended biomass concentration in the washed concentrate is determined using the standard method 2540 for the measurement of biosolids concentration.[6]

A measured volume of the sample is filtered through a preweighed glass fiber filter in the Gooch crucible using the suction flask. The filtered solids are rinsed with DI water. The filter-crucible assembly is dried in the drying oven for at least 1 h at 103–105°C, transferred to the desiccator, and reweighed after cooling. The difference between the weights divided by the sample volume yields the suspended solids concentration. The volatile suspended solids are obtained by igniting the residue after drying in a muffle furnace at a temperature of 550°C. The weight loss divided by the sample volume yields the volatile solids concentration.

Inoculation and incubation of the cultures

The inoculation of the cooled media is carried out in the anaerobic chamber. The details on the setup and operation of the anaerobic chamber are available in the manual supplied by the vendor and are not repeated here. The serum bottles are opened inside the anaerobic chamber, spiked with the washed SRB stock culture seed, and resealed. As mentioned earlier, the final concentration of the biomass in the seeded serum bottles should be approximately 100 mg/l. This is achieved by spiking an appropriate volume of the concentrated, washed culture. As described earlier, spiking 1 ml of washed culture having a suspended biomass concentration of 10,000 mg/l into 100 ml nutrient medium yields the desired concentration. Serum bottles should be set up at least in duplicate and preferably in triplicate for incubation at each metal concentration. The seeded serum bottles are removed from the chamber and incubated in the shaker-incubator at ca. 150 rpm to ensure well-mixed conditions. The temperature of incubation should be the same as the temperature of activation of the pure strain or the bioreactor temperature from which the SRB culture is derived.

Sampling and analysis

The toxic concentration of the heavy metal under investigation is determined from visual observation of blackening of the serum bottles. Samples are withdrawn at specified time interval (7-day, 10-day, etc.) for analysis of metal concentration and quantification of the sulfate reduction activity as mentioned in the section ''Introduction.'' The concentration data are used to obtain the toxic concentration and EC_{50} values (defined later) as discussed in the following section. A sample is obtained from the serum bottle using a B-D disposable needle attached to a B-D disposable syringe through a 0.20-μm syringe filter. The sample is acidified to a concentration of 2% nitric acid for storage at 4°C in a graduated polypropylene tube prior to the analysis. The metal concentrations in the sample are determined by ICP method as described in the standard methods.[6] Copper and zinc are determined at 324.75 and 213.8 nm, respectively. The standard operating procedures for the instrument are specific to the instrument, and the manufacturer's instruction should be followed for analysis. Two percent nitric acid is used as a diluent to adjust the metal concentration in the sample to within the range spanned by the calibration standards.

Results and discussion

Toxic concentrations

An acetate-utilizing mixed culture of SRB was exposed to copper and zinc in one series of experiments. The source for the mixed SRB culture was anaerobic digester sludge of a municipal wastewater treatment plant, and the SRB culture derived from this source was maintained at 35°C in a master culture reactor in the laboratory.[7] Consequently, the serum bottles were also incubated at 35°C. The toxic concentrations obtained in these experiments are shown in Table 9.2.

As seen from Table 9.2, the toxic concentration of copper was found to be 18.1 mg/l in the first set and 12 mg/l for the second set. The corresponding values for zinc were 23.4 and 19.4 mg/l, respectively. The discrepancy between the two toxic concentrations for each metal obtained in the two different serum bottle sets is attributed to the fact that the initial metal concentration is a discrete variable in the experiments. In the first set of experiments, the highest initial concentrations at which sulfate reduction activity (blackening of the inoculated bottles) was observed were 9 mg/l of copper and 10.6 mg/l of zinc. Sulfate reduction was not observed in the serum bottles containing the next higher initial concentrations of metals, which are the values shown in Table 9.2. The initial metal concentration ranges were reduced in the second set, such that the highest initial metal concentrations in the second set were 16 mg/l for copper and 20 mg/l for zinc. Further, serum bottles were set up at intermediate initial concentrations (12 mg/l for copper and 14.5 mg/l for zinc) to reduce the uncertainty in the determination of toxic concentrations. Sulfate reduction activity was observed in all serum bottles with initial concentrations less than 12 mg/l of copper and 19.4 mg/l (ca. 20 mg/l) of zinc. It can be concluded that the toxic concentrations of copper and zinc for this culture were 12 and 20 mg/l, respectively.

EC_{50}

The metal ions present in the system are consumed by the biogenic sulfide according to Equation (9.2). The toxicant metal ions, as well as the ferrous ion, present in the nutrient medium acted as sinks for the biogenic sulfide. The difference between the initial and final total metal ion concentrations (on a molar basis) was attributed to precipitation of the metal ion as sulfide, and thus served as a measure of the SRB activity. The dissolved ion concentrations were measured at the end of 7 days in tube set 2. The metabolic activity of the SRB can be correlated to ionic concentrations only when an excess of metal ions

Table 9.2 Toxic concentrations of copper and zinc

Serum bottle set	Toxic concentration, mg/l	
	Cu	Zn
1	18.1	23.4
2	12	19.4

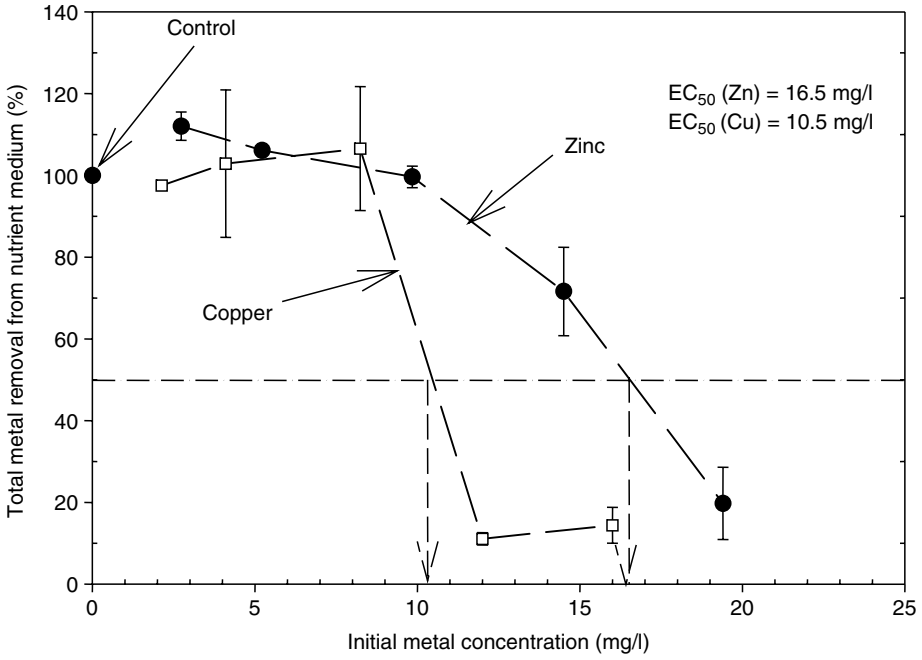

Figure 9.1 EC_{50} of zinc and copper.

(provided in this study through high ferrous ion concentrations) is present in the system. If the experiment is continued for a prolonged period of time, then all the metal ions will be consumed, and the measurements will be indistinguishable across the initial metal ion concentrations. Figure 9.1 shows the amounts of metal ions removed from the solution as a function of initial metal ion concentrations. The amount of metal removed is normalized with respect to the amount of metal removed in the control tubes and plotted as a function of initial metal concentration in the figure. The initial concentration of the metal ion at which the total metal removal was 50% of the control was defined as EC_{50} (7 days), and the values were found to be 10.5 and 16.5 mg/l for copper and zinc, respectively.

EC_{50} is an effective parameter that can be used to compare and rank different metal ions with respect to their toxicity to SRB. It can also be used to compare the toxicity of a toxicant (metal ions in this case) to SRB to the toxicity of the same toxicant to other microorganisms.

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section two

Techniques for measurement of cellular and subcellular toxicity

chapter ten

Cellular diagnostics and its application to aquatic and marine toxicology

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EnVirtue Biotechnologies, Inc.

Contents

Introduction

Theory

Biomarkers indicate the status or condition of a specific biological property. In principle, biomarkers are generally classified into three categories: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility.[1–3] Biomarkers of exposure indicate that the organism is or has been exposed to a specific class of xenobiotics or environmental conditions. Biomarkers of effect are indicative of alterations in molecular and cellular processes of an organism as a result of a xenobiotic or environmental exposure. Biomarkers of susceptibility reflect the risk for which an organism may acquire an altered physiological condition. The major difficulty in the application of biomarkers within toxicology is

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defining and linking different biomarkers within and between categories into an integrated system that can (1) identify a stressor or stressors affecting an organism, (2) distinguish between different physiological conditions, (3) elucidate the mechanism of toxicity as a result of the interaction, and (4) predict the fate of the interaction.[2]

Cellular diagnostics is a systematic approach to defining and integrating biomarkers of exposure, effect, and susceptibility based on their functionality within a cell and how alterations in the behavior of a single cellular parameter or set of cellular parameters (biomarkers) may affect overall cellular operation or performance. The cell is a dynamic system comprised of both macro- and microstructures and processes. Many of these sub-cellular processes are key metabolic pathways and cellular structural components that are essential for maintaining cellular operations and homeostasis. These cellular metabolic pathways and structural operations can be divided into categories or sub-systems of cellular integrity and function, which can be further defined by discrete parameters (Table 10.1). The behavior of these components and processes defines the physiological condition of the cell. Hence, changes in the behavior of the cellular components/processes are, in effect, changes in the physiological condition of the cell.

Adapting the concept of cellular diagnostics to aquatic toxicological assessments first requires defining cellular toxicity. The cellular diagnostic approach posits that changes in *cellular integrity* and *homeostatic responses* are fundamental end-points in discriminating whether a xenobiotic or environmental factor is affecting the cell. The terms *cellular integrity* and *homeostatic responses* can be partially and stipulatively defined by the some of the categories in Table 10.1. It should be noted that these *definiens* of cellular integrity and homeostatic responses are used because they are measurable by the present technology and are well understood. As our understanding and technology of cell biology evolves, so too will the categories and parameters that define cellular integrity and homeostatic response.

Each category is defined by a functional process and the physical components that are essential to that process. For example, protein metabolic condition can be defined, in part, by many of the RNA, protein, and enzymatic components (sub-systems) that play a role in protein synthesis, protein maturation, and protein degradation (Figure 10.1). To use the category of protein metabolic condition as an example, the three sub-systems of protein metabolic condition can be defined and measured by the following parameters:

Protein synthesis:

- Aminoacyl-tRNA synthase
- eIF1 (translation initiation factor 1; stress inducible A121/SUI1)
- p67 (modulator of eIF2 activation)

Protein chaperoning:

- Hsp72 B′ (inducible Hsp70 cytosolic homolog)
- Grp75 (inducible Hsp70 mitochondrial homolog)
- Grp78 (Hsp70 endoplasmic reticulum homolog)
- Hsp60
- Hsp90α (chaperonin)

Protein degradation:

- Ubiquitin (free and conjugated)
- Ubiquitin activase (E1)
- Ubiquitin ligase (E2)
- Lon (mitochondrial-localized protease)

Table 10.1 Categories of cellular integrity and homeostasis

Genomic integrity: The ability of the genomic process to maintain a functional state. Some parameters that are used as an index for this condition include DNA damage products (e.g., xenobiotic adducted to DNA, oxidized DNA, and DNA abasic sites), DNA repair systems (e.g., DNA glycosylases), occurrence of single and double strand breaks, chromosomal response, and mutation
Protein metabolic condition: The process of protein synthesis, protein maturation, and protein degradation. Parameters that are sensitive to changes in this category include p67, concentration and activation states of various Hsp90s, Hsp60, grp78, ubiquitin, LON proteases, and specific ubiquitin ligases
Xenobiotic detoxification: The process of preventing or reducing the adverse (toxic) effects of exposure to a xenobiotic. Some parameters of the detoxification condition of the cell include concentration and activity of various cytochrome P450 monooxygenase, glutathione-*s*-transferases, and members of the ABC protein family (P-glycoprotein 180/MDR)
Metabolic integrity: The process of a cell in maintaining a differentiated state from its environment and is the product of sub-processes or ''metabolic'' pathways. Parameters include the concentration and activity of specific enzymes (e.g., aconitase, PEPCase, ferrochelatase) and discrete organic products (e.g., pyruvic acid, ATP)
Oxidative damage and response: The process of maintaining a viable condition in an oxygen-laden environment. Parameters include changes in the concentration of oxidative damage products (e.g., protein carbonyl groups, oxidized DNA, aldehyde) and anti-oxidant enzymes and solutes (e.g., catalase and sorbitol)
Immuno-competence: The process of defending against microbial invasion. Parameters include anti-microbial peptides (*e.g.*, defensins), sterols, sulfonated glycolids, and polyphenols
Autophagy: The process of removing organelles and other cellular structures by self-digestion. This includes sub-processes, such as eradication of mitochondria, via lysosomal digestion and micronuclei deletion. Parameters that reflect autophagy state include dye retention of lysosomes (e.g., neutral red assay), activation state of Apg16p, Apg12p, and ceramide content
Endocrine competence: The ability of the cell to communicate with other cells to coordinate phenotypic expression and tissue function. This includes processes of differentiation, inflammation, and stress tolerance
Membrane integrity: The purpose of a membrane is to act as a selectively permeable barrier. Components and activities that can be used as an index for membrane condition include lipid peroxide and peroxidation breakdown products, sterol content and composition, fatty acid composition, and integral and peripheral membrane protein complexes

These molecular entities were selected as cellular diagnostic parameters for protein metabolic condition because changes in the concentration or activity of these entities may reflect a change in that sub-system. The other categories that define cellular integrity and homeostatic response are similarly structured (Table 10.1).

Shifts in the steady-state rates or levels of these parameters are indicative of a shift in the equilibrium of these sub-systems (Figure 10.2). These shifts contribute to defining the

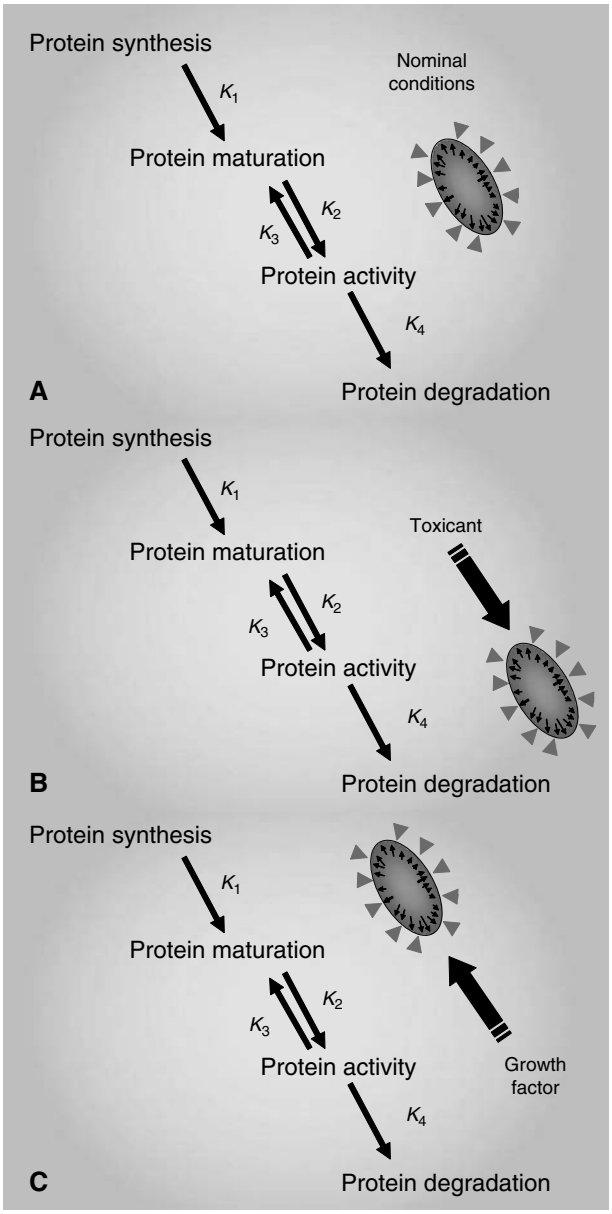


Figure 10.1 Detecting shifts in the kinetics of cellular sub-system equilibria. A primary objective of cellular diagnostics is to detect and understand the consequences of shifts in the steady-state rates of specific cellular sub-systems (e.g., Table 10.1). For example, in protein metabolic condition, there are four primary rates (K_1, K_2, K_3, K_4). Under nominal conditions (Panel A), an equilibrium is formed among the four rates. Introduction of a toxicant that either damages proteins directly, and/or up-regulates the production of specific proteins can alter the steady-state rates that were formed under nominal conditions (Panel B). The final result is a shift in the steady-state rates. Toxicants are not the only factor that can alter steady-state rates. Hormones, pheromones, cellular growth, and differentiation factors can alter the regulation of the physiological condition of the cell, possibly resulting in an increase in protein synthesis and maturation, but not degradation (Panel C). By measuring parameters of protein metabolic condition, one can determine if there has been a change in equilibrium kinetics, and in what direction the change occurred.

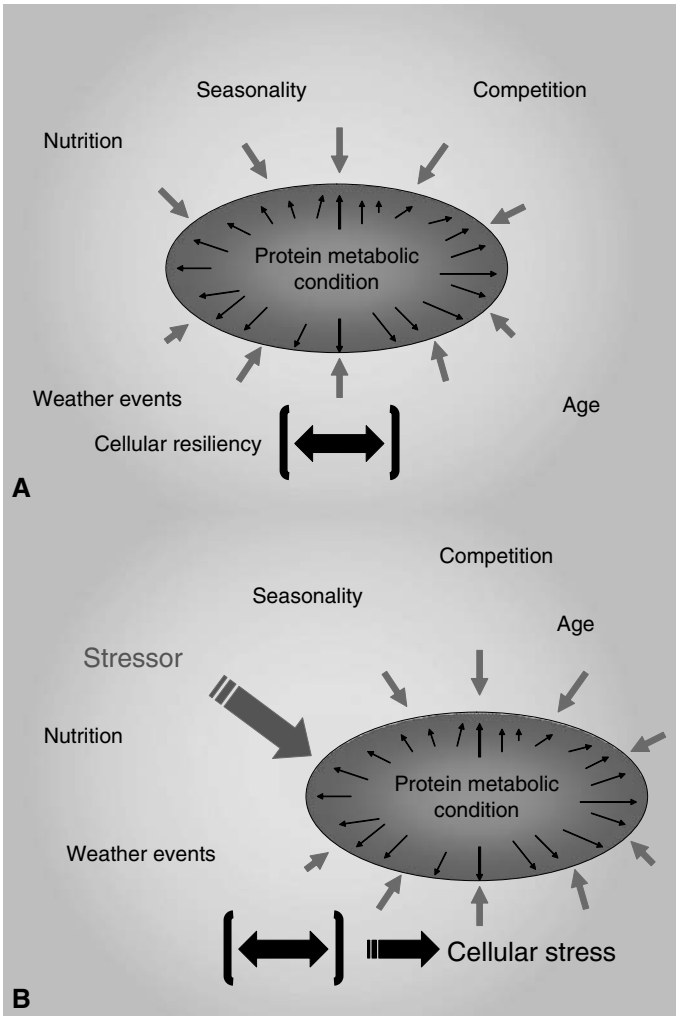


Figure 10.2 Natural variation, cellular resiliency, and cellular pathology. The steady-state rates of a cellular sub-system (e.g., protein metabolic condition) have a dynamic range. Internal and external factors shape the equilibrium of the cellular sub-system. Examples of internal factors include gene regulation, gene/protein structure (mutations), and availability of ligands and substrates. Examples of external factors include temperature, nutritional status, and age of the organism. Under most conditions in the life of the organism, the steady-state rates usually occur within a specific range. The cell has a specific range, in which the steady-state rates can exist and not incur an altered phenotype. This range is known as the range of cellular resiliency or the cellular stress capacity (Panel A). If a ''force'' factor affects the steady-state rates and so that an altered cellular condition emerges, then this shift in steady-state rates can be categorized as a stressed condition (Panel B). A stressed condition does not necessarily imply a pathological condition. Designation of a pathological condition requires detecting and associating decreases in aspects of cellular performance, such as a decrease free energy levels (e.g., ATP) or a decreases in the reducing/oxidation capacity (e.g., glutathione, NADH) of the cell.

cellular condition. Deviation of the behavior of a specific parameter from the reference is an altered state. An altered state is defined as a pathology or ''diseased condition'' for the individual (or population) only if that phenotype is associated with condition that adversely affects *performance*.[4] Performance is defined by qualitative states of reproductive fitness, metabolic condition, immuno-competence, genomic integrity, and other similar aspects (Table 10.1). Hence, a pathology or diseased condition may be defined by an abnormal vital function involving any structure, process, or system, whether it is for a cell, individual, or population. In the context of toxicology and cellular diagnostics, a diseased state is any condition where there is deterioration in cellular integrity, an inability or reduction to function, or a reduction in homeostatic capacity.

Cellular diagnostics is analogous in its methodology to clinical and engineering diagnostics, both in theory and practice. Three elements or ''concepts'' are inherent to the process of diagnosis, which include (1) mastery of the literature (scholarship) and mastery of technique, (2) observation (data) and history of the subject, and (3) the reasoning and interpretative process.[5] The criteria established in all three elements of the diagnostic process must first be met in order to achieve the goals of cellular diagnostics; to detect changes in the subject and to relate or designate an understanding of what those changes mean in terms of the quality of cellular ''health.''

The first element of the diagnostic process requires a thorough knowledge of the principles and facts of biochemistry, cell physiology, and physiology, and includes mastering the literature pertaining to each of the cellular parameters that are to be examined.[6] For example, ''Hsp70'' (heat-shock protein 70) is not just ''Hsp70''; there are constitutive and inducible isoforms and isoforms that have distinctive sub-cellular localizations that possess slightly different functional properties.[7] Knowing the functional role of each isoform is necessary for not only deriving an accurate interpretation/diagnosis of what the data means, but also to design the appropriate tests and assays in a test array. The other aspect of this element is achieving a level of skill and competence in performing the assay protocols and in handling of the specimen. Additionally, it requires the ability to modify or optimize these standardized protocols to account for the nuances inherent to the sample matrix. For example, to successfully (i.e., an acceptable level of confidence in the data produced) conduct a SDS-polyacrylamide gel electrophoresis (PAGE)/western blot immuno-assay for protein carbonyl residues on blood serum from sea otters and on polyp tissue of *Porites lobata* (a species of hard coral), different ingredients in the buffers as well as different PAGE running conditions are required for each species. An understanding of the matrix composition of the sample is crucial for optimizing assay protocols to provide an acceptable level of confidence in the accuracy of the data produced.

The second element of the diagnostic process is composed of two types of observations: *anamnesis* and test evaluation. *Anamnesis* or ''history'' is the process of recognizing and understanding the context in which the samples will be analyzed. Anamnesis is the act of taking into account a patient's history. In the context of cellular diagnostics and toxicology, it is the appreciation of an organism's life history, the organism's current state of development, the motives underlying the investigation, and the justification for sampling and testing specific tissue type(s) or the whole organism. Cellular diagnostics is based on the premise that there is a hierarchical relationship between cellular level behavior and phenomena that occur at the tissue, organismal, and population levels.[8] The toxicological investigation may be focusing on the question as to why an exposure to a particular xenobiotic decreases the fecundity of that organism or it may be focused on why mortality occurs after several weeks of exposure to a xenobiotic within a certain concentration range. The motivation of the investigation should provide a historical and conceptual context in

which the cellular diagnostic approach can operate. This is no different than the patient history that is essential to an accurate and confident medical diagnosis by the clinician.

The second component of this element is the *test evaluation*: this includes not only processing and assaying the samples but also experimental design and statistical analysis. The act of collecting, processing, and assaying the samples is the most visible component of cellular diagnostics. Designing the assay arrays and the competent execution of the assay protocols is only one aspect of the *test* evaluation. Appropriate experimental design and rigorous statistical methods are required to ensure validity of the data, and that the data can be used as legitimate premises to infer logical conclusions. Hence, not only must there be a level of confidence in the accuracy and precision of the data, but also there has to be a level of confidence in discriminating the differences between populations (reference versus treatments). Only after acquiring an acceptable level of confidence in both aspects of the test evaluation process can the clinician move to the third element of cellular diagnostics, the interpretative process.

The third element of the diagnostic process, reasoning and interpretation, can be divided into two different methodologies; mathematical theory-based methodology and an empirically driven methodology.[9] The mathematical-theory methodology focuses upon symbolic logic and probability theory. Similar to recent developments in bioinformatics, the mathematical theory-based methodology uses methods of Bayesian analysis, neural networks, and fuzzy sets to create algorithms for pattern recognition and discrimination of biomarker data and to test (correlate) for relationships between biomarker pattern and qualitative categories of cellular conditions (e.g., cellular resiliency verusus cellular stress versus cellular degeneration). The empirically driven methodology is a five-step process similar to that described by Ledley and Lusted (1959).[9] The first step is establishing the question being asked concerning the cellular condition. For example: Does exposure to a pesticide induce oxidative stress and to what extent? The second step lists the facts established from the test evaluation and the anamnesis. The third step is ranking or prioritizing the significance of the facts based on values perceived by the diagnostician. For example, the diagnostician may grade the accumulation of hydroxynonenal (HNE) in a pesticide-exposed population to be of great importance because of HNE's known role in cellular pathology and as a key indicator of oxidative damage. The fourth step is creating categories that qualify a cellular condition. In the context of oxidative stress, categories, such as ''nominal,'' ''responding to an oxidative stress, but not exhibiting damage,'' and ''exhibiting cellular damage,'' are crude but effective designations that provide a means to differentiate levels of physiological or process decline. The fifth step is the inclusionary and exclusionary categorizations of the data with the appropriate cellular condition. These two methods (mathematical versus empirical) are not exclusive of one another but are rarely used in conjunction, probably because of the lack of available exposure and formal training to these two methods. Recent work with *in silico* cellular modeling using biomarker data from field-based studies may provide an effective solution in combining the two methodologies for a more robust diagnosis.[2]

Materials required

Coral nubbins for experimental application

- Hammer or mallet
- Steel or titanium leather punch

- Metal, glass, plastic, or Teflon bolt with a head 1–2 cm in diameter
- Marine epoxy or biological glue

Sample preparation and assay protocols

Equipment:

- Ceramic or stainless steel mortar and pestle
- Microcentrifuge
- Vortex mixer
- pH meter
- Analytical balance
- Hot plate
- Container to boil water
- PAGE set
- Wet or semi-dry western transfer apparatus
- 96-well dot blotter or 48-well slot blotter
- Incubation chambers
- Rocker
- Micropipetters (10, 200, and 1000 μl)
- Multi-channel (10–200 μl) pipetter (8 or 12 tips placements)
- Scanner or camera imaging system
- Liquid nitrogen dewar
- Styrofoam box with lid (1 l)
- Computer

Supplies:

- Locking microcentrifuge tubes
- Cryovials (1.8–4 ml)
- Zinc-based spatulas
- Pyrex-based bottle (250–1000 ml)
- Micropipette tips (20, 200, and 1000 μl)
- Graduated cylinders (25 and 500 ml)
- PVDF membrane or nitrocellulose membrane with a pore size 0.2 μm or less
- Flat-plated forceps
- Microcentrifuge tube holders
- Whatman No. 5 filter paper

Reagents:

- Liquid nitrogen
- Dry ice
- Distilled water or distilled/deionized water
- Trizma base (ultra-pure grade)
- Sodium chloride
- Sodium dodecyl sulfate (ultra-pure grade)

  - *Note*: Use SDS from a bottle that has *not* been opened for more than 45 days. SDS can oxidize over time reducing its properties and compromise the sample preparation.
- Dithiothreitol (DDT) or B-mercaptoethanol (BME)
  - *Note*: DDT should be kept in the refrigerator and in a desiccator. When in solution, it should have a ''sweet'' smell to it when wafted; if it has a ''sour'' smell to it, the DDT is oxidized and its capacity as a thiol reductant is severely reduced.
  - BME has a shelf life of about 60 days after initially opened and will undergo considerable oxidation, reducing its effectiveness as a thiol reductant.
- Disodium ethylenediaminetetraacetate (EDTA)
  - *Note*: EDTA will dissolve into solution if it is titrated with 5 *M* NaOH, final pH of the solution should be 8.0
- Desferoximine methylate
- Phenylmethylsulfonyl fluoride (PMSF)
- Aprotinin
- Leupeptin
- Bestatin
- Pepstatin A
- α-amino-caproic acid
- Salicylic acid
- Polyvinylpolypyrrolidone (PVPP)
- Dimethyl sulfoxide (DMSO)
- Coomassie blue RR 250
- Methyl alcohol (methanol)
- Glacial acetic acid
- Non-fat dry milk
- Bovine serum albumin
- Tween-20

Procedures

Cellular diagnostics is a methodology that can be applied to almost any given situation in aquatic and marine toxicology. The important question is: Is its application in a given investigation appropriately and correctly applied? One of the pillars of toxicology is the investigation of a dose response of an organism or cell to exposure of a xenobiotic. In keeping with the theme of this book, a strategy is described for using cellular diagnostics to examine the serial dose effects of a single xenobiotic to the hard coral species, *P. porites*. The procedures needed to execute such an experimental design are also included.

Step 1: preparing the organism and experimental design

P. porites has a boulder-like coral structure that includes finger-like extensions from the main body. Unlike most branching coral species (e.g., *Acroporids* and *Stylophora*), the coral tissue extends well into the coralline skeleton. Tissue with an abundance of zooxanthallae (the algal symbiont found in coral) can go as deep as 11 mm into the skeleton, and Coomassie blue or Ponceau red staining suggests coral tissue devoid of obvious zooxanthallae may extend 1–2 mm deeper. Coral plugs should be 1–2 cm in diameter,

depending on how much tissue is needed for assaying and should also be 2–4 mm deeper than the zooxanthallae-tissue zone. A steel leather punch is one of the best and most inexpensive tools for creating the initial coral plug. Steel leather punches can be obtained from any local hardware store. Care should be taken to ensure that the leather punch is rust free, and washed with soap (dish soap is best) to remove any oils or silicon grease before use. For the most part, the skeleton of most *Porites* species can easily be cored without much damage to the edge of the leather punch. Extended use or denser skeletons can damage the edge of the leather punch. Jagged or bent edges on a leather punch should be sharpened or, if beyond repair, the leather punch should be discarded.

Once the coral plug is made, it can be affixed to an appropriate mounting fixture, such as the top of a clam shell, or better, the top of a plastic or Teflon-coated bolt with a 1- to 2-cm diameter head is preferable (Figure 10.3). Using a bolt-like fixture will allow for higher densities of coral plugs to be placed in the same chamber, as well as ease of manipulating the plugs between chambers. The nature of the xenobiotic should be taken into consideration. If using a fuel/oil or other types of polycyclic aromatic hydrocarbons (PAHs) as toxicants, Teflon-coated or solid-Teflon fixtures should be used because PAHs have a habit of adhering to ''non-stick'' materials, altering the working concentrations of the xenobiotic in the dosing chambers. Care should also be taken in choosing the type of Teflon used. PFE or PFA Teflon is best and PTFE and related Teflons should be avoided because of their susceptibility to degradation in saline environments. There are several types of adhesives that can be used to affix the coral plugs to the fixture. Most workers use a marine epoxy (suggest Mr. Sticky's Underwater Glue [Fairbanks, California, USA] for its low toxicity) or a biological glue. Biological glues include byssal-thread glue or collagen-based glues that are commonly used in the make-up industries that are saline resistant. Other glues that work well are medical glues, such as Pros-Aide (ADM Tronics), used in surgery or dentistry. It is important not to use standard marine epoxies because their potential toxicity, especially prolonged exposures to ultra-violet light.

The act of creating a coral core induces a stress response. Cellular damage products, such as lipid peroxides, malondialdehyde, hydroxynonenals, and protein carbonyl

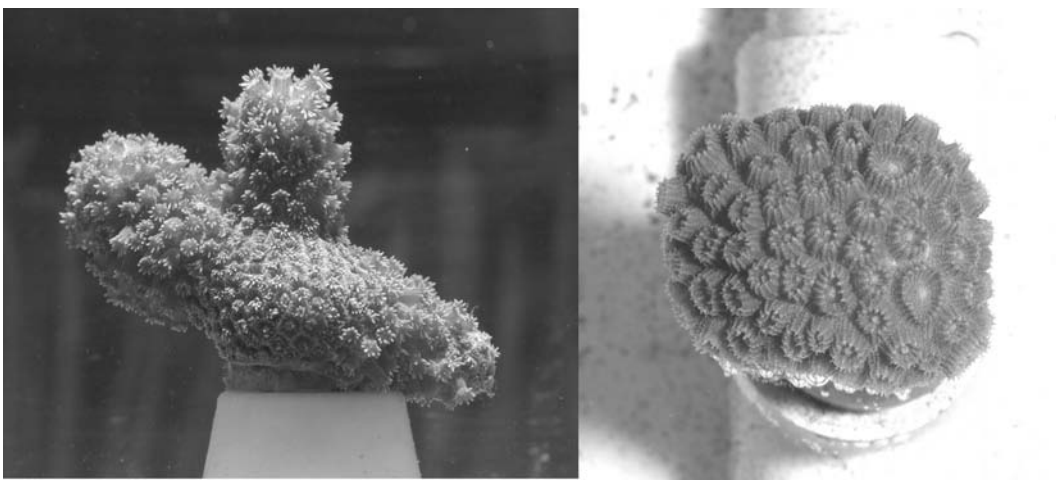


Figure 10.3 Coral plugs. Corals plugs can be mounted on an appropriate fixture so that they can be grown at high density and be easily handled. On the left, *P. porites* coral plug in the branching form. On the right, *P. porites* coral plug in a non-branching form. Photos courtesy of Tom Capo.

residues may take as long as 7–14 days to clear from the cellular system and return to basal levels. Chaperonins (e.g., Hsp60) and other stress-associated proteins may take as long as 21 days to return to basal levels. It is suggested that coral plugs be allowed to recuperate and acclimate for at least 2 months before being used in an experiment. Culture conditions like water flow, temperature, light, and feeding methods will not be discussed here, but the U.S. Coral Disease and Health Consortium can be used as a good resource for this information due to the participation of coral culturing experts from both commercial and academic sectors (http://www.coral.noaa.gov/coral_disease/cdhc.shtml).

The dosing system itself may be a significant source of artifact. Dosing corals in static chambers for even 24 h can produce significant artifact. Most coral species require a specific rate of fluid flow over them. In some coral species, the optimal flow rate is almost negligible, while in other species, the optimal rate may be as high as 4 ml min^{-1}. *P. porites* requires a high flow rate, and low flow rates can result in decreased growth rates, increased ammonia secretion, and a significant increase in many stress and growth proteins within 48 h. Ammonia/nitrogen toxicity is an important factor to consider, especially with static dosing systems. Frequent water changes (e.g., every 12 h) or a water flow-through system can control ammonia artifact effects. Light concentration is another significant source of artifact. Indoor ambient lighting is relatively low, even if it seems bright to our eyes (compare 50 μmol of photons m^{-1} s^{-1} in most laboratories versus 2100 μmol of photons m^{-1} s^{-1} outside at noon on a clear day). The rate of photosynthesis is dependent on the concentration rate of light, as are all of its potentially adverse by-products, such as active oxygen species. For photosynthetic organisms, many herbicides and toxicants are light dependent for acute toxicity (e.g., methyl violagen, atrazine, diuron). At least 500 μmol of photons m^{-1} s^{-1} should be used during an experiment. Light quality is also another factor. Like plants, coral zooxanthallae require certain wavelengths of light more than other wavelengths to have optimal photosynthesis. The quality of light emitted by a sulfur lamp or a standard fluorescent lamp is very different compared to the spectrum composition of natural light. Two options in resolving the issue of quality and quantity of light are to use natural light and alter the quantity of light by means of neutral density filters or to use a solar simulator generator. Though solar simulator generators can produce some of the most consistent light conditions from one experiment to another, their expense makes their use prohibitive.

One of the best systems for coral dosing is a recirculating system (Figure 10.4). The system can be placed on a standard laboratory cart that has two benches. On the top bench sits an insulated dosing chamber that is deep enough so that the coral core is submerged at least 4 cm below the surface. On one end of the chamber is a flow-in port, while on the opposite end of the chamber is the flow-exit port; seawater flow enters from the flow-in port and exists from the flow-exit port. On the bottom bench sits a 10- to 20-l reservoir. A variable flow-rate pump will pump water from the reservoir to the chamber. Water flows back to the reservoir from the dosing chamber via the flow-exit port by gravity. Shut-off valves on the tubing that interfaces between the reservoir and the dosing chamber can be installed to make it easy to switch out the reservoir at appropriate time intervals, depending on the length of the exposure. The top part or lid of the chamber can be left open to allow for lighting. A coil connected to a recirculating chiller/heater can be placed in the reservoir or dosing chamber to control for temperature.

Coral plug samples that are collected for testing should be rinsed free of excess mucus with fresh seawater, and quickly ''toweled'' or dried with a fiber-free towel for excess seawater. Excess seawater with the samples can dilute the final concentration of the

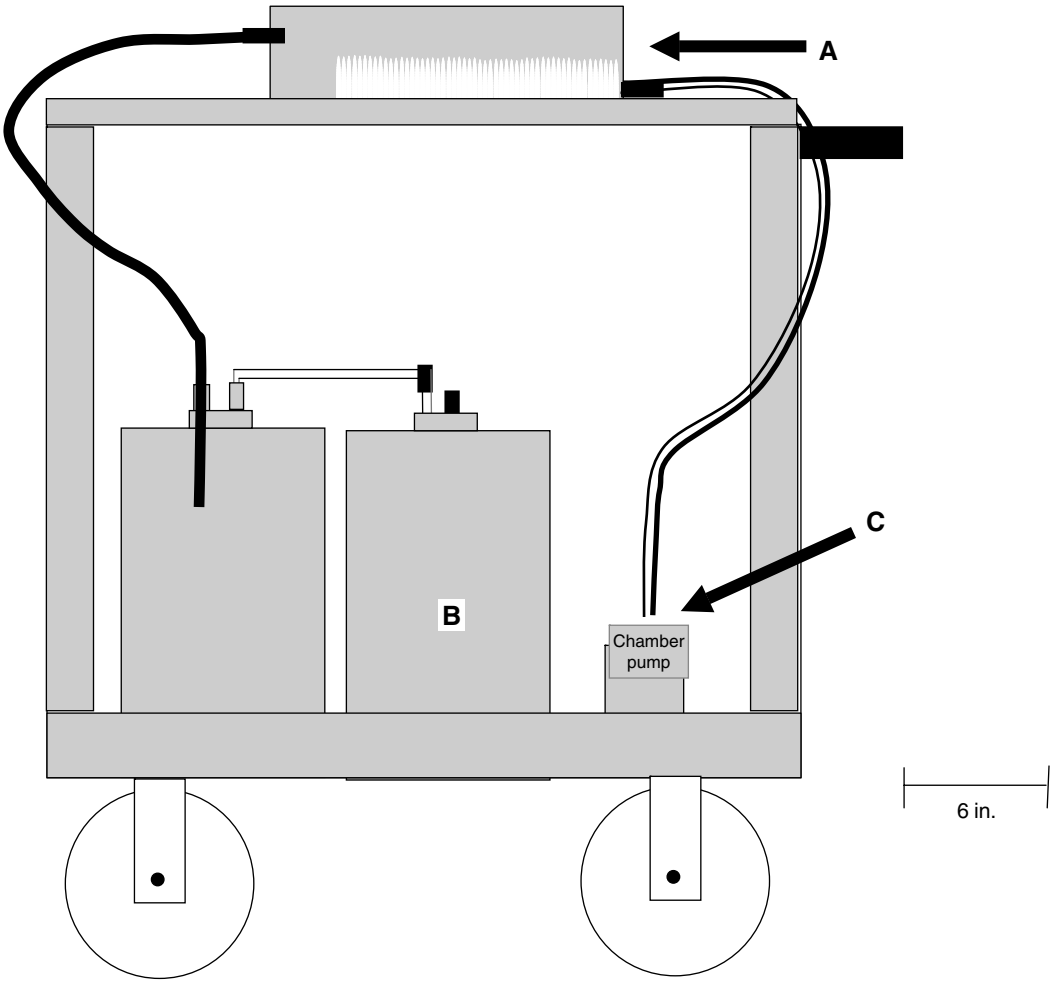


Figure 10.4 Dosing system for corals. Static systems can produce significant artifact, especially if the exposure to the xenobiotic is longer than 24 h. The entire system can be placed on a two-shelf cart. On the top shelf is the dosing chamber, a chamber about 2 l in volume, large enough so that the top of the coral plug is covered by at least 6 cm of water. The top of the dosing chamber is open to the air (light transmission). Water is pumped into the chamber from a set of 25-l reservoirs by a variable-flow pump that sits on the bottom shelf of the cart. Reservoirs can be changed out at designated time points (8–12 h) with fresh solution of seawater/xenobiotic.

sample during sample preparation and can cause artifact when running the sample on a SDS-PAGE. Coral should be quickly frozen in liquid nitrogen or dry ice and kept at –80°C or colder until sample preparation.

Sample preparation, quality control validation, and ELISA protocol

Tests

The protocols described in the following focus only on enzyme-linked immuno-sorbent assays (ELISA). There are a number of non-ELISA tests that can be included in a standard cellular diagnostic assay arrays. For example, spectrophotometric assays, such as those

for lipid peroxide, glutathione, and for measuring specific enzyme activities (e.g., esterase, EROD), can readily be included in an array of tests with some modification of the sample preparation protocols. Other innovative assay protocols, such as in this volume by Dr. Kouda and co-authors (Chapter 15), PCR-based assays, COMET assay, and HPLC-based assays, can readily be included in a cellular diagnostic assay array.

Sample preparation

Pre-chill the mortar and pestle with liquid nitrogen equivalent to two volumes of the mortar. Add half or all of a coral plug sample to the mortar. Immediately half-fill the mortar with liquid nitrogen, and after about 5 s begin grinding the sample with the pestle, making sure that the sample never thaws. The sample should be ground to a consistency between that of table salt and fine flour. The spatula and cryovial are pre-chilled in dry ice. Use the spatula to transfer the sample powder to the cryovial. The sample should be kept at –80°C or lower. Thawing and refreezing will cause the sample to solidify, making it impossible to use in further sample preparation steps unless it is reground.

The denaturing buffer should be made within 4 h of use and consists of 2% SDS, 50 m*M* Tris–HCl (pH 6.8), 25 m*M* DDT, 10 m*M* EDTA, 0.001 m*M* sorbitol, 4% PVPP (w/v), 0.005 m*M* salicylic acid, 1% (v/v) DMSO, 50 μ*M* desferoximine methylate, 0.04 m*M* Bestatin, 0.001 m*M* E-64, 2 m*M* PMSF, 2 m*M* benzamidine, 5 μ*M* a-amino-caproic acid, and 1 μg/100 μl pepstatin A. Transfer 50–100 mg of sample from the cryovial to the locking-cap microcentrifuge. Add 1400 μl of denaturing buffer to the microcentrifuge tube, lock the cap to the tube, and vigorously vortex the sample for at least 10 s. Incubate the tube in a 90°C water bath for 3 min, vortex the tube for 10 s, and then reincubate at 90°C for 3 min. Remove the tube and allow it to cool at room temperature for at least 5 min. Centrifuge the tube for 15 min at least 13,000*g*. Three phases will be evident after centrifugation; the bottom phase is the insoluble phase consisting predominantly of coralline skeleton and PVPP (Figure 10.5). The middle phase should be transparent, brownish in color, and free of a whitish film that characterizes the top phase. The top

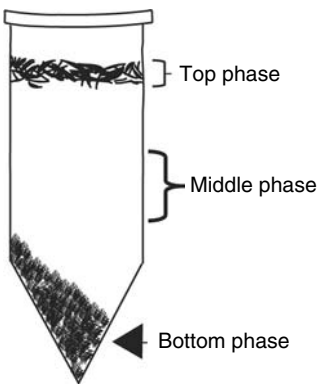


Figure 10.5 Sample preparation. Three phases are formed after centrifugation of the sample. The bottom phase is insoluble debris, primarily calcium carbonate skeleton, and insoluble PVPP. The middle phase should be relatively transparent and should be absent of residual mucus. It should be non-viscous. The top phase should be somewhat viscous and sticky, whitish in color. The demarcation between the middle and top phases is not always apparent, and care should be taken that when aspirating the middle phase, there should be no contamination from the top-phase matrix.

phase is a very viscous matrix composed predominantly of cross-linked polysaccharides. Aspirate 200–300 μl of the middle phase; be careful not to collect any supernatant that may be contaminated with the whitish matrix, which is the top phase. Deposit the middle-phase supernatant into a new tube.

Determining the protein concentration of the sample is necessary to equalize the sample for loading onto SDS-PAGE/western blotting assays, ELISA, and for normalizing the data when calculating the quantity of the parameter in the sample. Protein concentration is one of the most common parameters assayed, but unfortunately, it is one of the least understood assays. Besides sample preparation, assaying for protein concentration is a potential source of artifact and variation that can affect all subsequent assays. There are a number of good sources that explain both the method and theory of a number of different protein concentration assays that should be studied.[10] Many components of a denaturing buffer, as well as compounds inherent to the sample, may potentially interfere with a particular protein concentration assay. For example, chlorophylls and carotenoids in plant and photosynthetic material (e.g., coral, some species of gastropods and mollusks) can interfere with accurate measurements using Coomassie-based spectrophotometric assays. For corals, Ghosh et al.'s (1988)[11] protein concentration assay is a preferred method, because it is amendable to high-throughput, does not require a protein precipitation step to exclude interfering substances produced by the zooxanthallae, and is not affected by the harsh reagents found in the denaturing buffer recipe described in this chapter.[11] Using Whatman No. 5 filter paper rather than using a microtiter plate is a better medium for a blotting platform. Using SDS denatured bovine serum albumin or oval albumin is acceptable as protein concentration standards, though the highest concentration standard that should be used is 2.5 mg ml^{-1}. Sample protein concentrations above 2.5 mg ml^{-1} are suspect because the ratio of SDS to protein does not ensure adequate binding of the SDS to protein, which can lead to protein aggregation and precipitation as a result of freeze/thaws and during the heat-denaturation step of the sample preparation.

Apply 1 μl of sample in triplicate to the filter paper (5 or 10 cm diameter). Samples and calibration curves can be designated on the paper using a pencil (No. 2). Allow the samples to completely dry, either air dry for 10 min or with a hair dryer. Incubate the filter paper on a rocker in the stain solution for at least 10 min. Pour off the stain solution, and then apply 4–5 washes of the destain solution, incubating 5–8 min for each wash. The filter paper untouched by sample or calibrant should be white and not have any remnants of blue stain (Figure 10.6).

Stain solution:

- 200 ml of distilled water
- 50 ml of glacial acetic acid
- 250 ml of methanol
- 4 g of Coomassie blue RR 250

Destain solution:

- 800 ml distilled water
- 200 ml of glacial acetic acid
- 1000 ml of methanol

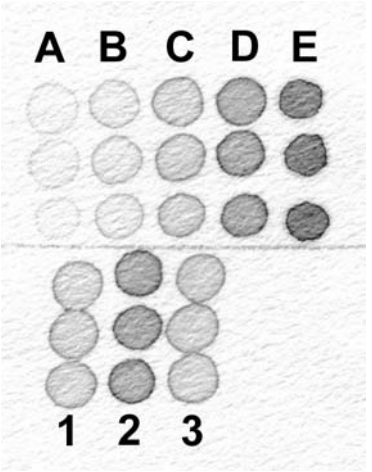


Figure 10.6 Protein concentration assay. Results of protein concentration assay using a modified method of Ghosh et al. (1988).[11] Concentration calibrants are plated on the Whatman No. 5 filter paper as in columns A–E. Column A = 0.125 μg μl^{-1}. Column B = 0.250 μg μl^{-1}. Column C = 0.5 μg μl^{-1}. Column D = 1.0 μg μl^{-1}. Column E = 2.0 μg μl^{-1}. Samples done in triplicate indicated in columns 1–3. All calibrants and samples are plated (hence assayed) on the filter paper in triplicate. Assay paper can be scanned by a scanner to create a digital image. Optical densities for each replicate can be determined using a densitometry program, such as NIH Image developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

Sample validation

Quality control step 1: Confidence that the assays are measuring the appropriate cellular marker is usually required for most scientific publications, government reports, or litigious discussions. SDS-PAGE/western blot analysis is the minimum level of quality control/quality assurance. Western blot analysis will demonstrate that (1) preparation of the sample was satisfactory, and that (2) the antibody for the set of samples can be validly used for ELISA. Criteria for confidence assurance are:

- There is no ''smearing'' of the sample lane
- Band(s) are present at the expected migration rate
- Absence of non-specific cross-reactivity

Aliquots of 100 μl from four random samples should be pooled, the protein concentration determined for the pooled sample and applied to SDS-PAGE. Concentration of acrylamide and other gel contents are specific to the type of parameter being examined. For example, assaying of small heat-shock proteins and metallothioneins will require specific reductants in both the denaturing buffer and in the separating gel. Adding reductants, such as TCEP (pH neutral) at 1 m*M* in the separating gel, will ensure thiol reduction as the thiol-rich proteins move through the gel. Altering the percent of acrylamide in the separating gel can enhance the resolution in seeing differences, depending on the molecular weight of the target protein. Finally, it is important to ensure that the gel has SDS in both the stacking and separating solution. Many pre-cast gels lack SDS, which can affect the migration rate of many of the target proteins.

Contents of gels are transferred to activated PVDF or nitrocellulose membrane using either a semi-dry or wet transfer system. PVDF blots are incubated in a blocking buffer and then transferred to the primary antibody solution for an allotted time at a specific temperature. Blots are washed four times with a wash buffer, incubated with an appropriate conjugated secondary antibody for 1 h, washed four times with a wash buffer, and then developed using an appropriate detection system. Replicates of the sample blots should also be assayed with only the conjugated secondary antibody with no exposure to a primary antibody in order to determine level of artifact caused by the conjugated secondary antibody (non-specific cross-reactivity).

Blocking solution:

- 1× Tris buffered saline (pH 8.4); 500 ml; 15 m*M* NaCl, 50 m*M* Trizma base; titrate pH of buffer with 3 *N* HCl to 8.4
- 30 g of non-fat dry milk

Wash solution:

- 2× Tris buffered saline (pH 8.4).
  - Tween-20, 0.01% (v/v). If the Tween is old (shelf life of 45 days), it may undergo oxidation, causing an increase in non-specific cross-reactivity.

Primary and secondary antibody solution:

- 2× Tris buffered Saline (pH 8.4)
- Pre-determine titer for optimal specificity

Blots can be developed either colorimetrically, chemiluminescently, or fluorimetrically. There are a number of good references that describe standard SDS-PAGE/western blotting procedures in detail.[10,12]

The purified protein of the target parameter should be run on the lane next to the sample to ensure that the migration rates are the same. Using only molecular weight standards is a practical means of approximating that the band is the right migration rate, but there is more confidence in using the purified protein or a sample that you know has the protein, because you can account for the migration nuances of the protein running under specific buffer and gel conditions.

There are five types of results that can be observed on a blot when working with coral or other invertebrates that house an algal symbiont and plant samples. The five types of results are:

1. Band at the expected migration position with no other non-specific cross-reactive bands (Figure 10.7A).
2. Band is *not* at the expected migration position, but bands can be observed at different migration positions. There is no confidence that the antibodies, the sample preparation, or the running conditions are optimal for ELISA (Figure 10.7B).
3. Band at the expected migration position, but with bands at unexpected migration positions. For example, if you are assaying for invertebrate manganese superoxide dismutase, you should see a band around 25 Ku (standard international designation for migration of proteins for gel electrophoresis). You may also see

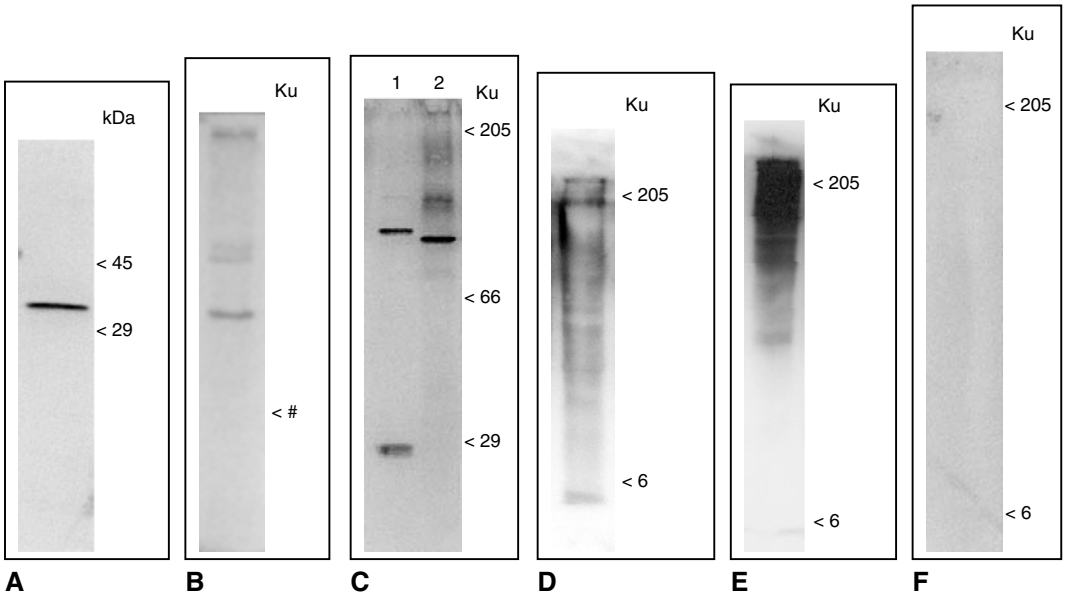


Figure 10.7 Quality control step 1: possible results for immuno-assaying with western blotting. Panel A: acceptable result of a sample assayed for heme oxygenase, which should have a band around 32 Ku. Panel B: unacceptable result of a sample assayed for glutathione-*s*-transferase, which should have a band at the position designated by #. Panel C: unacceptable result of a sample assayed for chloroplast small heat-shock protein (sHsp). In lane 1, the monomer of the chloroplast sHsp is detected at the 29 Ku migration distance, but there are also higher-molecular weight bands. The sample in lane 1 was made up in sample buffer with 2% SDS, 10 m*M* thiol reductant, and was not heat-denatured. In lane 2, the sample was made up in sample buffer with 0.5% SDS, no thiol reductant, and was not heat-denatured. Notice that the monomer is not present. Panel D: coral sample (*Montastrea annularis*) that was prepared in a sample buffer lacking PVPP and exhibits streaking as a result of extensive Maillard product contamination. Panel E: coral sample (*M. faveloata*) had extensive mucus contamination that was not removed during sampling. Panel F: coral sample that was assayed using only the HRP conjugated secondary antibody to ensure that the secondary antibody was not associated with any non-specific cross-reactivity.

bands around 40 Ku, and above the 190 Ku markers. There may be something wrong with the sample preparation, with the antibodies, or with the running conditions of the gel. For example, many enzymes exist in a multimeric structures and require harsh denaturing and reducing conditions to break the enzyme complex apart into its separate components. These slower migrating bands may be the non-denatured, multimeric structures, as in the case of homogenization buffer lacking SDS and run on a native gel. In any case, there is no confidence in the sample or conditions to conduct an ELISA (Figure 10.7C).

4. There is a dark smear going the entire length of the sample lane. This is most likely due to extensive Maillard product formation. Polyphenols from the algae are cross-linking proteins and carbohydrates. Increasing the concentration of PVPP or adding PVP (0.5% w/v maximum) may rectify this artifact. Additionally, Na tetraborate (5 m*M* maximum) may further help reduce the level of Maillard product formation. Nonetheless, there is no confidence that the samples can be subject to an ELISA (Figure 10.7D).

5. There is a dark smear running from the top of the lane to about the 66 Ku marker. This artifact is most likely the result of high polysaccharide contamination. When aspirating the middle phase during sample preparation, some of the top phase was also aspirated as part of the sample. Multiple centrifugations/aspirations, subjecting the sample to a polysulfone microcentrifugation filter, or increasing the level of DMSO or adding L-cysteine (1 m*M* maximum) may help to reduce this artifact. There is no confidence that the samples can be subject to an ELISA under their present state (Figure 10.7E).

When assaying a sample blot with only the secondary antibody, the blot should be clear. Any bands or smears appearing on the blot can mean that the secondary antibody used for both western assay and the ELISA is non-specifically cross-reacting with some epitope in the sample (Figure 10.7F). Fab fragments conjugated with the appropriate reporter enzyme usually give the best results under most situations.[13]

Quality control step 2 and ELISA optimization: The pooled sample from step 1 is serially diluted eightfold, the highest concentration is 6.667 ng μl^{-1}. A 16-fold serial dilution of the calibration standard is also created. The calibration standard is the purified protein or antigen being assayed. The concentration of the calibration standard should be known and designated either in moles or grams of the antigen. Both the sample serial dilution and the calibrant serial dilution should be plated on a 384-well microtiter plate or on a membrane dot or slot blotter. Because most labs can affordably access a dot or slot blotter, the protocol will focus exclusively on using a dot/slot blotter for carrying out the ELISA. If using a Bio-Rad or other brand of dot/slot blotter, activate the PVDF membrane in methanol and then equilibrate the membrane in 1× TBS for 1 min. If using a nitrocellulose membrane, equilibrate the membrane for 1 min in 1× TBS. Quickly place the membrane within the manifold of the dot/slot blotter and assemble the dot/slot blotter. Apply the serial dilutions of both the sample and the calibrant in triplicate on the dot/slot blotter. Contrary to most manufacturers' instructions, do not incubate the serial dilutions on the dot/slot blotter for 20 min or longer. Once the samples and calibrants are loaded onto the blotter, suctioning the samples through the membrane should be done immediately. A hand-held vacuum, such as the Nalgene hand pump, is one of the best instruments for creating a vacuum for a dot/slot blotter. You can control the vacuum pressure much more readily than with a motorized pump, as well as maintain better control in releasing the vacuum. A slow release of the vacuum pressure is best because it prevents backwash of the samples back across the membrane; a major source of artifact for dot/slot blotters. Immediately transfer the membrane to the blocking solution and carry out the development of the blot using the same protocols as done in ''quality control step 1.''

Once the blot is developed, scan or image-capture the blot. NIH Image is an easy to use and free optical density program that can be used on PC or Macintosh computers. Determine the optical density of each point and then plot the data. A trend line can be fitted using either a liner or polynomial (second order) regression, and the equation and R^2 for the trend lines are determined.[13] Lack of linearity (or curvilinearity), such as a low R^2 value, is an indication of artifact.[13] Sample loading, primary and secondary antibody titers, as well as incubation, and wash conditions can all be optimized during this step. Intra-specific variation for each triplicate concentration point should be less than 15%. Finally, the calibrant standard curve should be optimized with the sample loading, so that the signal of the sample falls somewhere in the middle of the calibrant standard curve. To increase the probability that all the samples will fall within the calibrant standard curve,

create a serial dilution of a sample from the reference treatment and a serial dilution of a sample from the highest concentration from the xenobiotic-exposure treatment.

A ''third quality control step'' is expensive, training intensive and time consuming but bestows the highest confidence that the antibody is truly detecting the correct protein or antigen in the samples. This step entails the construction of an immuno-column using the primary antibody, purification of the immuno-column elutant, extensive fractionation protocols if there is more than one protein eluted from the columns, and Edman degradation or Mass spectrometry peptide sequencing. This step is perceived as optional only because of the expense, extensive training, and accessibility to expensive supplies and equipment that is required to conduct the quality control step. Protocols for this step can be found in a number of good resources.[10]

ELISA: All samples and calibrants should be plated onto the dot/slot blotter in triplicate. Optimally, a single calibrant curve should be plated to the left, middle, and the right sides of the dot/slot blotter (Figure 10.8). Sample replicates can be plated in a similar fashion, or plated close together. The reason for dispersing the calibrant curves throughout the microtiter plate is to account for the potential of edge effects or differential staining from one side of the blot to the other. Data should be calculated as mole or gram per nanogram of total soluble protein (normalization). For example, when measuring Hsp60, the data should be calculated as moles of Hsp60 per nanogram total soluble protein or grams of Hsp60 per nanogram total soluble protein.

Statistical analysis of data

The first step of the analysis is to determine if the level of a specific parameter in any of the treatments are significantly different from the control population. Data should be first tested for normality and equal variance. If the data are normally distributed, apply a one-way ANOVA; but if the data are not normally distributed, consider the assumptions of other types of analyses, such as a one-way ANOVA on Ranks (e.g., WELCH ANOVA). If treatment differences are found, apply the appropriate planned comparison test[14] (Figure 10.9).

Canonical correlation analysis (CCA) has been used in the past as a heuristic tool to illustrate how biomarkers can be used to discriminate among xenobiotics and

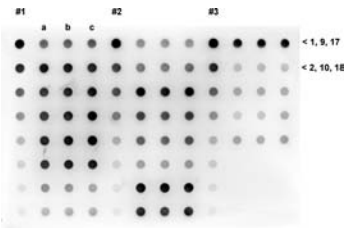
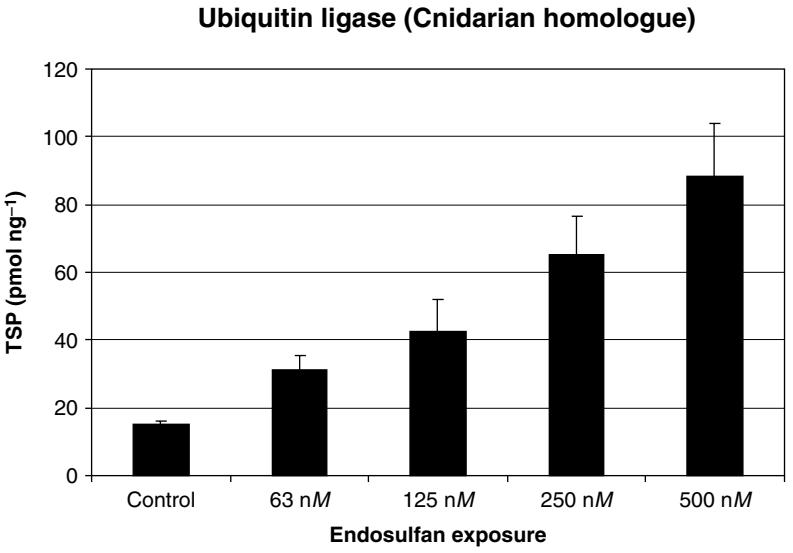


Figure 10.8 ELISA's result using a 96-well dot blotter. # = calibrant standard curve. The calibrant with the highest concentration in plated in row 1, the calibrant with the lowest concentration is plated in row 8. Optimally, replicates of the standard curve should be evenly distributed across the dot blotter. In row 1, triplicate assays of samples 1, 9, and 17. In row 2, triplicate assays of samples 2, 10, and 19. In column a, replicate one of samples 1, 2, 3, etc., are plated. In column b, replicate two of samples 1, 2, 3, etc., are plated. In column c, replicate three of samples 1, 2, 3, etc., are plated. Plate represents data for the chloroplast small heat-shock protein in samples of the hard coral, *M. annularis*.



Normality test: Passed ($P > 0.050$)
Equal variance test: Passed ($P = 0.057$)

One-way ANOVA

Source of variation	DF	SS	MS	F	P
Between groups	4	11619.839	2904.960	34.222	<0.001
Residual	15	1273.281	84.885		
Total	91	2893.120			

α=0.05

Mutiple comparisons versus control group (Holm-Sidak method):
Ovcaerall signifince level = 0.05

Comparsion	Diff. of means	t	Unadjusted P	Critical level	Significant?
Col 1 vs. Col 5	67.814	10.409	0.000	0.013	Yes
Col 1 vs. Col 4	50.314	7.723	0.000	0.017	Yes
Col 1 vs. Col 3	27.564	4.231	0.001	0.025	Yes
Col 1 vs. Col 2	16.377	2.514	0.024	0.050	Yes

Figure 10.9 *P. lobata* (tropical hard coral species) exposed to endosulfan (a pesticide) in a dose response experimental design. $N = 4$ per treatment. Coral plugs were exposed to differing concentrations of endosulfan for 12 h, then prepared and assayed using the protocol described in this chapter. Top panel is the data for the cnidarian homolog of ubiquitin ligase (E2), an enzyme that conjugated ubiquitin to a protein slated for complete proteolysis. Bottom panel is the statistical method used to test for differences in protein levels of ubiquitin ligase between the different treatments and the reference control.

environmental stressors.[15,16] CCA is an eigen analysis method that reveals the basic relationships between two matrices, in this case those of xenobiotic treatments and biomarker data. The CCA provides an objective statistical tool for determining which biomarker (or suite of biomarkers) best reveals the presence or response of a particular xenobiotic and environmental stressor. In the case of discriminating the effects of two or more xenobiotics/environmental exposures, two assumptions of CCA are made that result from the experimental design used: (1) stressor gradients are independent and linear and (2) biomarker responses are linear (Figure 10.10).

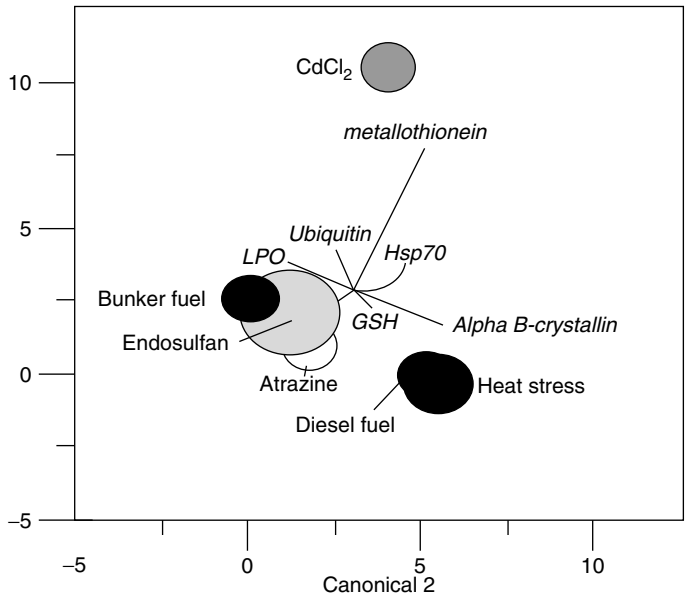


Figure 10.10 Canonical centroid plot of standardized biomarker responses in an organism exposed to a pesticide, an herbicide, a heavy metal, heat stress, and two different types of fuel. Original variates were biomarker levels expressed as a percentage of the control value in each experiment. Circles show the 95% confidence intervals around the distribution centroid of each stressor. Biplot rays radiating from the grand mean show directions of original biomarker responses in canonical space. Metallothionein, which responds to heavy metal stress, distinguished the cadmium chloride treatments from all others; αB-crystallin distinguished diesel fuel and heat stress versus the others.

CCA can discriminate between the effects of the different concentration doses using the same protocol as above, but CCA can also differentiate the patterns of system resiliency and system degeneration. Instead of randomly choosing or compiling all the biomarker data into a single matrix, biomarkers from only a specific cellular diagnostic category are included into the matrix. For example, only parameters of protein metabolic condition are included into one matrix, while the other matrix is the reference and xenobiotic treatments compose the second matrix (Figure 10.11).

Results and discussion

Case study: hexachlorobenzene and corals

The objective of the investigation is to begin to answer the question: ''What effect does an exposure to a hexachlorobenzene (HCB) have on coral cellular physiology?'' This inquiry concerning the mechanisms of toxicity can be further distilled into three questions:

- Does exposure to a HCB produce an oxidative stress?
- Does exposure to HCB affect heme metabolism?
- Is light a factor for HCB toxicity and pathology?

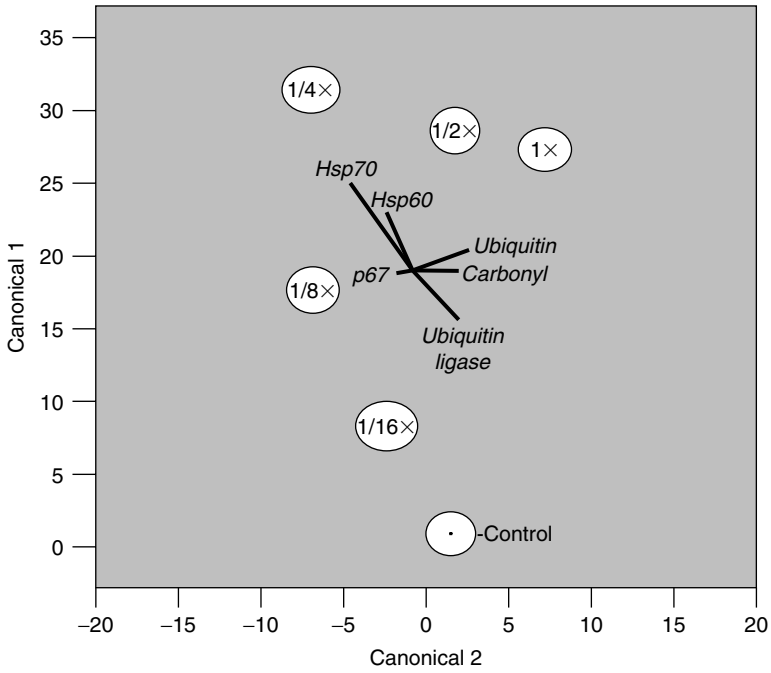


Figure 10.11 Canonical centroid plot using six parameters of protein metabolic condition in liver cells exposed to the different concentrations of the hepatotoxicant, thioacetimide. Exposure to thioacetimide was 24 h. Concentration of thioacetimide is: 1 m*M* at centroid plot 1×, 500 μ*M* at centroid plot 1/2×, 250 μ*M* at centroid plot 1/4×, 125 μ*M* at centroid plot 1/8×, and 63 μ*M* at centroid plot 1/16×. Biplot rays radiating from the grand mean show directions of original biomarker responses in canonical space. Notice that protein oxidative damage product (protein carbonyl) and a marker for protein degradation (ubiquitin) define differences between the 1× centroid and the control, indicating that at 1 m*M* thioacetimide induces protein oxidation (oxidative stress) and increase in the rate of protein degradation.

These three questions have particular relevance because an examination of the mammalian, vertebrate, and invertebrate literature indicates that oxidative stress and disruption of heme synthesis are critical aspects of HCB toxicity.[17] In this case study, we will only consider a 24-h exposure of 750 ng l^{-1} of HCB.

Coral nubbins the size of 1 cm^2 of *P. porites* were cultured in the laboratory under 800 μmol m^{-1} s^{-1} photosynthetic active radiation (PAR) for two months (12-h night-and-day cycles) and supplemented with *Artemia* on a tri-weekly basis. Coral nubbins came from a single coral colony from 6-m depth.

Corals nubbins were transplanted to a recirculating dosing system and allowed to acclimate for 1 week. Light levels and feeding schedule were unchanged, and there was an 85% volume complete water change-out of the recirculating dosing system every 12 h. Water temperature range was 25–27°C. A single coral nubbin was placed in a single recirculating dosing system; $N = 6$ per treatment, there was a total of 18 independent recirculating dosing units and 18 coral nubbins. There were three treatments: (1) control with light at 800 μmol m^{-1} s^{-1} PAR, (2) 750 ng l^{-1} of HCB at 800 μmol m^{-1} s^{-1} PAR, and (3) 750 ng l^{-1} of HCB at 50 μmol m^{-1} s^{-1} PAR. Exposure began 30 min before the beginning of the light period and ended 30 min before the end of the dark period.

Corals were collected and prepared as described earlier, with slight modifications to accommodate an assay that measures protein carbonyl.[18,19] The criteria for using the biomarkers, measured in this study, were partly constrained by technology and available resources. The cellular parameters included in this study are evolutionarily conserved, thus making it easy to produce and validate ELISAs for this species. Assays for biomarkers that are not genetically constrained, such as GSH and protein carbonyl, had to be modified to account for qualities inherent to coral sample matrix. As in any situation, resources are limited and assaying for 30–40 different parameters can be cost and time prohibitive. We measured seven parameters that are particularly relevant to HBC toxicity in both vertebrate and invertebrate species.[17] These seven parameters can be categorized into the cellular sub-systems of ''oxidative damage and response'' and porphyrin metabolism (Table 10.2).

Protein carbonyl formation increased in response to HCB exposure, and the rate of lesion formation was exacerbated by high-light. MnSOD was unchanged by exposure to HCB in both light conditions, suggesting that oxidative damage was not occurring in the mitochondria, or the ability to respond to damage was somehow hindered. In contrast, cytosolic Cu/ZnSOD showed an expression pattern similar to protein carbonyl, suggesting that the oxidative damage/response was, in part, localized to the cytosol, but that light was again an exacerbating factor. Catalase levels significantly decreased with exposure to HCB under both light regimes. This decrease in catalase can be interpreted as: (1) the ability of the cell's oxidative stress capacity is greatly reduced, especially its ability to deal with hydrogen peroxide; and (2) a mechanism is present that is specifically altering catalase steady-state levels. From these markers, we can conclude that the HCB exposure induces a significant oxidative stress, and that it is exacerbated by light. The pattern of the anti-oxidant enzymes, by themselves, does not confer a clear understanding of why this pattern exists. From a prognostic perspective, an increase in protein lesions by HCB suggests membrane integrity and even genomic integrity may be jeopardized by oxidative damage, suggesting further investigation into the mechanism of HCB-associated oxidative stress toxicity.

Scholarship of the literature surrounding HCB biochemistry and toxicology can help elucidate the pattern observed in the oxidative damage and response category. A classic

Table 10.2 Example data for diagnostic interpretation[a]

Cellular parameter	Control	High light HCB	Low light HCB
Oxidative damage and response			
Protein carbonyl (pmol mg^{-1} TSP)	147 ± 8	$1631 \pm 42^{A,B}$	$465 \pm 27^{A,B}$
Cu/Zn superoxide dismutase (fmol ng^{-1} TSP)	67 ± 8	$368 \pm 16^{A,B}$	$128 \pm 22^{A,B}$
Mn superoxide dismutase (fmol ng^{-1} TSP)	598 ± 120	628 ± 92	580 ± 39
Catalase (fmol ng^{-1} TSP)	142 ± 7	15 ± 3^{A}	22 ± 6^{A}
Porphyrin metabolism (subset of metabolic condition)			
Ferrochelatase (fmol ng^{-1} TSP)	66 ± 6	7 ± 2^{A}	12 ± 4^{A}
PPO IX oxidase (fmol ng^{-1} TSP)	32 ± 3	58 ± 3^{A}	62 ± 4^{A}
Protoporphyrin species (nmol ng^{-1} TSP)	28 ± 7	1855 ± 351^{A}	1695 ± 178^{A}

[a] Data from an experiment where corals were exposed to hexachlorobenzene for 24 h. $N = 5$ per treatment. All parameters except for protoporphyrin and protein carbonyl are for cnidarian homologs. Under these assay conditions, it is impossible to distinguish between dinoflagellate and cnidarian protein carbonyl and protoporphyrin. All parameters were analyzed by one-way ANOVA. Data are given as means and standard errors. Superscript ''A'' indicates that the treatments are significantly different from the control. Superscript ''B'' indicates that the treatments are not only significantly different from the control, but are significantly different from one another.

paper by Cam and Nigigisyan[20] showed that mammals exposed to HCB resulted in a pathology known as *porphyria cutanea tarda symptomatica* (PCTS). The more obvious manifestation of HCB exposure is a dark red hue discoloration of urine (described as a port wine color) and severe skin lesions when the individual is exposed to sunlight.[17,21] Experimentation of HCB in rats demonstrated that HCB produced porphyria as a result of interference in the pathway of heme metabolism.[22] These studies were done, in part, to help elucidate the toxicological mechanism associated with an acquired outbreak of porphyria in more than 3000 individuals in Turkey between 1955 and 1959, which was finally traced to the consumption of wheat contaminated with HCB.[21,23] Since the 1970s, studies investigating the toxicological mechanisms of HCB have been able to demonstrate that HCB inhibits a number of enzymes in the porphyrin synthesis pathway, producing an accumulation of protoporphyrin and porphyrinogen species.[21,24] These porphyria species can readily absorb light, react with divalent oxygen, and produce singlet oxygen, a noxious reactive oxygen species (ROS).[25]

This literature can aid in explaining *why* we see an increase in protein oxidation lesions, especially the increase of these lesions associated with light.[26,27] In fact, the phenomenon of light-induced ROS from a porphyrin-like compound has been capitalized on as a new and effective treatment of certain cancers (e.g., skin cancer, Barlett's esophageal cancer, colon cancer): hypericin-based photo-dynamic therapy.[28] The decrease in catalase can be explained by two separate but synergistic mechanisms. Catalase is a porphyrin-dependent enzyme whose accumulation and activity is known to decrease as a result of porphyrin synthesis interference.[29,30] In addition, catalase is extremely susceptible to oxidation and inhibition by both singlet oxygen and superoxide.[31,32] Both mechanisms are relevant to this situation and most likely acting to affect catalase levels.

To confirm that a porphyrin-based toxicological mechanism is occurring as a consequence of HCB exposure, three parameters central to porphyrin metabolism (a subset of the category, metabolic condition) were assayed: ferrochelatase, protoporphyrinogen oxidase (PPO) IX, and total porphyrin content. PPO IX is the second to the last enzyme in heme synthesis and converts PPO IX into protoporphyrin.[33] Ferrochelatase is the last enzyme of heme synthesis and insets the iron into the protoporphyrin to form heme.[33] Ferrochelatase levels decreased significantly in both HCB treatments, most likely resulting in decreased heme synthesis and increased protoporphyrin accumulation. Ferrochelatase is an iron-sulfur protein, and its activity and structure are sensitive to changes in redox state of its sub-cellular environment.[34] Ferrochelatase is the ''failure point'' of heme synthesis in response to a number of toxins and is not surprising that it would be adversely affected by HCB.[34] PPO levels significantly increased, probably as a result for cellular requirement for metalloporphyrins (e.g., heme). Increase of PPO with a decrease in ferrochelatase suggests that protoporphyrin species may be hyper-accumulating as a result of this defect in the heme synthesis pathway. A microplate fluorimetric method was used to detect total concentration of uroporphyrin, coproporphyrin, and protoporphyrin in the coral sample.[34] If enzymes in the porphyrin synthesis pathway are inhibited, porphyrin synthesis intermediates will accumulate.[35] Measurement of total porphyrin species (actually porphyrinogen and non-metal porphyrins) revealed that HCB in both the light treatments resulted in over a 20-fold increase compared to controls, supporting the argument that (1) HCB interferes with the porphyrin/heme synthesis pathway in corals, and (2) the presence of non-metal porphyrins and porphyrinogens is likely a primary source of oxidative stress.

From these seven biomarkers, we were able to address the three questions posed at the beginning of this case study, and more importantly, we established a foundation for the mechanism of HCB toxicity in corals; HCB exposure causes an oxidative stress that is exacerbated by light as a result of heme/porphyrin synthesis interference. In addition, the cellular diagnostic approach can immediately provided a basis for further investigation into the nature of HCB toxicity in corals. HCB causes an oxidative stress, and we know that it adversely affects protein function and steady-state levels. Does it also cause DNA lesions, resulting in increased mutation rates? Does this oxidative stress translate into a reduction in reproductive fitness? Is there oxidation of lipid membranes with HCB exposure? Is this lipid peroxidation associated with accumulation of malondialdehyde and hydroxynonenal-known mutagens and enzyme inhibitors? Examining biomarkers in the categories of genetic integrity and membrane integrity can provide answers to these questions.

A principal goal of any kind of systematic biomarker approach is to link behavior occurring at the molecular and cellular level to the population level and, perhaps, even to the community and ecosystem levels.[36] Predicting population responses and *ecological forecasting* is an esteemed aspiration of biomarker research.[37] Approaching this problem with a thorough understanding of biochemistry and cellular physiology is an essential prerequisite for accurate predictions. For example, if we know that HCB exposure causes or at least increases the risk for oxidative damage in corals, and that it also diminishes aspects of the cell's anti-oxidant enzyme defense, we could prognosticate that a HCB exposure would increase the risk of a coral bleaching event in corals experiencing conditions conducive to coral bleaching. This prognosis is further based on the knowledge that oxidative stress is a major mechanism for many forms of environmentally associated coral bleaching.[19] Hippocrates aphorism of ''he will manage the cure best who has foreseen what is to happen from the present state of matters''[38] best encapsulates the central tenet of cellular diagnostics and prognostics: to determine the present and future physiological condition of the cell through an understanding of the cell's functional components and processes.

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chapter eleven

A non-destructive technique to measure cytochrome P4501A enzyme activity in living embryos of the estuarine fish Fundulus heteroclitus

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Contents

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Introduction

An embryonic and larval bioassay using the estuarine fish, *Fundulus heteroclitus*, was developed to detect and measure the relative potency of bioavailable compounds that act through the aryl hydrocarbon receptor (AhR). AhR agonists, including poly-halogenated dioxins/furans, and some poly-aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs), are widely distributed globally and potentially toxic to the wide array of vertebrate species possessing this signal transduction pathway.[1,2] Exposure to this class of contaminants activates this toxicological pathway, resulting in induction of cytochrome P4501A (CYP1A). CYP1A induction is assessed typically as increased mRNA or protein concentration or enzyme activity, often measured as ethoxyresorufin-*O*-deethylase (EROD) activity.[3–5] In addition, CYP1A induction may be causally related to the early life stage effects produced by AhR agonists in fishes,[6,7] although probably not involved in all toxic effects produced by these compounds. Therefore, EROD activity is useful as an indicator of exposure and some adverse effects produced by this ubiquitous and important class of toxic contaminants.

In the embryonic and larval fish bioassay described here, EROD activity is measured using a novel *in ovo* non-invasive fluorescent technique applied to individual embryos. Because this endpoint is measured non-destructively, embryos for which EROD activity has been measured can also be assessed for traditional toxicity endpoints, including survival, development, and growth. Thus, this bioassay provides a simple, safe, and small volume whole animal method, which is highly responsive to AhR agonists. This *in ovo* method is applicable for use with single compounds and mixtures, as well as environmental media, such as water, sediment, sediment pore waters, and organic extracts. Therefore, the assay can be used to detect bioavailable contamination by compounds that act through the AhR, rank toxic potency of environmental media, and further understanding of the mechanisms of toxicity for compounds and environmental mixtures. In addition, lethal and sub-lethal effects of contaminants on fish early life stages can be included in species-specific population models used to project ecological effects of contamination.[8] A summary of procedures is shown in Figure 11.1, and a detailed description of materials and methods based on published methods[9,10] follows.

Materials required

- Water purification system — biologically pure deionized water (DI), e.g., Millipore Super-Q®, or equivalent
- Air pump for oil free air supply
- Air lines, plastic or Pasteur pipettes, or air stones
- pH and dissolved oxygen (non-stirring probe) meters, for routine physical and chemical measurements
- Light box for counting and observing embryos and juvenile culture animals
- Refractometer or salinometer for determining salinity
- Thermometers, glass or electronic, laboratory grade for measuring water temperatures
- Thermometers, bulb-thermograph or electronic-chart type for continuously recording temperatures
- Rinse bottles for DI and seawater
- Temperature-controlled room or chamber (23°C) for maintenance of *Artemia* and fish cultures

Embryo exposure (1–6 days post-fertilization)

- Collect fertilized eggs from parent tanks
- Transfer individual eggs into exposure vials
- Add contaminant and EROD substrate to exposure vials
- Maintain in sealed vials for 5 days
- Observe microscopically for developmental progress and abnormalities

Embryo development (7 days post-fertilization — hatching)

- Transfer eggs into uncontaminated sea water
- Observe microscopically for developmental progress and abnormalities
- Renew sea water on alternate days
- Quantify EROD activity using fluorescence microscopy/photometry (days 7–10 post-fertilization)
- Monitor daily for hatch (~14 days post-fertilization), feed hatchlings *Artemia*

Measurement endpoints on individual fish

- Embryonic survival
- Embryonic development: rate and normality
- Embryonic EROD fluorescence
- Hatch age
- Larval survival
- Larval length (7 days post-fertilization)
- Histological lesions (fixed samples)

Figure 11.1 Protocol for measuring cytochrome P4501A enzyme EROD activity in a standardized embryonic and larval fish bioassay with the estuarine fish *F. heteroclitus*.

- Adult *F. heteroclitus* for use as brood stock
- Aluminum minnow traps (20 cm diameter) for collecting adult fish
- Squid-bait for minnow traps
- Wooden stakes-hold minnow traps from drifting with tide
- Large (100 l) coolers for transport of field-collected adult fish to the laboratory
- Newly hatched Great Salt Lake *Artemia* nauplii for feeding larval and juvenile fish
- Pellet fish food (e.g., Biodry 1000®, Bio Vita FF Pellet Food, Bio-Oregon, Warrenton, OR) for feeding adult fish
- Flake fish food (e.g., TETRA Standard Mix SM-80®, Tetra Werke Baensch, Melle, Germany) for feeding adult fish
- Frozen adult *Artemia* for feeding adult fish
- Egg collection chambers constructed as two separable polyvinyl chloride pipe rings: one covered with large nylon mesh screening upon which adults spawn, and a second inner ring with fine screening that retains eggs and protects them from consumption (Figure 11.2)
- Crystallization dishes (20 cm diameter) for holding embryos
- Transparent plastic wrap for covering vials and dishes

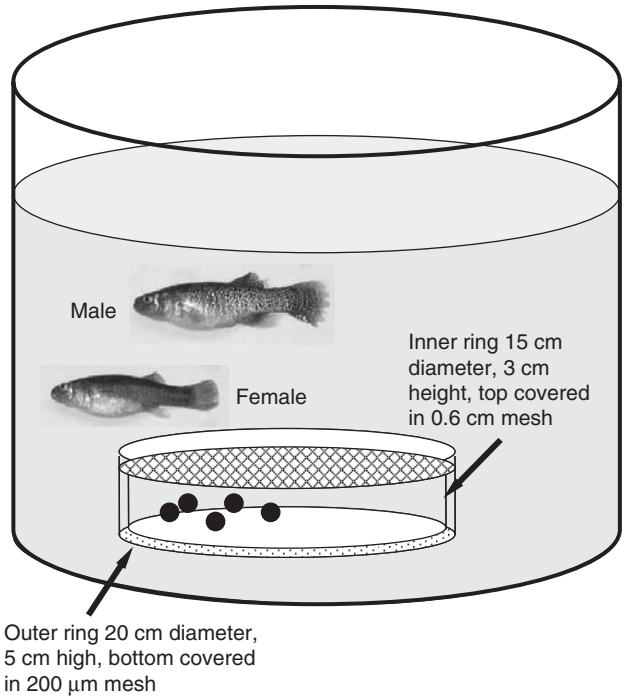

Figure 11.2 Collection of fertilized eggs from reproductively active *F. heteroclitus* into chambers made of readily available materials: large mesh nylon screening as top layer (spawning substrate), large-diameter polyvinyl chloride pipe for framework, and fine mesh nylon screening as bottom layer (egg retention).

- Natural or artificial sea water, 20–30 ppt dependent on local salinity of parental fish collection
- Glass scintillation vials (20 ml, e.g., ASTM Specifications E438 Type 1 Class A borosilicate) with plastic caps for embryo exposure and embryonic and larval maintenance
- Solvents, including acetone and dimethyl sulfoxide, ACS reagent grade or better
- Enzyme substrate: ethoxyresorufin (ER; Molecular Probes, Eugene, OR, USA) dissolved in dimethyl sulfoxide, and stored frozen prior to use
- Forceps and Pasteur pipets for transferring embryos
- Dissecting microscope for observation of embryos
- Depression microscope slides with coverslips
- Fluorescence microscope, equipped with G 365/LP 420 (UV excitation) and LP 510/LP 590 (or rhodamine) excitation/emission filters
- Microscope photometer to quantify fluorescence, or microscope image capture and analysis software
- Buffered formalin (10%) to preserve embryos and larvae after testing

Procedures

Spawning stock

Adult *F. heteroclitus* used as brood stock are collected using baited traps from Atlantic coast estuaries. Adult fish can also be acquired from commercial sources, or from young

fish raised to maturity in the laboratory. Adult fish can be maintained in natural seawater, flow-through or artificial, re-circulated aerated systems. Typically, the fish are maintained at salinities that reflect collection conditions, i.e., from 20 to 30 ppt, and a photoperiod of l4-h light:l0-h dark, and 23 $\pm$ 3°C. To maintain spawning condition, adults are fed commercial fish food *ad libitum*.

Egg collection

Spawning chambers are placed into tanks containing mature adult fish on nights preceding the full and new moons (Figure 11.2). The following mornings, eggs deposited into the lower portion of the chambers are rinsed with uncontaminated seawater, and gently washed into collection dishes. Up to about 200 eggs are selected randomly for each test. Each egg is transferred by forceps into a 20-ml glass scintillation vials, containing 7.5 ml filtered seawater at room temperature. These vials are covered loosely with transparent wrap and incubated at 23°C, under standard laboratory fluorescent lights, cycling 12 h ''on/off.''

Embryo-larval exposure and development

Embryo-larval exposure and assessment[9,11] is described briefly here. On the day following collection, each egg is reviewed under a dissecting microscope for development. Undeveloped or abnormal eggs are discarded (with percentage of viable eggs noted). Chemical exposure to embryos (aged 2 days post-fertilization) begins by introducing solvent (acetone) or toxicant in 0.1–1 μl volume contained in 2.5 ml seawater (i.e., final solvent concentration $\leq$ 0.01%). Seawater also includes ER dissolved in dimethyl sulfoxide to produce a final ER concentration of 21 μg/l, and final solvent concentration of 0.001%. Vials are capped, incubated at 23°C, and gently rolled or rotated daily. At 7 days post-fertilization, after 5 days static exposure, embryos are transferred (by forceps or Pasteur pipette) into clean vials containing uncontaminated seawater. These vials are left uncapped and renewed with fresh seawater every other day. Developmental progress is reviewed by observation using a dissecting microscope, and abnormalities are evaluated as described[12,13] (Figure 11.3).

After initiation of spontaneous hatching (beginning about 11 days post-fertilization using these laboratory conditions), eggs are checked every day. Beginning on the day of hatching, larvae are fed 24-h-old *Artemia* nauplii on a daily basis. Seawater is renewed every other day throughout larval development. On 7 days post-hatching, larvae are fixed by transfer into buffered formalin (10%) and preserved for future histological examination.

In ovo *EROD assay*

Embryos are observed for EROD activity using fluorescence microscopy (Figure 11.4). Although EROD can be measured in embryos as young as 3 days post-fertilization, embryos aged 7–10 days are observed typically.[9] Embryos of this age are convenient; bladders are large enough to find easily, and manipulations do not prompt premature hatching. To measure EROD, individual embryos are placed onto glass depression slides containing a small volume of seawater and covered with a glass cover slip. Embryos are

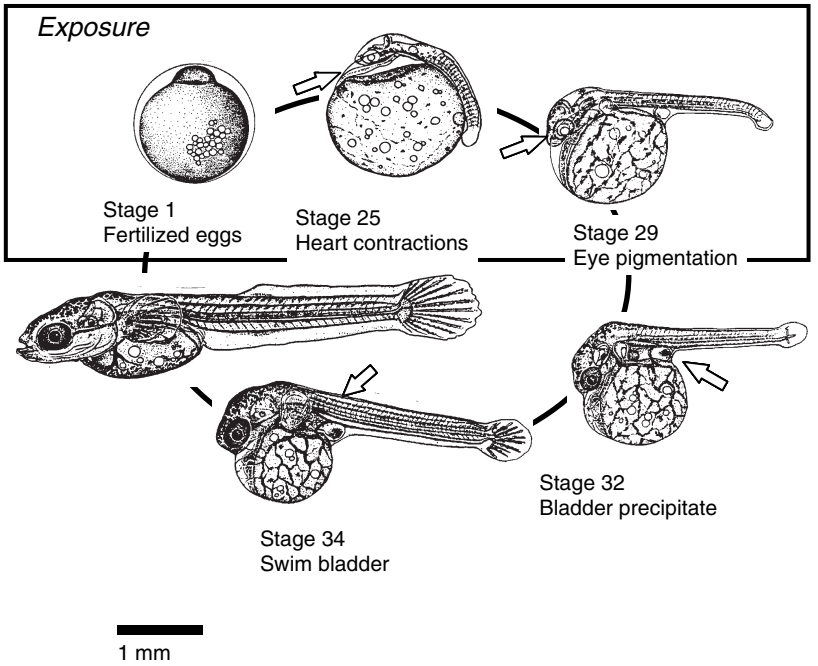

Figure 11.3 Embryonic development rate and features of estuarine fish *F. heteroclitus*. (Adapted from Armstrong, P.B. and Child, J.S., *Biol. Bull.*, 128 (2), 143–168, 1965.)

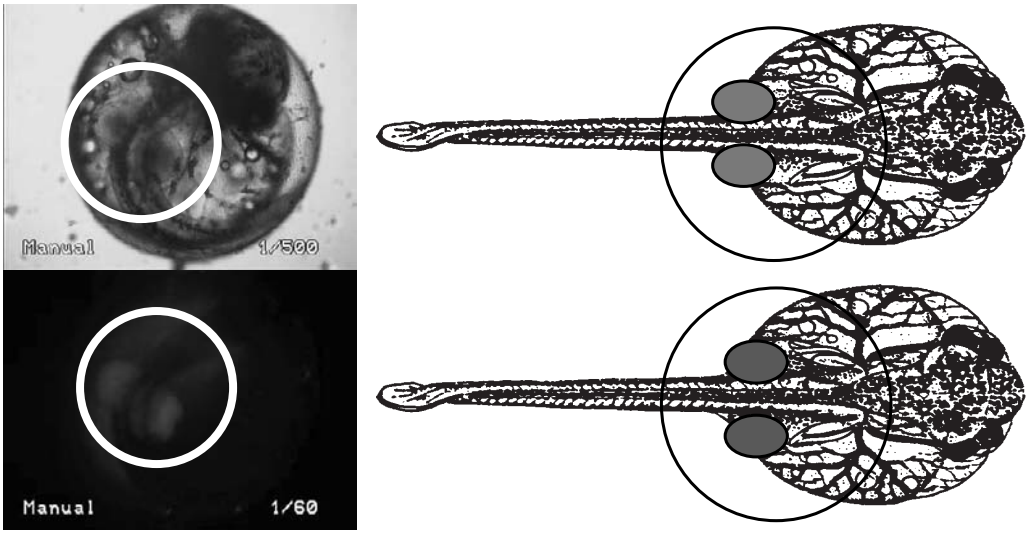

Figure 11.4 **(see color insert following page 464)** Bilobed bladder in *F. heteroclitus* embryo (10 days post-fertilization) fluoresces blue (420 nm) under UV excitation (365 nm); bladders in embryos exposed to aryl hydrocarbon agonists and the EROD substrate (ethoxyresorufin) accumulate resorufin and fluoresce red (590 nm) under green (510 nm) excitation.

observed using a fluorescent microscope, equipped with G 365/LP 420 (UV excitation) and LP 510/LP 590 (or rhodamine) excitation/emission filters. (Appropriate procedures for fluorescence microscope use should be followed. For example, fluorescence lamp should be warmed up and adjusted to produce stable, maximal output prior to test measurements.) Gentle pressure on the cover slip is used to rotate the embryo and localize the large, bilobed urinary bladder using low power (2.5×) magnification. Under UV excitation, the bladder is blue in color. This feature (and 10× magnification) allows easy localization, and orientation prior to fluorescence measurements (Figure 11.4).

EROD quantification

Once the bladder is located, the rhodamine filter set is used to observe the fluorescent product of the EROD reaction, resorufin. Red resorufin fluorescence, indicative of EROD activity, is quantified using image analysis or a photometer system operated as an attachment for the standard fluorescent microscope. For the latter method, fluorescent intensity is quantified using a standardized pinhole size or area of measurement, i.e., a pinhole aperture size 8 equivalent to 0.10 mm^2, centered over a bladder lobe. Fluorescence can be quantified as signal per unit area (V/mm^2) by normalizing to pinhole area. Alternatively, relative fluorescence can be reported by dividing measurements by average fluorescence values in control embryos.

For image analysis techniques, quantification of EROD intensity is based on captured images. Briefly, fluorescence intensity is quantified as pixel density in a small area over one lobe of the bladder, normalized by subtraction of background fluorescence and exposure time. While many data analysis programs can be used, image quantification has been accomplished using IP Lab Software (Scanalytics, Fairfax, VA).

Data management, storage and analysis

Test data include a unique test identification number, parental source of embryos, and date of fertilization for the batch of eggs tested. Daily logs include incubation temperature, observations by individual of developmental features, and presence and severity of lesions or abnormalities. Other useful endpoints include post-fertilization and post-hatching age of mortality, and age at hatching. In addition, length at hatching and histological lesions can be recorded after test termination. Typically, data are entered into spreadsheet formats and, 100% error-checked before statistical analysis by individual or treatment group.

Bioassays include 100–300 embryos in treatment groups of 20–40 embryos. To characterize the toxicity of a compound or substance replicate bioassays (2–3 per compound) are often conducted using different batches of embryos from the same parental stocks. Statistical procedures to develop concentration-dependent responses of EROD and health endpoints are described fully elsewhere.[11] Briefly, quantitative models are constructed using a non-linear least-squares regression procedure.[14] Survival response is described using this equation: $R_0 \times \phi\ [(\log(EC_{20}) - \log(C))/\phi + 0.8416]$. In this equation, C is the tested concentration and the estimated parameters are: R_0, the predicted control response; F, the cumulative area under the standard, normal distribution; s, the standard deviation of the normal distribution. A similar model is used to describe EROD responsiveness: $(R_m - R_0) \times \phi\ [(\log(EC_{20}) - \log(C))/\phi + 0.8416] + R_m$. The parameters are defined as for the previous model, with the addition of R_m, the predicted maximal response. To make

statistical comparisons between these concentration-dependent responses, data can be fit to logistic regression models as described elsewhere.[15] Variances of log-transformed EROD responses can be tested for normality using the Shapiro–Wilks analysis (univariate procedure) and if normal, can be tested to determine differences between treatments, e.g., using one-way analysis of variance followed by Duncan's multiple range test.

Troubleshooting

Poor condition of parental fish may produce embryos with decreased survival compared with eggs produced by parents in good condition. Toxic substances may be introduced by contaminants in dilution water or solvents, glassware, sample hardware, and testing equipment. Be sure that all materials that are in contact with test materials or animals are thoroughly clean, but rinsed free of any cleaning products, and then soaked in seawater. Optionally, glassware can be prepared using acid soaking (e.g., up to 1 h in 5% HCl) and then heating (250°C for 6 h).

Results and discussion

To compare results across methods and to evaluate the sensitivity, specificity, and applicability of the *in ovo* EROD method, tests have been conducted using single compounds and complex environmental mixtures. Specifically, positive controls include strong AhR agonists, such as ''dioxin,'' 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and dioxin-like compounds, i.e., 3,3′,4,4′,5-pentachlorobiphenyl (PCB126), and PAHs, including benzo(*a*)pyrene (BaP) and 3 methyl cholanthrene (3MC). Another PAH that has been used as a negative control is fluoranthene (FLU), a poor AhR agonist.[3] In addition to single chemicals, environmental samples have also been tested using this method. These samples include marine and estuarine waters and sediment-derived samples, i.e., pore waters and organic extracts. Examples of results from these tests are provided to demonstrate test attributes and uses.

Comparison with standard EROD method

Increased EROD activity has been shown to be a sensitive response of *F. heteroclitus* embryos exposed to AhR agonists.[16] Typically, EROD activity is measured *in vitro* using microsomal fractions of tissues or whole embryos.[4] To compare *in ovo* and embryonic *in vitro* EROD results directly, embryos from reference site populations were exposed to PCB126, then tested using the *in ovo* method, or sacrificed and measured using the *in vitro* method. For this purpose, *in vitro* enzyme activity was measured using fish embryonic microsomes prepared as described[17] and modified for embryonic *F. heteroclitus*[15] and is described fully elsewhere.[18] Results using both methods were correlated, showing significant increases at comparable concentrations (Figure 11.5).

Associations between EROD and developmental effects

Developmental anomalies are shown (Figure 11.6) that typify exposures to AhR agonists at concentrations that increase EROD activity. Characteristic developmental embryonic

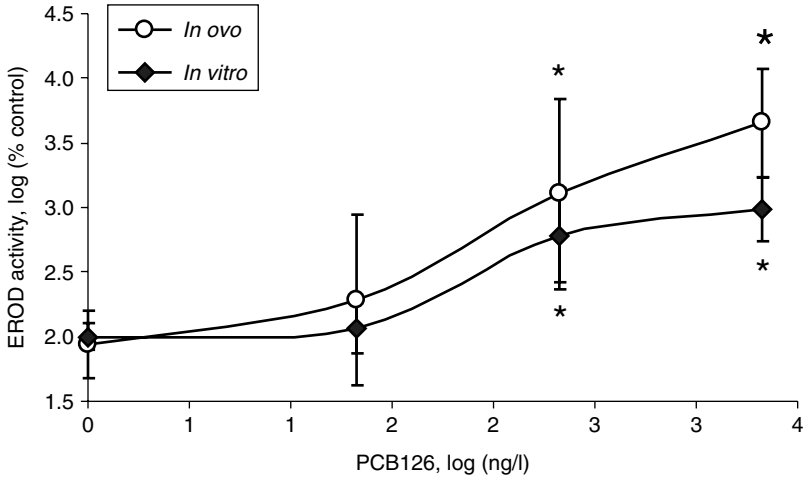

Figure 11.5 EROD activity in embryonic *F. heteroclitus* measured using microsomal *in vitro* production or *in ovo* fluorescence (mean ± SD), three determinations per treatment with PCB126. Significant differences from respective controls as determined using analysis of variance procedures are indicated (*).

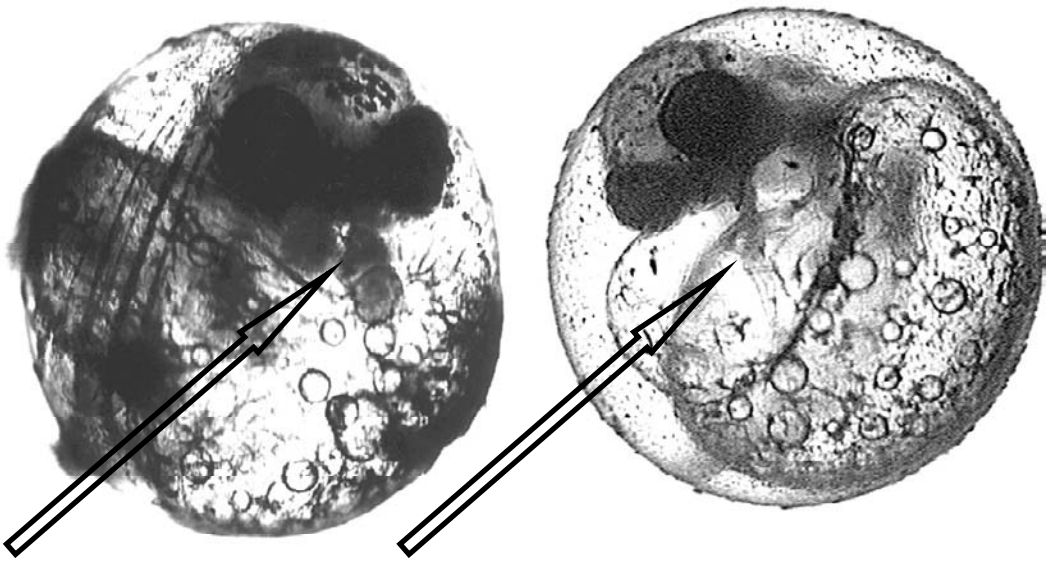

Figure 11.6 Photomicrograph of *F. heteroclitus* embryos (10 days post-fertilization). Arrows indicate normal heart development (left photo), and pericardial edema and ''tube heart'' (right photo) in an embryo exposed to an aryl hydrocarbon receptor agonist, PCB126.

anomalies are consistent with descriptions of ''blue sac'' like syndrome observed following exposures to AhR agonists of early life stages of *F. heteroclitus*[19,20] and other fish species.[21] These abnormalities included circulatory lesions, such as pericardial edema and ''tube heart,'' and tail hemorrhages.[7,19,22] While characteristically observed, this syndrome of lesions is not uniquely diagnostic of AhR agonist compounds.[19]

To explore the relationship between EROD induction and developmental deformities, reference site embryos were exposed to a range of PCB126 concentrations, and assayed for *in ovo* EROD activity on day 7 of development.[23] The same embryos were assessed for deformities on day 10 post-fertilization. The frequency and severity of deformities were correlated with EROD fluorescence (Figure 11.7), consistent with a mechanistic association between EROD and health effects. In addition, this relationship suggests that concentrations of test substances that produce EROD responses are predictive of concentrations that produce adverse effects on fish development and early life stage survival.

Method sensitivity and specificity

Table 11.1 provides a comparison of the sensitivity of the *in ovo* EROD method with some other published methods used to quantify the potency of AhR agonists. Test endpoints selected for comparison were related to CYP1A induction, i.e., EROD activity and AhR response via reporter gene-linked activation. Although species/tissues, exposure durations and test parameters differ among test systems, nominal exposure concentrations of TCDD and PCB126 producing responses using the *in ovo* EROD method are 1–2 orders of magnitude lower than those reported in *in vitro* systems. This limited comparison suggests that the *F. heteroclitus in ovo* method is relatively sensitive to AhR agonist effects.

In ovo EROD activity has been measured in *F. heteroclitus* embryos using chemicals that vary in their potency as AhR agonists, such as PCB126, 3MC, BaP, and FLU.[9] Test data and modeled response relationships show that embryonic EROD fluorescence is specific for AhR agonists (Figure 11.8). In the order of their potency to produce EROD fluorescence, EC_{50} values for PCB126, 3MC, and BaP were 74, 123, and 7112 ng/l,

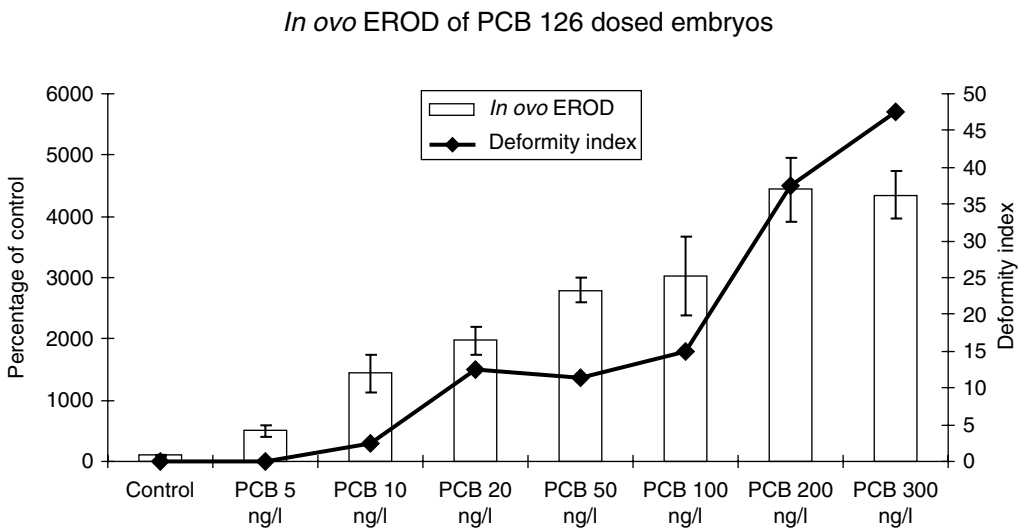

Figure 11.7 *In ovo* EROD analysis expressed as percent control fluorescence (left axis) and heart deformity assessment (right axis) of PCB126 exposed-embryos. Embryos were dosed on the day of spawning (day 0), EROD was assessed on day 7 post-fertilization, and deformities were assessed on day 10 post-fertilization. Heart deformities were scored for severity between 0 and 5 (no deformity to most severe). A deformity index was calculated by dividing the sum of the scores for a treatment group by the total possible score ($5 \times n$) and multiplying the quotient by 100.

Table 11.1 Comparative responses to aryl hydrocarbon agonists, TCDD and PCB126, measured or estimated using cell culture or *F. heteroclitus* embryo systems

Endpoint	Test population	TCDD EC_{50} (ng/l)	PCB126 EC_{50} (ng/l)	References
EROD, in ovo	*F. heteroclitus*, reference population	0.2[a]	36	(11)
EROD, in vitro	HepG2 (human)	32.2		(30)
	H4IIE (rat)	6.4	86	(29)
	PLHC-1 (fish)	41.9	121	(31)
	RTL (trout)	2.0	75	(32, 33)
Reporter gene, in vitro	HepG2 (human)	112.7		(34)
	101L (human)	32.2	3263	(35)
	H4IIE-Luc (rat)	1.9	94	(29)
	RTL 2.0 (trout)	20.6		(36)

[a] Estimated for TCDD using measured values for PCB126 and toxic equivalency in fish for PCB126 (i.e., 0.005).[37]

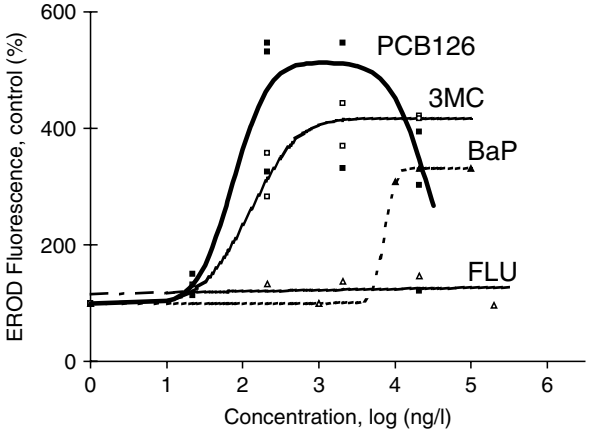

Figure 11.8 AhR-agonist specificity and concentration-responsiveness of EROD fluorescence in 10-day post-fertilization *F. heteroclitus*. Modeled response curves and mean ($\pm$ SD) responses for replicated tests of strong AhR agonists, PCB126 (triangles), 3MC (squares), BaP (circles), and FLU, a poor AhR agonist (diamonds). (Adapted from Nacci, D., Coiro, L., Kuhn, A., Champlin, D., Munns, W., Jr., Specker, J. and Cooper, K., *Environ. Toxicol. Chem.*, 17, 2481–2486, 1998. With permission.)

respectively.[9] Consistent with expectations for non-AhR-mediated effects, no significant increase in EROD fluorescence was detected for FLU (<20,000 ng/l). Taken together, these results demonstrate that embryonic EROD fluorescence is a specific indicator of AhR-mediated effects, and that fluorescence results are consistent with other methods to assess similar responses.

Testing mixtures and environmental samples

The *in ovo* EROD assay has also been used to explore how environmentally relevant mixtures affect EROD activity. For example, EROD activity can be inhibited by combinations of chemicals acting through the AhR and other toxicological pathways.[24] To test the effects of chemical combinations, embryos were exposed to BaP, and PCB126, agonists

alone in and in combination.[23] BaP and PCB126 both induced EROD activity, but when dosed in combination, resultant EROD measurements were less than those that would be predicted if the compounds were strictly additive (Figure 11.9).

Because the effects of chemical combinations cannot be predicted accurately, it is often desirable to test effects directly of environmental samples composed of chemical mixtures. Whole sediment testing is generally unacceptable because adhering sediment obscures the observation of *in ovo* EROD fluorescence. However, the *in ovo* EROD method has been used to test estuarine sediment-associated samples, including pore waters[10] and extracts.[25] Typically, results include positive and negative control exposures using chemicals, such as 3MC and FLU, respectively, and tested materials derived from sediments that range in severity and categories of chemical contamination (Figure 11.10). In the example shown, pore waters and organic extracts were prepared from sediments collected from a relatively uncontaminated reference site and one highly contaminated reference site with a complex mixture of contaminants, including TCDD. Test media from the highly contaminated sediment, both the pore water (tested as 71% of the exposure media) and organic extract (tested as 0.02% of the exposure media) produced strong *in ovo* EROD responses. Testing pore waters and organic extracts from the same sediment provides a strategy to estimate the bioavailability of AhR agonists in sediments. However, caution must be applied to the interpretation of results because exposure to complex mixtures often produces non-additive effects (as demonstrated in the previous example).

Investigating toxicity mechanisms

Relative to other species, *F. heteroclitus* is highly responsive to AhR agonists during early embryonic development.[9,16,22] However, some populations of *F. heteroclitus* indigenous to

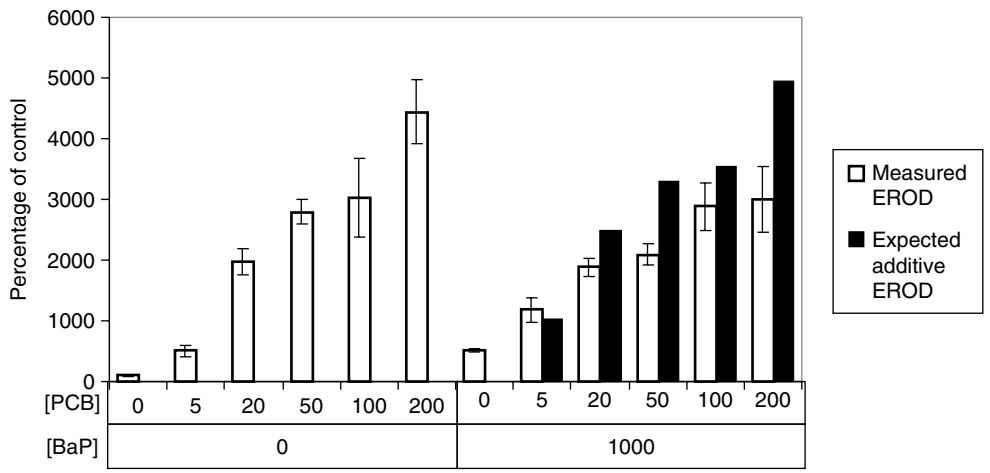

Figure 11.9 *In ovo* EROD activity (% solvent control, mean and standard error) for embryos treated with BaP (1000 ng/l, white bar), and a range of concentrations of PCB126 alone (white bars) or in combination with BaP (white bars), and expected additive EROD fluorescence (black bars). (Adapted from Wassenberg, D.M. and Di Giulio, R.T., Teratogenesis in *Fundulus heteroclitus* embryos exposed to a creosote-contaminated sediment extract and CYP1A inhibitors, *Mar. Env. Res.*, 54, 279–283, 2002. With permission.)

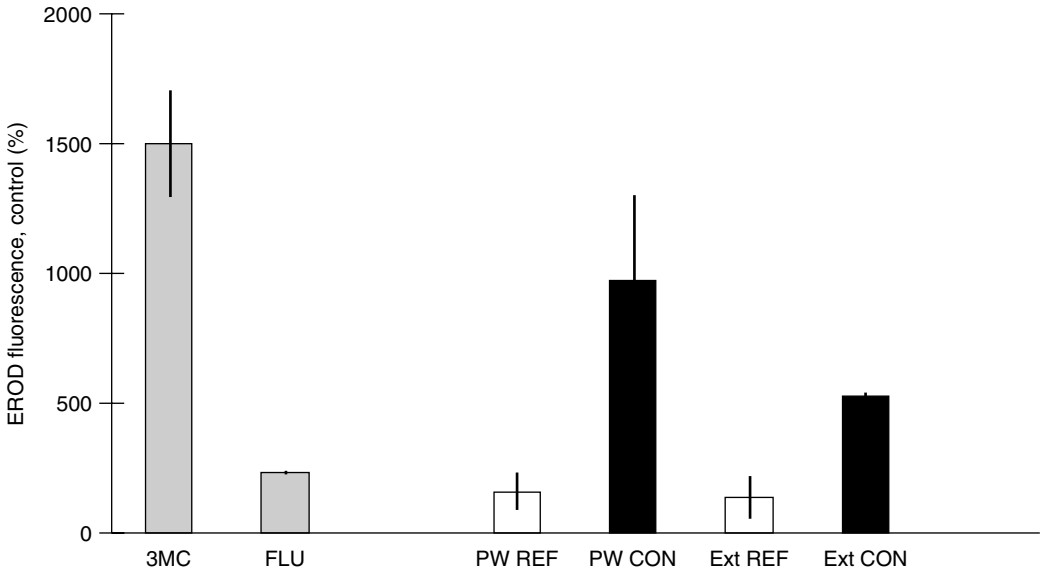

Figure 11.10 *In ovo* EROD responses of *F. heteroclitus* embryos exposed to positive control, 3MC (light bar), negative control, FLU (light bar), and sediment-derived materials: pore waters (PW) and organic extracts (EXT) from a reference (REF white bars) site or a site contaminated with complex mixtures of AhR agonists and other contaminants (CON dark bars).

contaminated sites are highly resistant to the effects of AhR agonists.[9,10,26] This tolerance is demonstrated as poor CYP1A inducibility, and resistance to the health effects associated with exposure to AhR agonists.[8,9] As shown, *in ovo* EROD fluorescence and embryonic deformities have been shown to increase when embryos from reference site populations are exposed to AhR agonists. However, embryos from populations of *F. heteroclitus* that have been characterized as tolerant to AhR agonists are relatively unresponsive to 3MC exposure (Figure 11.11).

These tolerant populations are indigenous to sites that are highly contaminated with complex mixtures that include PAHs (VA population) and PCBs (MA population). Similarities and differences between tolerant populations of *F. heteroclitus* are suggested by their intergenerational responses to 3MC. Specifically, F1 and F2 embryos from the population indigenous to the PCB-contaminated are unresponsive to 3MC exposure. However, while F1 embryos from the PAH-contaminated site are unresponsive to 3MC, their progeny (F2 embryos) are as sensitive to 3MC as those from a local reference population. Although the specific biochemical mechanism for reduced sensitivity in *F. heteroclitus* is not known (although see Reference 27), these differences suggest tolerance that has evolved in independent populations (like these) may have different mechanistic bases.[28] These examples demonstrate how the *in ovo* EROD method can be used in conjunction with other endpoints to elucidate mechanisms of toxicity and tolerance to contaminants that act as AhR agonists.

Summary

A non-destructive indicator of cytochrome P450A1 embryonic enzyme activity in fish has been developed that provides information relevant to exposure and effects of environmental contaminants. This information complements information obtained

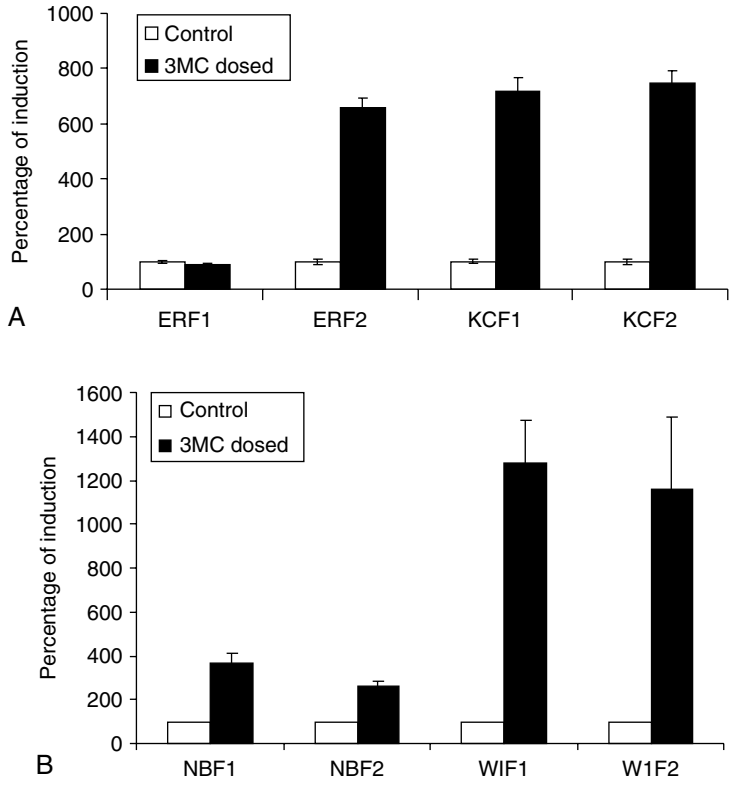

Figure 11.11 *In ovo* EROD activity in response to AhR agonist exposure in populations of *F. heteroclitus* that display tolerance to local contaminants in comparison to local reference populations: (a) from Virginia, tolerant (creosote-contaminated Elizabeth River, ER) embryos and reference (King's Creek, KC) embryos (F1 and F2 generations) exposed to 3MC (268 ng/l) or solvent control (From Meyer, J.N., Nacci, D.E. and Di Giulio, R.T., *Toxicol. Sci.*, 68, 69–81, 2002. With permission.); (b) from Massachusetts, tolerant (PCB-contaminated New Bedford, NB) and reference (West Island, WI) embryos (F1 and F2 generations) exposed to 3MC (2.68 μg/l) or solvent control.

using traditional fish embryonic and larval bioassays. In addition, because measurements using this technique are made non-invasively, they can be used to examine mechanisms linking AhR-mediated toxicity and long-term effects of environmental contaminants on individuals.

This method is based on the accumulation in the embryonic fish bladder of the fluorescent product of the EROD enzyme, indicative of cytochrome P450A1 enzyme activity. Results derived from this method are specific to AhR agonists and are correlated quantitatively with standard methods to measure EROD activity in fish embryos and larvae. This technique can be used diagnostically to identify bioavailable classes of contaminants in environmental media. However, complex mixtures may inhibit EROD responses, masking the occurrence of some toxic chemicals.

While many fish species are potentially suitable, this technique has been optimized using an estuarine fish species, *F. heteroclitus*, indigenous to estuaries along the east coast of the US. Adults of this species are easy to collect and can be maintained in the laboratory in fertile condition year round, producing many eggs per female on a semi-lunar cycle. Relative to other species, *F. heteroclitus* is highly responsive to AhR agonists, although

populations vary in their responsiveness to contaminants in manners that are adaptive to their home grounds.[10,11] This intra-specific variation in responsiveness (reflected as variation in *in ovo* EROD fluorescence[10,11]) makes this species and this measurement endpoint useful for the investigation of mechanisms of toxicity for an important class of environmental contaminants.

The methods described here were developed specifically for *F. heteroclitus*. However, with minor modifications, they are appropriate for other fish species (not shown). Simple experimental procedures using positive and negative control exposures provided as examples here can be used to validate the utility of these methods for other fish species.

Acknowledgments

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chapter twelve

Determination of lipid classes and lipid content in tissues of aquatic organisms using a thin layer chromatography/flame ionization detection (TLC/FID) microlipid method

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Contents

Introduction

Lipid content of aquatic animals is used frequently to normalize the concentrations of lipophilic contaminants.[1] In partitioning studies of lipophilic contaminants among various tissues in aquatic organisms, concentrations of these compounds are often

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lipid-normalized to elucidate how contaminants partition among the various tissues of an organism. This adjustment is also used when examining differences in contaminant levels among different species, as well as for modeling biomagnification of lipophilic contaminants in food webs and examining biota-sediment accumulation factors.

Tissues of marine biota consist of many different classes of lipids that differ significantly in polarity and may require specific solvents for their extraction. For example, blubber of cetaceans is comprised primarily of neutral lipids, such as triglycerides (TG) or wax esters,[2–5] whereas brain samples of these animals contain a much wider suite of lipids, including cholesterol and more polar lipid classes (e.g., phospholipids).[4] Solvents that have been used for lipid extractions range from non-polar/semi-polar solvents, such as hydrocarbon solvents (e.g., hexane, pentane) and chloroform, to polar solvents, such as methanol for the extraction of more polar lipids (e.g., membrane-bound phospholipids). Differences in lipid class composition can impact lipid normalization and, therefore, interpretation of results. For example, Ewald and Larsson[6] observed that fish lipids with high phospholipid content accumulated less PCB77 than did fish lipids with low phospholipid content. A review on the interactions among lipids and lipophilic contaminants in fish can be found in Elskus *et al*.[1] Kawai *et al*.[4] and Tilbury *et al*.[7] found lower lipid-normalized PCB concentrations in marine mammal brain compared to other tissues. The majority of lipids in brain are comprised of phospholipids and cholesterol rather than the neutral lipids that make up most of the total lipid in other tissues. Therefore, a lipid method that is capable of determining ''total lipids,'' as well as lipid class distribution, can be very valuable in understanding the differences in organochlorine (OC) concentrations among various tissues, species, and trophic levels.

A number of methods are used to extract and quantitate lipid content in a wide array of marine biota tissues[8–11] The most widely accepted gravimetric method, developed by Bligh and Dyer,[12] employs a chloroform/methanol solvent system to extract both neutral and polar lipids from a wide array of matrices. Although the most frequently used methods for lipid quantitation are based on gravimetric techniques, these analyses provide no information about the classes of lipids present in a tissue. A method has been developed to quantitate lipids using thin layer chromatography (TLC) coupled with flame ionization detection (FID), TLC/FID, system (see review in Reference 9). This method has been used to measure concentrations of lipid classes and calculate percent total lipid in phytoplankton, shrimp, bivalves, fish, and marine mammals.[5,13–15] Although this instrument system does have some limitations (e.g., time-consuming setup and calibration of instrument, variability of lipid quantitation due to sample spotting technique, a small range of linearity of the FID response), it provides rapid quantitation of lipid classes and lipid content in one analysis, including low-lipid tissues, as well as samples too small for good gravimetric quantitation.

Over the past decade, non-destructive collection of tissue samples (e.g., whale blubber biopsy, fish blood) from marine animals, especially populations of species that are threatened or endangered, has increased for biological and ecological studies. The tissue samples collected via biopsy are used to determine, for example, contaminant levels and profiles, stable isotope ratios and biomarkers.[15–18] However, due to the small size (<0.30 g) of some biopsy samples, only a selected number of analyses (e.g., contaminant and genetic analyses) can be completed. For contaminant analyses of small (<1.0 g) tissue samples, especially low-lipid containing samples, it is important to develop a single method that is capable of efficiently extracting both lipophilic contaminants, as well as lipids. Recently, we have developed a method that simultaneously extracts lipophilic OCs and lipids in tissue samples of marine organisms that range in size from 0.2 to 3.0 g using

an accelerated solvent extraction (ASE) method (see Chapter 35)[19]. With this method, the majority of the sample extract (>90% by volume) is used for OC analyses [e.g., high-performance liquid chromatography with photodiode array detection (HPLC/PDA) or gas chromatography/mass spectrometry (GC/MS)]. The remaining portion of the sample extract is then used to determine percent lipid and profiles of lipid classes using TLC with FID. In this chapter, the modifications made to determine lipids by ASE are compared to two previous analytical methods (ASE dichloromethane extraction/gravimetric quantitation and pentane/hexane extraction with TLC/FID analyses) to demonstrate the advantages and limitations of each lipid method.

Materials required

Tissue extractions

See Chapter 35[19] for details.

Lipid analyses

Equipment:

- Iatroscan MK-5®, Bioscan; Washington, D.C.
- Baxter Temp Con Oven
- A Dell Dimension 8300 computer or equivalent computer, which can run Microsoft Windows XP Pro®, has at least 2 GB hard disk space and 384 MB of RAM and is not a hard disk configured to be FAT 16
- Waters Empower® data acquisition system (Waters; Milford, MA)

Supplies:

- 100-ml Pyrex glass graduated cylinder (Fisher Scientific, Pittsburgh, PA, catalog #08-552E)
- 25-ml Pyrex glass graduated cylinder (Fisher Scientific, Pittsburgh, PA, catalog #08-552C)
- Chromarods®, type S-III (Shell-USA, Fredericksburg, VA, catalog #3248)
- Chromarod holder SD-5 (Shell-USA, Fredericksburg, PA, catalog #5321)
- Chromarod spotting guide (Shell-USA, Fredericksburg, VA, catalog #5231)
- TLC development tank, 6 in. wide × 7.5 in. high × 1.25 in. deep (Shell-USA, Fredericksburg, PA, catalog #3201)
- 32 in.-diameter Whatman Grade No. 1 filter paper, cut to 5.75 in. wide × 7.35 in. high (VWR Scientific, West Chester, PA, catalog #78450-229)
- Hamilton syringe 1 μl (Fisher Scientific, Pittsburgh, PA, catalog #14813112)

Reagents:

- Cholesterol (Sigma Chemical, St. Louis, MO, catalog #C-8667)
- Oleic acid (Sigma Chemical, St. Louis, MO, catalog #O-1008)
- L-α-phosphatidylcholine (Sigma Chemical, St. Louis, MO, catalog #P-3556)
- Triolein (Sigma Chemical, St. Louis, MO, catalog #T-7140)
- Lauryl stearate (Nucheck Prep, Elysian, MN, catalog #WE-1304)

- Diethyl ether (Fisher Scientific, Pittsburgh, PA, catalog #E197-1)
- Formic acid (88%) (Fisher Scientific, Pittsburgh, PA, catalog #A-118P-100)
- Dichloromethane, pesticide grade (VWR Scientific, West Chester, PA, catalog #BJ300-4)
- Ultra high-purity hydrogen, 5.0 grade (PraxAir, Danbury, CT)

Procedures

TLC/FID calibration standards

Stock solutions of lauryl stearate (10 mg/ml), triolein (10 mg/ml), oleic acid (10 mg/ml), cholesterol (2.0 mg/ml), and L-α-phosphatidylcholine (2.0 mg/ml) were prepared in dichloromethane and stored at −20°C. Four lipid calibration standards for the Iatroscan were prepared in dichloromethane and stored in a −20°C freezer. Calibration standard #1 contained the following compounds: lauryl stearate (10.0 mg/ml), triolein (10.0 mg/ml), oleic acid (10.0 mg/ml), cholesterol (2.0 mg/ml), and L-α-phosphatidylcholine (2.0 mg/ml). Calibration standard #2 contained the following: lauryl stearate (5.0 mg/ml), triolein (5.0 mg/ml), oleic acid (5.0 mg/ml), cholesterol (1.0 mg/ml), and L-α-phosphatidylcholine (1.0 mg/ml). Calibration standard #3 contained the following: lauryl stearate (2.5 mg/ml), triolein (2.5 mg/ml), oleic acid (2.5 mg/ml), cholesterol (0.50 mg/ml), and L-α-phosphatidylcholine (0.50 mg/ml). Calibration standard #4 contained the following: lauryl stearate (1.25 mg/ml), triolein (1.25 mg/ml), oleic acid (1.25 mg/ml), cholesterol (0.25 mg/ml), and L-α-phosphatidylcholine (0.25 mg/ml). We recommend making new calibration solutions every 4–6 months to ensure that the standards have not decomposed or oxidized.

Microlipid extraction of samples

Prior to use, all tubes and glass transfer pipettes are rinsed three times with acetone to remove any potential contaminants. As a safety precaution, all glassware rinsing, tissue extraction, and lipid spotting procedures are performed in fume hoods by personnel wearing nitrile gloves and safety glasses.

Lipids and OCs were extracted from tissues using an ASE as described by Sloan *et al*. in Chapter 35 of this volume. For HPLC/PDA and TLC/FID analyses, the amount of tissue extracted ranges from 0.20 to 5.0 g, depending on the expected lipid and water content. Briefly, a tissue sample was weighed (to the nearest 0.01 g) in a tared 10-oz jar. Sodium sulfate (15 cc) and magnesium sulfate (15 cc) were added sequentially to each sample jar and mixed thoroughly using a clean metal spatula. Each sample mixture was transferred to a 33-ml ASE extraction cell and an OC surrogate standard (see Chapter 25 of Reference 20 by Ylitalo *et al*.) was added to each cell. Lipids and OCs were extracted using dichloromethane (see Chapter 35 of Reference 19 for details on ASE extraction parameters).

After the ASE extracted a sample set, each 60-ml collection tube was weighed to the nearest 0.01 g and the weight was recorded. Using a solvent-rinsed 9-in. glass pipette, a 1-ml aliquot of each sample extract was transferred to a 2-ml GC vial, capped and stored at −20°C until TLC/FID lipid analysis. Each 60-ml collection tube was recapped and the remaining sample extract was weighed and recorded. For low-lipid tissues (<10%), each 1-ml lipid extract portion was reduced in volume to 100-μl using a gentle stream

of ultra-pure nitrogen. By concentrating the low-lipid tissue extracts to 100 μl, we are fairly confident that we can quantitate the lipid classes within the linear response ranges of the FID. The remaining sample extract was cleaned up and analyzed for OC contaminants by HPLC/PDA or GC/MS (see Chapter 25 of this volume).

Separation of lipid classes

Silica gel chromatography rods (type S-III Chromarods) were used for separation of each lipid class in the tissue extracts. The Chromarods are quite sensitive to humidity,[9] so we suggest storing the racks of Chromarods in a 60°C oven or desiccator until needed. Ten Chromarods were placed into a SD-5 Chromarod holder, and each Chromarod was blank scanned (scanned from top to bottom at a speed of 30 s/scan) three times by the Iatroscan MK-5 TLC/FID to remove any organic compounds that were present and to ''activate'' the Chromarod. While the Chromarods were blank scanned, a TLC development tank containing hexane/diethyl ether/formic acid [60:10:0.02 (v/v/v)] with a rectangular piece of filter paper (5.75 in. × 7.35 in., to help saturate the tank) was set up in a laboratory hood. The development tank is performed in a fume hood of a temperature-regulated laboratory (temperature range 20–25°C) to prevent shifts in retention times of the lipid classes. To ensure that the development tank was completely saturated, we added the filter paper and developing solvents to the tank at least 30 min prior to placing the first set of Chromarods in the tank. After blanking the Chromarods, the rod holder was removed from the Iatroscan and placed onto a spotting guide. A 1-μl aliquot of sample extract was carefully spotted onto each Chromarod near the base of the Chromarod using a 1-μl Hamilton syringe. The spotting technique was one of the most difficult techniques to master. For example, if the sample extract was spotted over a large area of the Chromarod, we have found that the concentration of phospholipids is 20–50% less than the phospholipid concentration in the same extract spotted over a smaller region. In order to provide better separation of the various lipid classes, we try to keep the sample spot as small as possible to avoid spreading at the origin of the Chromarod. Although we have not used a semi-automatic sample spotter (available from Shell-USA, Fredericksburg, VA, model SES 3202/IS-02), we have been told by colleagues that sample spots made with the spotter are more consistent and smaller compared to the spots delivered with a 1-μl syringe. After spotting each Chromarod with the sample extract, the rod holder was placed in a 60°C oven for 2 min to evaporate the sample solvent. Alternately, the sample solvent can be rapidly evaporated using a hair dryer. The rod holder was removed from the oven and placed in the chromatography development tank for 24 min in order to separate the various lipid classes (e.g., wax esters, TG, free fatty acids, cholesterol, phospholipids) in each sample extract. After 24 min in the development tank, the Chromarod holder was removed immediately from the tank and placed in a 65°C oven for 5 min to evaporate any remaining solvent. The rack should not stay in the tank any longer than 24 min because the separation of lipid classes can be altered with additional time in the development tank.

TLC/FID analysis

The FID was operated with hydrogen and airflow rates of 160 and 2000 ml/min, respectively. We have found that if the hydrogen flow on the Iatroscan was changed (e.g., hydrogen flow dial is inadvertently bumped or turned), the calibration curves of the lipid classes must be recalculated. Each Chromarod was scanned at 30 s/scan, which

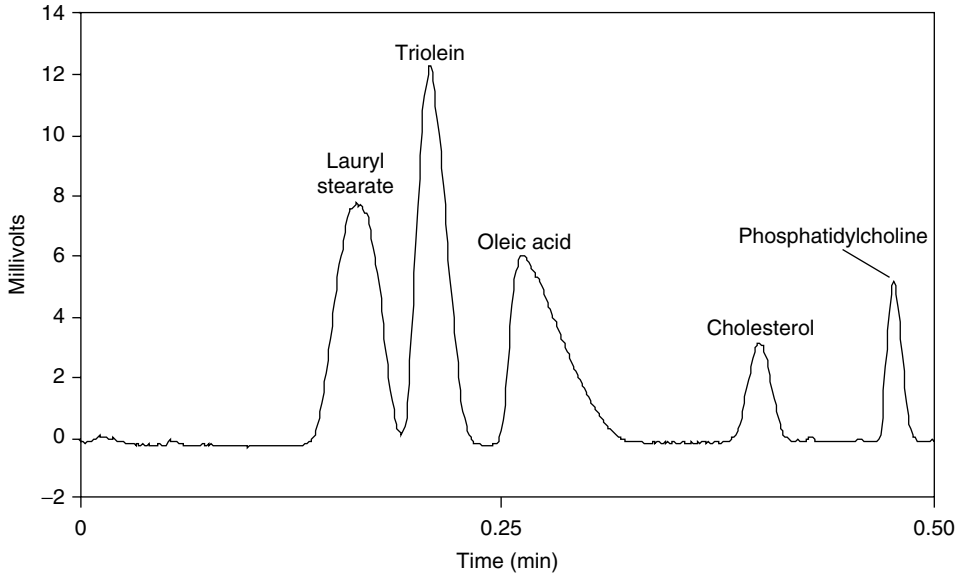

Figure 12.1 A TLC/FID chromatogram showing the separation of five lipids [lauryl stearate (wax ester), triolein (TG), oleic acid (free fatty acid) cholesterol, and L-α-phosphatidylcholine (phospholipid)] contained in a calibration standard.

allowed for complete combustion of all components on the rod. The Chromarods were then passed through the hydrogen flame to ionize the sample. The ionization caused changes in electric current flowing through the FID. The Iatroscan MK-5 was interfaced with a Dell Dimension 8300 computer and data were collected and reprocessed using the Waters Empower data acquisition system. The currents were recorded by the Empower data acquisition system. Each sample chromatogram was automatically integrated using the Empower software system. The integration of each sample peak was checked, reintegrated (if needed), and each sample report with corresponding chromatogram was printed.

Quality assurance

Each extraction sample set consisted of 11–14 field samples, a method blank, and a National Institute of Standards and Technology (NIST) standard reference material (i.e., SRM 1974b, SRM 1945, SRM 1946). To determine if the extraction solvents, glassware, or other supplies were free of lipid contaminants, a solvent blank was analyzed with every sample set. To monitor the accuracy of our extraction method, an NIST standard reference material was analyzed with each sample set and results met laboratory criterian.[21] The percent lipid concentrations of the SRM 1945, SRM 1946, and SRM 1974b determined by our TLC/FID method were compared to ''total extractable organic'' values published by NIST.

External standard curves for each lipid class were created every 3 weeks. To determine if the Iatroscan TLC/FID was operating properly, a calibration standard was analyzed with each Chromarod rack that consisted of 10 Chromarods. If the concentration of a lipid in the calibration standard was not within $\pm$ 15% of the known concentration of that compound, the field samples were reanalyzed. The limits of detection for each of the five lipids contained in the calibration standards were the following: lauryl stearate

(0.20 mg/ml), triolein (0.20 mg/ml), oleic acid (0.30 mg/ml), cholesterol (0.25 mg/ml), and L-α-phosphatidylcholine (0.25 mg/ml).

Calculations of lipid class concentrations and percent lipid

The Empower data acquisition software integrates the area under each peak. Once the calibration curves for the lipid classes are completed, the software automatically calculates the concentration of each lipid class. These data are then used to calculate the concentration of each lipid class per gram sample extracted. For example, to calculate the concentration of TG in a tissue sample, Equation (12.1) was used:

$$\mu g\ TG/g\ sample = (\text{amount of TG in sample} * \text{volume } (\mu l) \text{ of sample extract})/(\text{sample weight (g)} * \text{volume } (\mu l) \text{ of extract spotted on Chromarod}) \quad (12.1)$$

This concentration was then converted to g TG/kg samples extracted using Equation (12.2):

$$g\ TG/kg\ sample = \mu g\ TG/(g\ sample\ extracted * 0.001) \quad (12.2)$$

Percent total lipids were calculated by summing the concentrations (g of lipid class/kg sample) of the five lipid classes and multiplying this sum by 0.1. A four-point linear external calibration curve for each lipid class was used for quantitation. Duplicate TLC/FID analyses were performed for each sample extract, and the mean value was reported.

Results and discussion

Lipid classes are readily separated on the Chromarods and are rapidly quantitated using the Iatroscan TLC/FID system. In Figure 12.1, five lipids [lauryl stearate (wax ester), triolein (TG), oleic acid (free fatty acid), cholesterol, and L-α-phosphatidylcholine (phospholipid)] contained in a calibration standard were separated using a hexane/diethyl ether/formic acid development system. With this non-polar/semi-polar solvent system, the neutral lipids migrate up to the Chromarod further than with the polar lipids. Similar to our findings, other studies that determined lipid content of marine organisms and fish meal using Iatroscan TLC/FID system reported good separation and quantitation of various classes of lipids on the Chromarods.[11,13,14]

We compared the lipid content of various tissues of marine organisms determined by three different methods to evaluate the best method to extract and quantitate lipids. The three methods were the following: (1) dichloromethane ASE extraction/gravimetric analyses[20]; (2) pentane/hexane extraction with TLC/FID analysis (old method);[5] and (3) dichloromethane ASE extraction with TLC/FID analysis (new method). The percent lipid values of the NIST SRMs and various tissues of marine biota determined by these methods are reported in Table 12.1. The mean lipid values of the three SRMs determined by the ASE/gravimetric method or by either of the Iatroscan methods are comparable or lower than the mean values published by NIST. We also found that, although the percent lipid values determined by TLC/FID were comparable to or lower than the values measured gravimetrically, the lipid concentrations quantitated by these two methods were correlated. For example, the lipid values of various SRMs and marine tissues determined by the new method were correlated with the lipid concentrations measured gravimetrically ($r^2 = 0.990$, $P < 0.0001$). Similarly, Delbeke *et al.*[13] found that the lipid concentrations determined by TLC/FID of tissues of various species of marine biota were correlated but were

approximately half as great as those determined by the gravimetric method. Because the gravimetric method measures lipids, as well as other exogenous materials extracted from a tissue, it is likely that the lipid content determined gravimetrically is overestimated due to interferences from non-lipid substances. In contrast, non-lipid compounds do not substantially affect the TLC/FID lipid measurements. Therefore, our preliminary findings indicate that lipid concentrations can be readily measured using the new ASE/TLC/FID method.

To evaluate the extraction efficiency of the two solvent systems used to extract lipids and then quantitated by TLC/FID, we compared the lipid values in three SRMs and various marine biota tissues extracted with dichloromethane (new method) or pentane/hexane (old method). In the present study, it should be noted that the SRMs and fish tissue samples were homogenates, whereas the blubber of the California sea lion were individual subsamples. We found that the lipid values were highly correlated ($r^2 = 0.951$, $P < 0.0001$) between the two systems but did not find any consistent trends. For example, the dichloromethane-extracted blubber samples (SRM 1945 and blubber of sea lion) contained comparable or higher percent lipid values than did the same samples extracted with pentane/hexane, whereas the dichloromethane-extracted rockfish liver samples (14965 and 14968) had lipid values that were comparable or lower than the lipid concentrations of the same liver samples extracted by the pentane/hexane method (Table 12.1). Because a limited number of samples (<50 samples) have been analyzed by both the old and new methods, the results presented in this chapter should be considered primarily qualitative.

Data on lipid classes measured in tissues of marine biota can provide information on quality of the samples that are being analyzed for chemical contaminants. In a contaminant study on eastern North Pacific gray whales, Krahn *et al.*[5] found that the blubber samples of ''decomposed'' whales (decomposition state rated by field personnel) contained lower proportions of TG and higher proportions of free fatty acids, cholesterol, and phospholipids than the blubber of ''fresh dead'' whales. Because free fatty acids are generally attributable to hydrolysis of TG, increase in this lipid class is an indication of decomposition. In fact, whales showing advanced decomposition had higher levels of free fatty acids than were found in moderately decomposed whales. In the present study, blubber of the California sea lions ($n = 3$) contained high proportions of TG (~95%) and low proportions of phospholipids (~5%). These results are consistent with other studies that show that blubber of ''healthy'' marine mammals is comprised primarily of neutral lipids (e.g., TG, wax esters).[4,5,7,15] The lipid class profiles (no free fatty acids or cholesterol) of the California sea lion blubber samples (data not shown) indicated that these samples were stored properly. Over the past few years, we have determined the lipid profiles of a number of blubber samples of stranded marine mammals prior to OC analyses to determine if the tissue quality may be compromised. In the future, we will continue to screen blubber samples for quality using ASE with TLC/FID.

The TLC/FID analyses of the lipid extracts provide information on lipid classes, as well as percent lipid on a wide array of marine biota tissues, including low-lipid containing samples, such as rockfish muscle samples, and low-weight blubber biopsy samples. Data on lipid classes can provide valuable information on the quality of the tissues being analyzed. In addition, both contaminant and lipid analyses can be conducted on the same dichloromethane ASE extract — saving analyses time and reducing costs.

We recommend extraction of marine biota tissues using the dichloromethane ASE method — the lipid values of the NIST SRMs determined by the new method (ASE with dichloromethane) are more similar to the NIST published values than the lipid values measured by the old method (pentane/hexane). The advantages of lipid analyses by the Iatroscan TLC/FID are: (1) data on lipid classes are obtained that can be used to

Table 12.1 Percent lipid values measured gravimetrically and by TLC with FID in various NIST SRMs and tissues of marine biota

Sample number	Sample type	NIST published values	Iatroscan TLC/FID		Gravimetric GC/MS method, dichloromethane
			Pentane/hexane[a]	Dichloromethane[b]	
SRM 1974b ($n=5$)	Blue mussel homogenate	0.64 ± 0.13	0.32 ± 0.046[c]	0.20 ± 0.038	0.36 ± 0.03
SRM 1945 ($n=15$)	Whale blubber homogenate	74.29 ± 0.45	57 ± 4.9	63 ± 7.6[d]	74 ± 2.7
SRM 1946 ($n=5$)	Fish muscle homogenate	10.17 ± 0.48	7.2 ± 0.21	5.6 ± 0.34	10 ± 3.4
CSL 4951	CA sea lion blubber		39	56	Not determined
CSL 4955	CA sea lion blubber		50	49	Not determined
CSL 4914	CA sea lion blubber		48	54	Not determined
SK-XM2	Coho salmon muscle		5.0	4.1	4.7
NQ-XM9	Coho salmon muscle		3.2	3.9	5.3
14965	Rockfish liver		4.2	4.1	7.6
14968	Rockfish liver		13	8.3	13
PO-PHW10	Pacific herring whole body		Not determined	5.3	6.0
CH-PHW07	Pacific herring whole body		Not determined	1.3	3.0
02SM-PHW04	Pacific herring whole body		4.4	2.6	6.6
1572	Rockfish muscle		Not determined	0.55	0.94
1583	Rockfish muscle		Not determined	0.19	0.27

[a] Old method.

[b] New method.

[c] $n=6$.

[d] $n=5$.

determine the quality of certain tissue samples (e.g., blubber); (2) overestimation of percent lipid due to extraction of exogenous substances is eliminated; and (3) lipid content can be determined for low-lipid tissues and small tissue samples. However, there are some limitations: (1) the initial setup is more costly than for gravimetric quantitation; (2) calibration of the TLC/FID is fairly time consuming compared to gravimetric analyses; and (3) sample spotting technique is difficult to master. We recommend a performance-based quality assurance program for lipid analyses. For example, we include a method blank and an SRM or control material, for which there are published or certified percent lipid values, with each sample set to determine how well the dichloromethane ASE with TLC/FID system is operating.

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chapter thirteen

Larval molting hormone synthesis and imaginal disc development in the midge Chironomus riparius *as tools for assessing the endocrine modulating potential of chemicals in aquatic insects*

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Contents

Introduction

In metazoans, hormones play a crucial role in control and fine-tuning of physiological processes. Such diverse and complex functions as energy metabolism, cellular homeostasis, reproduction and development, behavioral patterns, or phenotypic characteristics are governed by endocrine regulation.[1] Thus, endocrine disruption, the impairment of one or more of these processes by exogenous compounds, may well have uncalculable consequences in the living environment, including human health. Consequently, in the last decade,

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considerable research effort has been directed towards this issue.[2–4] Most of these studies focussed on vertebrate hormone systems, and although invertebrate species represent about 95% of all animals, effects in invertebrates have never been thoroughly investigated.[5–8] Probable reasons are that invertebrate hormone systems are much more divergent and much less examined (and understood) than those of vertebrates, and that the high degree of conservation within vertebrate hormone systems makes these better animal models with respect to possible impacts of endocrine-disrupting chemicals on human health.

In selecting a suitable test endpoint for detecting endocrine disruption, it is important to consider the multitude of levels of interaction, in which a chemical may impact hormonal function in an organism. These may comprise production, metabolism, release, transport, receptor interaction, or metabolism of natural hormones.[5] The present chapter describes a method that uses a combined *in vivo* and *in vitro* approach for detecting endocrine modulation of development in aquatic larvae of the non-biting midge *Chironomus riparius* Meigen. The test endpoints, *in vitro* synthesis of ecdysteroidal molting hormones by prothoracic glands and imaginal disc development *in vivo*, represent different levels of biological organization, but both functions are clearly correlated with endocrine regulation.

The choice of *C. riparius* as a test organism is based on a variety of factors that make working with these animals advantageous. First, as insects, their endocrinology is well understood when compared to other invertebrate taxa. Furthermore, chironomids are widely used as test organisms in ecotoxicology, culturing is easy and standard methods have been developed.[9,10] Chironomids are sexually reproducing animals (in contrast, e.g., to daphnids) and their larvae are of a size that allows dissection of hormone-secreting glands. Finally, particularly in the older literature, a wealth of information is available on developmental biology and endocrinology of this species.

Wülker and Götz[11] have suggested a method for exact determination of sex and developmental stage of fourth-instar larvae, which will be summarized in the following. King et al.[12] first described the analysis of molting hormone production by *in vitro* incubation of prothoracic glands and, since then, it has been applied in a huge variety of problems, including neuroendocrine control of ecdysteroid synthesis[13] and the effects of synthetic insect growth regulators.[14]

Materials required

A prerequisite for the experiments described in this chapter is a healthy laboratory culture of *C. riparus* or related *Chironomus* species (e.g. *Camptochironomus* (*Chironomus*) *tentans*). Methods for setting up and maintaining a culture have been described, e.g., in References 9 and 10.

General:

- A set of 0.2 µl to 5 ml pipettes
- 25, 100, and 300 µl and 1.5 ml repeater pipettes
- Tubes, tube racks, volumetric flasks (10 and 100 ml)

For staging:

- Microscope or stereomicroscope equipped with bottom illuminator
- Glass slides
- A set of tweezers, including spring steel and self-locking tweezers
- Vials or dishes to collect the staged animals

For dissection:

- Stereomicroscope
- Fiber optic illuminator with dual light pipes
- Very fine curved micro-scissors
- Precision tweezers Dumont No. 5 (available through laboratory suppliers; be sure to have some spare tweezers available because they are very sensitive to inadvertent damage)
- Tweezer set, including spring steel and self-locking tweezers
- Insect minute pins
- Ice pack for cooling the dissection dish from below
- Paper tissue
- Ice-cold water for anesthesia
- Cold 70% EtOH
- Pasteur pipettes
- Sterile glass Petri dishes, approximately 50 mm diameter (the use of plastics should be avoided because several plastic additives are suspected to possess endocrine activity)
- Autoclaved 8 × 40-mm GC sample vials (Merck Eurolab, or comparable product) (avoid autoclaving the caps unless you are sure that they are resistant)
- Water bath or electronic heating block
- Beadle's ringer solution: 7.5 g/l NaCl, 0.35 g/l KCl, 0.21 g/l $CaCl_2$, as given in Reference 15, autoclaved or sterile filtered
- Cannon's ringer solution as given in Reference 16, see Table 13.1 for details
- Finally, for a quiet hand, I have found it helpful to renounce the morning cup of coffee when dissections are planned

For ecdysteroid extraction:

- Methanol HPLC grade or better
- Table top centrifuge (1000 *g*)
- Ultrasonic bath
- Nitrogen
- Water bath or heating block
- Evaporator manifold (this can easily be self-made using plastic tube and injection needles). Alternatively a vacuum concentrator can be used (''Speed-Vac'' or similar product)

For radioimmunoassay:

- Access to a working place where handling and storage of substances with activities of up to 50 μCi tritium is allowed
- Tritium-labeled ecdysone (New England Nuclear, #NET621)
- Unlabeled ecdysone standard (Sigma-Aldrich, #E9004)
- Ecdysone-specific antiserum (Trifolio, Lahnau, Germany, DBL-1)
- Normal sheep serum (ICN Biomedicals, #642952)
- Sodium azide
- RIA buffer: 100 m*M* boric-acid/sodium tetraborate, 75 m*M* NaCl, pH 8.4
- Scintillation fluid
- Refrigerated centrifuge capable of a speed of at least 5000 *g* and capacity for 24 or more tubes

- Ice-cold saturated ammonium sulfate solution
- Refrigerator
- Scintillation counter

Procedure

1. Staging and sexing of the developmental phase of 4th larval instars

A method for exact determination of sex and developmental stage of fourth-instar chironomid larvae was described by Wülker and Götz.[11] By examining the larval imaginal discs they characterized nine morphologically distinct phases of equal duration, considering developmental characteristics of gonads, genital and thoracic imaginal discs. For the method presented here, only the genital imaginal discs were used for determination of the developmental stages. This was found to provide sufficiently detailed information to classify a single larva within a reasonable amount of time.

Single specimens are collected from the culture vessels containing larvae from a synchronized culture (i.e., having left their egg masses within 24 h or less), usually on day 11 or day 12 after hatching (*C. riparius* at 20°C). For sexing and staging, larvae are blotted on tissue and placed dorsal surface down on a microscope slide. Another slide is carefully placed on top to keep the larva in place. Using a dissection microscope (light from below) at 50-fold magnification, the genital imaginal discs in the 8th and 9th abdominal segment are easily observable. No symptoms were found to indicate that intact larvae were hurt by this procedure. However, in a few cases, the larvae did not stay intact and hemolymph leaked out of the body. These larvae are moribund and should be excluded from any further experimentation. A detailed description of the developmental characteristics of the single phases is given in Figure 13.1 (from Reference 11, with permission).

After the determination, larvae are collected in appropriate vessels, one for each sex and stage. For the experiments of substance-induced alteration of development, larvae in phases 5 and 6 should be chosen, as these larvae usually do not enter pupal stage within the test duration of 48 h. For dissection and *in vitro* incubation of prothoracic glands, animals in phase 9 were most suitable because molting-hormone production in insects reaches its maximum just before molting begins (Figure 13.2, and Reference 17).

2. In vivo *development*

Single larvae to be used in these experiments are transferred into 20-ml test vessels that contain 2 ml medium to which the desired test substance was added, and are exposed for 48 h under rearing conditions, but without aeration, food, or sediment. After this time, the larvae are removed from the vessels and staged again as described earlier. For each larva, the individual 48-h development is then computed by subtracting the larval phases' value at the start of the experiment from the value determined after 48 h of exposure.

3. *Dissection of larvae and* in vitro *incubation of prothoracic glands*

Larvae are sexed and staged as described earlier, and males and females only from the 9th (= last) larval stage are used for dissection. It was long known that ecdysteroid release by the prothoracic glands reaches its maximum activity directly prior to the molting process. This was also established for chironomids by Laufer et al.,[17] who determined the whole-body ecdysteroid titer in relation to days of larval age after hatching. In

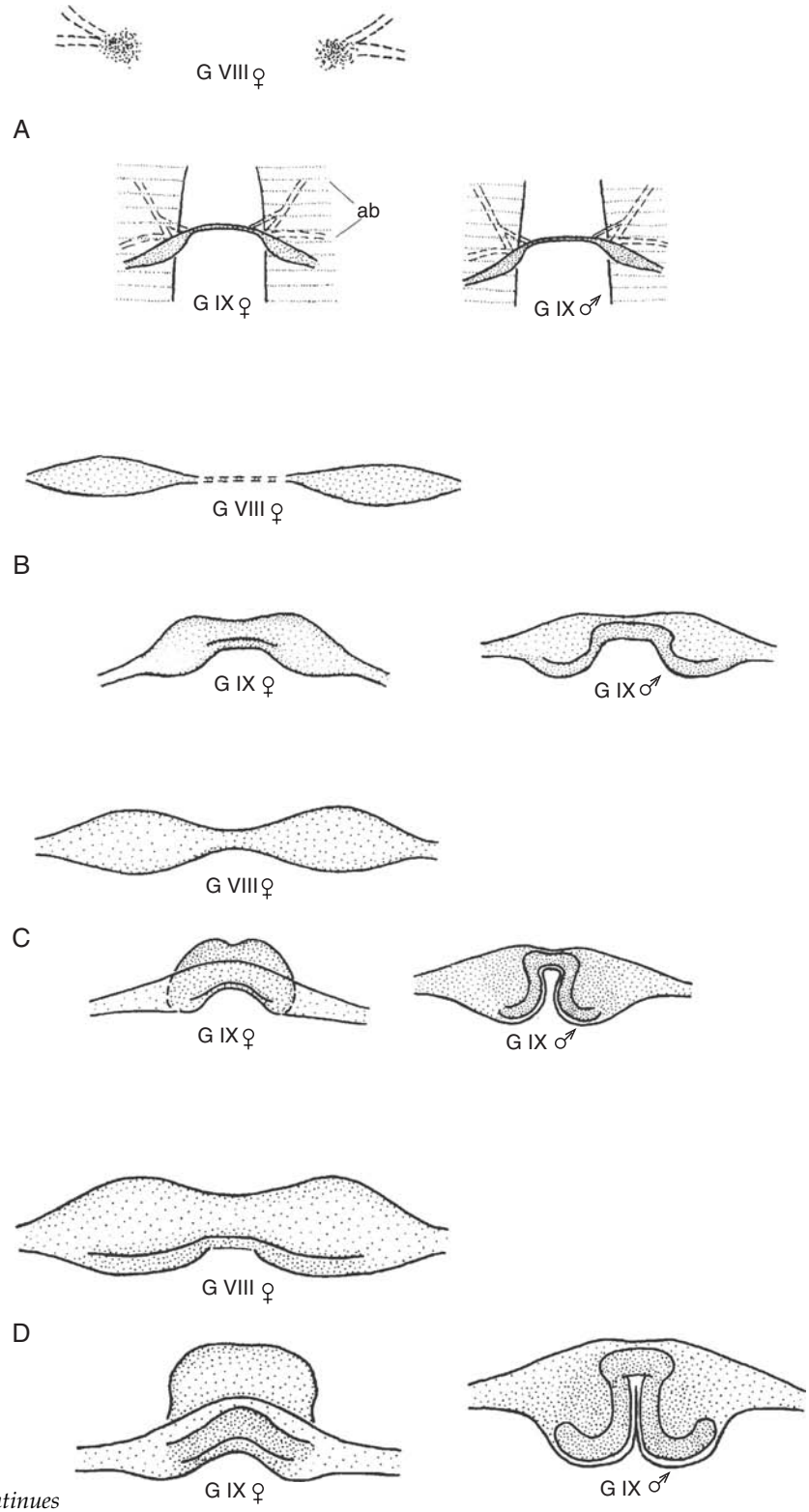

Figure 13.1 *A–D Continues*

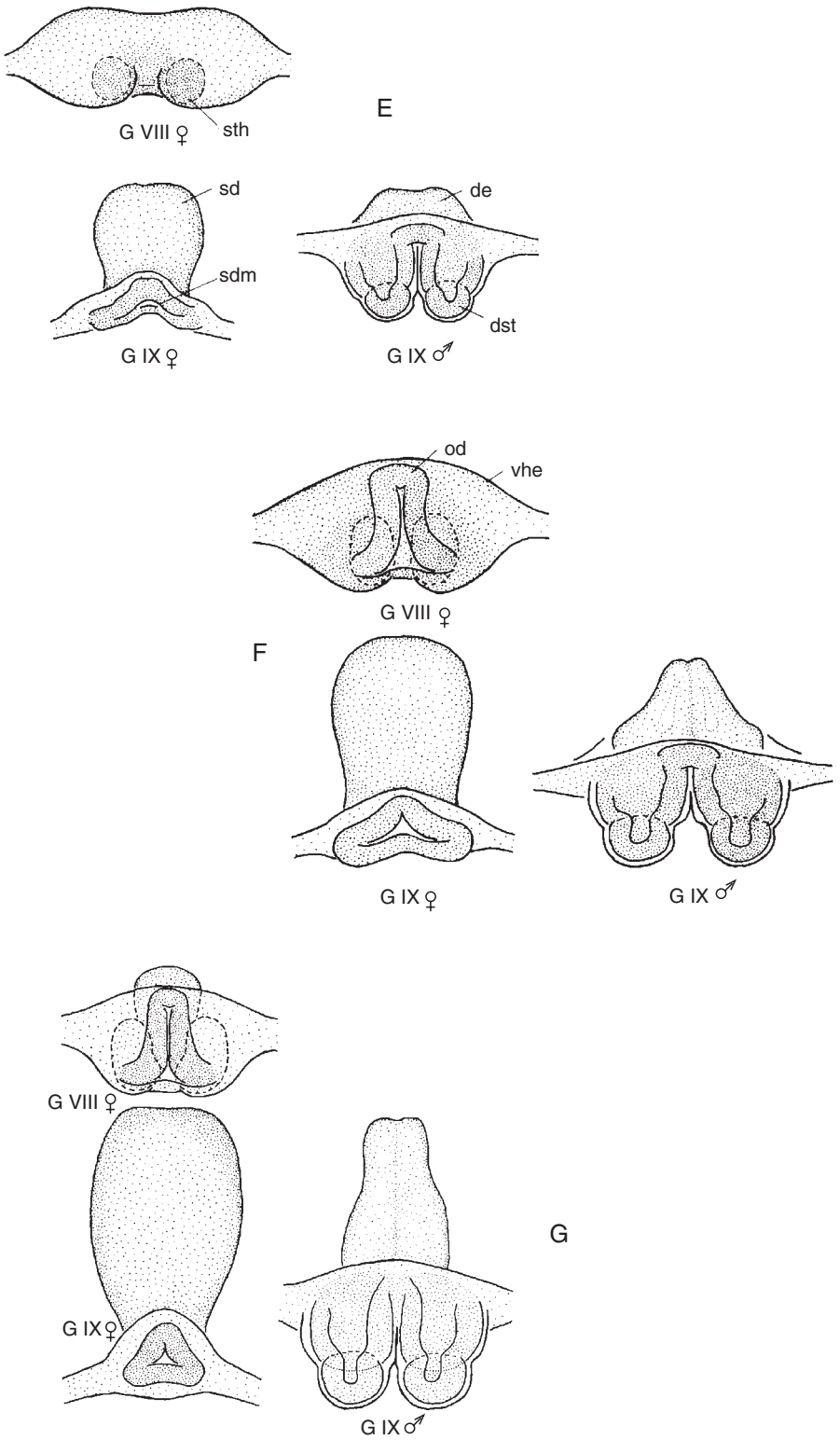

Figure 13.1 *E–G Continues*

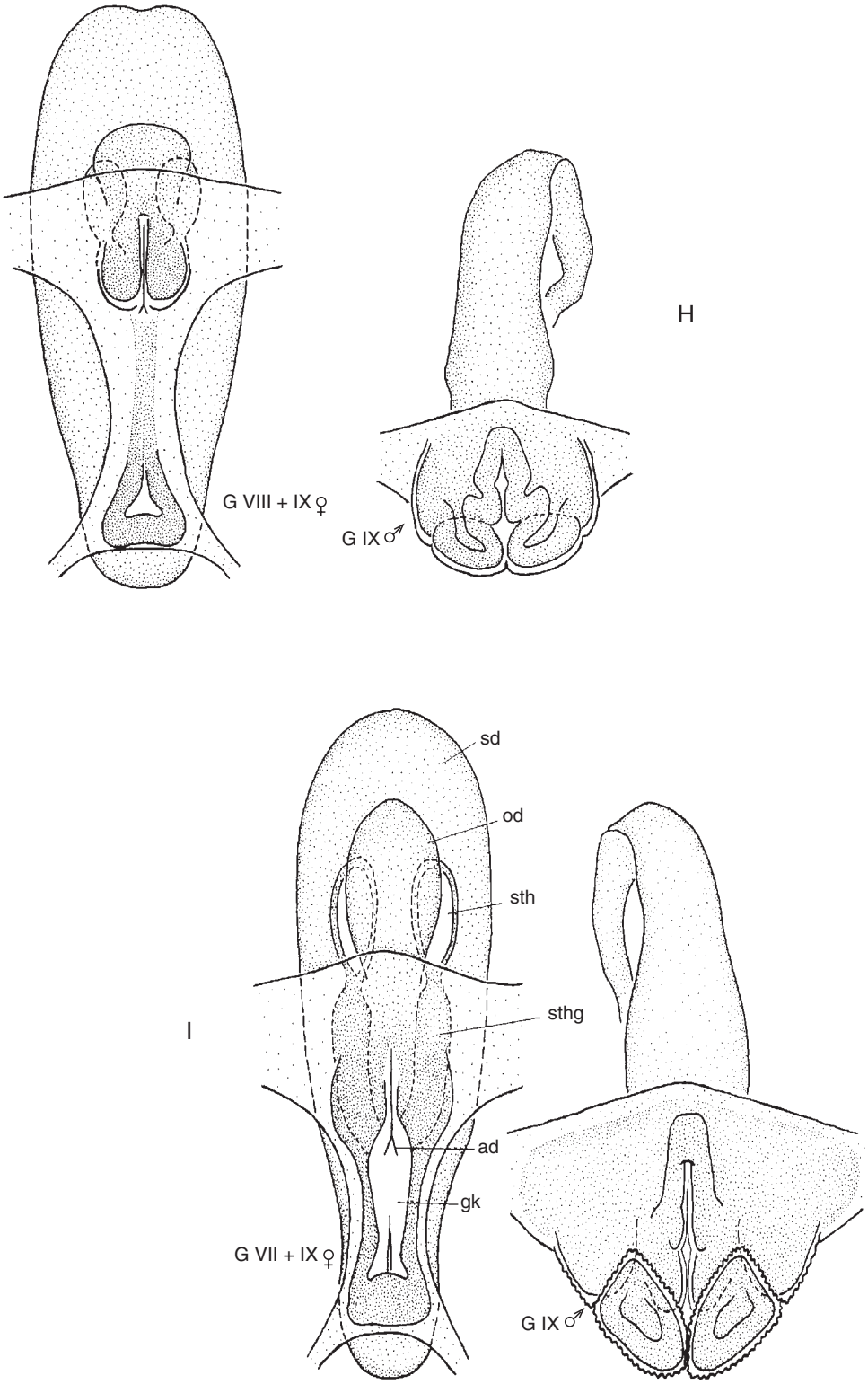

Figure 13.1 *H and I Continues*

preparation for the present experiments, tests of ecdysteroid titers in hemolymph reverified these results and additionally confirmed that 9th-phase midges display the highest molting hormone content (Figure 13.2).

Late larvae in phases 8 and 9 can, without any microscopical observation, easily be recognized by a thick white ''collar'' in the first thoracic segment, but for an unequivocal determination, sexing and staging by imaginal disc characters is necessary. The collar is formed by the pupal respiration organ, which has already developed under the larval cuticle. Because late-fourth-instar larvae already contain the developing or fully developed pupae, they are often referred to as pharate pupae. However, before final ecdysis these animals are still true larvae, in overall physical state and in their response to their environment.[18]

Staged animals are immersed in ice-cold water for anesthesia until they stop moving. (I have no experience with CO_2 anesthesia but this also might be an option.) They are then briefly washed in cold 70% ethanol and blotted dry on tissue. The dissection dish is placed on an ice pack for continuous cooling and filled with ice-cold Beadle's ringer solution. Under a stereomicroscope the larva is fixed ventral surface down with a minute pin, which is inserted directly between the head capsule and the first thoracic segment. This is crucial because the head capsule itself is too brittle and may break if the larva should move, and the thoracic segments contain the central nervous system and adhering neuroendocrine glands, which should not be harmed. An overview of the organization of the chironomid retrocerebral system[19,20] is provided in Figure 13.3. For

Figure 13.1 Overview of the developmental phases in 4th-stage *Chironomus* larvae, depending on the differentiation grade of the genital imaginal discs. (Reproduced from Wülker, W. and Götz, P., *Z. Morphol. Tiere*, 62, 363–388, 1968. With permission.) (A) Phase 1: G VIII ♀, in some cases, groups of enlarged hypodermis cells at the typical site. G IX ♀ and ♂, hypodermal depressions in the region of the terminal ampullae, linked together by a narrow hypodermal ridge. Sexes cannot be differentiated. (B) Phase 2: G VIII ♀, the two parts of the anlage connected by a narrow, often incomplete band. G IX ♀ and G IX ♂, anlage parts larger and connected by a broad bridge. Sexes cannot be differentiated. (C) Phase 3: G VIII ♀, anlage parts oval, with a wide connecting strip. G IX ♀, anlage expanded and fused medially; mucus gland sometimes visible as transverse slit. G IX ♂, anlage spectacles-shaped; medial and posterior edge reinforced by a ridge. (D) Phase 4: G VIII ♀, anlage has formed a unit, with indented oral margin. G IX ♀, mucus gland growing out like a tongue; ratio of tongue length to minimal width (L:W) < 1.2. G IX ♂, caudal parts of the anlage medially apposed to one another over a large area. (E) Phase 5: G VIII ♀, anlage a wide oval; spermathecal anlagen emerging dorsally as round bulges. G IX ♂, mucus gland, L:W = 1.2–2.0. G IX ♂, ejaculatory duct growing out at the front edge, as a two-peaked hillock; in the back part dististyli already stand out slightly. (F) Phase 6: G VIII ♀, anlage of the oviduct extends to the front edge of the hypodermal infolding, but not beyond. G IX ♀, mucus gland, L:W = 2.0–3.0. G IX ♂, in ventral view, the anlage of the ejaculatory duct appears about half as long as the hypopygal anlage; dististyli clearly demarcated. (G) Phase 7: G VIII ♀, oviduct anlage projects beyond the hypodermal indentation. G IX ♀, front edge of mucus gland extends to the same level as G VIII. G IX ♂, anlage of the ejaculatory duct is at least as long as the hypopygal anlage. (H) Phase 8: G VIII ♀, an elongated oval. G IX ♀, front edge of mucus gland extends far beyond G VIII; the regions around the openings (G IX and G VIII) are beginning to fuse; apodeme already visible. G IX ♂, anlage of ejaculatory duct twice as long as hypopygal anlage; dististyli form tongue-like inward projections. (I) Phase 9: G VIII ♀ and G IX ♀, in the fusion region of G VIII and G IX a forked apodeme and complete genital chamber have formed. Spermathecae have long excretory ducts. G IX ♂, Hypopyge in conspicuously curled pupal sheath. *Key to lettering*: ab, suspension bands; ad, apodeme; de, ejaculatory duct; dst, dististylus of the male genital anlage; G VIII, GIX, genital imaginal disks of the 8th and 9th abdominal segments; gk, genital chamber; od, oviduct; sd, mucus gland; sdm, opening of mucus gland; sth, spermatheca; sthg, spermathecal duct; vhe, front edge of the hypodermal infolding.

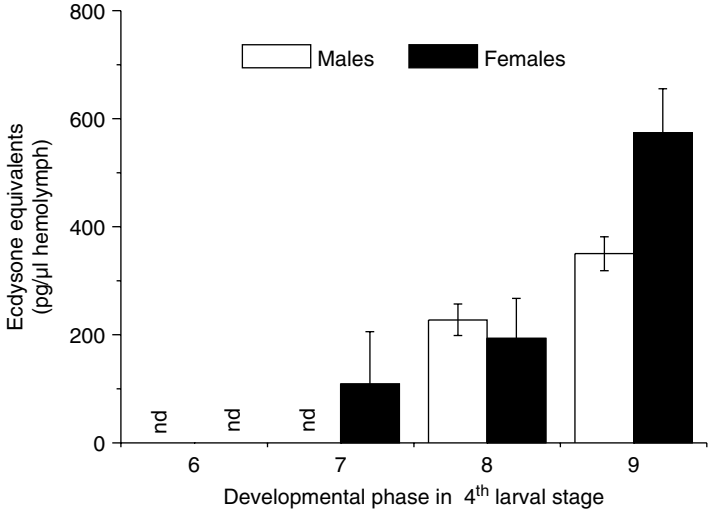

Figure 13.2 RIA-analyzed hemolymph-ecdysteroid concentration in relation to larval developmental phase. Between 1 and 2 µl larval hemolymph, measured precisely ($\pm$ 0.05 µl), were collected, extracted, and RIA-analyzed as described in the text. Columns represent the means of data from 4 to 8 larvae $\pm$ S.E.; n.d., not detectable.

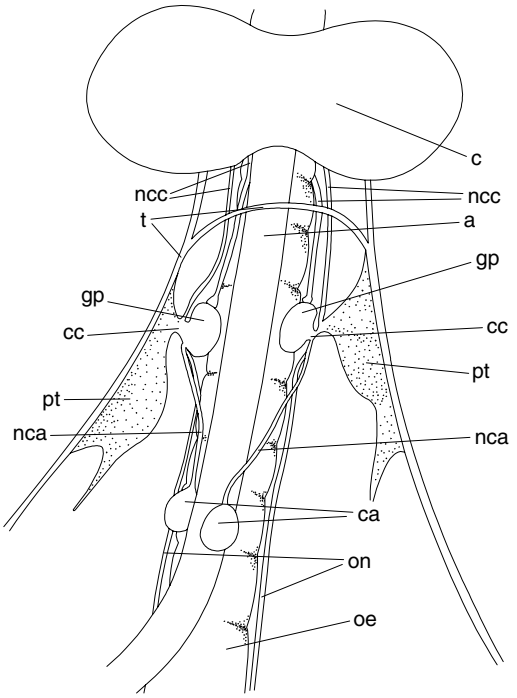

Figure 13.3 Overview of the organization of the retrocerebral glands in *Chironomus* larvae. (Lettering in accordance with Possompès, B., *Arch. Zool. Exp. Gen.*, 89, 201–364, 1953; Credland, P., Scales, D. and Philipps, A., *Zoomorphologie*, 92, 65–75, 1979.) *Key to lettering*: a, aorta; c, brain; ca, corpus allatum; cc, corpus cardiacum; gp, post-cerebral gland; nca, nervus corporis allatum; ncc, nervus corporis cardiacum; oe, oesophagus; on, oesophageal nerve; pt, peritracheal tissue; t, trachea.

further dissection, the first or second abdominal segment is gripped with a pair of fine tweezers, and the larva is carefully elongated. The whole abdomen can now be severed directly behind the last thoracic segment. Usually, the midgut and parts of both salivary glands then emerge from the opened body cavity. The midgut is *carefully* pulled out until the transition to the oesophagus appears, where it is severed. The salivary glands are also pulled out and the salivary ducts are severed. Be careful not to damage the glands; the tissues are very sticky and may cause problems during the following dissection steps. If removal of the glands is difficult in this stage of the dissection, it is better to leave them in place.

To fix the tissue, a second needle is now placed in the ventral part of the opened thorax, so that the dorsal skin of the thoracic segments can be opened by a proximad longitudinal cut up to the recess of the first needle. Be sure not to make the cut too deep, so that only the integument but no deeper tissues are hurt. The fine white structures of the paired pupal respiratory organs may hamper the view inside the thoracic segments. However, they should not be removed, as the main trachea at their bases lies close to the almost invisible peritracheal tissues (the equivalent to prothoracic glands, the source of the ecdysteroids), which it is important not to damage. If possible, the silky-white transversal trachea directly behind the brain should be cleaved to facilitate the opening of the segments. After removal of the head capsule, the opened thoracic segments are carefully flushed with Beadle's ringer using a Pasteur pipette to remove residues of hemolymph. If salivary glands have not been removed yet, they can now easily be accessed to complete the dissection. Tissues are then placed carefully in a sterile Petri dish containing Cannon's medium for an acclimation period of 30–60 min at room temperature. With some practice, a single dissection can be performed in about 5 min.

After acclimation single tissues are transferred to autoclaved 8 × 40-mm sample vials filled with 80 µl Cannon's medium, in which the appropriate amounts of test substance had previously been dissolved. We have always used pure ethanol as a carrier solvent and not exceeded a solvent concentration of 0.01%, but if one wishes, other combinations remain to be tested. The vials are closed and incubated at 30°C in a water bath for 24 h.

Preliminary experiments on *in vitro* ecdysteroidogenesis revealed that Cannon's medium was indeed best suited for chironomid tissue. Attempts to use Beadle's ringer solution, Grace's insect medium, and Medium 199, pure or supplemented with 25 m*M* HEPES, 1.73 m*M* $CaCl_2$, and 1% (w/v) Ficoll 400 (chemicals from Sigma-Aldrich, Taufkirchen, Germany)[21] resulted in low or undetectable ecdysteroid concentrations after 24 h of incubation. This might be due to the relatively high osmolality of the commercial media (around 400 mos*M*/kg). For Cannon's medium, a value of merely 260 mos*M*/kg was found (Table 13.1). This is supported by the findings of Scholz and Zerbst-Boroffka,[22] who measured an osmolality of not more than 180 mos*M*/kg in *C. plumosus* hemolymph.

4. Ecdysteroid extraction

At the end of the incubation period, 320 µl cold methanol are added to each vessel to give a final concentration of 80% (v/v). Vials are then briefly vortexed and the tissues are removed. Samples can now be stored at –20°C or directly processed.

For ecdysteroid extraction, the vials are vortexed and sonicated in an ultrasonic bath for 2 min and then centrifuged at room temperature at 1000 *g* for 5 min to remove any solid tissue residues. The supernatant is transferred to a reaction tube, and the solid residues extracted once with 80% aqueous methanol as above. The supernatants are

Table 13.1 Cannon's modified insect medium,[a] as described in Ringborg and Rydlander[16]

Na_2HPO_4	356	L-Arginine	140	Thiamin–HCl	0.004
$MgCl_2 \times 6\ H_2O$	93.5	DL-Lysine	250	Riboflavin	0.004
KCl	82	L-Histidine	500	Nicotinic acid	0.004
NaCl	420	L-Aspartate	70	Pantothenic acid	0.004
$Na_2SO_4 \times 10H_2O$	2000	L-Asparagic acid	70	Biotin	0.004
$CaCl_2$	42	L-Glutamate	120	Folic acid	0.004
		L-Glutamic acid	120	Inositol	0.004
		Glycine	130	Choline	0.004
Glucose	140	DL-Serine	220		
Fructose	80	L-Alanine	45		
Sucrose	80	L-Proline	70	Cholesterol	6
Trehalose	1000	L-Tyrosine	10	Penicillin	100 U/ml
		DL-Threonine	70	Streptomycin	0.1 mg/ml
		DL-Methionine	180	Phenol red[b]	20
Malic acid	134	L-Phenylalanine	30		
α-Ketoglutaric acid	74	DL-Valine	40	pH	7.2
Succinate	12	DL-Isoleucine	20	Osmolality[c]	260 mos*M*/kg
Fumarate	11	DL-Leucine	30		
		L-Tryptophan	20		
		L-Cystine	5		
		L-Cysteine–HCl	16		

[a] All concentrations as mg/300 ml water (cell culture grade).

[b] Omitted.

[c] Measured.

combined and evaporated to dryness. I have found that, with respect to speed, evaporation at 55°C under a stream of nitrogen is superior to the use of a vacuum centrifuge (Speed-Vac). The dry residues are resuspended in 300 μl RIA buffer (vortex, ultrasonic bath 2 min), and two aliquots of 100 μl are used for RIA analysis. (The volumes given in this chapter have in our hands yielded precise results; however, transferring a method from one laboratory to another often causes weird problems, so method optimization may be necessary in preparation for the RIA.)

5. *Radioimmunoassay*

A RIA for the determination of ecdysteroids was first introduced by Borst and O'Connor,[23] and since then it has been applied and methodically improved by many researchers, too numerous to be reviewed in detail in this volume. For a general treatise on theory and practice of immunoassays, the reader may refer to, e.g., References 24 or 25. In brief, the procedure applied here is as follows: an antiserum, raised against ecdysone or 20-hydroxyecdysone, is added to a solution containing known amount of radioactive-labeled ecdysone and an unknown amount of unlabeled ecdysone. The antiserum will now competitively bind both the labeled and the unlabeled ecdysone in a ratio that reflects the ratio of the ligands' concentration. After incubation the antibodies are precipitated by centrifugation in the presence of 50% ammonium sulfate, and the supernatant is removed. Radioactivity originating from labeled ecdysone bound to the pelleted antibodies can now be determined by liquid scintillation counting. Results are quantified

by a standard curve, in which known amounts of ''cold'' ecdysone are analyzed instead of unknown samples.

Antiserum DBL-1 can be purchased from Trifolio GmbH, Lahnau, Germany. This antiserum was originally produced by the group lead by Prof. J. Koolman in Marburg, Germany, and displays high affinity to ecdysone and its active metabolite 20-hydroxyecdysone. A detailed characterization can be found in References 26 and 27. The freeze-dried material is resuspended in 0.5 ml bidistilled water with 0.05% sodium azide added as a preservative. From this stock a 1:1000 working solution in RIA buffer with 5% (v/v) normal sheep serum (ICN Biomedicals GmbH, Eschwege, Germany) is freshly prepared for every assay. (If, for any reasons, you wish to replace the sheep serum, be sure not to use any bovine protein source in an assay with DBL-1.)

Tritium-labeled ecdysone (α-[22, 23-^{3}H(N)]-ecdysone) was obtained from New England Nuclear, Zaventam, Belgium. Specific activity of the batch used in our laboratory was 53 Ci/mmol. A working solution with an activity of 0.1 μCi/ml was prepared in RIA buffer as follows: from the original package of 50 μCi in a volume of 500 μl ethanol, 25 μl were pipetted to a volume of 25 ml RIA buffer. A volume of 25 μl ($= 0.0025$ μCi) yielded an activity of approximately 2700 counts/min, when 1.5 ml of Wallac HiSafe2 scintillation liquid were added and samples counted in a Wallac 1409 scintillation counter (Wallac, Freiburg, Germany).

Pure ecdysone was obtained from Sigma-Aldrich, Taufkirchen, Germany. An amount of 1 mg ecdysone is weighed into a 10 ml volumetric flask and then dissolved in pure methanol to give a stock solution of 100 μg/ml and stored at −20°C. Because it was difficult to precisely match the desired nominal weight of 1 mg, it was necessary to approximate the weight as near as possible and then to read the exact weight from the balance's display and to use this for further calculations. Stock solutions in our laboratory were between 95 and 106 μg/ml. From this stock, a working solution of (about) 50 pg ecdysone per microliter RIA buffer is prepared by dissolving 50 μl in 100 ml RIA buffer.

Following the scheme given in Table 13.2, an ecdysone standard curve is pipetted into 2-ml reaction tubes (Eppendorf SafeLock). Non-specific binding (NSB) is the amount of labeled ecdysone that unspecifically (i.e., not by antibodies) binds to the protein matrix in the reaction mixture. This should generally not exceed 10% of the maximum bound radioactivity (B_0).[26] There are two ways of preparing NSB samples, either by omitting antiserum so that the mixture will only contain the sheep serum as protein matrix, or by adding a large amount (50× the highest concentration applied in the standard curve) of unlabeled ecdysone, which will readily prevent antibodies from binding significant amounts of labeled ecdysone.[26] Under the assay conditions described here, I have found no qualitative differences between these possibilities, but usually applied the latter one.

Volumes of 100 μl unknown sample, NSBs, or standards, 100 μl radioactive tracer solution (0.1 μCi/ml), and 100 μl of 1:1000 DBL-1 antibody dilution are combined in this order in 2-ml reaction tubes (Eppendorf SafeLock), and after mixing the samples are incubated overnight (>12 h) at 4°C. Incubation is stopped by adding 300 μl ice-cold saturated ammonium sulfate solution to each tube to yield an ammonium sulfate concentration of 50% (tests using polyethylene glycol 6000 instead of ammonium sulfate[24,25] were not successful in this assay). Samples should be mixed well soon after addition of the ammonium sulfate. For all the above steps it is extremely helpful to use a repeater pipette or a suitable multichannel pipette. Samples are then placed on ice for 30 min and centrifuged 12 min at 5000 *g* or more and 4°C to precipitate the protein-bound ligands, both labeled and unlabeled. In the centrifuge, make sure to orientate all tubes in such a way that the position of the almost invisible pellet is still known after removing

Table 13.2 Preparation of a standard curve for quantification of ecdysteroids by radioimmunoassay

Unlabeled ecdysone per well (pg)	Ratio ecdysone standard[a] to RIA buffer (μl), to give a sample volume of 100 μl	Suggested number of replicates
NSB	—[b]	6
Maximum amount of bound radioactivity (B_0)	0.0/100	6
12.5	0.25/99.75	2
25	0.5/99.5	2
50	1/99	2
100	2/98	2
200	4/96	2
400	8/92	2
800	16/84	2
1600	32/68	2
3200	64/36	2
5000	100/0.0	2

[a] 50 pg/μl.

[b] For the preparation of NSB samples, see text.

the tubes (e.g., hinge up). When the centrifuge's capacity is too small for precipitating all samples at one time, temperature control is crucial because centrifugation temperature may rise when several batches of tubes are processed. This could severely affect RIA results.

After centrifugation the supernatants are removed by vacuum aspiration using a Pasteur pipette or a smoothed flat-bottomed injection needle. While doing so, be careful not to injure the pellet. The collected supernatants must then be discarded as radioactive waste. To speed up the procedure, I have found that a washing step with 50% saturated ammonium sulfate solution, as prescribed by Gelman et al.,[28] can be omitted without loss of accuracy when the liquid phase is aspirated twice; after removal of the bulk of liquid allow each tube to stand for 2 min, during which the remainder of the viscous ammonium sulfate solution will collect at the tubes' bottom, then aspirate again.

To the pelleted antigen-antibody complexes 25 μl cell-culture-grade water and a suitable volume of scintillation liquid are added. We have used 1.5 ml of Wallac HiSafe2 scintillation liquid, Wallac, Freiburg, Germany. For other products it may be necessary to optimize the added volume. Though time consuming, each single tube should now be thoroughly vortexed. A white ''flash'' in the tube during vortexing indicates that water and scintillation fluid have formed a homogeneous suspension.

The tubes are placed in a scintillation counter and antibody-bound radioactivity is determined as counts per minute. Studies to determine the optimal counting time have revealed that 2 min are enough to achieve an adequate accuracy under the conditions given here. Mean NSB activity, which should not exceed 10% of the mean B_0 value, is subtracted from all samples and standards. Results are expressed as percent radioactivity bound (B/B_0), and concentration is plotted on a logarithmic scale. A sigmoidal regression model, e.g., of the form

$$f(x) = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} + A_2$$

can be used for analysis, with A_1 and A_2 determining the initial and final y-values, respectively, x_0 determining the concentration where $B/B_0 = 50\%$, and p determining the curve slope at x_0. This regression can be generated with any suitable statistical package for personal computer. Generally, quantifiable results are in a range of B/B_0 between 20% and 70%,[26] which in this assay corresponded to 100–1000 pg ecdysone. Inter-assay stability in terms of x_0 values was found to display a coefficient of variation of 7.6%, and the correlation coefficient of each single standard curve was always better than 99%. Because of the cross-reactivity of the antiserum with 20-hydroxyecdysone and other free ecdysteroids,[27] results should be expressed as picograms ecdysone-equivalents synthesized per hour. For the above procedure, a recovery rate of 99.8 $\pm$ 9.4% ($n = 10$) was determined by extracting known amounts of ecdysone dissolved in Cannon's medium. Duplicate values that differ by more than 10% from their means should be rejected as a criterion of assay validity.

In our laboratory, the above method has proven to provide accurate results with acceptable efforts in experimental time and handling. However, if a higher throughput of samples is planned, the methods described in Reference 28 may provide useful hints for further optimization.

Results and discussion

In a combined experiment, both endpoints were investigated for the effects of the endocrine-disrupting biocide tributyltin oxide.[29] As described above, male and female ecdysteroid production was monitored in tissues exposed to 50, 500, and 5000 ng/l tributyltin oxide (as Sn). In a parallel experiment, single larvae in phases 5 or 6 were exposed to 10, 50, 200, and 1000 ng/l tributyltin oxide, and the development within 48 h was observed. The results are presented in Figures 13.4 and 13.5. These concentrations were in the sublethal range, as under the same test conditions the mean lethal concentration was estimated to be 25 μg/l.[29]

This experiment revealed that mean ecdysteroid-synthetic rates were different between males and females from the control groups (Figure 13.4), which is consistent with earlier findings by Laufer et al.[17] and with the results presented in Figure 13.2. Furthermore, in treated males, the ecdysteroidogenic activity was significantly increased in the 500-ng/l exposure, but in the other two test concentrations no significant differences from the control were found. In females a different effect occurred (Figure 13.4) because in all three groups ecdysteroid production was significantly lowered, by about 50% compared to the control group. In both sexes, therefore, the influence of tributyltin oxide was not dose dependent. A hypothesis focussing particularly on endocrine disruptors has recently raised the possibility that dose–response curves in the endocrine system might sometimes differ from traditional paradigms in toxicology, due to the possible involvement of various mechanisms of action and multiple target sites.[30]

The *in vivo* endpoint, larval development over a 48-h exposure period, also revealed a sex-specific influence of tributyltin oxide (Figure 13.5). In both males and females, the proportions of larvae in distinct developmental phases at the end of the experiment exhibited significantly different patterns, depending on TBTO dose. In general, exposed

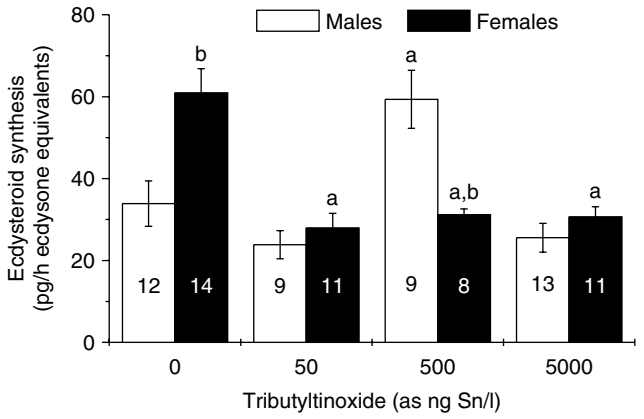

Figure 13.4 *In vitro* ecdysteroid biosynthesis in male and female *C. riparius* larvae exposed to tributyltin oxide. Numbers in columns show number of analyzed animals, error bars indicate $\pm$ S.E.; a, significantly different from the respective control; b, significantly different from the respective male group (Scheffé's test, $p < 0.05$). (From Hahn, T. and Schulz, R., *Environ. Toxicol. Chem.*, 21, 1052–1057, 2002. With permission.)

males tended to develop faster, i.e., within 48 h most of the larvae reached a more advanced developmental phase than in the control. TBTO-treated females, in contrast, clearly showed retarded development relative to the controls. Again a significant difference between the sexes was also observed in the untreated control. This probably reflects protandry, which is typical of chironomidae; that is, male emergence starts before that of females.[11,18] This might enhance mating success in species with short-lived adults.[18] In tributyltin oxide-exposed animals, this natural asynchrony in larval development increased, but whether this is of ecological relevance or not is difficult to decide from these results alone.

However, the results from the *in vitro* and *in vivo* experiments hint at a connection between larval development and molting-hormone production, as in females lowered ecdysteroid synthesis coincided with slower development. In males a partly opposite pattern was present. Exposed male larvae developed faster, which was consistent with the elevated ecdysteroid synthesis *in vitro* found in the 500-ng/l group but not in the groups exposed to 50 and 5,000 ng/l. It cannot be judged from these results whether the effects are connected, either directly or indirectly (or not at all). Several modes of action, either alone or in combination, may be responsible for these effects,[29] and for a clearer elucidation of the mechanism(s), more detailed experiments have to be carried out.

However, rather than to study target–site interactions, it is the aim of this combined *in vitro* and *in vivo* approach to provide a screening tool for the detection of a chemical's endocrine modulating potential by combining two levels of biological organization. In this context, the preparation and incubation of almost complete thoracic segments, containing the intact neural complex of cerebral and subesophageal ganglia, corpora allata, corpora cardiaca, and prothoracic glands, is advantageous for this method. Thus, multiple substance-induced modulations in a variety of neuroendocrine organs and functions within the complex hierarchy of the hormone system can be visualized in only two endpoints, ecdysteroid synthesis and imaginal disc development.

Depending on the size of the midge culture and the number of larvae in a suitable 4th stage larval phase available simultaneously, the *in vivo* tests on imaginal disc

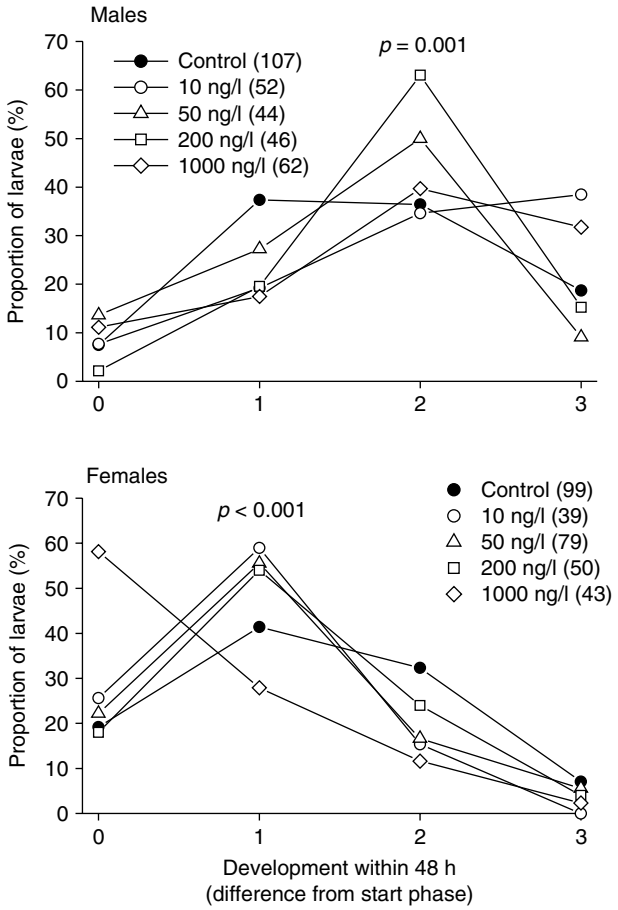

Figure 13.5 Development of fourth larval instar *C. riparius* within a 48-h period of exposure to different concentrations of tributyltin oxide. Numbers in parentheses show number of analyzed animals. *p*-Values indicate statistical significance when distributions of larvae in developmental phases (given as the numerical difference of phase at start and end of the experiment) were different between the experimental groups, due to tributyltin oxide treatment (contingency table tests). (From Hahn, T. and Schulz, R., *Environ. Toxicol. Chem.*, 21, 1052–1057, 2002. With permission.)

development can be performed in relatively short time. Also a sufficient number of *in vitro* ecdysteroid synthesis experiments can be performed in a reasonable time period, although sample extraction and radioimmunoassay are time-consuming steps, and successful dissections require prior training of a researcher's surgical skills.

A disadvantage is certainly the unavoidable remaining subjectivity connected with the microscopic identification of the larvae's developmental stage. Minimizing this subjectivity to ensure reliable and consistent work through several experiments requires a certain degree of practice, which can be acquired only by previous training. If larvae are to be staged by more than one person, all persons involved in this work should harmonize with their definitions of diagnosis of imaginal disc differentiation. This is crucial because the drawings of Wülker and Götz[11] (Figure 13.1) only provide snapshots of a continuous process of development, and the persons involved should agree in staging intermediate

appearances. Therefore, repeatability controls (e.g., staging of the same larvae by different persons) should be carried out at regular intervals.

The combined methods described earlier are focussed on interaction of xenobiotics with ecdysteroid metabolism. However, the *in vitro* part does not include ecdysone receptor interaction of the test substance, which should not be ignored as a further mode of action. For this endpoint, another test for hormonal activity of xenobiotics in insects has recently been applied by Dinan et al.,[31] who were able to detect agonistic and antagonistic effects of a wide array of tested chemicals using an ecdysteroid-responsive cell line in a microplate assay.

Furthermore, the focus of experiments dealing with insect endocrine disruption could be extended to another class of molting hormones, the juvenile hormones. Using the experimental processes described in this chapter as a model, it should be possible to establish a similar method for measurements on *in vitro* juvenile hormone production, e.g., using a radiochemical assay[32] or an antibody-based method.[33]

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chapter fourteen

The electro-olfactogram: An in vivo *measure of peripheral olfactory function and sublethal neurotoxicity in fish*

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Contents

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Introduction

There is an emerging realization that environmental contaminants can have important sublethal impacts on fish and other aquatic organisms.[1] While traditional measures of acute mortality (i.e., lethal concentrations, or LC_{50}s) may be useful for predicting responses to accidental chemical spills or similar scenarios that release unusually high concentrations of a contaminant into fish habitats, most forms of pollution in natural systems do not result in acute fish kills. This is particularly true for stormwater runoff and similar kinds of non-point source pollution. Consequently, a current challenge in the field of aquatic toxicology is to develop and refine sublethal endpoints for fish. Such endpoints are most useful for the purposes of natural resource management if they can ultimately be related to survival, reproduction, or other life history parameters that are important for the dynamics of natural populations.

It is important that experimental measures of sublethal toxicity be sufficiently sensitive to detect changes in physiological function in response to low level, environmentally realistic contaminant exposures. The approach should also be quantitative, technically sound, and reproducible. Finally, measures of physiological function should be matched, to the extent possible, to the known or suspected mechanism of action for the contaminant (e.g., reproductive measures for endocrine disruptors or neurobehavioral measures for neurotoxicants).

Olfaction is an important sensory system that underlies several critical behaviors in fish. For example, olfaction plays a key role in predator detection and avoidance,[2,3] kin recognition,[4] reproduction,[5] and navigation.[6] Consequently, chemicals that damage the olfactory system have the potential to reduce the survival and/or reproductive success of individual animals. The peripheral portion of the olfactory system in fish (reviewed in Reference 7) consists of a pair of olfactory rosettes, each located within a chamber on either side of the rostrum of the fish. A single rosette from a juvenile coho salmon (*Oncorhynchus kisutch*) is shown in Figure 14.1A. The surface of the rosette is covered to a large extent by a sensory epithelium (Figure 14.1B) containing, among other cell types, olfactory receptor neurons (ORNs). These cells are in direct contact with the surrounding environment, and thus they are vulnerable to the neurotoxic effects of dissolved contaminants. The ORNs are responsible for detecting odorant molecules in the surrounding aquatic environment. The odorants bind to membrane-associated receptor proteins in the apical cilia or microvilli of ORNs. Odor binding results in a second messenger cascade that ultimately leads to the propagation of action potentials in the individual ORN axons projecting to the olfactory regions of the brain. The overall process of olfactory signal transduction has been described in considerable detail (reviewed in Reference 8). Some of the principal components are highlighted in Figure 14.1C. Contaminants that interfere with any part of this process could potentially impair peripheral olfactory function and, by extension, olfactory-mediated behaviors crucial for survival or reproduction.

The electro-olfactogram (EOG) is an established technique for measuring peripheral olfactory function in fish[9] and other vertebrates (reviewed in Reference 10). The odor-evoked EOG is an extracellular field potential that consists of a large, negative voltage

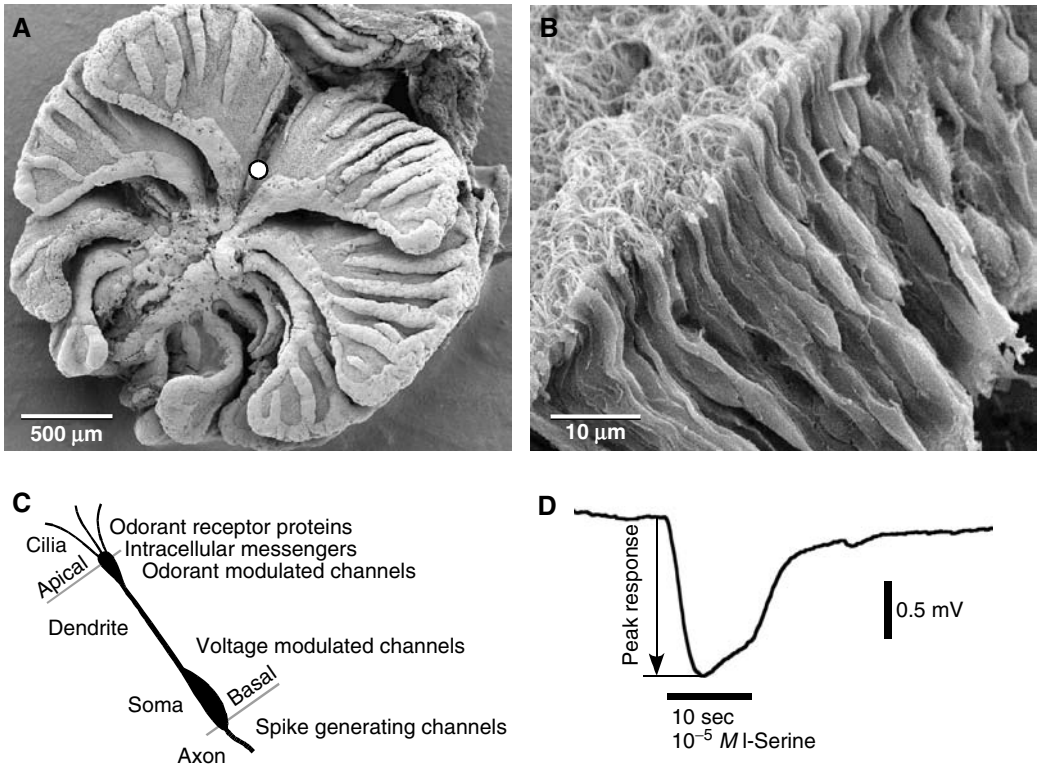

Figure 14.1 Features of the coho salmon peripheral olfactory system. (A) Scanning electron micrograph showing an entire olfactory rosette from a juvenile coho salmon. Each of the lamellae (major folds) is covered in an epithelium that includes regions of sensory neurons. The open circle denotes the location and approximate size of the tip of a standard recording microelectrode. (B) Scanning electron micrograph showing a cross-section of the sensory epithelium along a single lamella. In the upper left is the apical surface containing the cilia and microvilli of the ORNs. The dendrites connect the apical (or ciliated) ends of the ORNs with the somata, which are evident in the center of the epithelium. The axons of the ORNs emerge from the basal surface at the lower right and eventually produce the olfactory nerve (not seen). (C) Schematic of a single ORN showing the major anatomical compartments of the cell (labeled to the left). Major biochemical components of the olfactory transduction process are labeled to the right. Gray lines indicate the apical and basal surfaces of the epithelium. (D) Typical odor-evoked EOG obtained from a juvenile coho salmon. The horizontal bar indicates a 10-s switch from clean background water to water containing 10^{-5} *M* L-serine. The L-serine evoked EOG consists of a negative deflection in the transepithelial voltage potential. This includes a transient peak followed by a decay towards a tonic plateau that persists for the duration of the odor pulse. Scanning electron micrographs are courtesy of Carla Stehr, Northwest Fisheries Science Center.

transient measured with an electrode positioned near the surface of the sensory epithelium (dot in Figure 14.1A). The amplitude of the EOG reflects the summed electrical response of many ORNs as they bind to dissolved odorants.[7] An example of an EOG is shown in Figure 14.1D. The EOG is a robust and direct measure of ORN function in the intact animal. Because of this, the technique has been widely used to investigate the fundamental mechanisms of olfactory signal transduction (reviewed in Reference 10). However, while the EOG was recognized early as a potential tool for monitoring the

effects of contaminant exposure on olfactory function in fish,[9,11] it has been used only infrequently over the past two decades (e.g., References 12–18). This may be due, in part, to a lack of well-described, consistent experimental procedures. Also, neurophysiological recording methods are somewhat complex from a technical standpoint. Advances in technology over the last two decades, however, have reduced much of this complexity and made EOG recordings more tractable.

This chapter provides a detailed and updated description of standard EOG recording methods for fish. Experiments recently performed in our laboratory[17,18] will be used to highlight the application of the technique. This research investigated the effects of dissolved copper on the olfactory function of juvenile coho salmon (*O. kisutch*). Copper is used here only as an example, and the EOG approach should apply equally well to investigations involving other neurotoxicants and other fish species. For toxicology, the overall aim of the EOG recording method is to detect contaminant-induced changes in the responses of primary sensory neurons to odorants that are significant in terms of the behavior and life history of the species of concern.

Materials required

Equipment

- Fiber-optic Illuminator
- Stereoscopic zoom microscope (SMZ645, Nikon Instruments, Melville, NY, USA)
- Boom stand for microscope (SMS6B, Diagnostic Instruments, Sterling Heights, MI, USA)
- Vibration isolation table with magnetic top (Newport, Irvine, CA, USA)
- Air compressor or air cylinder and regulator to pressurize the vibration isolation table

This equipment collectively provides a magnified view of a stable, illuminated surface upon which to position the animal and the micromanipulators.

- Aquarium chiller (PowerCooler 1/5 HP Chiller, CustomSeaLife Inc., San Marcos, CA, USA)
- Aquarium pump (Mag-Drive 7, EG Danner Mfg, Central Islip, NY, USA)
- Water reservoir (a large plastic garbage will work)
- Fish holder (see the following)

This equipment provides the life-support system that maintains the fish during the experiments. The system used in our laboratory consists of a fish holder made from Plexiglas sheets forming a watertight container with a ''V'' cross-section to hold the fish upright. Small pieces of foam alongside the fish help support it within the holder. The inflow at one end of the holder includes a mouthpiece made from a Y-adaptor fitting normally used with Tygon tubing. The aquarium pump recirculates water between the aquarium chiller and the water reservoir to maintain a supply of chilled water. Fish requiring a temperature above ambient will need a heater instead. A PVC T-adaptor and valve divert some of the output of the aquarium pump to the fish holder for the continuous perfusion of the gills with chilled, oxygenated water containing anesthetic via the mouthpiece. For experiments that expose the fish to contaminants while in the holder

(via a separate perfusion of the olfactory chamber described in the following), the outflow of the holder is collected in a separate container and disposed of in a manner appropriate for the contaminant. This requires periodically refilling the water reservoir. In situations where the fish is exposed prior to being placed in the holder, and therefore no exposure will occur in the holder, the outflow can be routed back to the water reservoir for recirculation.

- Perfusion system (ValveLink8 pinch valve, Automate Scientific, San Francisco, CA, USA)

The perfusion system consists of a set of eight pinch valves connected to a micromanifold. A system is also available with 16 valves. Solenoid valves can be used instead, but pinch valves allow the silicone tubing (the part in contact with the solution) to be replaced if desired. This is routinely done between experiments in our laboratory to reduce contamination from previous experiments. Switching between the valves is accomplished manually using controls on the perfusion system or by external signals (e.g., from a computer) connected to the digital inputs of the valve controller.

- Thermoelectric chiller (Z-max prototype kit, Tellurex, Traverse City, MI, USA)
- Aluminum plate (3 × 3 in., 1/2-in. thick)
- Thermistor
- Ohmmeter

This equipment is used to chill the solution as it flows from the micromanifold to the olfactory rosette. For fish requiring a temperature greater than ambient, a thermoelectric device can be configured to heat instead of cool. The compact nature of this system allows it to be placed on the vibration isolation table next to the fish holder, thus reducing the length of the delivery tube and minimizing any warming that may occur before the solution reaches the rosette. Also, with this configuration, the individual solutions upstream of the micromanifold do not have to be chilled. The system constructed in our laboratory includes an aluminum plate with a serpentine groove (several back-and-forth channels connected by hairpin turns) milled into one side. A length of tubing lies within the groove with short lengths of the tubing emerging from two holes in the side of the plate. The entire groove is packed with heat sink grease to maximize thermal conduction from the plate to the tubing. The plate is securely clamped, with a good layer of heat sink grease, to the face of the thermoelectric chiller. The temperature of the solution flowing out of the aluminum plate is measured using an ohmmeter connected to a thermistor sealed into a T-adapter with aquarium sealant. This positions the thermistor in the solution as it flows through the tubing. The thermistor reading (resistance) is used to set the controller of the thermoelectric chiller to maintain the desired solution temperature. If the controller lacks feedback input from the thermistor (as does the unit in our laboratory), the thermoelectric chiller needs to be turned off when there is no flow through the perfusion system. Otherwise, the thermoelectric device will continue chilling and may ultimately freeze the solution within the aluminum plate.

- Electrode puller (P-30, Sutter Instrument, Novato, CA, USA)
- Microelectrode holders (MEH1S, WPI, Sarasota, FL, USA) mounted to Plexiglas rods
- Micromanipulators (MM-3, Narashige International, East Meadow, NY, USA)

- Magnetic bases for micromanipulators (M9, WPI, Sarasota, FL, USA)
- AC/DC differential amplifier (Model 3000, A-M Systems, Carlsborg, WA, USA)
- Oscilloscope (TDS224, Tektronics, Wilsonville, OR)

This is standard electrophysiological equipment for making and using microelectrodes. The large diameter (low impedance) of the microelectrode tips that are used for recording EOGs (see the section ''Procedures'') obviates the need for a headstage pre-amplifier sometimes used in electrophysiology.

- Computer (desktop with a PCI slot, PowerMac G4, Apple Computer, Cupertino, CA, USA)
- Data acquisition hardware (6035E, National Instruments, Austin, TX, USA)
- Data acquisition software (LabVIEW, National Instruments, Austin, TX, USA)
- Data analysis and graphing software (KaleidaGraph, Synergy Software, Reading, PA, USA)

The computerized data acquisition system serves to both control the perfusion system and to record the odor-evoked EOG. This requires hardware with at least one analog input and eight digital outputs and software that can simultaneously collect analog input and change digital outputs. This can be accomplished with a variety of hardware/software combinations that are commercially available. The system used in our laboratory (listed earlier) includes a custom LabVIEW program (available upon request from the authors) that allows for data collection to be largely automated (see the section ''Results and discussion''). Data analysis can also be performed using a variety of software programs that are commercially available. Our laboratory uses a second custom LabVIEW program (also available upon request from the authors) to measure the waveform and amplitude of odor-evoked EOGs. Conventional commercial software is used for graphics, statistics, and other standard analyses.

Reagents

- Agar (Sigma, St. Louis, MO, USA)
- MS-222 stock solution

5 g/l MS-222 (tricaine methane sulfonate, Sigma, St. Louis, MO, USA) and 1 g/l sodium carbonate (as a pH buffer) in distilled water

- Physiological saline (for freshwater-phase coho salmon)

140 m*M* NaCl, 10 m*M* KCl, 1.8 m*M* $CaCl_2$, 2.0 m*M* $MgCl_2$, and 5 m*M* HEPES in distilled water (pH to 7.4 with NaOH)

- Gallamine triethiodide solution

300 mg/l gallamine triethiodide (Sigma, St. Louis, MO, USA) in physiological saline

- Microelectrode saline

140 m*M* NaCl, 10 m*M* KCl, 1.8 m*M* $CaCl_2$, and 2.0 m*M* $MgCl_2$ in distilled water

- Microelectrode holder filling solution

3 *M* KCl in distilled water

- Odorant stock solutions

Prepare as appropriate for the desired odorants (including a carrier if one is needed). For the experiments shown here, the solutions consisted of taurocholic acid (TCA, 10^{-2} *M*), L-serine (10^{-2} *M*), and an amino acid mixture (L-arginine, L-aspartic acid, L-leucine, and L-serine each at 10^{-2} *M*) all in distilled water.

- Contaminant stock solution

Prepare as appropriate for the contaminant being studied. For the data shown here, the solution consisted of 3.4 g/l copper chloride (Sigma, St. Louis, MO, USA) in distilled water (pH to 3.0 with HCl).

Supplies

- Microelectrode glass (borosilicate 1 mm OD/0.75 mm ID w/filament, TW100F-3, WPI, Sarasota, FL, USA)
- Metal ground electrode (a hypodermic needle connected to ground will work)
- Syringes (having a variety of sizes is useful)
- Hypodermic needles (having a variety of sizes is useful)
- Microfil syringe tip (MF34G-5, WPI, Sarasota, FL, USA) for filling microelectrode holders
- Dissecting tools, including scalpels, small scissors, and small forceps
- PVC piping and valves
- Tygon tubing and fittings
- Silicone tubing
- Electrical wiring and connectors

Procedures

Fish

The data presented in this chapter are from experiments on juvenile coho salmon reared at the Northwest Fisheries Science Center in 2400-l fiberglass tanks supplied by a filtered, recirculating water system (11–13°C, pH 7.1, buffered to 120 ppm total hardness as $CaCO_3$). Fish were fed commercial salmon pellets (Bio-Oregon, Warrenton, OR, USA). Whatever the method of animal husbandry and handling it is important to avoid introducing any chemicals into the tanks housing the fish that might impair the olfactory system. This might include, for example, contaminants in the source water for the hatchery or metals originating from certain kinds of plumbing. Also, fish rearing facilities occasionally use algicides or fungicides to control the growth of algae in the aquaculture system or fungi on the fish. While these chemicals may not have any discernible effects on the overt behavior of fish in the rearing system, they may nevertheless impair the olfactory system. This may lead to uncontrolled variation in EOG recordings among all

fish in a given study. Avoiding this requires knowing, and perhaps modifying, the protocols used for animal husbandry.

While the procedures in the following are based on specific studies by our laboratory and others,[9,12,17] they can be adapted to a range of fish species, sizes, and ages. Different sized fish might require changes to the dose and duration of anesthesia, physical modifications of the fish holder and tubing, and changes to the temperature and/or flow rate over the gill and rosette. For the experiments presented here, fish ($n = 77$) were age 1+ with an average size ($\pm$ 1 SD) of 22.7 $\pm$ 0.4 cm and 143 $\pm$ 8 g.

Solutions

Prepare concentrated stock solutions (odorants and contaminant) each week in the appropriate carrier (e.g., distilled water for amino acids) and store appropriately. Prepare odorant stimulus solutions daily by diluting the stock into the background water (filtered, dechlorinated municipal water for the experiments shown in this chapter). Prepare contaminant exposure solutions as needed based on the experimental protocol. For the examples presented in this chapter, copper solutions were made daily by diluting copper chloride stock into background water. The selection of olfactory stimuli (odorants) will depend on the fish species of interest. Ideally, they should be naturally occurring olfactory cues with relevance to olfactory-mediated behaviors. In the case of salmon, the rationale for selecting four amino acids and TCA as odorants will be discussed.

Exposure prior to electrophysiological recording

Odor-evoked EOG recordings are typically stable for several hours.[17] Exposures lasting longer than this should be conducted separately, before preparing the fish for electrophysiological recording. Since EOG responses are recorded from fish after the exposure, the exposure protocol can vary (e.g., flow-through versus static renewal) without requiring changes to the EOG procedure itself.

Microelectrode preparation

Microelectrodes should be prepared each day. Adjust the electrode puller to make glass microelectrodes with gentle tapers to ~100-μm diameter tips. An easy approach is to pull electrodes with relatively fine tips and manually break the tips under a dissecting microscope to the desired diameter. Gently heat a 2% agar-saline solution (0.1 g agar in 5 ml of electrode saline) until the agar dissolves completely. Remove from the heat and load the solution into a small syringe connected to narrow diameter Tygon tubing that snugly fits over the microelectrode glass. The agar solution will begin to cool, so this needs to be done quickly. Using the syringe/tubing, carefully fill the microelectrodes from the rear (non-tip) end until solution comes out of the tip. Make sure that there are no gaps or air bubbles in the electrode. Gently remove excess agar from the tip of the microelectrode. Fill several microelectrodes in the approximately 5 min before the solution cools. Microelectrodes can be stored for use later in the day.

Microelectrodes are fitted to holders and then connected to the electronics prior to use in recording. Fill a microelectrode holder with 3 *M* KCl using a microfil needle attached to

a syringe. Gently insert a microelectrode halfway into a holder and tighten the screw to seal the o-ring. Make sure that there are no air bubbles trapped in the holder. Mount a second microelectrode in the same way. Attach each electrode holder to a micromanipulator and temporarily secure the micromanipulators via their magnetic bases on the vibration isolation table away from the fish holder. If desired, the electrical properties of the microelectrodes can be easily tested at this point by inserting them both into a beaker of physiological saline. The method for microelectrode testing is not described here, since it will be specific to the amplifier and should be described in the associated manual.

Fish preparation

A schematic diagram and photograph of a coho salmon with the perfusion and electrophysiological recording systems are shown in Figure 14.2. Anesthetize a fish with MS-222 (10 ml of MS-222 stock per liter of water, 50 mg/l final concentration) for approximately 20 min. The fish should be unresponsive yet still show some opercular movement. Inject the paralytic gallamine triethiodide (1 ml of paralytic solution per kg body mass, 0.3 mg/kg final concentration) into several locations in the muscles along the side of the fish. The anesthesia and paralytic are necessary to prevent movement during recordings. We tested several other procedures for anesthetizing fish, including chilling, and observed no differences in terms of the odor-evoked EOGs. Thus, we use MS-222 as a standard anesthetic. Also, any effect of the anesthetic/paralytic will occur in both treatment and control fish.

Place the fish into a Plexiglas holder on the vibration isolation table with a mouthpiece (an appropriately sized Y-adaptor for the Tygon tubing works) inserted into the

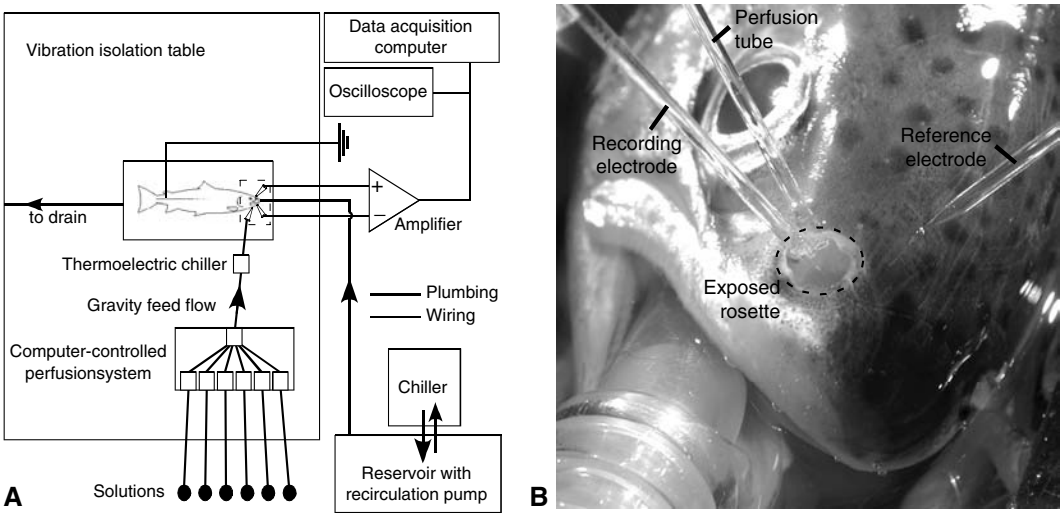

Figure 14.2 Electophysiological recording system used to measure odor-evoked EOGs from the sensory epithelium of juvenile coho salmon. (A) Schematic diagram showing the major components of the apparatus. The dashed box denotes the area shown in more detail in (B). (B) Photograph showing the rostrum of a coho salmon, the mouthpiece providing water to the gills, and the positioning of the perfusion tube and glass microelectrodes. See the sections ''Materials required'' and ''Procedures'' for more information. Figure adapted from Reference 17.

mouth, just anterior to the gill arches. Deliver chilled (12°C), oxygenated water containing MS-222 (50 mg/l) through the mouthpiece at a flow of 120 ml/min. To improve water flow across the gills, clip a piece of each operculum and hold the remaining portion open with a piece of foam. Place a wet paper towel over the body of the fish (and in contact with the water) to keep it moist. For larger fish, add ice to keep animals cool.

Remove the skin overlying the olfactory chamber to expose the olfactory rosette. Care must be taken to minimize bleeding, particularly from the blood vessels near the rosette. Excessive blood loss will deprive the rosette of circulation and render the olfactory epithelium essentially unresponsive to odorant stimulation. In coho salmon, this is readily apparent as a change in the color of the rosette from pink to pale white.

Electrophysiological recording

With the aid of a stereomicroscope, position the recording microelectrode along the midline of the rosette at the base of the large, posterior-most lamella (see Figure 14.1A) using a micromanipulator. Place the reference microelectrode in the skin of the rostrum, dorsal to the rosette (see Figure 14.2B). The two microelectrodes and the fish need to remain stable to avoid movement artifacts in the recording. Insert a separate grounded metal electrode (a hypodermic needle will work) into the muscle near the tail. Using a differential amplifier, amplify (500×) and filter (100 Hz low pass) the differential signal from the two microelectrodes (voltage) and display the signal on an oscilloscope. The output of the amplifier should also be connected to the computerized data acquisition system for recording.

Direct delivery of odorants and contaminants to the olfactory epithelium

During recordings, deliver a continuous flow of chilled water (12°C) to the exposed rosette at a rate of 5 ml/min through a perfusion tube that terminates in the olfactory chamber. Since gravity drives the perfusion flow, the length and diameter of the tubing and height of the storage syringes relative to the table will determine (and limit) the rate. A microelectrode glass mounted to a micromanipulator can be used as the terminal end of the perfusion tube (Figure 14.2B). Fill the individual storage syringes of the perfusion system with the desired solutions (e.g., water only, water plus odorant, water plus contaminant, or water plus odorant and contaminant). Opening a pinch valve of the perfusion system allows the selected solution to flow (via gravity feed) to the rosette. One line to the manifold should be dedicated to background water alone. The remaining lines can vary depending on the experimental design. The pinch valves allow for automated, rapid (40 ms) switching between the different solutions. Pass the single output from the manifold through a thermoelectric chiller (see the section ''Materials required'') to chill the solution (12°C) just prior to delivery to the rosette. Using this approach, a perfusion source can be selected from as many as eight different solutions (one line for background water and seven lines for odorant or contaminant solutions) by setting the appropriate digital output on the computerized data acquisition system. If necessary, a line can be changed during a recording to deliver a different solution as long as the line is carefully flushed of the previous solution. Use the data acquisition system to record the electrical response from the rosette, while simultaneously switching the perfusion system to produce odor pulses (see the following).

Testing procedure

Following the placement of electrodes, allow the fish to acclimate for approximately 15 min before testing. EOG responses are elicited by briefly switching from background water to an odorant-containing solution. Blank (or control) responses are obtained by switching between background water delivered through the dedicated line and background water from a line normally used for odorant-containing solutions. Blank pulses should be delivered routinely to test for any responses that may reflect mechanical switching artifacts or residual odorants in the lines or micromanifold.

In our laboratory, a computerized system allows for EOGs to be evoked by multiple odor pulses that can be easily varied in terms of stimulus solution, pulse duration, or pulse interval. At the onset of any new experiment, typical responses to a standard set of odor pulses should be determined. This is best done empirically, since species, size, flow rate, and other experimental parameters can influence odor-evoked responses from the olfactory epithelium of fish (see Figures 14.4–14.6). Once the optimal conditions for delivering stimuli have been determined, the computerized system can reproducibly present the odor pulses to multiple fish within and between exposure groups. For brief exposures (one to a few hours), measure baseline responses to the odor pulses, and then change the solution perfusing the rosette to a separate line containing the contaminant. Wait for the duration of the exposure, switch back to clean background water, and then obtain a second set of responses to the same set of olfactory stimuli (Figure 14.3). For experiments in which fish are exposed for longer durations, it is typically not possible to compare pre-exposure versus post-exposure EOG responses of individual animals. In these situations, only post-exposure EOG responses can be collected. These data are then compared to the responses obtained from control (unexposed) fish.[18]

Data analysis

Quantify the EOG responses by measuring the peak negative amplitude relative to the pre-odorant baseline (Figure 14.1D). Since the EOG represents the summed activity of the ORNs,[7,10] the peak amplitude is presumably proportional to the electrical impulses (action potentials) that are transmitted to the olfactory forebrain via the olfactory nerve. EOG responses to an odor pulse can be reported in absolute terms (mV) or as a relative response. Relative responses may be intra-animal (pre-contaminant versus post-contaminant exposures) or inter-animal (exposed versus unexposed treatment groups). EOG responses should not be expressed relative to responses to a standard odor pulse (e.g., 10^{-5} *M* L-serine), since the responses to the standard pulse may also change with contaminant exposure. It is relatively straightforward to collect contaminant dose–response data by monitoring the EOG responses of fish in different treatment groups. Several methods exist for determining effects thresholds from these types of data. As an example, a regression-based approach is presented in the section ''Results and discussion''.

Determining an appropriate pulse duration

For sufficiently long pulse durations, a typical EOG consists of a negative phasic peak that decays slowly to a tonic plateau (see Figures 14.1D and 14.4A). If the pulse duration is too short, there will be insufficient time for the phasic peak to develop, and the true peak

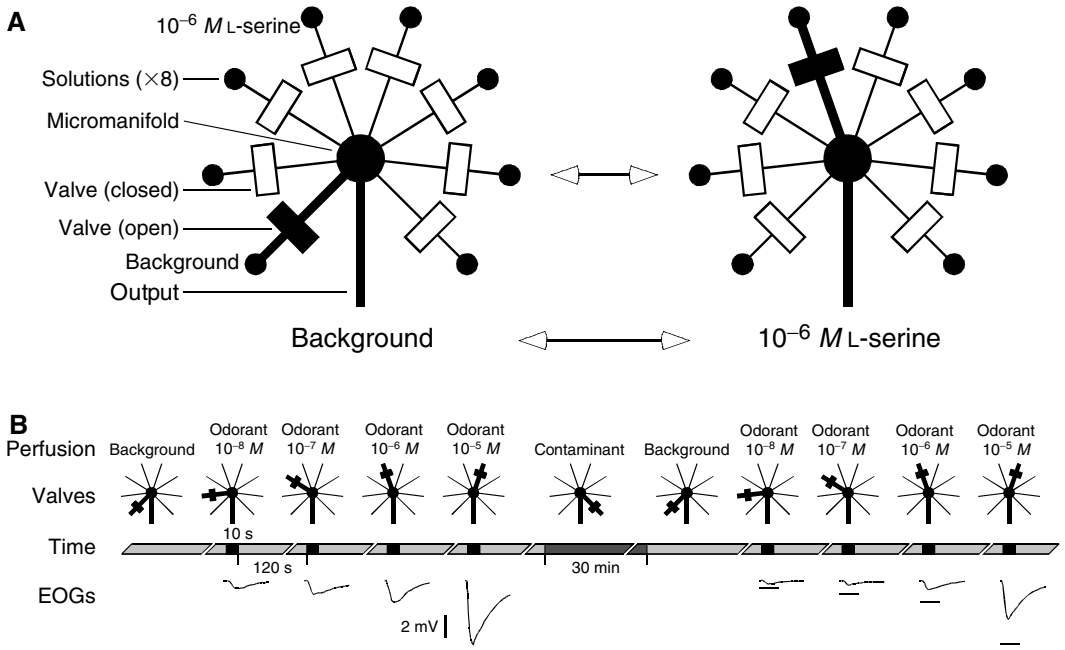

Figure 14.3 Schematic diagrams illustrating the perfusion apparatus and an example of a solution delivery sequence used to expose the olfactory epithelium to a contaminant and to record odor-evoked EOGs pre-exposure and post-exposure. (A) A schematic representation of the perfusion system showing the switch between solutions. Each solution container (a large syringe or glass bottle) is connected to a single micromanifold with a silicone tube that passes through a pinch valve. Switching from one open pinch valve to another changes the solution flowing out of the manifold to the olfactory rosette. As an example, a switch from the background solution to a solution containing background plus 10^{-6} *M* L-serine is illustrated. The double arrow denotes a switch back to background water, which terminates the odor pulse. (B) A schematic representation of the delivery of multiple odor pulses and a dissolved contaminant. Although four separate odor pulses are shown here as an example, the number and combination of odor pulses may vary for different experiments. In the present example, odor pulses are 10 s long (black bars) and separated by 120-s washes with background water (light gray bars). Each stimulus is represented by a different valve and line to the manifold. Here, a single odorant is presented in an ascending order of four concentrations. Diagonal white gaps denote breaks in the time bar. The four pulses are used to gauge the pre-exposure response, and then the perfusion is switched to a line containing a dissolved contaminant (note the different valve). After a brief exposure period (30 min), the perfusion sequence for the four odor pulses is repeated to determine the post-exposure olfactory response. The EOGs shown are responses to a mixture of four amino acids (see the section ''Materials required'') before and after a 30-min exposure to 10 μg/l copper (see the section ''Results and discussion''). The horizontal bars below each post-exposure EOG shows the peak amplitude of the corresponding pre-exposure EOG for that odorant.

amplitude of the olfactory response to the odorant will not be recorded. The kinetics of the phasic peak will depend on the properties of the delivery system (e.g., flow rate, diffusion at the leading edge) and on the neurophysiological properties of the responsive ORNs. Figure 14.4A shows EOG recordings from a single fish using various pulse durations. Pooled data from several juvenile salmon (Figure 14.4B) empirically show that pulse durations of 10 s are sufficient to reach maximum amplitude given the fish and stimulus delivery apparatus used during these experiments. Shorter pulses produce smaller peaks

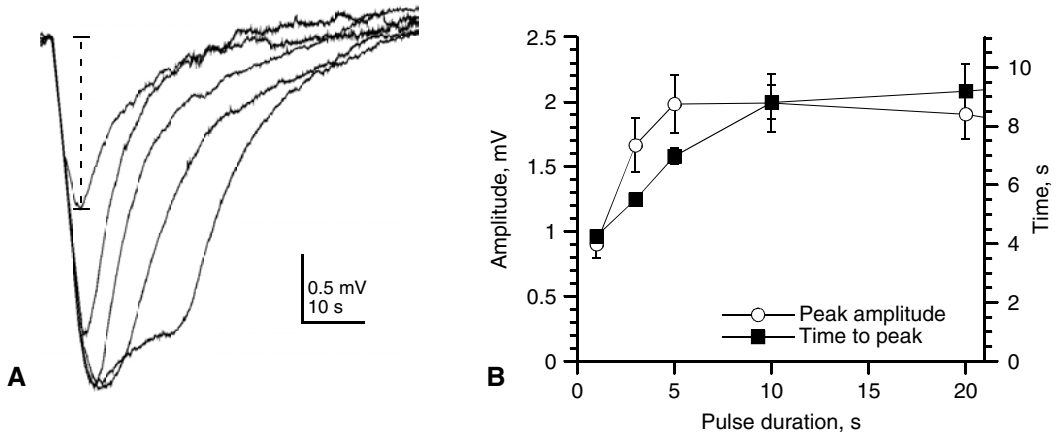

Figure 14.4 Varying the duration of odorant pulses changes the waveform and amplitude of evoked EOGs. (A) Superimposed EOG responses to 10^{-5} *M* L-serine pulses delivered with durations of 1, 3, 5, 10, and 20 s (obtained from the same fish). The dashed vertical line indicates the peak amplitude (as measured from the baseline) for the EOG evoked by a 1-s pulse. (B) Double *Y*-axis plot showing the effect of pulse duration on peak amplitude and time to peak (mean $\pm$ 1 standard error, $n = 4$ fish). Time to peak was measured from the initial deflection from the baseline to the peak of the EOG. Figure adapted from Reference 17.

and/or reduce the time to peak. Longer pulse durations do not increase peak amplitude, but they do increase the duration of ORN activity and thus the potential for sensory adaptation of the olfactory responses.

Determining an appropriate pulse interval

If two successive odor pulses are presented too close together, the amplitude of the second pulse will be less than the first (even if the stimulus pulses are the same). This is a form of sensory adaptation, and it limits the minimum rate at which a set of odor pulses should be presented. Figure 14.5A shows the consequences of varying the interval between two pulses (10^{-5} *M* L-serine) on the EOG responses of a single fish. When odor pulses are presented in sequence, the amplitude of the second pulse increases with the duration of the interpulse interval. The pooled data from several fish (Figure 14.5B) shows that the responses of the olfactory epithelium to identical odorant pulses are equivalent if the pulses are separated by at least 120 s. Therefore, in this specific example, individual odor pulses should be separated by at least a 2-min interval.

Selecting odorants and concentrations

Fish use distinct ORNs to detect different classes of odor molecules that vary in terms of their molecular structure.[7] These include bile salts (e.g., TCA), amino acids (e.g., L-serine), and steroid hormones (e.g., prostaglandins). For example, amino acids have been shown to activate at least four non-overlapping populations of ORNs.[19,20] The ability of specific odorants to evoke EOGs can be relatively species specific (e.g., the prostaglandins[21]) or generalized across species (e.g., bile salts and amino acids[7]). Since individual contaminants could potentially have different effects on different populations of ORNs, EOG-based

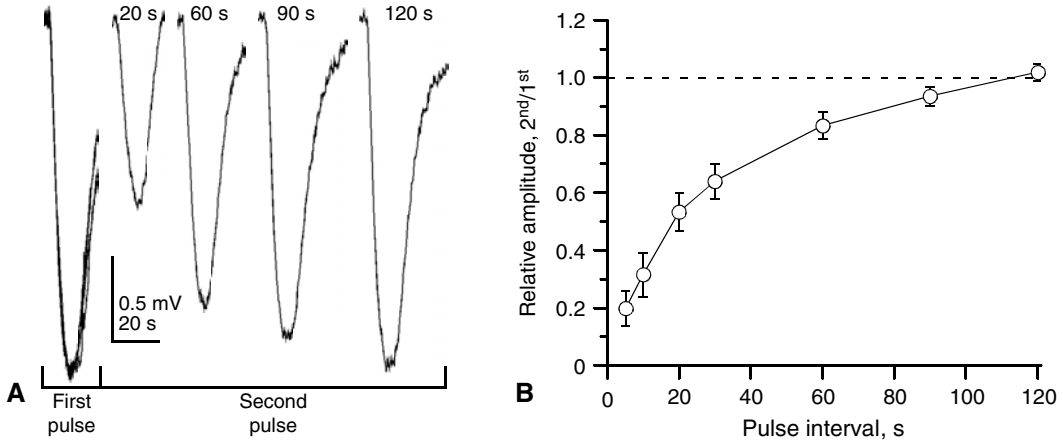

Figure 14.5 Varying the relative timing of two odor pulses changes the amplitude of the evoked EOG for the second pulse. (A) Traces showing pairs of 10^{-5} *M* L-serine pulses (each 10-s long) presented with inter-pulse intervals of 20, 60, 90, and 120 s (obtained from a single fish). For clarity, the intervening data have been deleted and only the evoked EOGs are shown. The first pulses (four total) are superimposed and the second pulses have been arbitrarily positioned horizontally and aligned vertically by their initial downward deflections. (B) Pooled data (mean $\pm$ 1 standard error, $n = 4$ fish) showing the effect of pulse interval on the amplitude of the second pulse relative to the first pulse. Figure adapted from Reference 17.

experiments should, to the extent possible, use multiple and dissimilar odorants as olfactory stimuli. The bile salt TCA and four amino acids serve as examples here since they are known to stimulate distinct olfactory pathways,[19,20] and they do not appear to be species specific.[7] Ultimately, of course, the choice of odorants will depend, in part, on the species being studied and on the olfactory-mediated behaviors of interest.

Odorants can be presented individually or as mixtures. Using odor pulses with a single odorant will only measure the effect of contaminant exposure on the population of ORNs sensitive to that odorant. Using mixtures of odorants will stimulate multiple populations of ORNs and mean that any effect of contaminant exposure on the EOG represents the combined effect on multiple populations of ORNs. Whether the contaminant exposure had an effect on any specific populations of ORNs cannot be discerned. In fact, a population of ORNs stimulated by the mixture may have been unaffected by the exposure. However, since the EOG response represents the summed activity of the multiple independent pathways, the mixture will produce relatively large EOG responses while limiting the amount of stimulation to any one population of ORNs, thus reducing the chance of saturation or adaptation. This makes the EOG responses easier to resolve relative to background noise and blank responses while at the same time reducing the chance of saturation or adaptation of the ORNs.

In addition to selecting odorants, consideration should be given to the concentrations at which the odorants are presented to the sensory epithelium. Again, initial range finding experiments are recommended here as there may be differences in olfactory sensitivity due to differences in species, age, etc. Figure 14.6A shows EOG responses to several concentrations of a single odorant (L-serine). Figure 14.6B shows a range of concentrations of several odorants producing EOG responses above the noise in the measurements of amplitude and the responses to blank pulses, but below concentrations that saturate the response. Using concentrations in this range will produce robust measurements and

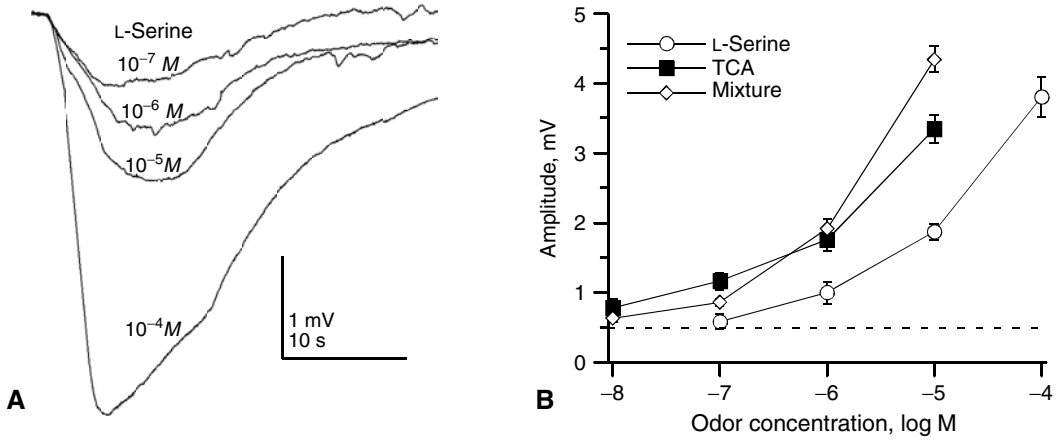

Figure 14.6 Varying the concentration of odor pulses changes the amplitude of evoked EOGs. (A) The amplitudes of EOGs evoked in response to 10 s L-serine pulses increase with increasing odorant concentration (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} *M*). EOGs from a single fish have been superimposed. (B) Dose–response data (mean $\pm$ 1 standard error, $n = 6$ fish) for three odorants (L-serine, TCA, and the amino acid mixture). The dotted line shows the mean response to a blank odor pulse (background water; $n = 4$ fish). Figure adapted from Reference 17.

reduce the likelihood of changes in the sensory physiology of the ORNs, such as adaptation. For the amino acids, these concentrations may also reflect actual levels seen in the natural environment.[22] In summary, the effect of contaminant exposure on the olfactory response should ideally be evaluated for multiple odorants over a range of stimulus concentrations. The use of a multi-line perfusion system, such as the one described earlier, makes these types of experiments more tractable.

Determining recording stability

Preliminary experiments should be performed to test the stability of the EOG response over time. Experimental recordings may last for a few hours if they involve a pre-exposure series of odor pulses followed by a delivery of contaminant, and then a post-exposure odorant presentation. Changes in the condition of the fish (rundown) or the properties of the microelectrodes may lead to non-specific changes in EOG amplitude. Prior to the experiments presented here, preliminary recordings were performed to show that EOG responses to repeated pulses of 10^{-5} *M* L-serine do not significantly change over a 2-h recording interval (linear regression, $n = 6$, data not shown). This stable recording interval is typically sufficient to conduct most kinds of experiments.

Sources of artifacts

Several sources of artifacts can cause non-olfactory signals to be recorded. These include a high level of electrical noise, or an unstable recording baseline.[10] Electrical artifacts may also originate from the operation of the valves. These will appear as transient spikes coinciding with the opening/closing of the valves. Improving the electrical isolation between the wiring to the valve controller and the wiring from the amplifier can reduce these artifacts. However, they usually do not interfere with the measurement of EOGs.

Any movement of the rosette relative to the recording microelectrode will cause a change in the baseline. This can occur due to movement of the fish or microelectrode. It can also occur due to changes in the perfusion flow rate that will arise if there are differences in the heights of the solutions in the supply syringes. Problems with the microelectrodes, such as blockage with tissue or an air bubble, will lead to increased noise in the recordings. Make extra microelectrodes, so that a faulty microelectrode can be changed if needed. Finally, there will almost always be a slight cross-contamination between the lines and micromanifold when multiple solutions are used. To gauge the magnitude of this artifact, periodically conduct tests with blank stimulus solutions. A small response is likely even when switching between two lines that each contain source water.

Results and discussion

Copper exposure inhibits EOG responses

Dissolved copper is a common non-point source pollutant in fish habitats. Recent measurements of the effect of copper on the odor-evoked EOGs in coho salmon[17,18] will be used as an example of EOG recordings from contaminant-exposed fish. Figure 14.7 shows EOG responses obtained from a single fish before and after a 30-min perfusion of 10 μg/l copper chloride over the rosette. Seven odor pulses of 10-s duration were presented at 2-min intervals in the following sequence: 10^{-5} *M* L-serine, 10^{-6} *M* TCA, 10^{-8} *M* amino acid mixture, 10^{-7} *M* mixture, 10^{-6} *M* mixture, 10^{-5} *M* mixture, and 10^{-5} *M* L-serine. The perfusion was then changed to the copper-containing solution for 30 min. After the exposure interval, the perfusion was changed back to background water, and the series of seven odor pulses was repeated. A schematic illustration of the solution delivery is shown in Figure 14.3B. For each set of odor pulses, only the first L-serine odor pulse is shown in Figure 14.7. For data analysis, the data (post/pre-exposure ratios) from the two responses were averaged. Whereas the effect of copper on the responses to L-serine and TCA are point estimates (i.e., inhibition of an olfactory response to an individual odorant at a single concentration), the EOGs evoked by the amino acid mixture captures the effects of copper on multiple receptor populations over three log units of stimulus intensity. The effect of copper was quantified by averaging the reductions measured for the four mixture concentrations. For the fish shown in Figure 14.7, the 30-min exposure to 10 μg/l copper reduced the L-serine response by 57%, the TCA response by 67%, and the response to the amino acid mixture by 35% (all relative to the pre-exposure EOG amplitudes for the same fish). EOGs recorded from fish exposed to copper for 96 h show reduced EOGs similar to those seen in Figure 14.7.[18]

For brief exposure durations, such as the example above, the fish can serve as both the treatment and a control. Comparing post-exposure EOG responses to pre-exposure responses in the same fish provides some control over the biological variability between fish in the recordings. A separate control group exposed to uncontaminated water rather than copper should still be tested. Recordings from this unexposed treatment group represent an additional control for any effects of time and/or artifacts in the apparatus.

Estimating thresholds for sublethal copper neurotoxicity

The EOG recording method is particularly useful for determining sublethal thresholds for copper-induced neurotoxicity. This is true for fish exposed to copper for relatively short

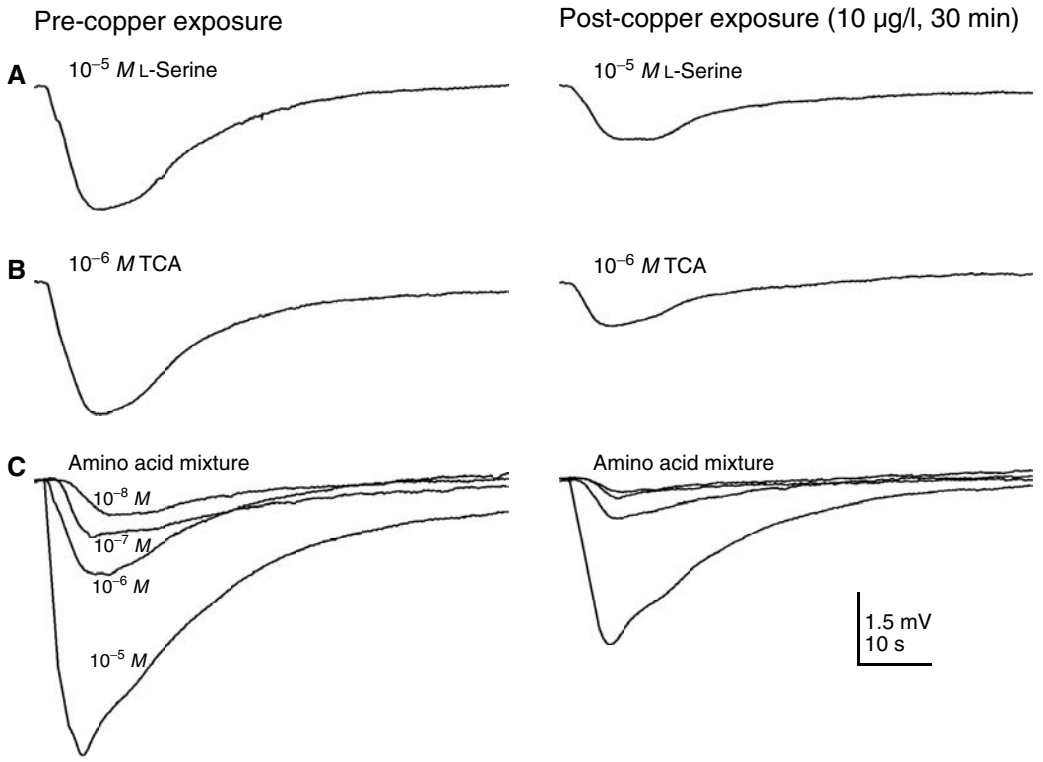

Figure 14.7 Short-term copper exposure diminishes the responsiveness of the olfactory epithelium to natural odorants. EOGs obtained from the same fish before and after copper exposure are shown. A 30-min exposure to 10 µg/l copper reduced the EOG evoked by 10^{-5} *M* L-serine by 57% (A) and the response to 10^{-6} *M* TCA by 67% (B). Similarly, the EOG responses to all four concentrations of the amino acid mixture (C) were reduced. The average reduction in the responses to the mixtures was 35%. Not shown are EOG responses to blank pulses, which were also reduced, and by a similar percentage. Figure adapted from Reference 17.

intervals (30 min) while on the recording apparatus (as shown here and in Reference 17), and also for animals exposed for longer intervals (i.e., days) in separate tanks (e.g., Reference 18). For the short-term experiment shown here, where copper was delivered directly to the olfactory chamber for 30 min, EOG responses were collected from six different exposure groups (see the section ''Procedures'' and Figure 14.7). These consisted of an unexposed (control) group and fish exposed to copper at nominal concentrations of 1, 2, 5, 10, and 20 µg/l ($n = 6$ fish per group). During the exposure interval for the control group, the perfusion was switched from the dedicated background water line to the line normally used to deliver copper-containing solutions, but this time used to deliver background water instead. This was done, in part, to monitor for any residual copper contamination in the delivery line.

For the purposes of demonstration, only data from the EOGs evoked by L-serine are shown here. Notably, the inhibitory effects of copper on the olfactory responses to other odorants are similar to those for L-serine.[17,18] The inhibitory dose–response relationship for the effect of short-term (30 min) copper exposure on the EOG is shown in Figure 14.8. Notably, there was a slight reduction in EOG responses to the odorants (expressed as the post-exposure/pre-exposure ratio) for the control fish

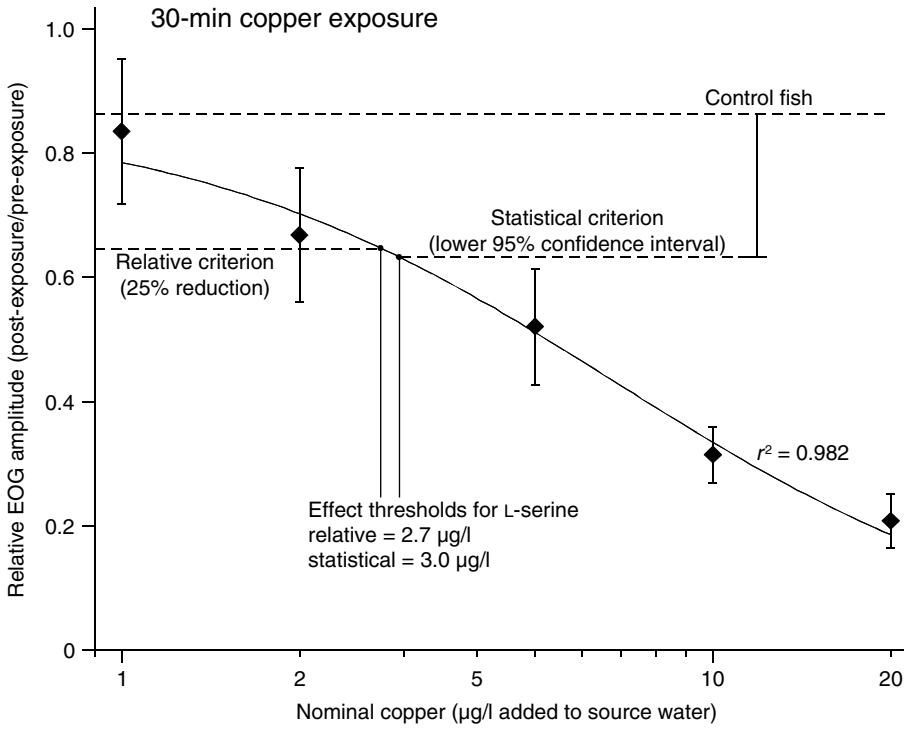

Figure 14.8 Threshold determination for copper-induced neurotoxicity to the fish olfactory epithelium. Data were obtained from six treatment groups (control and five copper exposures; $n = 6$ fish per group) exposed for 30 min. For each exposure group, the mean (± 1 standard error) relative EOG amplitude in response to 10^{-5} *M* L-serine is shown. The upper dashed line indicates the mean reduction in EOG responses seen in control fish (a reduction discussed in the text). A dashed line indicates the relative threshold criterion (a 25% reduction relative to controls). The vertical line in the upper right shows the lower limit of the 95% confidence interval for the control fish. A dashed line indicates the resulting statistical criterion. See the section ''Results and discussion'' for the equation used to fit the data. Filled circles indicate the relative and statistical threshold concentrations of 2.7 and 3.0 µg/l (respectively) for copper's effect on L-serine olfactory sensitivity. Note that these threshold values are nominal concentrations above background, or an increase from the approximately 3 µg/l copper present in the source water for the NWFSC hatchery. Adapted from Reference 17.

(0.84 ± 0.09, mean ± 1 standard error). This intra-animal reduction in evoked EOGs was not statistically significant (one-group *t*-test, hypothetical mean $= 1$, $p > 0.05$). However, it does indicate the potential for slight residual contamination in the lines used to deliver copper to the olfactory chamber. Care should therefore be taken to flush the lines between experiments.

Several methods have previously been used to describe toxicological effects thresholds (e.g., lowest observable effect concentration, inhibition concentration, benchmark dose). In this example, the effect threshold for a 30-min copper exposure was estimated by fitting the data for L-serine with a sigmoid logistic model:

$$y = m/[1 + (x/k)^n]$$

where m is the maximum relative EOG amplitude (fixed at the control mean of 0.84), y the relative EOG amplitude, x the copper concentration, k the copper concentration at half-maximum relative EOG amplitude (IC_{50}), n is the slope.

Figure 14.8 shows that the model is a good fit for the range of responses to L-serine. The values ($\pm$ standard error) for k and n were 6.8 ± 0.6 and 1.2 ± 0.1, respectively. Similar fits with the model were found for the copper-induced reductions in EOGs evoked by the other odorants (TCA and the amino acid mixture; Reference 17). The threshold concentration was then determined based on the nominal concentration at which the model crosses a criterion level. The criterion was set to a relative departure of 75% of the control mean (or a 25% reduction relative to controls), a level very close to a statistical departure based on the lower 95% confidence interval for the control group (Figure 14.8). Using this approach, the nominal threshold concentration for copper yielding 25% inhibition (IC_{25}) was found to be 2.7 μg/l for L-serine. This inhibitory threshold is very similar to the relative thresholds for TCA (2.3 μg/l) and the amino acid mixture (3.0 μg/l).[17]

Additional applications of EOG recordings to aquatic toxicology

The case study presented earlier used the EOG recording method to determine thresholds for copper-induced reductions in the olfactory response of coho salmon. The application of the EOG technique to other fish species, other contaminants, and other natural odorants has already been discussed. The EOG can also be used to monitor the sublethal effects of dissolved contaminants in terms of time-to-effect and time-to-recovery. For contaminants that impact the sensory epithelium relatively quickly (e.g., copper[17]), an advantage of the EOG technique is that olfactory function can be measured in a single fish before, during, and after the exposure of the olfactory rosette to track both time-to-effect and time-to-recovery over the short term. Additionally, due to the importance of olfactory-mediated behaviors mentioned earlier, effects of contaminant exposure observed in EOG studies can be used to infer a contaminant's effect on higher endpoints, such as survival or reproduction, where data may be lacking or difficult to obtain. When experiments on a contaminant's effect on higher endpoints are undertaken, aspects of the experimental design, e.g., the exposure concentrations to test, can be guided by the results from EOG studies. The EOG recording technique, therefore, can serve both as a standalone measure of the sublethal impact of contaminant exposure on peripheral olfactory physiology and as a link between contaminant-induced disruptions of physiological function and disruptions of behavior, reproduction, or survival.

Acknowledgments

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chapter fifteen

Enzyme-linked immunosorbent assay for screening estrogen receptor binding activity

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Contents

Introduction

Endocrine disrupter chemicals (EDCs) include those that mimic estrogen (xenoestrogens) and cause reproductive problems.[1] Xenoestrogens can produce adverse effects by binding to the estrogen receptor (ER).[2] In addition, they may also alter hormone metabolism, affect various neural centers or the pituitary, and modify serum hormone binding proteins.[2] We need an efficient screening strategy for xenoestrogens because a large number of chemicals are commercially produced and widely used, and this type of information is not generally available.

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A final assessment of endocrine disrupting ability must be based on whole animal studies[3–5]; however, alternative tests, such as high through-put assays of receptor-dependent responses, can be used to prioritize chemicals before conducting time-consuming and expensive *in vivo* assays. Receptor binding assays and other *in vitro* tests, including cell proliferation assays, expression of specific proteins, yeast two-hybrid assays, and reporter gene assays, are examples of these high through-put methods.[1,2,6–13] We considered that from these tests, a receptor binding assay was a rapid and cost-effective screening technique. Therefore, we establish an enzyme-linked immunosorbent assay (ELISA) for assessing the binding ability of chemicals to an ER.[14,15] Our competitive ELISA used estrogen labeled with horseradish peroxidase, as opposed to radioisotopes, was safe and very easy to perform.

Materials required

ELISA kit for EDCs

- Human ERα[16]
- Anti-human ERα mouse IgG (monoclonal antibody); the antibody was fixed to microplate by physical absorption
- Unlabeled ligand, which was usually 17β-estradiol
- Horseradish peroxidase labeled 17β-estradiol
- Substrate was 3,3′,5,5′-Tetramethylbenzidine (Cas No. 54827-17-7)
- 1N sulfuric acid
- Buffer solutions: aqueous buffer (10 m*M* phosphate-buffered saline, pH 7.2) and washing solution (50 m*M* disodium phosphate, 150 m*M* NaCl, 0.05% [w/v] Tween-20)
- Microplate (96 wells)

The materials listed above are available as a kit (Ligand Screening System, ERα, Code No. ERA-101, Toyobo Co., Biochemical Department, 2-2-8 Dojimahama, kita-ku, Osaka, 530-8230, Japan. Tel.:+81-6-6348-3786, fax:+81-6-6348-3833, e-mail: order lifescience@bio.toyobo.co.jp).

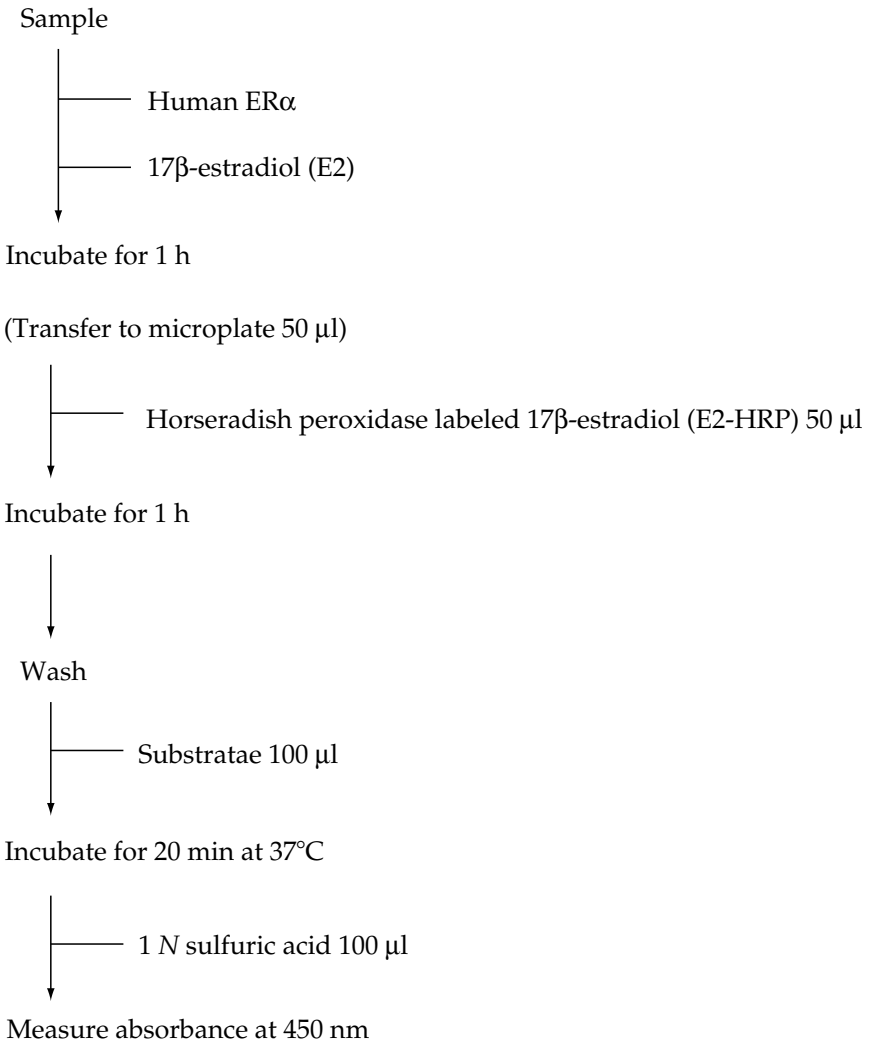
Equipment

- Immuno Wash MODEL 1575 (Bio-Rad Laboratories, Arakawa, Tokyo, Japan)
- Microplate reader (Benchmark, Bio-Rad Laboratories, Arakawa, Tokyo, Japan)
- Incubator

Chemicals for analysis

Chemicals for analysis were purchased from the following sources: bisphenol A (BPA), 3-*tert*-butylphenol (*m*-*t*-BP), 4-*n*-pentylphenol (*p*-*n*-PeP), 4-*tert*-pentylphenol (*p*-*t*-PeP), 4-octylphenol (*p*-*n*-OP), *p*-*t*-OP, and 4-*n*-nonylphenol (*p*-*n*-NP) from the Wako Pure Chemical Industries, Osaka, Japan; diethylstilbestrol (DES) from the Sigma Chemical Co., St.Louis, MO, USA; 4-nonylphenol (*p*-NP) from the Kanto Chemical Co., Tokyo, Japan; 4-*tert*-butylphenol (*p*-*t*-BP) from Nacalai Tesque, Kyoto, Japan. The 4-*n*-nonylphenol consisted of only *n*-isomer, the 4-nonylphenol consisted of an undefined mixture of isomers.

Procedures



The method involves two steps of competitive reactions. The first step is the competition between the ligand (E2) and test chemicals on the ERα. When test chemicals have affinities for ERα, the concentration of the free ligand in the medium increases depending upon the binding affinities of test chemicals for ERα. If a test chemical does not have any binding affinity for ERα, the ligand in the medium is completely absorbed by the ERα. The second step is the competition between the free ligand, which was obtained at the first step reaction, and E2-HRP on the anti-E2 monoclonal antibody. When the free E2 exists in the medium, the competition against E2-HRP occurs on the antibody. Thus, color development by HRP decreases depending upon the concentration of the free ligand. If the free ligand in the medium is absent, the color development by HRP occurs fully. A scheme of the assay is shown in Figure 15.1.

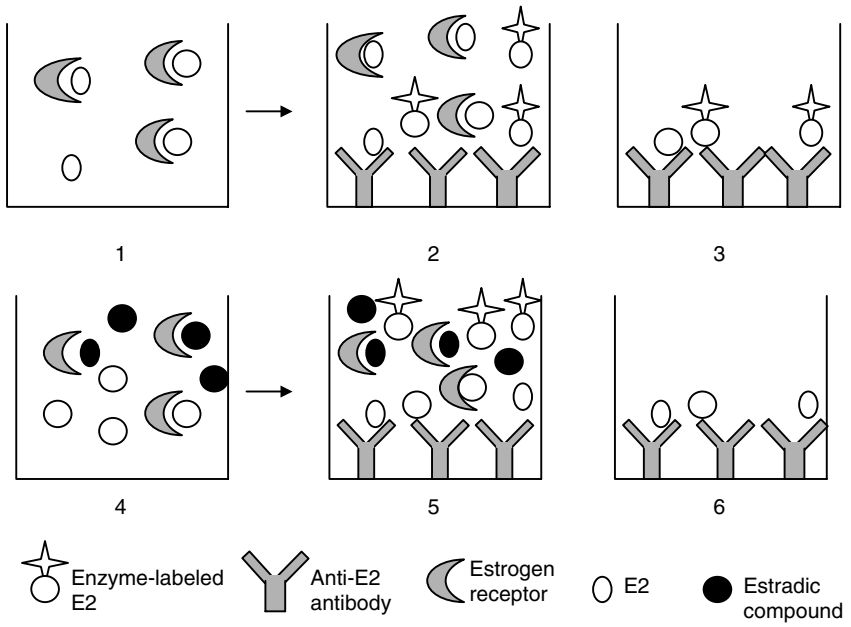


Figure 15.1 Scheme of ELISA method for measuring ERα binding activity of chemicals. Wells 1–3 show binding of 17β-estradiol to antibody and receptor. Wells 4–6 show how an estrogenic compound decreased the binding of enzyme-labeled estradiol to the anti-estradiol antibody on the microplate. Wells 2–3 and 5–6 were coated with anti-estradiol antibody. Well 1 (or 4) was different from wells 2–3 (or 5–6). Estradiol, compounds and ERs were mixed in well 1 (or 4), and they were transferred in well 2 (or 5), and then enzyme-labeled estradiol was added. After incubated, the plate was washed before the substrate was added. The compound competed with 17β-estradiol for binding to the ER, and the free 17β-estradiol then bound to the antibody and decreased the amount of the enzyme-labeled estradiol available to bind to the anti-estradiol antibody on the microplate. The antibody dose not bind other estrogenic compounds apart from estradiol. The enzyme-labeled estradiol does not bind to the receptor.

We usually used three concentrations of test chemicals in a range that ideally included 50% inhibition of each chemical. Test chemicals were dissolved in dimethylsulfoxide and diluted in aqueous buffer to a dimethylsulfoxide concentration of 1%. This sample solution (30 μl), receptor solution (20 μl), and E2 solution (30 μl) were added together to wells in the reaction plates (96-well polystyrene plates). The reaction plates were incubated at 4°C for 1 h, and 50 μl of the reaction solution and 50 μl of E2-HRP were added onto antibody plates that were coated with the E2 antibody, and incubated at 4°C for 1 h. Each well was washed three times with 150 μl of washing solution using an Immuno Wash MODEL 1575. Washing solution was removed from each well, 100 μl of substrate solution that contained 3,3′,5,5′-tetramethylbenzidine was added to the well and the antibody plates were incubated at 37°C for 20 min in the absence of light. Sulfuric acid (1 *N*, 100 μl) was added to each well to stop the reaction and the absorbance of each antibody plate well was measured at 450 and 655 nm using a microplate reader

Dimethylsulfoxide (1% in aqueous buffer) and DES (300 n*M*) were used as negative and positive controls, respectively.

Calculation of binding affinity

ERα inhibition was calculated based on the following equation:

$$\text{inhibition } (\alpha) = ([B] - [S])/([B] - [P])$$

where [*B*] is absorbance measured in the absence of the test chemical, [*S*] is absorbance measured in the presence of the test chemical, and [*P*] is absorbance measured in the presence of the positive control chemical (DES).

Calculation of receptor binding index

ERα index was calculated based on the following equation:

$$\text{ER}\alpha \text{ index} = (1 - \alpha) \times (S \times 3/8 - R \times \alpha)/\alpha$$

where α is inhibition, *S* is sample concentration (n*M*), 3/8 is the volume correction factor, and *R* is the receptor concentration (n*M*). ERα index, which is calculated using the above equation, is approximately equal to the dissociation constant, and we use ERα index as an approximation for Kd.

Calculation of IC_{50}s

Regression was made of inhibition (α) on sample concentrations, and IC_{50} (concentrations that cause 50% inhibition of binding of E2 to ERα) was calculated from the regression curve. When α α zwas lower than 0.5, IC_{50} was calculated using the equation:

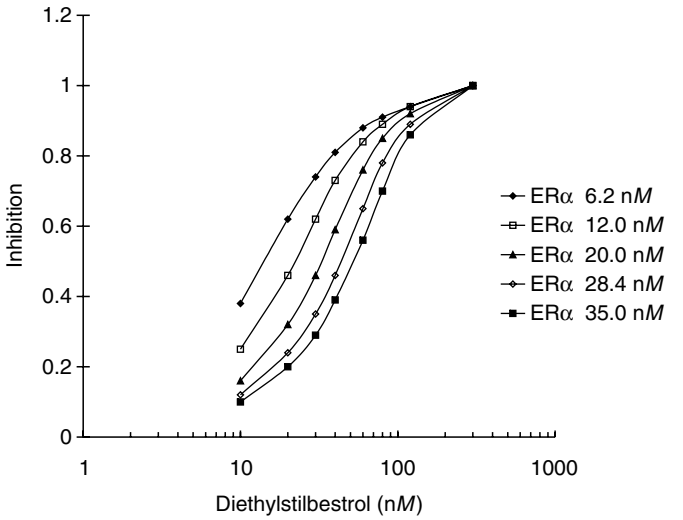


Figure 15.2 Inhibition curves in the presence of 6.2, 12.0, 20.0, 28.4, and 35.0 n*M* ERα. Ligand concentration was 12.5 n*M*, reaction temperature was 37°C, 17β-estradiol antibody concentration was 62.5 ng/well, 17β-estradiol-horseradish peroxidase was diluted 1/1000.

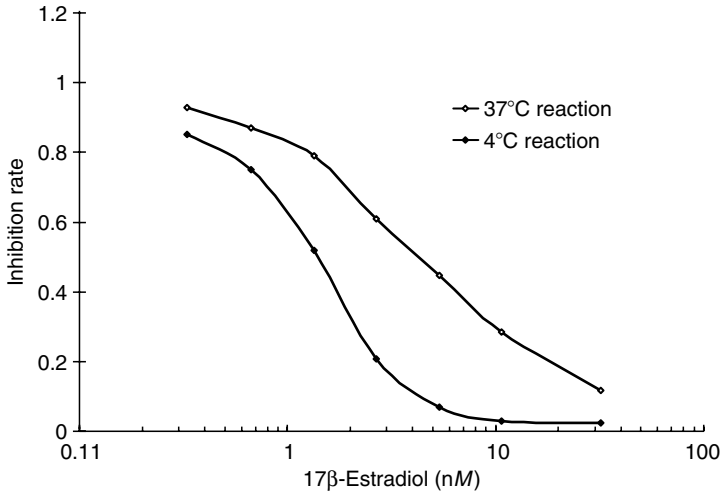


Figure 15.3 Inhibition curves for the 17β-estradiol and 17β-estradiol-horseradish peroxidase reaction for 17β-estradiol antibody at 4°C and 37°C. 17β-Estradiol antibody concentration was 62.5 ng/well and 17ββ-estradiol-horseradish peroxidase was diluted 1/1000. The inhibition rate was set at 1 when the concentration of 17β-estradiol was 0.

$$IC_{50}\ (nM) = (ER\alpha\ \text{index} + R \times 0.5) \times (8/3)$$

Calculation of relative binding affinity

The relative binding affinity (RBA) was calculated based on the equation:

$$RBA = IC_{50}\ \text{of DES}/IC_{50}\ \text{of test chemical}$$

Results and discussion

Factors that affected results

- Concentrations of ERα

When the concentrations of ERα at the first step varied between 6.2 and 35.0 n*M*, the binding rate of DES to ERα (the competition rate against E2) differed, as shown in Figure 15.2. When the ERα concentration was decreased, the quantity of the test chemical required to produce the same degree of inhibition also decreased. The concentration of the ligand (E2) was 12.5 n*M*, the reaction temperature for the first step reaction was 37°C, HRP-E2 was diluted 1/1000, and the concentration of the anti-E2 antibody was 62.5 ng/well.

- Temperature of the reaction between E2 and HRP-E2

The reaction rate depends on reaction temperature, as shown in Figure 15.3. When reaction temperature decreased, the reaction of E2 or HRP-E2 with coated E2 antibody also decreased. Since we anticipated that the measured concentration range would also decrease, we compared reaction temperatures of 4°C and 37°C. The measured concentration range was decreased from 1.5 approximately 20 n*M* at 37°C to 0.5 approxi-

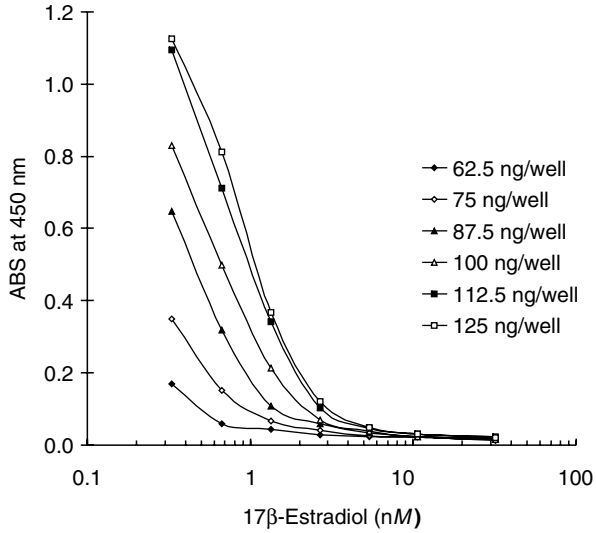


Figure 15.4 17β-Estradiol and 17β-estradiol-horseradish peroxidase reaction for 17β-estradiol antibody. 17β-Estradiol antibody concentrations were 62.5, 75, 87.5, 100, and 112.5 ng/well. 17β-Estradiol-horseradish peroxidase was diluted 1/1000.

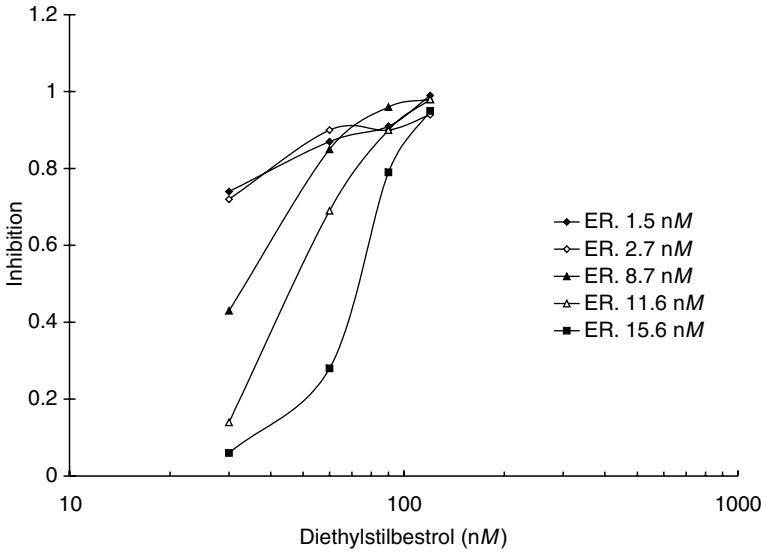


Figure 15.5 Inhibition curves in the presence of 1.5, 2.7, 8.7, 11.6 and 15.6 n*M* ERα. Ligand concentration was 4.2 n*M*, reaction temperature was 4°C, 17β-estradiol antibody was 125 ng/well and 17β-estradiol-horseradish peroxidase was diluted 1/1000.

mately 3 n*M* at 4°C. HRP-E2 was diluted 1/1000 and the concentration of E2 antibody was 62.5 ng/well.

- Concentration of the E2 antibody

The reaction rate (absorbance of 450 nm) between E2 and HRP-E2 depended on the concentration of the E2 antibody, as shown in Figure 15.4. When the solid-phase antibody

Table 15.1 IC_{50} and RBA values of each chemical[a]

Compound	IC_{50} (n*M*)	RBA
DES	8	1.13E + 02
BPA	883	1.00E + 00
m-*t*-BP	28,776	3.07E − 02
p-*t*-BP	31,651	2.79E − 02
p-*n*-PeP	27,487	3.21E − 02
p-*t*-PeP	9757	9.04E − 02
p-*n*-OP	32,792	2.69E − 02
p-*t*-OP	2832	3.12E − 01
p-*n*-NP	25,625	3.44E − 02
p-NP	699	1.26E + 00

[a] DES, diethylstilbestrol; BPA, bisphenol A; *m*-*t*-BP, 3-*tert*-butylphenol; *p*-*t*-BP, 4-*tert*-butylphenol; *p*-*n*-PeP, 4-*n*-pentylphenol; *p*-*t*-PeP, 4-*tert*-pentylphenol; *p*-*n*-OP, 4-*n*-octylphenol; *p*-*t*-OP, 4-*tert*-octylphenol; *p*-*n*-NP, 4-*n*-nonylphenol; and *p*-NP, 4-nonylphenol.

concentration was increased, absorbance also increased. HRP-E2 was diluted 1/1000 and the reaction temperature was 4°C.

Finally, the receptor concentration was optimized while other parameters were constant (Figure 15.5). When the receptor concentration was low, the same inhibition could be achieved with less test chemical. Thus, conditions can be optimized to require less test chemical and reduce cost. We usually performed the assay with 12 n*M* of ERα, 4.2 n*M* of E2, 4°C at the reaction, 125 ng/well of E2 antibody, and HRP-E2 diluted 1/1000.

ERα binding abilities of endocrine disrupting chemicals assessed using this method

IC_{50}s and RBA are shown in Table 15.1. The advantages of this method are: (1) it does not use radioisotopes, (2) many test compounds can be screened simultaneously in a short time at low cost, (3) specific apparatus is unnecessary, (4) the method is simple and easy to use, and (5) it can be applied with or without metabolic activation using rat liver S9 mix.

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chapter sixteen

Lysosomal destabilization assays for estuarine organisms

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Contents

Introduction

Estuarine ecosystems are subject to increased stress associated with human population growth, in some cases nearly explosive, in coastal areas of the United States.[1,2] Estuaries provide essential functions, including serving as nursery grounds, for a variety of shellfish and fish species.[3] There is no question that organisms in these habitats are being exposed to contaminants, and bioaccumulation can be readily documented.[4] While acute toxicity incidents (e.g., fish kills, depauperate communities) are highly visible occurrences, it is more difficult to determine the impacts associated with chronic or recurring exposures to sublethal levels of contaminants. In some cases, compensatory mechanisms may function to sequester, detoxify, or ameliorate the effects of stressors, so exposures do not always translate into adverse effects. In other cases, individual stressors or combinations of stressors may cause chronic stress that can compromise basic physiological functions and long-term population sustainability. Sensitive biomarker tools should facilitate our ability to recognize when habitat conditions adversely affect biotic integrity before the effects are irreversible or very expensive to remedy. Cellular biomarker responses are valuable for identifying when conditions have exceeded compensatory mechanisms and the individuals and populations are experiencing chronic stress, which, if unmitigated, may progress to severe effects at the ecosystem level.

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Lysosomes are intracellular organelles that are involved in many essential functions, including membrane turnover, nutrition, and cellular defense.[5,6] The internal acidic environment of lysosomes, integral for the optimal activity of acid hydrolases, is maintained by a membrane-bound, ATPase-dependent proton pump. Lysosomes sequester metals and other contaminants, which make them prone to oxidative damage, etc., that can lead to membrane destabilization. Contaminants can also impair lysosomal function by disruption of the proton pump. Either mode of action can lead to the impairment of vital functions and cell death.[7–10]

Lysosomal function assays of hepatic or blood cells are regarded as valuable indicators of pollutant-induced injury, and there is a substantial body of literature validating that environmental pollutants cause destabilization of lysosomes (e.g., see References 11–15). Neutral red (NR) techniques for assessing lysosomal destabilization have been used successfully in fish and invertebrate taxa, and have been incorporated into major European programs (BIOMAR, Black Sea Mussel Watch). In this chapter, the methods that we developed and our experiences with a lysosomal destabilization assay based on NR retention in three common estuarine species (oysters, *Crassostrea virginica*; grass shrimp, *Palaemonetes pugio*; and mummichogs, *Fundulus heteroclitus*) are described.

NR retention assays are relatively simply assays for assessing lysosomal stability. The most common sources of cells for the assay are blood cells or hemocytes and hepatic cells. Hepatic tissues are especially rich in lysosomes. Since liver or hepatic tissues are a common site of accumulation and detoxification of contaminants, they are particularly relevant for identifying sublethal impacts of contaminants. Moreover, hepatic preparations typically yield a large number of cells and are the only real option for small organisms or those that are difficult to bleed. Cells incubated in NR accumulate the lipophilic dye in the lysosomes, where it is trapped by protonization. In healthy cells, NR is taken up and retained in stable lysosomes for extended periods; whereas in damaged cells the NR leaks out of lysosomes and into the cytoplasm, and are referred to as cells with destabilized lysosomes. Therefore, the presence of NR in the cytoplasm reflects the efflux of lysosomal contents into the cytosol, which ultimately causes cell death.[13] We use a single-time-point assay, in which the endpoint for a site or treatment is based on microscopic examinations after a set incubation period (e.g., 60 min) of cell preparations from multiple individuals in which cells are scored as stabilized or destabilized, and the percentages of cells that are destabilized are determined. Other investigators use a NR retention time endpoint, a multiple-time-point assay in which individual cell preparations are examined repeatedly, and the time duration required until approximately 50% of the cells are destabilized cells is determined. The initial processing steps for both approaches are the same; only the endpoints are slightly different.

Materials required

- Ultrapure deionized water should be used for all buffers, and all chemicals should be cell biology grade.
- Calcium magnesium free saline (CMFS), used for all species; 20 m*M* HEPES, 360 m*M* NaCl, 12.5 m*M* KCl, and 5 m*M* tetrasodium EDTA
  - 4.766 g HEPES, 20.00 g NaCl, 0.932 g KCl, and 1.901 g EDTA in 995 ml DI H_2O
  - Adjust pH to 7.35–7.40 with 6 *N* NaOH
  - Adjust final volume to 1000 ml and filter through a 0.45-μm screen
  - Check pH and salinity just prior to use (salinity should be approximately 25‰)
  - Store refrigerated up to 5 days

- Magnesium free saline (MFS), used for shrimp and fish; 20 m*M* HEPES, 480 m*M* NaCl, 12.5 m*M* KCl, and 5.0 m*M* $CaCl_2$
  - 0.477 g HEPES, 2.661 g NaCl, 0.093 g KCl, and 0.055 g $CaCl_2$ in 99.5 ml DI H_2O
  - Adjust pH to 7.50–7.55 with 6 *N* NaOH
  - Adjust final volume to 100 ml with DI H_2O and filter through a 0.45-μm screen
  - Check pH and salinity just prior to use (salinity should be approximately 25‰)
  - Store refrigerated up to 5 days.
- Trypsin (bovine) (1.0 mg/ml), prepare just prior to use
- Collagenase (Type IV) (1.0 mg/ml), prepare just prior to use
- NR dye, Prepare just prior to use.
  - Make a 1° stock solution by adding 4 mg of NR powder to 1 ml of DMSO
  - Make a 2° stock solution (NR concentration of 0.04 mg/ml) by adding 100 μl of the 1° stock solution to 9.90 ml CMFS; wrap in foil to protect from light and keep at room temperature
- Hepatic or hepatopancreatic tissues (small piece, approximately 2–4 mm square, cleaned of extraneous tissues, and minced)
- 24-well cell culture plates
- Nylon mesh
- Reciprocating shaker
- Microcentrifuge tubes (2 ml)
- Pasteur pipettes
- Pipetter and tips
- Centrifuge
- Compound microscope
- pH meter
- Refractometer

Procedures

While we have more experience with oysters, like other investigators, we have been able to successfully modify the assay for other species, including marsh mussels (*Geukensia demissa*), as well as grass shrimp and mummichogs. In general, these methods have been readily applicable to a wide range of organisms with fairly minor modifications, a valuable attribute for cellular response assays, so these techniques should be transferable to other mollusks, crustaceans, and fish species. As part of our routine sampling protocols, the animals are kept cool (not cold) in aerated water collected on site from the field to the laboratory. We have also conducted assays with oysters that have been shipped cool (e.g., approximately 5–10°C) or held under refrigeration overnight; we have conducted holding studies and found that oysters can be kept cool in the refrigerator for up to 3 days before significant increases in lysosomal destabilization are observed; we routinely keep our analysis time within 2 days, preferably within 1 day of collection.

The basic protocols used for oysters are summarized in Figure 16.1 and described in more detail in the following. Only minor modifications were required for shrimp and fish, indicated with a numerical superscript in Figure 16.1, and described in the following:

1. The first series of steps involves dissection of hepatopancreatic (oysters and shrimp) or liver (fish) tissues and chemical dissociation of the tissues. Since in oysters the gonadal tissues are closely associated with the hepatopancreatic tissues, it is important to trim away as much gonadal tissue as possible and

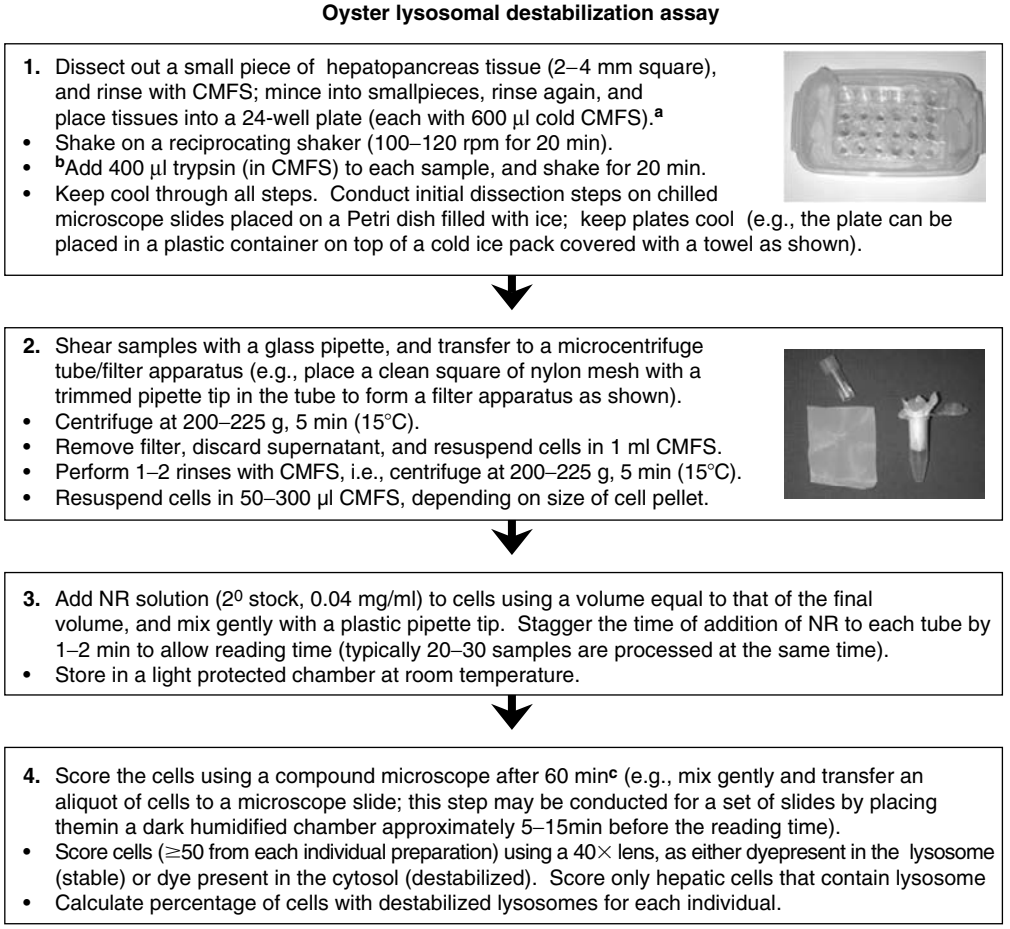

Figure 16.1 Annotated flow diagram of the lysosomal destabilization assay with oysters. The superscripts indicate the steps that must be modified for use with grass shrimp and mummichogs as follows: [a]Use 500 µl CMFS for shrimp and fish; [b]add 500 µl collagenase in MFS for shrimp and fish; [c]score shrimp and fish cells after 90 min.

rinse well (i.e., by pipetting the tissues on the cold microscope slide) with CMFS to remove extraneous gametes. For the fish tissues, extensive rinsing is required to remove excess blood cells. For all species, CMFS is used to initiate dissociation of cell junctions (while the CMFS solution described here was suitable for these three estuarine species, it may be necessary to adjust the osmolality, primarily by adjusting the NaCl concentrations, for species from higher or lower salinity habitats). Then for oysters, trypsin is used to further facilitate dissociation. However, shrimp and fish tissues have more collagen in their connective tissues, so collagenase rather than trypsin is used. The activity of collagenase requires calcium and also has a somewhat higher pH requirement, so it is made up in an MFS, pH 7.5, which contains the necessary calcium.

2. The next series of steps involves physical dissociation of the tissues and rinsing of the cellular preparation. The cells should be gently sheared as they are drawn in and out of a glass Pasteur pipette, and then transferred to a microcentrifuge tube/

filter apparatus. The entire apparatus is then gently centrifuged (approximately 500*g*) and the cells are pulled through the nylon screening during centrifugation to complete the dissociation. The cells are then rinsed and resuspended in CMFS. A microcentrifuge placed in a cooled chamber can be used, as well as a refrigerated centrifuge.

3. An equal volume of the NR solution (2° stock, 0.04 mg/ml) is then added to each cell suspension and then the cells should be mixed gently with a pipetter (vortexing is not recommended). NR is a light-sensitive vital dye, so it is important to minimize light exposure through all steps.
4. After the incubation period in NR (60 min for oysters; 90 min for grass shrimp and mummichogs), the cells should be scored as either stable (NR contained within lysosomes) or destabilized (NR leaking out of enlarged and damaged lysosomes and diffusing into the cytoplasm) (Figure 16.2). For fish and shrimp, a slightly longer incubation period (90 min) is required because the initial uptake of NR into the lysosomes is somewhat slower. Since these are mixed cell preparations, it is important to establish rules or criteria for scoring the cells. The rules that we typically use are:

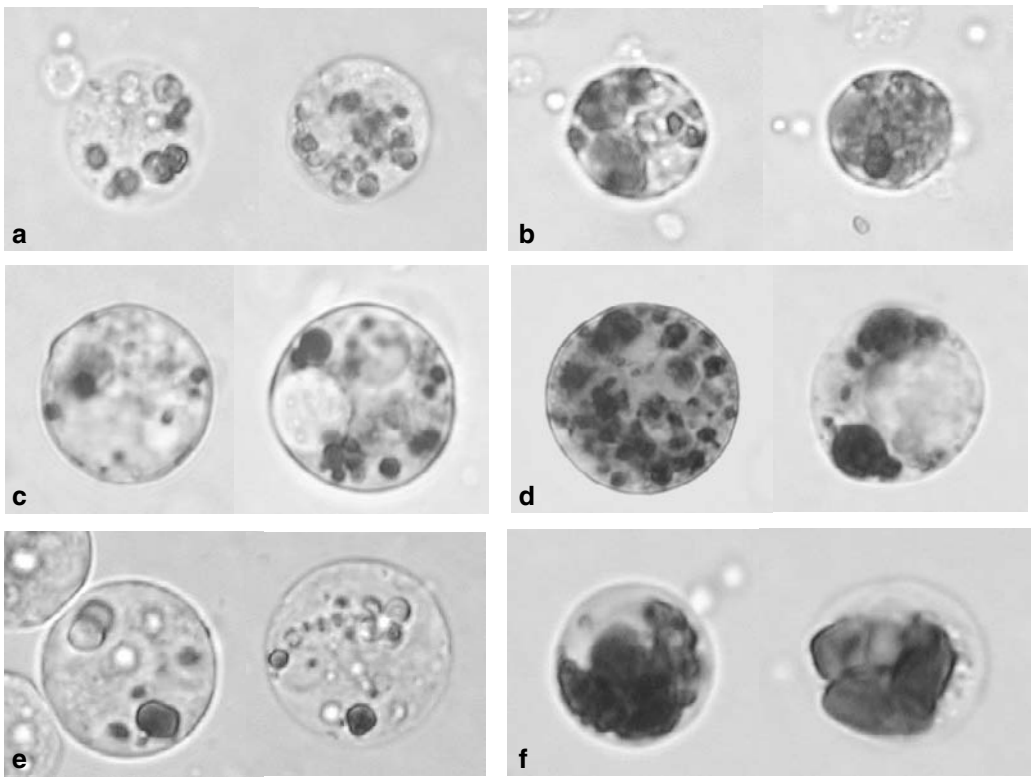

***Figure 16.2* (see color insert following page 464)** Photographs of examples of hepatic cells with stable and destabilized lysosomes for oysters, grass shrimp, and mummichogs: (a) oyster hepatopancreas cells scored as stable; (b) oyster hepatopancreas cells scored as destabilized; (c) grass shrimp hepatopancreas cells scored as stable; (d) grass shrimp hepatopancreas cells scored as destabilized; (e) fish liver cells scored as stable; (f) fish liver cells scored as destabilized.

- Score only those cells within a certain size range (oysters, 25–40 μm; shrimp, 60–75 μm; mummichogs, 35–50 μm).
- Score only those hepatic cells that accumulate NR with distinct lysosomes (the preparations sometimes also contain extraneous egg or yolk cells that will appear almost solid red especially during reproductive periods). Since the cells are still in CMFS at this stage, the cells will remain in a somewhat rounded state, so it is important to focus up and down a little with the microscope to observe the different planes of each cell.
- Score at least 50 cells from each individual preparation.
- Score conservatively. Most cells are readily scored as stable or destabilized. However, there may be some cases where there is greater uncertainty, but we typically score the somewhat marginal cells as stable. This assay is a microscopic assay, in which the live cells are evaluated by a reader, so there is a level of subjectivity. Reader training is critical, and routine QA is required. At least one sample from each preparation should be reviewed by a second reader. A library of photographic images can also be maintained for reference.

Using the single-time-point assay, studies with field-collected animals are typically based on as many animals as possible, preferably 20 individuals, but as few as 10 have been used. For laboratory studies, we typically use 3–5 individuals from three or more replicated treatments. The percent destabilized lysosomes are calculated for each individual, and then summary statistics are generated for each site (mean, median, standard deviation, quartiles). It is often recommended that percentage-based parameters be transformed (e.g., arcsin transformation) prior to statistical analyses, primarily to ensure that the data set is normally distributed. Generally, we have found that even though the data are based on percentages, the data are still generally normally distributed, and that the interpretation of site-specific comparisons using analysis of variance (ANOVA) is the same using untransformed or transformed data. Normality and homogeneity of variances should be confirmed for the parametric analyses, or non-parametric analyses should be used if these assumptions are violated. Examples of the spreadsheet formats (e.g., Excel) that we routinely use for raw data include information on organism height, length, sex, or gonadal index (an index of gonadal development based on 1 for little to no gonad present, to 4 for fully developed gonad), as well as the biomarker response for each individual (Figure 16.3a). The ''summary'' data tables should include enough redundant information that they can be clearly linked to the raw data tables (such as the site name or code, species name, dates) and also provide overall summaries (e.g., statistical values, such as the mean, standard deviation, median, 25th and 75th percentiles) (Figure 16.3b).

Results and discussion

The NR assay is a relatively simple assay that is readily adapted for a variety of species. The single-time-point assay described here is very similar to the NR retention time assay that uses repeated evaluations of the same individual preparations over time to define the time at which 50% of the cells are destabilized. Both approaches can be used as effective biomarkers of contaminant exposure. In our laboratory, we were faced with processing resident animals or those from caged deployments from multiple sites over a limited window of time, so the retention time approach was logistically difficult and would allow only a small sample size. After a careful review of the existing data on mussels, there was

(a) Example of raw data table for the lysosomal destabilization assay.

Site	Species	Sampling date	Animal #	Height (cm)	Length (cm)	Gonadal index	Percentage of lysosomal destablization	Lysosomal analysis date	QA code
CIAAAW00	crasvirg	2/9/2000	1	9.3	4.1	4	24.53	2/10/2000	
CIAAAW00	crasvirg	2/9/2000	2	9.0	4.4	3	26.56	2/10/2000	
CIAAAW00	crasvirg	2/9/2000	3	9.1	3.5	3	29.63	2/10/2000	
CIAAAW00	crasvirg	2/9/2000	4	8.6	3.6	4	26.23	2/10/2000	
CIAAAW00	crasvirg	2/9/2000	5	9.4	3.8	3	25.97	2/10/2000	
CIMOSW00	crasvirg	4/12/2000	1	6.7	2.6	4	47.17	4/13/2000	
CIMOSW00	crasvirg	4/12/2000	2	2.9	2.2	2	39.22	4/13/2000	
CIMOSW00	crasvirg	4/12/2000	3	2.9	2.3	4	49.06	4/13/2000	
CIMOSW00	crasvirg	4/12/2000	4	5.2	2.2	3	63.64	4/13/2000	
CIMOSW00	crasvirg	4/12/2000	5	6.9	2.5	4	36.67	4/13/2000	

(b) Example of summary data table for the lysosomal destabilization assay.

Site	Species	Assay	#Animals	Mean (%) Lysosomal Destabilization	STD	Median (%) Lysosomal Destabilization	25%	75%	QA code
CIAAAW00	crasvirg	Lyso	5	26.58	1.87	26.2	25.61	27.33	
CIMOSW00	crasvirg	Lyso	5	47.15	10.59	47.1	38.58	52.71	

Figure 16.3 Examples of raw data and summary tables used for the lysosomal destabilization assay for oysters for two sites. Site and species codes are followed by ancillary data, such as information on size, gonadal condition, etc.

a consistent general pattern that those from polluted sites had NR retention times of less than 60 min, often less than 30 min, whereas those from reference sites were often stable for hours. After some preliminary laboratory studies, we decided that a single-time-point of 60 min should provide good discrimination between treatments and sites. By using this approach, we were then able to substantially increase our sample size. Furthermore, it has been our experience that in most cases, cells harvested from oysters from control conditions or clean reference sites have very low destabilization rates for many hours (good morning-preparations can often be used for afternoon demonstrations or laboratory exercises); those exposed to contaminants often begin to show significant destabilization within 30 min.

The primary difference between the processing of the different species was that CMFS and trypsin are used as the primary means of chemical dissociation, whereas with the shrimp and fish, CMFS and collagenase are used due to the higher collagen content of their tissues. In general, oysters and mussels are the easiest to process because both have substantial amounts of hepatopancreatic tissue, and generate high quality cell preparations. Grass shrimp were the hardest to process because they have smaller hepatopancreases. With grass shrimp, there were sometimes problems with consistency in acquiring high quality cell preparations, as some preparations were very sticky and cell recoveries were sometimes very low while most preparations were of very high quality. Mummichogs also have very small livers, but generally enough cells could be obtained for the assay. Tissue pieces had to be carefully rinsed to avoid contamination by excess blood

cells. The NR incubation period for grass shrimp and mummichogs is a little longer than that used for mollusks. In general, more work is needed with both of these species to continue to refine cell-handling protocols, but these studies have served to establish the feasibility of the general approaches.

The most challenging aspect of any biomarker is developing a framework for interpretation. To do this effectively requires a sound basis for interpreting cellular data, including expected values and an appreciation of the potential variation, as well as the sources of the variation. In the biomedical context, this is analogous to defining the normal range of responses. With a good baseline knowledge of what is normal or what is expected, cellular assays are used as diagnostic tools in medical applications, as warning signals of early disease conditions, for prognosis, and evaluating the effectiveness of remedies. Our overall goal has been to apply this kind of framework to estuarine organisms as a means of characterizing organismal health and habitat quality. In the medical arena, it is sometimes necessary to define different normal ranges based on age, sex, etc. In estuarine habitats, species-specific responses may be affected by habitat differences, such as salinity regimes, seasonal differences, etc., as well as age and sex. Some physiological processes, such as overall metabolic rate and growth of oysters, are known to be affected by a variety of natural habitat factors,[16] which can make it difficult to distinguish contaminant stress from differences associated with natural factors. Therefore, one of the goals of our biomarker studies has been to identify essential cellular functions that are not readily affected by ''natural stressors'' (e.g., have less variation over broad environmental regimes) but are affected by contaminants. Ultimately, our ability to correctly interpret the responses is based on the robustness of the normal range criteria. In human medicine, the normal ranges are based on an extensive database from a wide range of sources; and likewise we draw on both laboratory studies and field studies from a wide range of habitats to delineate the ''normal'' responses that are characteristic of a healthy estuarine organism.

Examples of some of the ways that we have tried to address these important interpretation issues, as well as some of the kinds of data generated, are presented, particularly with regard to distinguishing contaminant effects from potential salinity effects. Laboratory studies were used to determine the effects of salinity, a variable that can range from nearly fresh water to full-strength seawater within an estuary. Oysters are typically found in low, as well as high, salinity regimes. Laboratory studies were conducted with oysters and shrimp (and also marsh mussels), in which animals were collected from a reference, mid-range salinity site (e.g., approximately 25‰), returned to the laboratory, and immediately placed in one of three treatments: 10‰, 20‰, or 30‰ seawater, with no acclimation. Then the lysosomal destabilization responses were evaluated over time, e.g., within 24 h in some cases and for extended periods up to 2 weeks. The results of these kinds of studies have consistently indicated that lysosomal destabilization rates are not different between any of the treatments in both oysters and grass shrimp (Figures 16.4a and b); results with marsh mussels are virtually the same as that observed with oysters.

Similar studies have been conducted with pollutants (e.g., Cu, benzo-*a*-pyrene), in which oysters and shrimp were exposed to a range of concentrations and sampled after various time points (from 18 h to 14 days). The patterns with both oysters and shrimp were very different from those observed for the salinity studies. After only 18 h, there were significant increases in oyster lysosomal destabilization rates that persisted over time, in a dose–dependent manner (Figure 16.4c). Significant elevations in lysosomal destabilization rates were also observed in 7-day exposures in grass shrimp (Figure 16.4d). Numerous laboratory studies have supported the model that lysosomal destabilization in

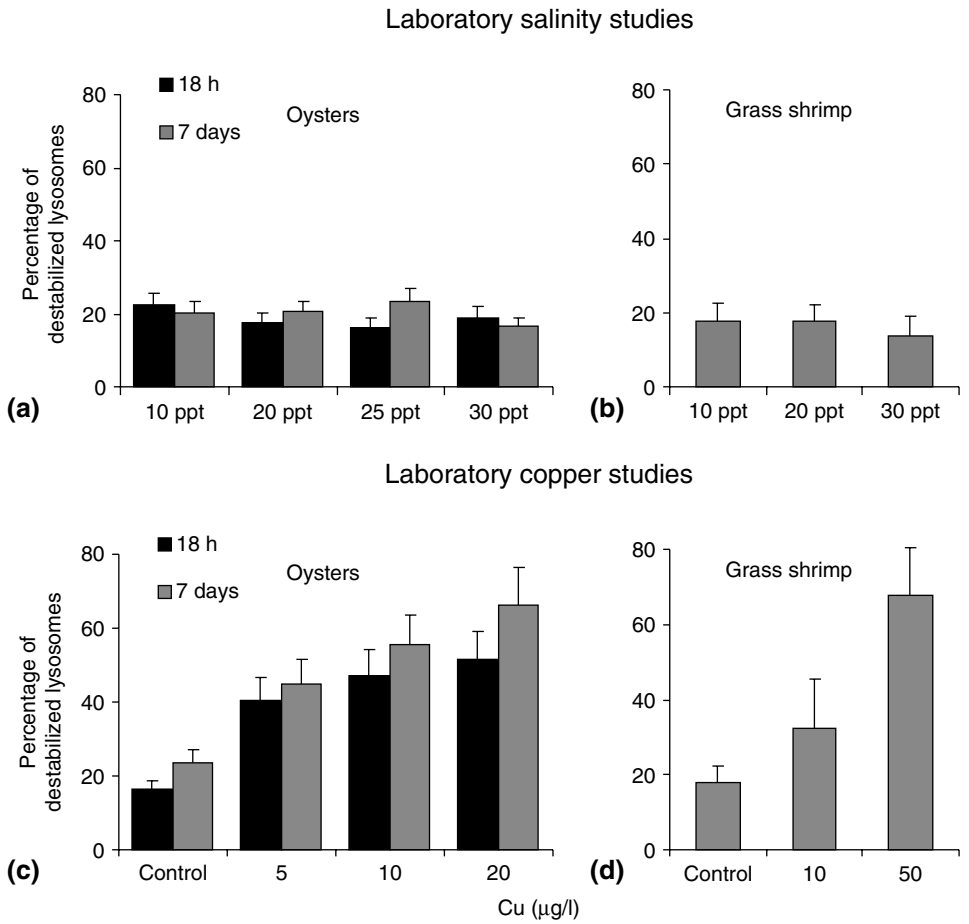

Figure 16.4 Results of laboratory studies regarding the effects of salinity on lysosomal destabilization in (a) oysters (modified from data published in Ringwood et al., 1998) and (b) grass shrimp. For the salinity studies, animals collected from 20 to 25 ppt (%) salinity regimes were placed in lower and higher salinities, and lysosomal destabilization was evaluated over time. Results of laboratory studies regarding the effects of copper exposures on lysosomal destabilization in (c) oysters (modified from data published in Ringwood et al., 1998) and (d) grass shrimp. All Cu treatments were significantly higher than controls. Data are means + standard deviations.

mollusks and grass shrimp is not significantly affected by different salinity conditions but increases dramatically when animals are exposed to toxic chemicals.

Extensive field studies with both caged oysters deployed *in situ* and organisms collected from resident populations have also been conducted. Lysosomal destabilization rates of organisms from reference sites remain low over broad salinity regimes; this is consistent with the results of the laboratory studies. Also consistent with the laboratory studies, oysters from polluted sites have significantly higher lysosomal destabilization rates as shown in Figure 16.5; similar results have been observed in oysters collected from polluted sites in Galveston Bay, Texas.[17] Therefore with oysters, field data further support the model that lysosomal destabilization is not significantly affected by salinity regimes but increases in response to contaminants. More details of field and laboratory work have been published and include additional issues, e.g., lysosomal responses were

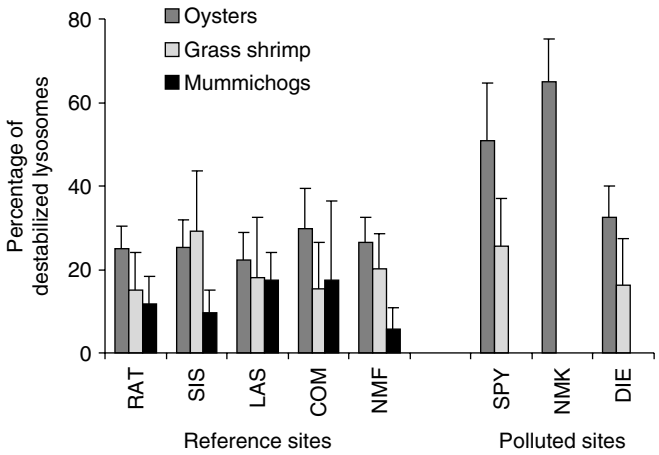

Figure 16.5 Results of lysosomal destabilization assays in field-collected oysters, grass shrimp, and mummichogs during the summer of 2000. The first five sites were classified as reference sites. The last three sites were polluted sites from Charleston Harbor, SC; the missing data for shrimp and mummichogs were due to very low collection numbers of these species in polluted systems.

significantly correlated with sediment and tissue contaminants.[15,17–19] We have seen no evidence of significant differences based on sex or size or effects of different temperature regimes. Some seasonal differences have been observed, e.g., higher lysosomal destabilization rates in the winter, but the rates are highly related to tissue metal levels, which also tend to be more bioavailable during the winter; since there are no differences at some very clean reference sites, the assay may truly reflect contaminant loads more than seasonal issues.[20]

With grass shrimp and mummichogs, the site-specific differences have been less clear-cut, e.g., organisms from polluted sites do not consistently have higher destabilization rates. Currently, there are only limited data for these species, in part, because we often had difficulty collecting shrimp and fish at polluted sites, in spite of repeated visits, different sampling techniques, etc., we caught only a few or no organisms. It could reflect that the animals have already died from the conditions or that they are mobile and have left the system. For those collected, there is generally not an easy way to know if the animals have been exposed for short or extended periods of time. Certainly, one of the advantages of working with sessile organisms like oysters and mussels is that they can not escape exposure to contaminants when they are present, so their responses reflect the integration of exposure and detoxification responses.

Overall, this body of work conducted since 1995 reinforces the value of the lysosomal assay as a valuable indicator of contaminant exposure. We have worked extensively at a range of contaminated sites, but have also focused on working at as many reference or uncontaminated sites as possible to be able to identify and characterize the variation associated with the normal ranges of responses. Using a much larger data set based on the kinds of data illustrated in the figures, we have begun to define normal ranges, as well as toxicologically significant levels, for various biomarker responses, including lysosomal destabilization. The single-time-point percentage endpoint results are highly repeatable between assays, so our interpretation can be based on a larger data base and not have to rely on specific assay controls or a limited number of reference sites. Summaries of data (means and standard deviations) can be used for estimating normal ranges for oysters,

grass shrimp, and mummichogs for lysosomal destabilization. Based on data for field-collected organisms during the summers of 1999 and 2000, compiled from 10 to 17 reference sites and >200 individuals per species, the normal ranges (means and standard deviations) were:

Oysters: 25.21 $\pm$ 4.08
Grass shrimp: 20.92 $\pm$ 10.33
Mummichogs: 16.97 $\pm$ 16.61

Overall, oyster lysosomal destabilization rates <30% are associated with reference or control conditions, e.g., normal responses of healthy oysters. These levels are believed to represent the normal background levels of lysosomal destabilization, and probably reflect a combination of low normal turnover rates of hepatic cells, as well as some low level damage associated with the assay itself. For grass shrimp and mummichogs, these levels may be a little lower, around 20%; although the variation was greater for shrimp and fish, and more work is needed for these species.

Therefore, we have some confidence with the oysters that based on the data from contaminated sites and laboratory exposure studies, as well as those under reference conditions, various toxicity criteria can be established. For example, consider the following criteria:

Oyster	Normal range	Concern	Stress
Lysosomal destablization	<15–30%	30–40%	>40%

The ''Normal range'' defines the optimum conditions and indicates that there is no evidence of stress. The ''Stress'' levels are believed to indicate that homeostatic and detoxification mechanisms have been overwhelmed, and that the oysters are significantly stressed. From our experience, these levels are consistently observed at the most polluted field sites or at the highest contaminant exposures. The ''Concern'' values represent levels that are somewhat outside the normal range limits, and should be regarded as indicating that the animals are experiencing some exposures or stressful conditions. These are levels that are often observed at moderately polluted sites. We hypothesize that lysosomal destabilization levels at or above the 40% stress level would represent serious damage that would translate into significant organismal effects. It is not difficult to comprehend that organisms with more than 40% dysfunctional hepatic cells would experience significant physiological impairment or death. Recent studies regarding relationships between gamete viability and lysosomal destabilization have supported this hypothesis, e.g., very poor rates of normal development are observed with gametes from parents whose lysosomal destabilization rates are >40%.[21] Oyster levels in both field and laboratory studies have gone as high as 60–70%, which could represent a threshold for acute toxicity. The laboratory studies with shrimp also support this. In the shrimp studies shown in Figure 16.4b, there was also a 100-ppb treatment, and the sole shrimp that survived had 70.4% destabilized lysosomes. An important question is whether or not the levels that we have defined as levels of concern reflect reversible effects if conditions improve.

It is important to consider how issues regarding statistical and biological significance may be relevant for interpretation of biomarker studies. For example, in some specific

cases, control or reference conditions may be sufficiently low and have a low variance term, so that statistical differences may be observed at levels less than the 30% normal criteria. Based on our broader experience at reference sites, lysosomal destabilization rates of <30% are not believed to represent biologically significant perturbations even though the results may be statistically significant. Therefore, we prefer to use a combination of biologically and statistically significant requirements for identifying significant toxicological effects, e.g., >30% and statistically significant from reference conditions.

Finally, as with any biomedical tool, the techniques, utility, and interpretation of biomarker responses will continue to be refined and characterized. One of the obvious future directions that are currently being explored is the development of an image analysis system for the lysosomal destabilization assays. Photographic analyses and records would provide an archival record of the assay and allow more rigorous quality control for scoring the cells. However, there are some important issues that need to be considered. For example, since the cells are held in CMFS and remain somewhat rounded and three-dimensional, it is difficult to appropriately evaluate cells by still photography. Real-time video scans of the slides with up-and-down focus would be better but can require more time (and requires higher equipment funds) than is typically needed for analyzing an individual slide; a trained reader typically can read a slide in 1–2 min, and since the assessments are with live cells, it is desirable to process the slides as quickly as possible. There have been some discussions about using the quantity of NR in cells as an index of destabilization, but it is important to realize that the distribution of NR is more important than the quantity. Cells with lots of lysosomes may accumulate a lot of NR, but as long as it is confined to the lysosomes, the functional integrity is maintained.

While there are a number of valuable reasons for using hepatic preparations, we like a number of investigators have used these techniques with circulating hemocytes and blood cells. Circulating hemocytes of bivalves and crustaceans are particularly relevant immunologically. Bivalve hemocytes may be sampled either destructively via intracardial puncture or nondestructively by withdrawing hemolymph from the adductor muscle. Nondestructive sampling techniques (i.e., the organism is not killed) have a variety of advantages, including the allowance for repeated sampling on a single individual and sampling of rare or threatened species.[22] Good candidates for these approaches include the freshwater Unionid mussels, some of which are among the most imperiled fauna in North America.[23] Moreover, adapting these techniques for fresh water species should serve to improve our ability to monitor and manage those groups, as well as estuarine species.

In summary NR techniques are relatively simple techniques for evaluating lysosomal function and organismal health that can be used in a variety of organisms. The ability to interpret the results requires a substantial effort under non-polluted conditions in order to build a sound database for defining criteria associated with toxicological effects. It should be appreciated that these ranges represent a combination of ''scientific art'' that draws on knowledge of mechanisms and experience, as well as statistical rigor and lots of samples. More data, from both laboratory and field studies, are needed for grass shrimp and mummichogs. With oysters, the considerable laboratory and field efforts have enabled us to conduct assessments and begin to interpret the results with a high degree of certainty (it has been used recently for oil spill, superfund, and other habitat assessments). It has a number of valuable attributes, including relatively inexpensive, rapid, and is believed to provide high value information regarding organismal health. Broader use of cellular biomarkers should improve our ability to monitor and manage important estuarine species, and protect these important habitats.

Acknowledgments

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chapter seventeen

IMCOMP-P: An assay for coral immuno-competence

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Contents

Introduction

Corals have recently been afflicted with epizootic outbreaks in the Caribbean, the Great Barrier Reef, and in the Indian Ocean.[1,2] These disease outbreaks have caused mortality rates as high as 90% on some reefs.[1] Only recently have some of the pathogens been identified.[1,3,4] *Vibrio shiloi* or similar species/strains[5] can cause bleaching or rapid tissue lysis in corals. Recent evidence indicates that *Serratia marcescens* causes white pox on corals.[4] Some evidence suggests that environmental factors may alter pathogen physiology, inducing a more infectious or pathogenic state[3] or alternatively that environmental conditions compromise coral defense mechanisms, rendering them more susceptible to infection. Unfortunately, little is known of coral defense systems (immunology), other than having allorecognition[6] and phagocytic cells.[7,8] Microbial pathology of coral disease is an issue that

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continues to baffle scientists and resource managers. Is the disease occurring because of an introduction of a novel pathogen into the environment, addition of abiotic factors that induce pathogenicity (e.g., increased iron availability or increased temperature), or factors influencing a decrease in cnidarian immuno-competence? Resolving this issue is paramount in effectively understanding and managing coral disease outbreaks.

Innate immunity and antimicrobial peptides

Innate immunity is an ancestral defense system found in all multicellular organisms (including plant and animal phyla) and is considered the first line of defense against invading microorganisms. In contrast to the vertebrate's adaptive immunity with its complex network of humoral and cellular responses, the innate immune system, in general, is characterized by three types of processes: (1) phagocytosis, (2) initiation of proteolytic cascades, or (3) synthesis of antimicrobial peptides (AMPs).[9]

Due to their rapid production and diffusible nature, AMPs compose one of the most important elements in lower invertebrate defense systems.[10] The discovery of the first AMPs, gramicidin and tyrocidine,[11] catalyzed a whole discipline of research that has made an impressive progress in the past 60 years. Antimicrobial peptides are distributed throughout the entire animal kingdom. Some AMPs are as small as three amino-acid residues long, while others are as large as 45 kDa.[12] AMPs are diverse in structure and function[13] with some AMPs permeabilizing cell membranes or blocking ion channels, while others inhibit metabolic electron transport or protein chaperoning.[14,15]

Anti-microbial peptides have distinct secondary structures that lend potency to their function and are commonly categorized into four groups according to their secondary structure (Table 17.1).[16] AMP structures often contain a region of cationic (i.e., positively charged) amino acids. This cationic region allows the AMP to bind to negatively charged microbial membranes, providing a simple but effective targeting system. In non-polar solvents, such as microbial membranes, AMPs often form separate hydrophobic and hydrophilic domains, making AMPs *amphipathic* proteins.

There are several mechanistic models that describe how AMPs function.[16] The exact mechanism varies from AMP to AMP depending on the mode of antimicrobial action. The barrel-and-stave model used to describe the activity of some AMPs consists of the AMPs aggregating into barrel-like structures within the microbial membrane to form pores in the membrane (Figure 17.1, Panels A–C).[10,16] These barrel structures will increase in size as more AMPs bind to the membrane, increasing both the frequency and size of pores. These pores begin a process of diffusion of vital intercellular components from the microbe and culminate in cell death. The aggregate channel model, which describes the activity of AMPs, such as histatin 5, is similar in that the AMPs cause the formation of small transient pores in the membrane.[16] However, the function of these pores is not to let vital components out as in the barrel-and-stave model. Instead, the pores facilitate the invasion of the microbes by the

Table 17.1 Common classification of antimicrobial peptides according to structure (Adapted from Olano, C.T. and Bigger, C.H., *J. Invert. Pathol.*, 76, 176–184, 2000.)

Group	Structure
I	Linear peptide with an α helical structure
II	Linear peptide with high representation of specific amino acids
III	Peptides with loops
IV	Peptides that contain β-strands or other structural restraints

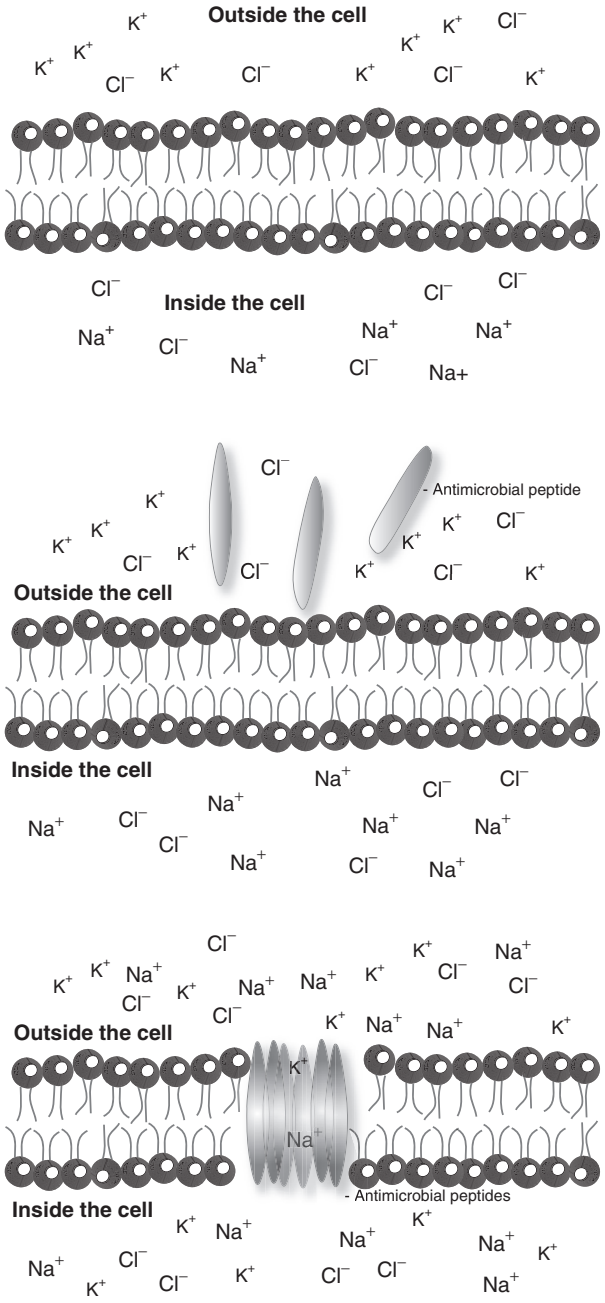

Figure 17.1 Panel (a) Cell membranes function to create a barrier between solutes outside the cell and solutes inside the cell. There are cellular pumps in the cell membrane that maintain this difference in solute composition and concentration on either side. Panel (b) AMPs (anti-microbial peptides) are produced by the coral and significantly contribute to the protein composition in coral mucus. AMPs can also be found within the cell, and within the extracellular space that exists between coral cells. Panel (c) Some types of AMPs can aggregate together and ''punch'' holes in the bacterial cell membrane, but not in the coral or zooxanthellae membranes. These AMPs create channels across the bacterial cell membrane.

AMP causing the pore formation. It is unknown if the pores are utilized by other AMPs to enter the cell. Once inside the microbe, the AMPs are hypothesized to inhibit a variety of cellular processes, from DNA expression and synthesis to protein folding to intercellular signaling events, resulting in inhibition of growth or death for the microbe.[16] In both of these models, the amphipathic structure of the AMPs is a key element to their function.

IMCOMP-P assay

To advance scientific research into coral immunity and coral epidemiology, we have developed an easy to use, inexpensive, quick, and accessible assay that is functional and quantitative. This assay measures one aspect of coral innate immunity, permeability of bacteria by a protein fraction of coral tissue: IMCOMP-P (immuno-competence-permeability). This assay is based on two fluorescent DNA probes, each having different accessibilities to microbial DNA. The total soluble protein fraction is extracted from the coral sample. The protein fraction is precipitated with acetone, and resolubilized in a non-denaturing buffer. This homogenate is filtered through a 10,000 molecular-weight cut-off filter, and the collected filtrate contains an abundance of putative coral AMPs. This filtrate is added to a normalized concentration of bacteria, and incubated for 30 min at room temperature. A solution containing two different probes are added to the bacterial culture. The green probe (SYTO 9™) is permeable to the bacterial membrane and can readily bind bacterial DNA, thereby fluorescing. Only if AMPs are present in the coral filtrate, thus permeabilizing the bacterial cell, can the red probe (propidium iodide) pass through the bacterial membrane. Once the red probe is within the bacterial cell, it will preferentially bind to the bacterial DNA and displace the DNA binding of the green probe, thereby quenching the green probe's fluorescent abilities. Thus, a decrease in the fluorescence of the green probe indicates the presence and action of AMPs (Figure 17.2 Panels A–D). This assay can be adapted to any bacterial, fungal, or algal species and requires only altering the bacterial growth conditions and using a different DNA binding fluorescent probe.

Materials required

Reagents:

- Propidium iodide from Molecular Probes (catalog #3566, Molecular Probes, OR, USA). A stock solution of 20 m*M* propidium iodide (Component A) in dimethyl sulfoxide (DMSO) was used for the assay
  - SYTO 9™ from Molecular Probes as a stock solution of 3.34 m*M* in DMSO (Component B; catalog #7012)
  - DMSO
  - Guanidine HCL, EM Sciences
  - Trizma base, EM Sciences
  - EDTA, EM Sciences
  - Deferoxamine mesylate, Sigma-Aldrich
  - Phenylmethylsulfonyl fluoride (PMSF), Sigma-Aldrich
  - Acetone, EM Sciences
  - Sorbitol, Sigma-Aldrich
  - Millipore Microcon diafiltration tube with a molecular weight cutoff of 10,000 Da

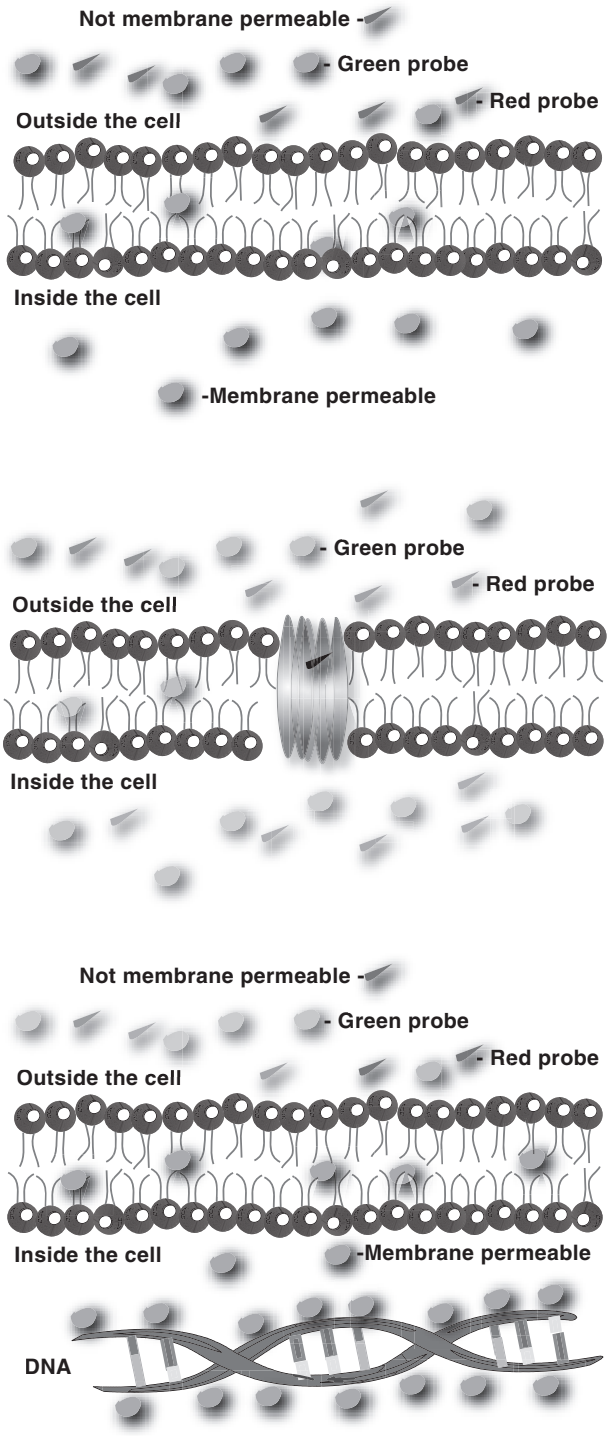

Figure 17.2 *Continues*

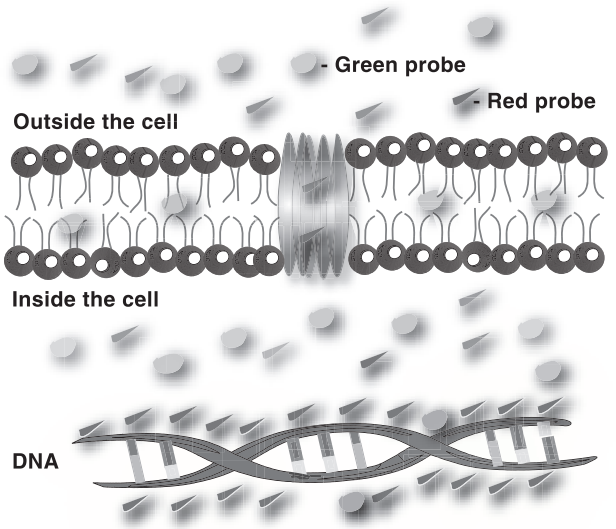

Figure 17.2 *Continued* Panel (a) Two different fluorescent probes are added to bacteria grown in culture, a green fluorescent probe and a red fluorescent probe. The green fluorescent probe is permeable to bacterial cell membranes; the red fluorescent probe is not. These two probes will *only* fluoresce when bound to DNA. Panel (b) The green fluorescent probe crosses the bacterial cell membrane and binds to the bacterial DNA. Once bound, the green fluorescent probe will emit light, or fluoresce. The red probe, which cannot bind to the bacterial DNA, does not fluoresce. Panel (c) Extract from healthy corals that have an abundance of AMPs will permeabilize the bacterial cell membrane, allowing the red fluorescent probe to diffuse into the bacterial cell. Panel (d) Once inside the cell, the red fluorescent probe will exclude the green fluorescent probe from binding to the bacterial DNA. This will do two things: (1) decrease the fluorescence emitted by the green fluorescent probe, and (2) once bound to the DNA, the red fluorescent probe will fluoresce.

- Tryptic soy broth
- Isopropanol
- *S. marcescens* cultures (American Type Culture Collection; www.atcc.org)
- *Supplies*:
- Sterile culture test tubes (8 ml)
- Sterile tooth picks
- Clear-bottom 96-well microtiter plates
- Micropipette tips (20, 200, and 1000 μl)
- Micropipettors (10, 200, and 1000 μl)
- Microcentrifuge tubes

Equipment:

- Vortex mixer
- pH meter
- Analytical balance
- Microcentrifuge tubes
- Temperature-controlled shaker
- Microplate Fluoresence Reader

Method

Sample preparation:

1. All coral samples should be ground frozen to a fine powder in a mortar and pestle using liquid nitrogen. About 200 mg of frozen coral powder is placed in a locking microcentrifuge tube.
2. To each tube, 1.4 ml of denaturating buffer 140 (DB140) containing 4 *M* guanidine HCl, 10 m*M* EDTA, 50 u*M* deferoxamine mesylate, and 1 u*M* PMSF were added. Tubes were vortexed for 30 s and then incubated at 40°C for 20 min, vortexed every 5 min.
3. Tubes are then centrifuged at 14,000 *g*, for 15 min.
4. After centrifugation, 300 ul of the middle phase, with care to avoid all lipo-sulfopolysaccharides, are aspirated and placed in a new tube.
5. Acetone that had been chilled at –80°C for over 1 h is added to the aspirant at a 4:1 ratio (v/v; acetone/aspirant).
6. Vortex the tubes for 20 s, and then incubate the tubes at –80°C for 1 h.
7. After the incubation, the tube is given a quick vortex then centrifuged at room temperature for 15 min at 14,000 *g*.
8. The acetone fraction is aspirated and discarded, and the pellet air-dried for 15 min at 36°C.
9. The dry pellet is then solubilized in a buffer containing 50 m*M* Tris/HCl (pH 8.5) and 50 u*M* sorbitol. Solubilization of the pellet may take between 10 min to over an hour, but the pellet must be completely solubilized. Mixing with a pipettor may accelerate the rate of solubilization.
10. Tubes are centrifuged for 5 min at 14,000 *g*.
11. The supernatant is transferred into a Millipore Microcon diafiltration tube with a molecular weight cutoff of 10,000 Da.
12. Filter tubes with sample are centrifuged at 6000 *g* for 8 min. The filtrate was collected into a new tube.
13. Determine the protein concentration of the filtrate. We suggest the protein concentration method of Ghosh et al. 1988 (see Chapter 10, this volume), though other methods may be used because the acetone precipitation step should have removed all interfering carotenoids.
14. Samples can be stored at –20°C for 1 week.

Microbial conditions

Bacteria used in the IMCOMP assay should be growing exponentially in tryptic soy broth. A growth curve must be generated to determine the range of OD_{640} that correlates to exponential growth. To generate the growth curve, incubate a 2-ml culture of *S. marcescens* in tryptic soy broth as below. Determine and plot OD_{640} every 2 h. The exponential fraction of the resulting sigmoid curve will determine the OD_{640} range for exponential growth. Use only bacteria within this OD_{640} range to generate the OD_{640} 0.157 stock suspension.

- To a sterile culture tube with a sterile stopper, add 2 ml of tryptic soy broth.
- With a sterile toothpick, dab a single colony of *S. marcescens* that had been plated and cultured on standard microbial agar media.

- Drop the toothpick into the culture tube with the tryptic soy broth, stopper the tube.
- Place tube in a temperature-controlled shaker (temperature set at 36°C) and incubate for at least 12 h until cloudy.
- After the culture has reached an appropriate amount of turbidity, generate a 1:10 dilution by taking 100 ul of culture and adding it to 900 ul of tryptic soy broth. Determine OD_{640}.
- Calculate the necessary volume of culture required to make sufficient 0.157 OD_{640} stock suspension for assay, i.e., 2 ml of 0.785 OD_{640} is required to generate 10 ml of 0.157 OD_{640}. Continue incubation and OD_{640} determination until the required OD_{640} is cultured.
- Centrifuge the exponentially growing cells at 10,000 rpm for 10 min.
- Decant the tryptic soy broth, resuspend the pellet in 1× PBS and diluted to 0.157 OD_{640} to generate a stock suspension of *S. marcescens* for the propidium iodide/SYTO 9 competition assay (see the following). A standard curve for this assay also requires dead *S. marcescens* cells. To generate the dead cell stock solution, *S. marcescens* cells were spun down at 10,000 rpm for 10 min and resuspended in 70% isopropanol for 1 h. The isopropanol-killed *S. marcescens* were then pelleted again, resuspended in water, and diluted to 0.157 OD_{640}.

Standard live/dead curves

- Mix stock solutions of live and dead *S. marcescens* in ratios of 100:0, 90:10, 50:50, 10:90, and 0:100 of live and dead bacteria.
- Aliquot 35 ul of each ratio into a 96-well plate. To account for possible artifact of ''plate'' effects, aliquot a replicate of the live dead curve in columns 1, 5, and 9 on the 96-well plate.
- Add 20 ul of water to each sample well to bring the final OD_{640} to 0.1.
- Add 55 ul of the fluorescent stock solution, incubating the cells on a platform shaker in the dark for 20 min. Take special care not to expose the sample to light before reading fluorescence to avoid bleaching of the fluorophores.
- Read the microtite plate using Flx800 Microplate Fluorescence Reader (Bio-Tek Instruments) using KCJunior software (v. 1.31.2, Bio-Tek Instruments). Excitation was 485 nm and emission was 528 nm for Component A and 485 nm excitation and 620 nm emission for Component B (Figure 17.3).

Experimental curves

In a 96-well plate, add 35 ul of live *S. marcescens* ($OD_{640} = 0.157$), 15 ul of water, and 5 ul of coral extract was added to each well, for a final $OD_{640} = 0.1$. To account for sample variation, each coral extract and bacteria sample must be repeated on the plate in triplicate. To minimize variability due to plate positioning, the placement of each sample should be randomized as much as possible. Finally, inclusion of a standard live/dead curve, in triplicate, on each experimental plate will minimize the variability when comparing results from plate to plate.

Incubate the samples on a platform shaker at 30°C for 1 h to allow cells to recover from the stress of sample preparation. Remove the samples from the shaker, and add 55 ul of 0.6% fluorescent stock solution to each well. Incubate the plate on a rocker at room

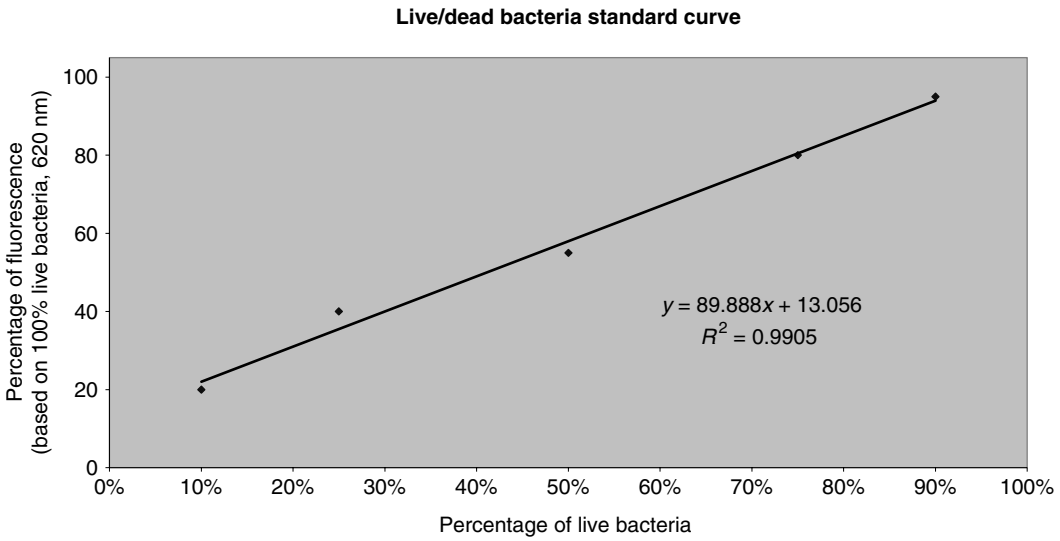

Figure 17.3 Live/death curve. Bacteria incubated with isopropanol for 1 h, then mixed in proportions to live bacteria. Mixed bacteria are then incubated with the probe solution, incubated for 15 min at room temperature. Bacteria are then plated on a 96-well microtiter plate, and read on a fluorescent plate reader at the appropriate excitation/emission wavelengths.

temperature in the dark for 20 min. Taking care to avoid light exposure, read the sample fluorescence as stated above.

Results and discussion

IMCOMP-P was developed to address certain deficiencies in discerning the nature and processes of coral pathology. Reports of disease outbreaks in corals have increased in the past ten years.[17] The Caribbean is notorious for extensive outbreaks of black-band and white-plague disease.[1] In the Indian Ocean, there are increasing reports of a ''red plague'' of an unknown etiology that can kill coral tissue along a front at a rate of 10–15 cm a day, and as fast at 35 cm a day (Dr. R. Jeyabaskaran, personal communication). Are these recent outbreaks the result of the introduction of opportunistic pathogens, changes in the environment that affect pathogen physiology, or changes in host immunity? Changes in host immunity can be the result of host age, nutritional status, seasonal physiology (i.e., spawning seasons), cost of intra- and inter-species competition, and environmental pressures. Because of the alarming declines of coral reefs in the Caribbean and around the world and of the increase of anthropogenic factors associated with these declines (i.e., agricultural run-off, water-shed alterations, residential and urban development), understanding the relationship between environmental factors and coral health is becoming vitally important.

Invertebrate physiology relies solely on innate immunity as a means of protection against microorganisms. IMCOMP-P is one of several technologies we have developed to better define cnidarian immunology. IMCOMP-P assesses the antimicrobial activity of small antimicrobial peptides or compounds that have a molecular weight smaller than 10,000 Da. In the format described in this chapter, IMCOMP-P cannot determine the source of the antimicrobial compounds (host versus dinoflagellate versus resident

microbial community). Many of the antimicrobial peptides and algal phenolic secondary compounds can cause permeablization of the microbial cell membranes, either by altering membrane–protein channel pores or creating channels.[16] IMCOMP-P capitalizes on the characteristics of two fluorescent probes that have different affinity constants to DNA and different membrane permeability constants. SYTO 9™ is permeable to bacterial cell membranes, while propidium iodine (PI) is not permeable to the cell membrane. If the integrity of the cell membrane is compromised, PI can enter the cell and compete for binding of DNA with SYTO 9™. Because PI has a higher binding affinity, it can outcompete SYTO 9™ for DNA binding sites. Hence, SYTO 9™ is excluded from binding to DNA, and the fluorescence signal of SYTO 9™ will decrease proportionally with the permeabilization of bacterial cell membrane. Therefore, an increase in antimicrobial membrane permeabilization peptides or compounds will decrease the fluorescence of SYTO 9™ fluorescence.

To test the applicability of this assay to different species of coral and preliminarily investigate the possible effects of relevant environmental factors on coral immunity, we examined the treatment effects of hydrogen peroxide on *Porites porites*, endosulfan on *Pavona gigantea*, and Irgarol 1051 on *Madracis mirabilis*.

Rates of oxidative damage increase in coral experiencing elevated sea-surface temperatures, hypo-salinity, high-light stress, and high ultraviolet light exposure. Exposure to exogenous hydrogen peroxide (H_2O_2) can induce intracellular oxidative damage because of the permeability of H_2O_2 to membrane lipid bi-layers.[18] Oxidative stress shifts the equilibrium of a cell's protein metabolic condition, by denaturing enzymes and proteins, decreasing transcriptional and translational efficiency, and altering primary amino-acid structure through mutagenesis.[19] Data in Figure 17.4 supports the hypothesis that increased oxidative stress decreases immuno-competence, either by decreasing antimicrobial compound production and/or decreasing exocytosis rates.

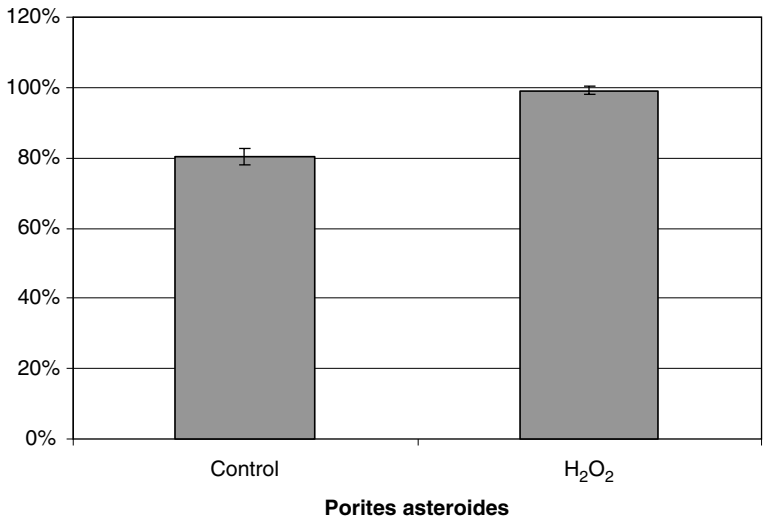

Figure 17.4 The percent of bacteria alive after a 1-h incubation with coral protein extract from *Porites porites* exposed to hydrogen peroxide for 8 h and controls. Exposure to H_2O_2 significantly decreased AMP activity, potentially making the coral more susceptible to infection ($p < 0.05$). $N = 3$ per treatment.

Endosulfan is an agricultural pesticide used world-wide, especially in districts whose watersheds spill into regions of coral reefs (i.e., Great Barrier Reef, Belizian Barrier Reef, Florida Keys). Exposure to endosulfan decreased the antimicrobial activity of corals (Figure 17.5). Endosulfan inhibits cholinesterase activity and induces massive oxidative damage in invertebrates.[20] The half-life of endosulfan is longer in marine environments than in aquatic environments.[21] Exposure of 100 u*M* endosulfan in *Pavona* and in *Montastrea annularis* induces extensive oxidative stress, decreasing protein metabolic condition, metabolic homeostasis, and increasing destabilization of genomic integrity [Downs et al., manuscript in preparation (*Pavona*); Downs et al., preliminary results (*Montastraea*)]. Endosulfan could be reducing immuno-competence via the oxidative stress mechanism as seen in *Porites* exposed H_2O_2, or it could also be reducing immuno-competence as an inhibitor. Endosulfan is a sulfonated chlorinated phenol, which can be oxidized either metabolically or by photolysis to create a chlorinated phenol with an active thiol group. A number of AMP classes have abundant cysteine residues whose thiols are functional groups. Thiolated endosulfan could bind to these thiol groups on the AMPs, thereby inhibiting activity.

Irgarol 1051 is an herbicide component used in boat antifoulant paints. Work from Owens and colleagues demonstrated that 100 ppb Irgarol in seawater can significantly reduce net photosynthesis of coral dinoflagellate symbiont after an 8-h exposure.[22,23] Further studies into the effects of Irgarol on both host and dinoflagellate symbiont cellular physiology show that an 8-h exposure to 10 ppb Irgarol can significantly reduce host levels of catalase and ferrochelatase, indicating a decrease in antioxidant capacity, as well as possible decrease in host immuno-competence (Owens et al., in preparation). Because of increasing concentrations of Irgarol in waters associated with recreational marinas near or on coral reefs, Irgarol may be considered a possible risk factor for compromising coral immuno-competence. However, in these preliminary experiments, there was no significant difference in antimicrobial activity between control and Irgarol exposed coral in the IMCOMP-P assay (Figure 17.6). This finding should not however be interpreted as Irgarol 1051 having no effect on coral immunity, but rather that IMCOMP-P does not assay all coral immune system components and other components may indeed be affected by this compound.

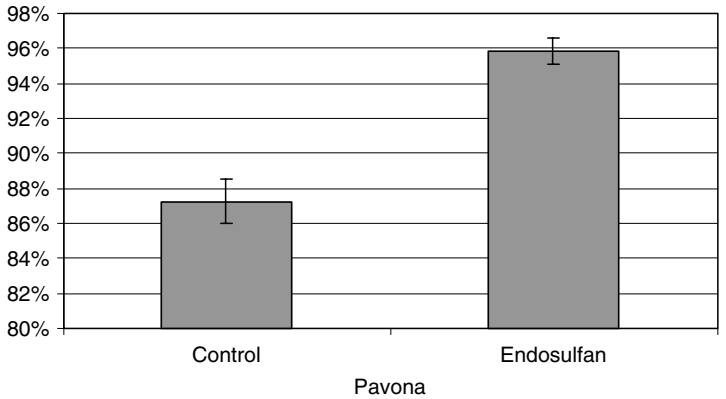

Figure 17.5 The percent of bacteria alive after a 1-h incubation with coral protein extract from *P. gigantea* exposed to an 8-h exposure of 100 u*M* endosulfan. Exposure to endosulfan significantly decrease AMP activity ($p < 0.05$). $N = 4$ per treatment.

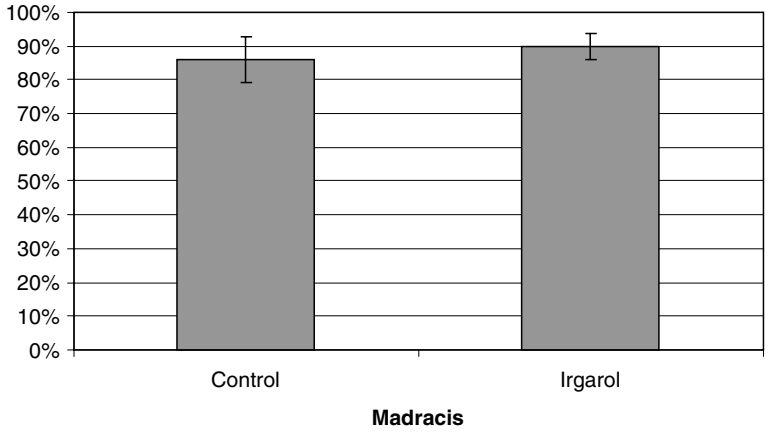

Figure 17.6 The percent of bacteria alive after a 1-h incubation with coral protein extract from *Madracis* controls and exposed to 10 ppb Irgarol 1051 for 24 h. There was no significant difference between control and coral exposed to 10 ppb Irgarol 1051 for 24 h. $N = 3$ per treatment.

Aspects of this assay and its protocol can easily be modified to meet end-user's needs. Instead of acetone precipitation, an acid or salt precipitation could be used to ensure better precipitation of more hydrophilic, short polypeptides that might remain soluble in a partial acetone environment. Care must be taken though, with a trichloroacetic acid or trifluoroacetic acid precipitation because of potential acid hydrolysis of polypeptides. For many samples, the final protein concentration may be too dilute to properly determine; hence, samples can be concentrated via vacuum centrifugation or lyophilization. We used extracts of whole coral tissue for antimicrobial activity. It may be possible to use just the mucus produced from the coral to determine the level of antimicrobial activity in coral, thereby avoiding tissue damage created from the biopsy, as well as alleviating the need to obtain collection permits.

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chapter eighteen

Monitoring gene expression in Rana catesbeiana *tadpoles using a tail fin biopsy technique and its application to the detection of environmental endocrine disruptor effects in wildlife species*

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University of Victoria

Contents

Introduction

Wildlife species are being exposed to an ever-increasing number of chemical compounds that are generated as byproducts of human activity.[1–4] These chemicals encompass a large group of environmental pollutants that include pharmaceuticals, pesticides, herbicides, and industrially relevant chemicals. Many of these compounds, while not overtly toxic, have the potential or have been demonstrated to interfere with the normal growth and development of a variety of species and could directly interfere with regulatory endocrine systems.[3–6] Exposure to such chemical contaminants during critical life stages could result

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in permanent deleterious effects to the reproductive potential[7] or physiological fitness[8,9] of species at risk. Of great concern is the increasing usage of these potential endocrine disrupting chemicals (EDCs) and a concomitant rise in their concentrations in surface and groundwater sources within the environment.[10–13]

With the number of chemicals registered for commercial use in the United States alone estimated to exceed 70,000 (Toxic Substances Control Act Inventory; U.S. E.P.A., www.epa.gov/opptintr/opptdiv), a toxicity assessment method must be applicable to a rapid, high throughput experimental format and allow for sample collection in both laboratory and field settings. While mortality and morphometric analyses have proven useful in the characterization of toxic exposure to chemical contaminants, such endpoints are limited in their sensitivity and processivity. In addition, design of non-lethal, minimally invasive procedures for sample collection and assessment of contaminant exposure is warranted for endangered or threatened species.[14–18] The techniques employed must also provide an increased predictive ability and be amenable to regulatory standardization. To this end, we have developed a biopsy procedure combined with genetic biomarker analysis that can be performed on live animals. This technique takes advantage of advances made in the stabilization of tissue samples prior to experimental processing and allows for sample collection in the field using an animal capture-and-release methodology. The use of the biopsy procedure standardizes the amount of tissue to be analyzed, thereby helping to minimize sampling variance and can be applied to a high throughput screening process. The introduction of genetic biomarkers as an analytical endpoint allows for early detection of chemical contaminant exposure effects prior to overt morphometric changes during development.[5] Comparison of the resulting gene expression information from test versus reference sampling locations can help in the determination of whether individual species have been exposed to potentially deleterious substances.

To demonstrate the utility of the biopsy procedure, we have collected tissue samples from North American bullfrog, *Rana catesbeiana*, tadpoles undergoing natural metamorphosis and from premetamorphic tadpoles exposed to exogenous thyroid hormone (TH). In addition, the effects of exposure to the potential EDC, acetochlor, on TH-induced metamorphosis of live animals were assessed. Biopsies were obtained from tadpole tail fins under three different scenarios: from the dorsal tail region of live animals, from tadpoles euthanized immediately after experimental exposures, and from cultured tadpole tail tips. Steady-state levels of TRβ mRNA, a sensitive and early biomarker of the TH-induced metamorphic genetic program,[5,17] were determined using reverse transcription followed by real-time quantitative polymerase chain reaction (QPCR) analysis. Such genetic assessment in conjunction with morphometric and population analyses can contribute to the identification of potentially hazardous chemical contaminants in the environment that disrupt normal developmental processes. The utility of the biopsy procedure across a variety of wildlife species is also discussed.

Materials required

- *R. catesbeiana* (Taylor–Kollros (TK) Stages VI–VIII, collected locally)
- Sterile disposable 2-mm dermal punch (catalog #162-33-31, The Stevens Company, www.stevens.ca)
- Extra fine-point curved dissecting forceps (catalog #25607-890, VWR International, www.vwr.com)
- 20 cm × 20 cm plastic Plexiglas plate
- Capture net

- Paper towel
- Protective latex gloves
- 1.5-ml sterile safe-lock plastic sample tubes (catalog #0540225, Fisher Scientific, www.fisherscientific.com)
- Tissue preservative (RNAlater, catalog #7021, Ambion, www.ambion.com)
- Tissue homogenization reagent (TRIzol, catalog #15596-018, Invitrogen Life Technologies, www.invitrogen.com)
- Retsch MM300 mixer mill (catalog #85110, QIAGEN, www1.qiagen.com)
- 2 × 24 position shaker racks (catalog #69998, QIAGEN, www1.qiagen.com)
- 3-mm tungsten-carbide homogenization beads (catalog #69997, QIAGEN, www1.qiagen.com)
- Glycogen (catalog #901-393, Roche Molecular Biochemicals, www.biochem.roche.com)
- $pd(N)_6$ random hexamer oligonucleotide (catalog #27216601, Amersham Biosciences, www1.amershambiosciences.com)
- Moloney murine leukemia virus (MMLV) RNase H^- reverse transcription kit (Superscript II reverse transcriptase, catalog #18064-014, Invitrogen Life Technologies, www.invitrogen.com)
- Ribonuclease inhibitor (catalog #15518-012, Invitrogen Life Technologies, www.invitrogen.com)
- Thermopolymerase (Platinum Taq DNA polymerase, catalog #10966-034, Invitrogen Life Technologies, www.invitrogen.com)
- Deoxynucleotide set (catalog #10297-018, Invitrogen Life Technologies, www.invitrogen.com)
- SYBR Green I fluorescent DNA-binding dye (catalog #S-7563, Molecular Probes, www.probes.com)
- ROX passive reference dye (catalog #600536, Stratagene, www.stratagene.com)
- MX4000 quantitative real-time thermocycler system (catalog #401260, Stratagene, www.stratagene.com)
- Sequence-specific DNA primer sets (see Table 18.1)

Procedures

Tissue biopsy

Tissue biopsies are taken from the dorsal tail fin of whole animals or from the fin region of dissected and cultured tail tips using a dermal punch that isolates a 2-mm diameter tissue sample. For amphibian organ culture conditions, see Reference 19. During collection of

Table 18.1 Primer sequences and target *R. catesbeiana* genes used in DNA amplification

Gene target	Genbank accession #	Amplicon size (bp)	Primer sequences
TRβ	L27344	538	UP 5′-AGCAGCATGTCAGGGTAC-3′ DN 5′-TGAAGGCTTCTAAGTCCA-3′
16S ribosomal RNA	M57527	533	UP 5′-AGAAGGAACTCGGCAAAT-3′ DN 5′-CCAACATCGAGGTCGTAA-3′
ribosomal protein L8	AY452063	270	UP 5′-CAGGGGACAGAGAAAAGGTG-3′ DN 5′-TGAGCTTTCTTGCCACAG-3′

tissue samples from live tadpoles, the biopsy is taken quickly in order to minimize stress to the animals. Tadpoles are placed briefly on a paper towel positioned on top of a plastic Plexiglas biopsy support plate. Each live animal must be gently immobilized body-first into the palm of the hand to prevent sudden movement that could result in tearing of the biopsy region. Figure 18.1A This is accomplished by one investigator immobilizing the animal, while a second investigator retrieves the biopsy sample. Figure 18.1B Up to four biopsies can be obtained from the dorsal fin of a live premetamorphic (TK Stages VI–VIII; Reference 20) *R. catesbeiana* tadpole allowing for time-course studies with a repeated measures design (Figure 18.1C). Due to the membranous nature of the tadpole tail fin sampled, biopsies collected as described do not cause any observable discomfort for live animals or affect their swimming behavior. Tissue biopsy collection from dissected and cultured tail samples are performed in a disposable plastic weigh-boat. Approximately two fin biopsies are available from a cultured 2-cm tail tip (Figure 18.1D). All collected samples are immediately immersed in the RNA preservative, RNAlater and stored at 4°C for up to a few months prior to RNA isolation.

Tissue homogenization

Homogenization of the biopsies was performed using the TRIzol reagent as described by the manufacturer. Mechanical disruption of tadpole tail fin tissue utilized 100 μl TRIzol reagent, a 3-mm diameter tungsten-carbide bead, and safe-lock Eppendorf tubes in a

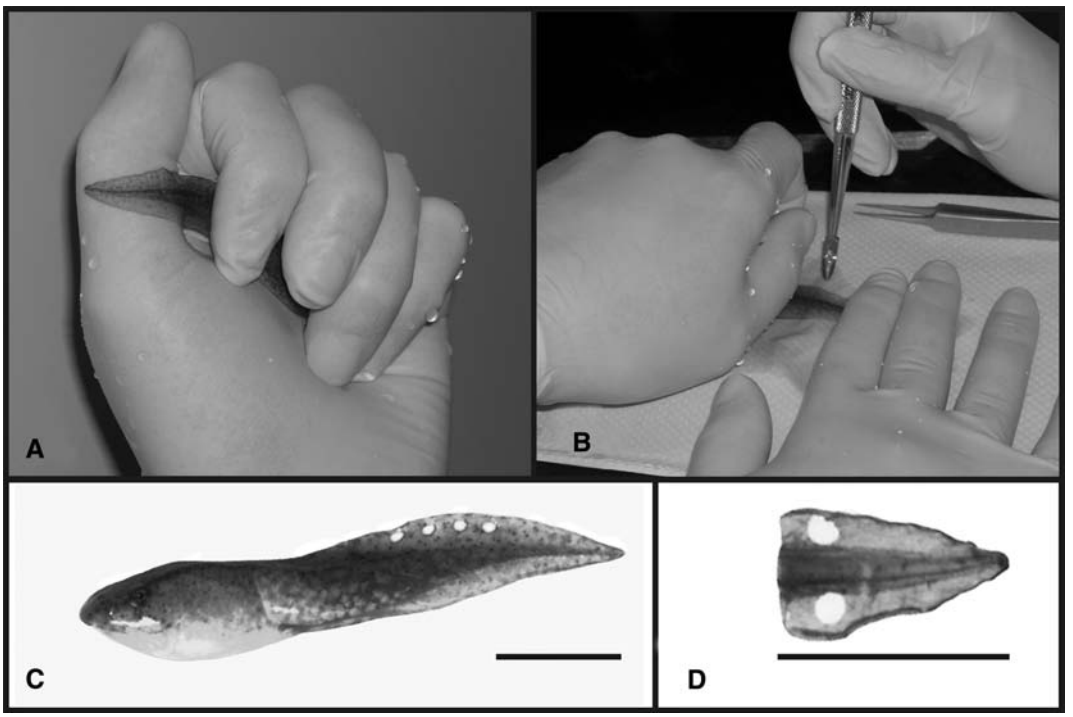

Figure 18.1 Collection of *R. catesbeiana* tail fin tissue using a biopsy procedure. Tissue for genetic analyses can be collected from live animals handled in an appropriate manner (A and B). The location of sampled tissue is shown within the dorsal tail region of live tadpoles (C) or from cultured tadpole tail tips (D). Bars in C and D represent a 2-cm length.

Retsch MM300 mixer mill at 20 Hz for 6 min. Mixing chambers are rotated 180° halfway through the homogenization procedure. Following phase separation, 20 μg glycogen was added as a nucleic acid carrier. Isolated RNA was subsequently resuspended in 10 μl of diethyl pyrocarbonate (DEPC)-treated RNase-free water and stored at −70°C. RNA is very sensitive to conditions that promote degradation. To maintain the integrity of RNA samples, ensure that proper laboratory methods that minimize potential RNase contamination are employed during the isolation procedure.[21] All plasticware used, such as pipette tips and sample tubes, should be guaranteed RNase-free.

Preparation of cDNA

A typical RNA yield was 1 μg per tail fin biopsy, and all of this RNA was annealed with 500 ng $pd(N)_6$ random hexamer oligonucleotide at 65°C for 10 min followed by a quick cool-down on ice. RNA was converted to cDNA using 200 units of MMLV RNase H^- Superscript II reverse transcriptase as described in the manufacturer's recommended protocol. The 20-μl reaction was incubated at 42°C for 2 h and diluted 20-fold prior to DNA amplification.

DNA amplification

All DNA amplification primers were designed with Primer Premier Version 4.1 software (Premier Biosoft International, www.premierbiosoft.com) and synthesized by AlphaDNA (www.alphadna.com) (Table 18.1). Amplification of specific cDNA targets was performed using real-time QPCR performed on an MX4000 system as described by the manufacturer. One major advantage of QPCR is a non-reliance on collection of endpoint expression data. Information associated with a given DNA amplification reaction is collected during each thermocycle, and differences in gene expression can be determined using the highly sensitive early phase of the amplification process. All reagents are prepared in distilled water, which has been autoclaved. The 15 μl QPCR reaction contained 10 m*M* Tris–HCl pH 8.2 at 20°C, 50 m*M* KCl, 3 m*M* $MgCl_2$, 0.01% Tween-20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I, 200 μ*M* dNTPs, 83.3 n*M* ROX reference dye, 10 pmol of each primer, 2 μl of diluted total cDNA, and 1 unit of Platinum Taq DNA polymerase.[5] The thermocycle program for all target DNA sequences under investigation included a denaturation step at 95°C (9 min); 40 cycles of 95°C (15 s), 55°C (30 s), 72°C (45 s), and a final elongation step at 72°C (7 min). Cycle threshold (Ct) data obtained from QPCR reactions were compared to standard curves of Ct versus DNA copy numbers that are generated for each individual target DNA using known amounts of plasmid containing the amplicon of interest. Data obtained from quadruplicate QPCR reactions performed for each sample are averaged and normalized to the invariant ribosomal protein L8 gene control (Table 18.1). The choice of a given normalizer gene product is crucial for correct interpretation of QPCR-derived data and must be supported by statistical determination of its invariant nature throughout the sample population. A trend or fluctuation in the expression of the normalizer could indicate a treatment or exposure-related effect. In general, the chosen normalizer gene should also display an amplification profile within the same dynamic range of detection to that of the target genes of interest. Controls that lack cDNA template (non-specific amplification control) or thermopolymerase enzyme (no amplification control) are included to ensure the specificity of target DNA amplification. In addition, QPCR reaction products that utilize newly designed primer pairs are initially

separated on an agarose gel and visualized with ethidium bromide. This is done to ensure that the SYBR dye-based signal derived during QPCR is specific for the target amplicon under investigation and is distinct from noise created through primer dimer effects or spurious non-specific DNA amplification. An alternative DNA amplification method that does not require use of a real-time QPCR system can also be employed.[17] This earlier study used endpoint collected data from standard DNA amplification reactions separated by agarose gel electrophoresis and visualized using ethidium bromide staining. Densitometric values for each amplicon band were quantified from captured digital images, and relative gene expression determined using 16S ribosomal RNA to normalize expression data between samples following the DNA amplification procedure (Table 18.1).

Results and discussion

TH action plays a crucial role in growth, development, and homeostasis of humans and other vertebrates. Of particular importance is the association of TH with brain development and normal cognitive function.[22,23] Exposure to chemical contaminants has been associated with changes in TH levels in human populations[24] and is also linked to neurotoxicity and modulation of gene expression in brain tissue of laboratory animals.[25,26] With respect to wildlife populations, amphibians represent a recognized sentinel species for the detection of EDC-associated effects due to their aquatic larval life stage and a dependence on hormone activity for metamorphosis.[27–30] TH plays a critical role in regulating the tissue remodeling events that occur during metamorphosis, and it is this process that could potentially prove a sensitive target for EDC action.[31–33] TH acts primarily through nuclear TH receptors (TRα and TRβ) that initiate tissue-specific genetic programs by the activation and repression of specific genes.[34–36] It is remarkable that this single hormone signal can induce such diverse tissue fates as resorption of the larval tadpole tail (involving cell apoptosis) and development of the limbs (involving cell proliferation). In addition to TH, the actions of other hormones, such as estrogen and corticosterone, also modulate this critical developmental period making metamorphosis a prime target for endocrine disruption.[37–39] The number of developmentally abnormal frogs observed from the wild and population declines suggests that some form of disruption in gene expression programs crucial for normal development may exist in certain amphibian populations.[40,41]

To test the potential of the biopsy procedure to detect changes in gene expression associated with amphibian development, the steady-state levels of TRβ mRNA in tail tissue were investigated during natural metamorphosis of *R. catesbeiana* tadpoles. This represents a period of increased TH-dependent gene expression and marked changes in target tissues that result in gross anatomic and physiologic changes culminating in the metamorphosis to a juvenile froglet. The steady-state levels of TRβ transcript increased approximately 9-fold during the prometamorphic phase of development up until metamorphic climax (TK Stages XIV–XXII) (Figure 18.2). Increased TRβ gene expression correlates with increased levels of endogenous TH during metamorphosis.[36,42] A similar upregulation in TRβ gene expression during metamorphosis has been reported previously for a number of amphibian species that undergo metamorphosis.[19,35,43]

Induction of precocious metamorphosis can occur following exposure of premetamorphic tadpoles to exogenous 3,3′,5-triiodothyronine (T_3).[44] At this stage of development, tadpoles are functionally athyroid (Regard et al., 1978), yet are highly sensitive to TH exposure[44] thus providing a useful physiological baseline from which to study both

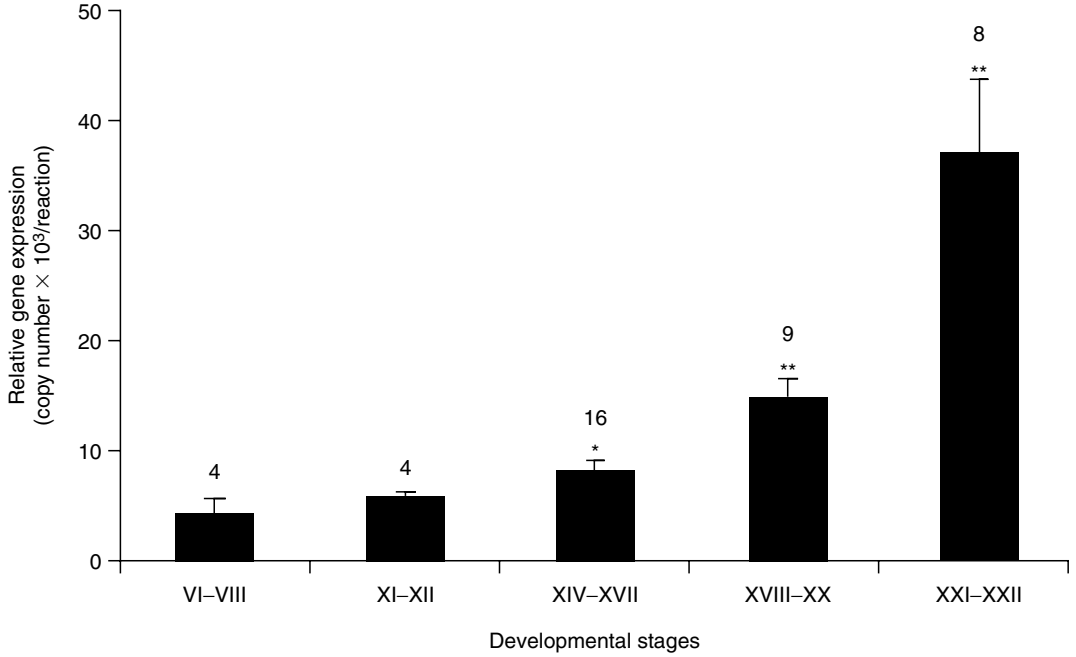

Figure 18.2 Expression of the TRβ gene in tadpole tail fin tissue during natural metamorphosis of *R. catesbeiana*. Biopsy samples were taken from the tail fin of euthanized tadpoles, which had reached the indicated developmental stage. The level of TRβ gene expression was determined using QPCR analysis and normalized across samples using the invariant ribosomal L8 gene expression. The means and standard errors are shown and the number of animals assessed per developmentally staged group is indicated above each bar. Significant differences compared to TK Stages VI–VIII are denoted by a single asterisk ($p < 0.05$) or a double asterisk ($p < 0.001$). Determination of standard errors and one-way analysis of variance (Tukey–Kramer multiple comparisons test) for significance were carried out using InStat V3.01 (GraphPad Software, www.graphpad.com).

hormone-dependent development and the effects of potential EDCs. Using this hormone-inducible system, TH-dependent changes in TRβ mRNA expression were assessed in tail fin tissue obtained from T_3-exposed whole animals that were sacrificed immediately prior to the biopsy (Figure 18.3A) and from dissected tail tips cultured in the presence of T_3 (Figure 18.3B). A significant increase in hormone-dependent TRβ mRNA expression is detected within 24 h for both intact animals exposed to hormone and with the isolated tail tissue. A similar upregulation in TRβ expression is also observed in tail fin samples collected from live tadpoles following a 24-h exposure to T_3 (Figure 18.4).

In order to test whether this method is capable of detecting a disruption of gene expression associated with EDC exposure during precocious metamorphosis, we treated tadpoles with the environmentally relevant dose of 10 n*M* acetochlor[10] in the presence or absence of 100 n*M* T_3 for 24 h (Figure 18.4). This pre-emergent herbicide has been shown to modulate TH-dependent gene expression and accelerate precocious metamorphosis in *Xenopus laevis*.[5] Acetochlor alone has no effect on TRβ mRNA expression in premetamorphic tadpole tails; however, the combination of T_3 with acetochlor results in a statistically significant elevation of the T_3-induced response. This observation in *R. catesbeiana*, in addition to effects on mRNA expression and morphometric endpoints observed in *X. laevis*, identifies acetochlor as a potential environmental EDC that targets

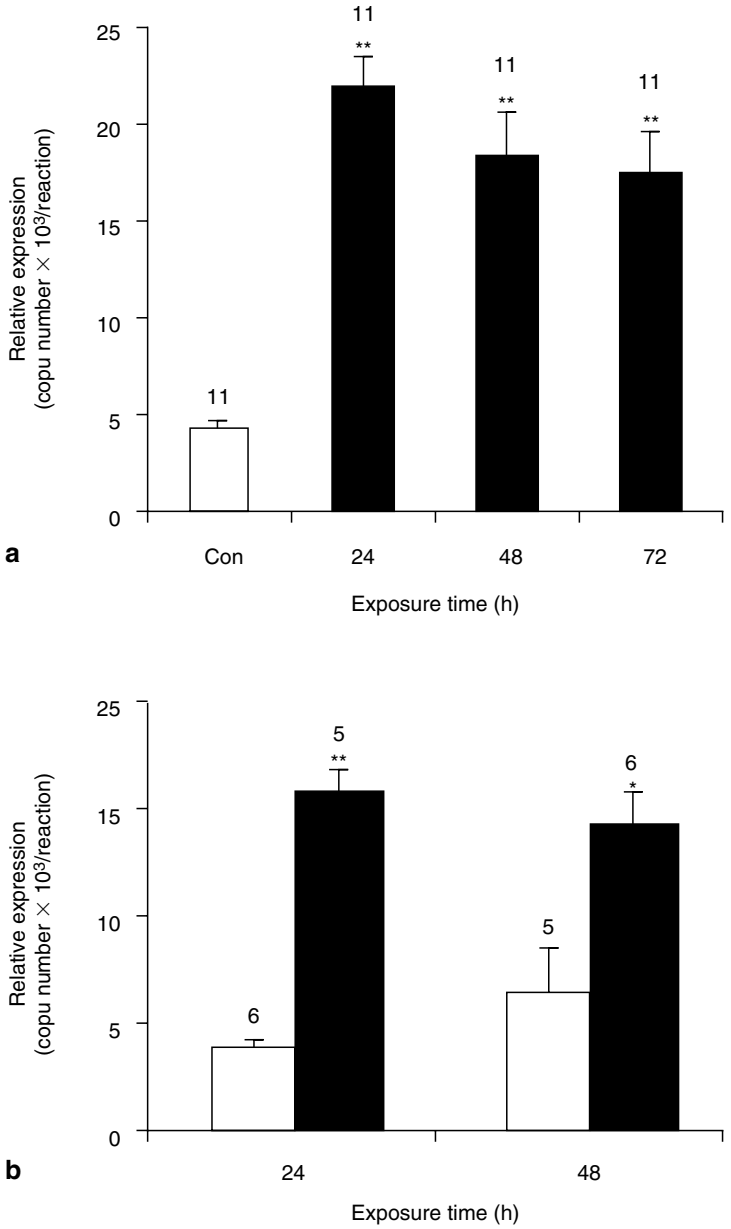

Figure 18.3 Expression of the TRβ gene in tail tissue of intact *R. catesbeiana* tadpoles or isolated cultured tail tips following exposure to exogenous TH. Biopsy samples were collected from the tail fin of tadpoles treated with 100 n*M* T_3 and euthanized immediately following treatment (a) or from cultured tail tissue exposed to 10 n*M* T_3 (b) for the noted time period. Control exposures included treatment with the DMSO vehicle (white bars). The level of TRβ mRNA expression was determined using QPCR analysis and normalized across samples using the invariant ribosomal L8 gene expression. The means and standard errors are shown and the number of animals used per time point is indicated above each bar. Significant differences compared with the vehicle control (Con) are denoted by a single asterisk ($p < 0.05$) or a double asterisk ($p < 0.001$). Statistical analyses were performed as described in Figure 18.2.

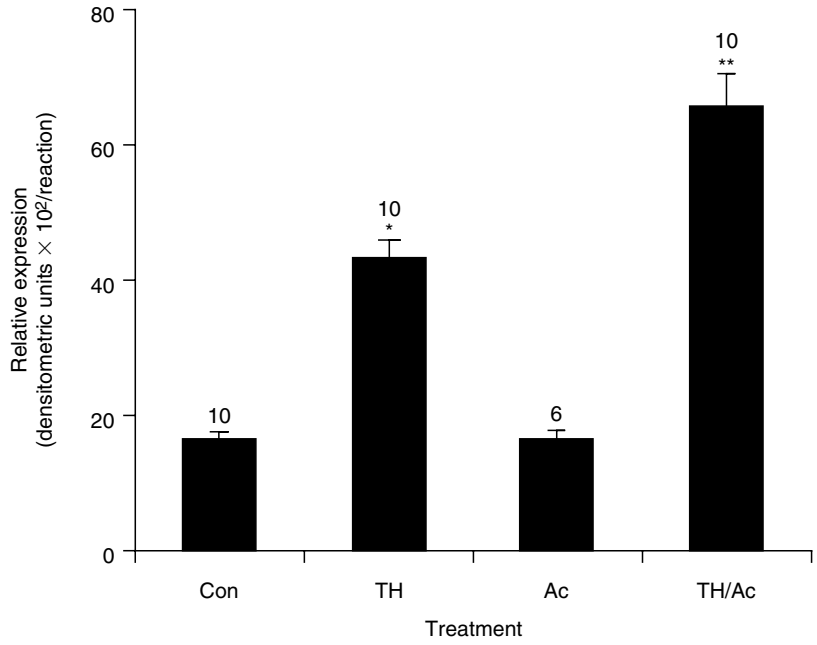

Figure 18.4 Identification of EDC action of the herbicide acetochlor in live *R. catesbeiana* tadpoles. Animals were acclimatized to 20°C for 2 days prior to treatment with 100 n*M* T_3 (TH), 10 n*M* acetochlor (Ac), or a combination of both (TH/Ac) for 24 h. Tissue biopsy samples were collected from tadpole tail tips. The level of TRβ mRNA expression was assessed using standard PCR analysis and normalized across samples using the invariant expression of the 16S ribosomal RNA gene. The means and standard errors are shown and the number of animals used per treatment is indicated above each bar. A single asterisk denotes a significant difference when compared with the control (Con) and acetochlor (Ac) treatments ($p < 0.001$). A double asterisk shows a significant difference compared with all other treatments (Con, $p < 0.001$; Ac, $p < 0.001$; TH, $p < 0.01$). Statistical analyses were performed as described in Figure 18.2. (From Veldhoen, N. and Helbing, C.C., *Environ. Toxicol. Chem*., 20 (12), 2704–2708, 2001. With permission.)

TH-associated gene expression during frog development. This gene-screen could be extended to evaluate the effects of potential EDCs that target other hormone-dependent developmental processes in amphibians (e.g., testosterone and estrogen in sex determination and the contribution of corticosterone and retinoids during metamorphosis). It is important to note that significant acetochlor-associated effects on TRβ gene expression following exposure to the herbicide are observed prior to any measurable changes in morphological endpoints.[5] Thus, the use of biopsy sampling in combination with genetic biomarkers may allow for greater predictive capability and earlier assessment of EDC action in wildlife species compared with established methods that rely solely on morphometric or mortality endpoints.

While invaluable for repeated measurement-based analyses within a controlled laboratory setting, the biopsy procedure can also be easily applied to the collection of samples from the field in an attempt to address the effects of acute and chronic environmental contaminant exposure on the health of wildlife species. This becomes particularly important in the context of exposure to complex mixtures of contaminants (e.g., effluents) and the contribution of environmental factors to the generation and/or propagation of bioactive chemical components. The equipment required is highly portable (Figure 18.5),

Figure 18.5 Equipment required for field collection of tissue samples. The portable kit includes: a dermal punch biopsy tool (1), a Plexiglas biopsy support (2), paper towel (3), fine-tip forceps (4), RNA preservative (5), plastic storage tubes (6), cooler container (7), capture net (8), and latex gloves (9).

and samples retrieved in this manner are stable at 4°C for several months prior to RNA isolation. It is estimated that a single 2-mm biopsy of tadpole tail fin yields enough RNA to perform 200 QPCR reactions allowing for repeated measurement of targeted gene expression. In addition, genetic material obtained from the biopsy sampling procedure can be used, in combination with recently developed amplified RNA (aRNA) techniques and DNA array technology, to obtain extensive multi-gene expression profiles of the effects of a suspected EDC on the growth and development of species at risk.[5,45–48]

It is also conceivable that this gene-screen procedure could be adapted for any species of interest by selecting the appropriate biopsy instrument for the size and location of tissue to be sampled and identifying appropriate genetic biomarkers for use in QPCR or DNA array-based assessments. Tissue samples could be obtained from wildlife species as diverse as rodents (ear), waterfowl (foot webbing), fish (fin), bats (ear or wing membrane), and amphibians (tail fin and foot webbing). Indeed, biopsy collection of skin and blubber tissue samples from marine mammals is well established.[14,15,49,50] By examining a number of different animal species that occupy both terrestrial and aquatic environments, a greater profile of the potential impact of chemical contamination within an ecosystem may be generated.

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section three

Techniques for identification and assessment of contaminants in aquatic ecosystems

chapter nineteen

Coral reproduction and recruitment as tools for studying the ecotoxicology of coral reef ecosystems

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Contents

Introduction

Aquatic toxicology is a discipline that deals with the effects of chemicals and potential pollutants on individual organisms, usually with the intent of applying such data to understanding effects on populations and community structure and function. Aquatic organisms have a number of different life-history stages through which they must successfully pass, in order to become part of a population, and hence, assays that deal with only one stage, such as the adult form, may miss critical effects and have limited predictive value.

Many benthic aquatic organisms in both the protostome and deuterostome phyla, including Porifera, Cnidaria, Annelida, Crustacea, Echinodermata, and Mollusca, spawn gametes (eggs and sperm) into the water column, with subsequent external fertilization and development into planktonic larval stages, which eventually settle on specific substrata prior to metamorphosis into the juvenile form. These organisms respond to

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chemical cues for synchronization of spawning events among conspecifics, for mediation of egg–sperm interactions and for metamorphic induction in response to particular substrata, aggregations of adults or preferred prey.[1] Since so many steps in the life cycles of marine organisms are chemically mediated, the lack of a measurable effect of a xenobiotic on adult organisms at a range of concentrations does not necessarily mean the chemical is safe from a population or community perspective if it interferes with chemical cues at earlier life-history stages. Additionally, 100% successful fertilization of eggs and development of larvae in a bioassay with a subsequent 0% recruitment rate has the same overall effect on the population as 100% mortality at the fertilization stage. Finally, the duration of the observation period following exposure of an early life-history stage of an organism is important, as initial survival does not necessarily mean growth, development and reproductive ability will be unaffected later on.

Scleractinian corals provide an excellent set of organisms for use in aquatic toxicologic studies in tropical marine environments. Many species are simultaneous hermaphrodites that release combined egg–sperm packets during highly predictable annual spawning events. The gametes are easy to collect and manipulate, the large numbers available lend themselves to experiments with large sample sizes and hence rigorous statistical testing, and the larvae that result from successful fertilizations often have specific cues necessary for settlement and metamorphosis, allowing for multiple stage testing. Additionally, specific genetic crosses can be controlled and as a result, the confounding effects of genetic variability among individuals can be controlled. Genetic lines can be reared for later exposure and determination of biomarker expression. Gametes can be collected from the same coral colonies over several years if care is taken in collecting and maintaining ''donor'' corals.

Materials required

Material	Source	Comments
Gravid corals; at least two different colonies of the same species	Local coral reef communities	See (Richmond and Hunter, 1990; Richmond, 1997) Reference 2, for timing and location. Collecting permits is usually necessary
Sea water system	Field station, aquarium, or laboratory; can be open, closed, or recirculating	Corals have specific requirements for water quality, light, pH, temperature, and water motion
Basins/containers large enough to hold individual colonies	Aquarium store, hardware store, department store	Containers should be conditioned prior to use; glass is preferable for some experiments; plastic basins should be soaked to remove plasticizers
Plankton netting (Nytex) 105-μm mesh	Aquatic Ecosystems catalog #M105 (www.aquaticeco.com)	The netting is used to make sieves to collect gamete bundles. Coral eggs typically range in size from ca. 180 to 360 μm. A mesh size of 105 μm works for most species

Material	Source	Comments
2-in. PVC pipe	Hardware stores	Good diameter for making sieves for separating egg–sperm clusters. Nytex is attached to end of PVC pipe with hot melt glue
Hot melt glue and glue gun	Hardware or department stores	Used to attach plankton netting to PVC pipe sections for sieves
250-ml specimen cups, beakers or equivalent	VWR, Scientific Products or most laboratory suppliers	Glassware is preferable over plastic
Pasteur pipettes and bulbs	VWR, Scientific Products, etc.	Both 5- and 9-in. pipettes are useful
1-l glass beakers, fleakers or jars	Most labware suppliers	1200-ml vessels allow for raising batches of 1000 larvae at a density of 1 larva/ml
Air pumps (aquarium type or system), lines and airstones	Any pet shop or store with pet department	Need gentle aeration to keep floating eggs from getting trapped in the surface tension of the water
Hemocytometer	VWR, Scientific Products, etc.	Necessary for determining sperm density if the separation technique is used

Procedures

Selection of coral species

Coral species are selected based on their reproductive behavior and timing, growth rate, growth form, sensitivity to environmental parameters, cultivation characteristics, and biogeographic distribution patterns. Our laboratory has been able to routinely raise larvae from 13 species of corals that represent a variety of growth forms, habitats, and range of sensitivities to stress. *Pocillopora damicornis*, a brooding species, is widely distributed across the Pacific Ocean and produces viable planula larvae monthly, throughout the year, in Micronesia and Hawaii.[3] These larvae contain a full complement of zooxanthellae upon release from the parent colony. As such, this coral is good for studies requiring competent larvae but not for experiments to test the effects of chemical compounds on fertilization, larval development, or acquisition of symbiotic zooxanthellae. *P. damicornis* is a non-perforate species, meaning tissue is relatively easy to remove from the carbonate exoskeleton, which is a helpful characteristic for assays requiring subsequent colony tissue analysis. This species also releases relatively low quantities of mucus and is easy to maintain and transplant.

Several broadcast spawning acroporids (e.g., *Acropora humilis*, *A. tenuis*, *A. valida*, *A. danae*, *A. surculosa*, and *A. wardii*) and favids (*Goniastrea retiformis* and *Leptoria phrygia)* have proven to be useful in fertilization, development, symbiotic algal uptake, and recruitment bioassays. Species in these two groups, along with corals in the family Poritidae, are among the most commonly studied coral species. Since our research aim is to develop protocols that are widely applicable, we continue to add coral species that have appropriate biogeographic distribution patterns.

Collection of corals

Corals are collected from the field several days prior to predicted planulation or spawning. For the brooding species *P. damicornis*, colonies are collected 2–3 days after the new moon, as this species has been found to planulate within several days of the lunar first quarter, each month of the year, in Micronesia.[3] Timing is different in Hawaii and Australia, demonstrating the need for accessing data on the reproductive timing of local populations.

Spawning species are collected 2 days after the June–August full moons on Guam (Richmond and Hunter, 1990),[1] and in March–May in Palau. In all cases, corals are checked in the field for the presence of gametes each month leading up to spawning. Generally, if a coral is ripe, the eggs will be pigmented (pink, red, or orange) and are easily seen on the colony side of a broken branch or fragment (Figure 19.1). If no color is observed, the branch tip or fragment is brought back to the laboratory for examination under a compound microscope. For the acroporid and favid corals studied on Guam to date, eggs can be observed developing up to 6 months prior to spawning (January), appearing as white growths along the mesenteries. As spawning approaches, the eggs increase in size and eventually become pigmented. In the species that are simultaneous hermaphrodites (containing both eggs and sperm within the same colony and polyp), sperm begin to develop within a month or two of spawning, after eggs have already formed.

When ripe colonies are found, they are gently removed from the substratum with a hammer and chisel, and kept submerged until they are transferred to coolers filled with seawater and quickly transported to running seawater tables or aquaria. It is important to collect at least two colonies of each species, preferably some distance apart (to increase the probability of the two being genetically different) to allow for outcrossing in fertilization assays. Experiments have demonstrated self-fertilization rates for the simultaneously hermaphroditic corals are low or non-existent.[1,4]

Collection of gametes and larvae

The acroporid corals proven most appropriate for toxicology studies are all simultaneous hermaphrodites that release combined egg–sperm bundles[2] (Richmond and Hunter, 1990;

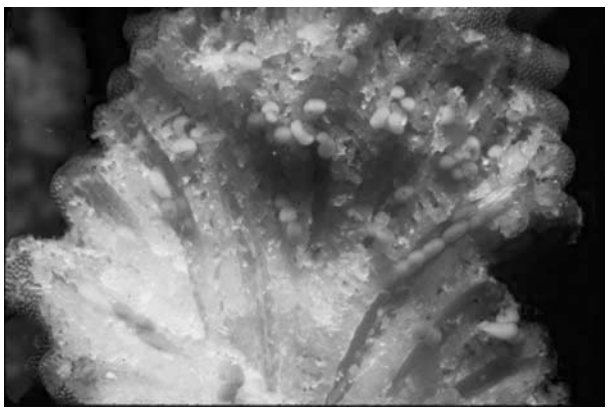

Figure 19.1 **(see color insert following page 464)** Cross-section of coral colony with pink eggs and white spermaries.

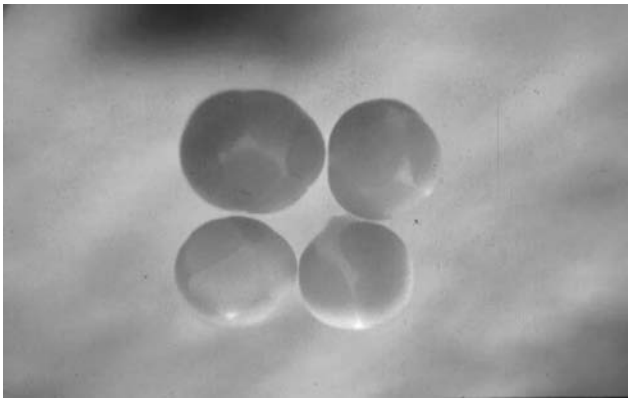

Figure 19.2 **(see color insert)** Combined egg–sperm clusters.

Figure 19.3 **(see color insert)** Floating gamete bundles collected in sieves.

Figure 19.2). Upon release, these float to the surface and can be collected on sieves with a mesh size of 105 μm or, alternatively, collected individually with a Pasteur pipette and placed intact into a 250-ml container with UV-sterilized/filtered seawater (Figure 19.3).

Two different systems have been developed, depending upon the experiments that will be performed. In fertilization studies, or those testing the effects of water-soluble pollutants, the clusters are washed with 0.45 μm (Millipore) UV-sterilized/filtered seawater, with the sperm being collected as the wash. The eggs are re-suspended in filtered/sterilized seawater, and fertilized with sperm from another colony of the same species, at a sperm concentration of ca. 10^5 sperm/ml.[4] Outcrossing has been found to be necessary as self-fertilization rates are relatively low. Higher concentrations of sperm often result in polyspermy and non-viable embryos. Fertilized embryos are placed into 1- and 2-l glass or plastic culture flasks with aeration, to provide a degree of water motion. Densities of eggs and developing larvae should not exceed 1/ml of seawater. Water is changed every 12 h during the first 48 h by siphoning from the bottom of the vessel, and daily afterwards until the larvae are ready to be settled onto appropriate substrata. Antibiotics are not necessary and handling of larvae should be minimized.

In studies using larval development, recruitment, zooxanthellae acquisition, and survivorship, we have simplified the fertilization procedure, and simply added the appropriate number of gamete clusters from two different colonies of the same species to 1 l of filtered seawater to achieve the proper sperm density. For example, for most acroporids, 50–100 gamete clusters from each of two colonies yield a sperm density of ca. 10^5 sperm/ml, and yield between 800 and 1200 larvae, with over 90% fertilization success. For the favid corals, crossing 150–200 clusters from each of two colonies in 1 l of seawater has been effective. The ''cluster'' technique makes a number of experiments logistically feasible at high levels of replication, and hence statistical rigor.

The planula larvae of the brooding coral *P. damicornis* are approximately 1 mm in diameter. Adult colonies measuring 15–20 cm in diameter are placed into 3- to 9-l vessels that overflow into containers constructed from plastic beaker bases with walls of 80-μm Nytex plankton netting. The corals are kept under continuous flow conditions of 1 l/min, and the buoyant larvae, which float out of the bowls, are collected in the mesh of the cups. This species has been found to planulate at night, so collectors are checked each morning for the presence of larvae.

Bioassays

Fertilization bioassays address the first critical, chemically-mediated step in the reproductive process and can be performed in a variety of vessels and a using a range of concentrations based on the question being asked and the characteristics of the substance being tested. When gamete quantity is limited, 15-ml prewashed glass scintillation vials are used with ca. 100 eggs (50 each from two different colonies; 5–8 clusters from each, for a variety of acroporid and favid corals) per vial, in 10 ml of UV-sterilized/filtered seawater, with a sperm density of ca. 10^5 sperm/ml. With the expectation that outcrossing will occur when using the ''cluster'' technique, developing larvae will be of two different genotypes. If follow-up experiments are to be performed on the larvae that form from the fertilization assays, using 100 eggs collected from a single colony and fertilized with sperm separated from a conspecific colony will presumably yield larvae of a single genotypic cross, and may reduce variability in experimental results.

Experiments are scored by viewing samples under a dissecting microscope for the number of eggs fertilized, the number of embryos reaching the planula larval stage versus the number of eggs/embryos that are non-viable.[5] A watch glass or other concave dish makes counting easier. Careful and limited handling allows for the larvae that do form to be reared further for recruitment and algal acquisition assays.

Recruitment assays

Coral larvae from spawning species become competent to settle between 18 and 72 h following fertilization, depending on species and egg size.[1] The smaller eggs (from favids) develop cilia and become competent more quickly than the larger *Acropora* eggs. The brooded larvae of *P. damicornis* are fairly non-specific and will settle on a variety of substrata pre-conditioned with bacterial/diatomaceous films. The *Acropora* and *Goniastrea* larvae are more selective, and our previous experiments indicate that several species are highly specific, settling only on particular species of crustose coralline algae (*Hydrolithon reinboldii*).

For recruitment bioassays, chemical effects of potential pollutants are tested by exposing preferred substrata to the xenobiotic, rinsing the substrata, then placing them

into 250-ml beakers with clean, filtered seawater or by exposing larvae to the chemical and providing them with untreated substrata. A known quantity of larvae is added to each beaker (50–100 per replicate), and the substrata scored daily under a dissecting microscope for 5 days for larvae that have settled (come into contact with the substrata), metamorphosed (cemented to the surface and displaying evidence of calcification; Figure 19.4), remained swimming or died. Alternatively, larvae are exposed (generally for 12–24 h) and subsequently added to beakers with the appropriate untreated substrata. Our research has demonstrated that pollutants can interfere with metamorphic inducers associated with the substrata and/or with the inducer receptor of the larvae. It is important to address both possibilities. While fertilization and embryological development, stages in corals are particularly sensitive to water-soluble chemicals; lipophilic substances appear to have a greater effect on larval recruitment.

In assays designed to study the effects of chemicals on zooxanthellae uptake, competent larvae are placed into basins or aquaria containing cleaned coral rubble in 0.45 μm (Millipore) filtered seawater. Pieces are checked daily for the presence of settled larvae, and if present, fragments are transferred to separate containers for exposure to chemicals and the addition of zooxanthellae. Zooxanthellae for use in these assays can come from pieces of donor colonies of the same species placed in the container, from cultured lines, from zooxanthellae centrifuged from tissue preparations of conspecific colonies, or from zooxanthellae collected and filtered from ''induced expulsions.'' One known stress response of corals is the breakdown of the animal–algal symbiosis. A by-product of cyanide bioassays performed to determine the effects of cyanide fishing on reefs was the discovery that exposure of coral branches to cyanide at 0.1 g/l for 5 min resulted in free-swimming zooxanthellae evacuating the host cells and tissue within hours of exposure. Since zooxanthellae appear to have the same cyanide-resistant respiratory pathway found in other plants, clean zooxanthellae free of coral tissue residue can be acquired this way that have found to be functional and capable of colonizing coral recruits.

For bioassays using adult corals, colonies are cultivated from larvae to a size of approximately 5–10 cm in diameter. Depending on the question, corals are either

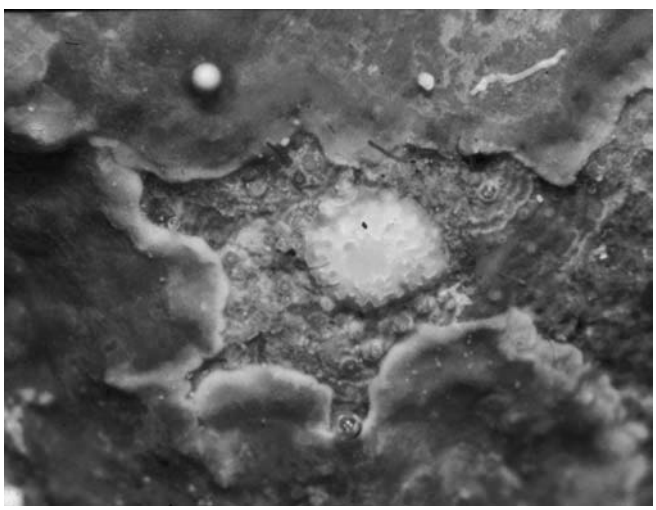

Figure 19.4 **(see color insert)** Coral larval recruit, settled and metamorphosed.

''pulsed'' and transferred to grow-out tanks with flowing seawater or maintained under test conditions in a closed system with air stones. We use separate containers for each coral, 10 corals per concentration (for replication, avoiding pseudoreplication) in a randomized block design. In pulse experiments, after exposure, tagged, treated and control corals are placed into a flowing seawater tank with a flushing rate of 2 l/min and observed for signs of bleaching, tissue loss, and death for a period of 30 days.

A great advantage of using corals is the ability to control genetic variability by using larvae from a single cohort. A single colony can be repeatedly ''harvested'' for gametes and larvae over a period of years with careful handling. For spawning species, once gametes are released and collected, colonies are tagged and transplanted back into the field, by cementing them in a marked area using a mixture of 7 parts cement to 1 part plaster of paris. Previous experiments have demonstrated that for several types of stress assays, genetic considerations are important, as variability among coral colonies across different genotypes was greater than the effect measured within a single genotype.

In conclusion, corals have proven to be valuable tools for ecotoxicological studies. Their reproductive behavior provides opportunities for studying effects of chemicals on cueing between conspecific colonies during spawning events, for studies of egg–sperm recognition, fertilization, embryological development, metamorphic induction, and acquisition of symbiotic zooxanthellae. The ability to rear corals of known genotypes in large numbers allows for statistically rigorous testing and for monitoring specific sites over time. Recent advances in the development of biomarkers of exposure in corals hold promise for determining the effects of pollutants at sublethal levels, and for measuring responses to mitigative measures. Such tools are proving useful to help resource managers address the effects of human activities on coral reef ecosystems, presently under threat world-wide.

Acknowledgments

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chapter twenty

Using the stickleback to monitor androgens and anti-androgens in the aquatic environment

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Contents

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Introduction

Over the past few years there has been increasing evidence of the hormone-like effects of environmental chemicals, such as pesticides and industrial chemicals, in both wildlife and humans. These so-called endocrine disruptive chemicals (EDCs) mainly act by mimicking or antagonizing the effect of the endogenous hormones estradiol and testosterone but may also disrupt the synthesis and metabolism of endogenous hormones and/or their receptors.[1] A large number of compounds have been reported to possess endocrine modulating activity. These include natural products, pesticides, fungicides and insecticides, medical drugs, and commercial and/or industrial chemicals. Although a causal relationship between exposure to these substances and human/wildlife reproductive health has not been fully established, the characterization of the adverse effects of EDCs, at environmentally relevant concentrations, is very desirable.

Much research appears to have been done already on the role of estrogenic xenobiotics. The most widely used biomarker for estrogenic exposure is the presence of the female specific protein, vitellogenin in the plasma, liver or whole-body homogenates of male fish.[2–6] The role of androgenic xenobiotics has not been studied in nearly so much detail, despite the fact that there is an increasing concern for clinical implications of these chemicals in humans.[7] One of the clearest observations of androgenicity in the aquatic environment has been made in female mosquito fish (*Gambusia* sp.), living downstream of kraft mill effluent discharges.[8–10] While the detection of androgenic compounds in the environment is presently restricted to pulp mill effluents (PMEs) and sewage treatment works without secondary treatment,[11] compounds with anti-androgenic activity appear to be more widespread. At the moment, the only official *in vivo* test able to provide information on the androgenic or anti-androgenic effect of suspected chemicals is the Hershberger castrated male rat assay.[12]

The three-spined stickleback (*Gasterosteus aculeatus*) offers a great potential for the assessment of reproductive disturbances caused by androgenic xenobiotics due to its pronounced androgen-dependent male secondary sexual characters that present during its breeding season (late spring and summer). These characters include development of nuptial coloration, kidney hypertrophy, territorial and nest-building behavior. The kidney hypertrophies under the control of androgens to produce a ''glue'' protein that is used to build the nest out of algae, plant material, sand, and detritus. This glue protein was first characterized by Jakobsson et al.[13] and was given the name spiggin from the name of the stickleback in Swedish, the spigg. Spiggin is assembled from three subunits in the urinary bladder[14] and is deposited on suitable nest material by contractions of the urinary bladder. The production of the glue protein by the male stickleback has potential as a biomarker for androgenic and anti-androgenic xenobiotics because (a) it is well established that is androgen-dependent,[15–18] and (b) it has easily measurable response parameters that include kidney weight changes (nephrosomatic index), histological changes on the height of the epithelial cells,[17,19,20] spiggin changes,[21,22] and spiggin mRNA changes.[14]

To date, spiggin is the only androgen-induced protein that has been isolated from fish. Recent research at Centre for Environment, Fisheries and Aquaculture Science (CEFAS) has firmly established that female stickleback kidneys can produce spiggin in response to several model androgens, such as 17α-methyltestosterone (17α-MT), dihydrotestosterone (DHT), testosterone (T), and 11-keto-testosterone (11-KT), added to the ambient water. As we show in this chapter, spiggin production is inhibited in males (and in 17α-MT-stimulated females) by flutamide (FL; a well-known anti-androgenic

drug that is used in the treatment of prostate cancer.[23] A number of other xenobiotics with suspected anti-androgenic activity have been tested and most showed clear inhibition of spiggin production.

Materials required

Husbandry

Glass aquaria of various sizes for maintaining fish stock and experimental populations; de-chlorinated freshwater and/or seawater supply; multi-channel peristaltic pumps; air (O_2) supply; tubing (high quality Portex or similar polypropylene); pipe work fittings; flow meters; oxygen and pH meters; equipment for determining water hardness; thermometers; under-gravel bacteria filters; coarse gravel (approximately 5 mm); controlled day-length light; siphoning tube; nets; various sized beakers; Pasteur pipettes.

Histological processing

Dissection kit; glass vials (50 or 20 ml); 10% neutrally buffered formalin (BDH); industrial methylated spirit (IMS); xylene or the safer alternative Histosolve™ solvent (Thermo electron/Shandon); ethanol (reagent grade); cassettes (5 × 3 cm uni-cassette, Tissue Tek); paraffin wax (W1 formula wax, RA Lamb or similar with a melting point of 57–58°C); Historesin (Leica or Taab, UK, comes with extensive manufacturers instructions to use); molds; vacuum infiltration processor (VIP; Tissue TEK VIP 2000 or similar); tissue embedding center (Tissue TEK II or similar); microtome (Shandon Finesse E or similar); standard cryostat; disposable microtome blades (80 × 0 mm, RA Lamb, Catalog E53.37/S35); stainless steel knife; routine histological stains (see Tables 20.2 and 20.3); histology glass slides and cover slips; xylene-based polymer mountant (DPX or similar); standard microscope equipped with a graticule or an eyepiece micrometer; microscope (Nikon E800 or similar) linked to camera (DXM 1200F or similar) and PC; image analysis software (Lucia G LIM screen measurement or VIDS or other similar software).

ELISA for spiggin

Polystyrene plates (high protein-binding, flat-bottomed, Costar® EIA/RIA 96-well); polypropylene plates (low-binding, round-bottomed); plate covers (Mylar sealing tape from Sigma); plate washer (e.g., MRW, 8-channel plate washer made by Dynex Technologies, The Microtiter® Company); plate reader (e.g., MRX microplate reader made by the same company as the washer).

Coating buffer (0.05 *M* sodium bicarbonate–carbonate, pH 9.6); plate wash buffer (0.1 *M* sodium phosphate (di-basic salt, 0.072 *M*, mono-basic salt, 0.028 *M*), 0.14 *M* sodium chloride, 27 m*M* potassium chloride, 0.05% Tween-20); assay buffer (same as plate wash buffer plus 0.1% bovine serum albumin (Sigma) and 0.15 m*M* sodium azide); second antibody (affinity isolated anti-rabbit IgG whole molecule conjugated to alkaline phosphatase (Catalog A-3937, Sigma Immuno-Chemicals); alkaline phosphatase substrate (1 mg/ml 4-nitrophenyl phosphate (*p*-NPP) in 0.2 *M* Tris buffer (SIGMA FAST™ tablets). There are also specific reagents (that have to be produced in-house). These include polyclonal antisera against spiggin and a spiggin standard.

Procedures

Fish collection and husbandry

Three-spined sticklebacks are ubiquitous in the whole of the North hemisphere and can be found in almost all aquatic habitats from freshwater until full seawater. However, they spend the best part of their lifetime in freshwater systems where they reproduce, and it is relatively easy to catch them with simple netting devices or traps.

Although juvenile fish can also be used as a model for endocrine disruption research, the proposed tests are designed to use adult fish that are 8–18 months old and weigh at least 0.8 g. Fish used in the experiments should be disease-free and have no obvious signs of parasitism. The fish can be maintained in the laboratory in either freshwater or brackish or even full seawater. However, it is preferable that they are kept in brackish water at 10°C and short photoperiod, 8 h light and 16 h dark (*L:D* 8:16) until used for the experiments and in some cases during the exposure period. The fish are fed *ad libitum* with a variety of fresh or frozen food [mosquito larvae, bloodworm, *Daphnia*, brine shrimp (*Artemia*), etc.] once daily throughout the exposure period. Stock populations can be fed on dried food (TetraMin) also but not exclusively. Dry food however, whenever used, should be estrogen free.

Stock populations can be kept at a density of 2–10 fish per litre of water, while the experimental tanks should not exceed 1 fish/l. The duration of the proposed tests is 21 days.

Exposure to test compounds

Test compounds can be delivered in a number of ways, such as injections (IM, IP), implantation, and via the food. However, fish will readily absorb compounds added to the water, and this is the preferred method of exposure. Water-borne exposure to androgenic and anti-androgenic compounds/effluents can be accomplished using two systems, namely, continuous flow and semi-static. The former requires the use of peristaltic pumps that can be adjusted to deliver the desired flow rate/concentration of test compound to the water inflow. The semi-static system that we use at CEFAS involves a 70% water change (by siphoning out the water slowly and refilling the aquaria) and addition of fresh test solution(s) every 48 h. For establishment of actual (as opposed to nominal concentrations) of the compounds, water samples (1–2 l) are taken at least once a week into dark glass bottles when using a continuous flow system to verify the concentration of the test compound(s) in the water. When using a semi-static system of exposure, the frequency should be increased to include water samples just prior to and immediately after the water change in order to establish the variation of test compound concentration in the 48-h period. Extraction and then measurement of the tested chemicals in the water can be achieved by a number of methods that are outside the scope of this chapter.

Light microscopy

At the end of the exposure period, the fish are humanly killed and their weight recorded. The kidneys and gonads should be dissected out and immediately placed in 10% neutrally buffered formalin. Kidney dissection allows better fixation and embedding. It is advisable that the gonads are processed along with the kidneys to allow determination of

the reproductive status of the fish and to allow easy and unmistakable identification of fish gender.

If whole fish sections are preferred, then an incision should be made across the ventral lining to allow penetration of fixative. The ratio of tissue: formalin should be 1:10 (w/v). Fixed dissected tissues (or whole fish) should then be placed in perforated plastic cassettes and processed using a VIP as described in Table 20.1. The times and solutions can be varied to specifically suit the size of the samples. If using a rotary processor or immersion technique, duration of steps need to be extended. Samples can be placed into 70% IMS before processing begins. Solutions described as ''sitting'' solutions (stations 1 and 14) are called this, as these are the stages immediately before and after the processing schedule commencement. The processor is normally run overnight will finish as set on the processors timer. In the case of our VIP, station 14 will not empty out at the end of processing, and cassettes can be allowed to stand until removal for embedding. Station 1 may be replaced by 10% neutral buffered formalin to aid fixation or to speed up sample processing. Thermo electron/Shandon Histosolve™ is used as a safer alternative to xylene. Following impregnation, tissues are ready to be embedded. The lids of the cassettes are removed, and molten paraffin (58°C) is poured into the cassette. Paraffin is allowed to solidify around and within the tissue forming blocks, which are then rapidly cooled over a cold plate.

Although paraffin wax is a standard embedding material, resin embedding allows for a more detailed picture of renal histology and is therefore recommended. For this, fixation and dehydration steps are the same as for paraffin wax embedding, followed by the steps pre-infiltration, infiltration, polymerization, and embedding, which are described in great detail in the product instructions.

Both paraffin and resin sections are cut at 5 μm using a standard microtome and disposable blades or a cryostat and stainless steel blade, respectively. The sections are mounted on glass slides and stained.

Lee's methylene blue-basic fuchsin stain is an inexpensive, one-step simple stain that offers great detail in the renal epithelium. Table 20.2 describes the staining schedule. The

Table 20.1 Fish tissue processing schedule for VIP

Station No.	Solution	Duration (min)
1	70% I.M.S.	35 (sitting solution)
2	90% I.M.S.	35
3	100% I.M.S.	35
4	100% I.M.S.	35
5	100% I.M.S.	35
6	100% I.M.S.	35
7	Histosolve solvent	35
8	Histosolve solvent	35
9	Histosolve solvent	35
10	Histosolve solvent	35
11	W1 paraffin wax at 60°C	45
12	W1 paraffin wax at 60°C	45
13	W1 paraffin wax at 60°C	45
14	W1 paraffin wax at 60°C	45 (sitting solution)

Table 20.2 Staining schedule for Lee's methylene blue-basic fuchsin (500 ml)

Step No.	Procedure	Chemicals
1	Dissolve stain in 100 ml water	0.128 g methylene blue
2	Dissolve stain in 100 ml water	0.128 basic fuchsin
3	Dissolve chemicals in 175 ml water	0.52 g NaH_2PO_4 $2H_2O$, 2.52 g Na_2 HPO_4 $2H_2O$
4	Combine solutions (steps 1–3)	
5	Add ethanol and mix	125 ml ethanol
6	Filter stain (can be used for 4 days)	
7	Immerse slides into stain and agitate gently for 10–15 s	
8	Rinse in de-ionized water (DW)	
9	Air dry	
10	Mount under cover slip	

stain is useful for 4 days when kept at 4°C and can be used for both paraffin and resin sections. Standard hematoxylin–eosin staining schedule can also be used.

When greater detail is desired, then resin sections and periodic acid Schiffs–Mallory trichrome (PAS) schedule can be used. This stain highlights the presence of carbohydrates in purple red (and since spiggin is a glycoprotein, it can be clearly seen in renal sections). Details are provided in Tables 20.3 and 20.4.

In each renal section, the height of the cells that line the secondary proximal kidney epithelium height (KEH) should be measured under a microscope equipped with a video camera and linked to a PC with image analysis software. If such a system is not available, then a standard microscope equipped with a graticule or an eyepiece micrometer can be

Table 20.3 Staining schedule for Schiffs–Mallory trichrome schedule (resin sections)

Step No.	Procedure/solution	Duration
1	Immerse in 5% (w/v) periodic acid	4.5 min
2	Immerse in DW	Wash 5×
3	Immerse in Schiffs reagent (Table 20.4)	60 min
4	Place under running tap water	10 min
5	Immerse in 1% (w/v) acid fuchsin	1 min
6	Wash with DW	30 s
7	Wash with DW	30 s
8	Immerse in 1% (w/v) phosphomolybdic acid	1 min
9	Wash with DW	10 s
10	Immerse in Mallory trichrome stain (Table 20.4)	15 s
11	Wash with DW	10 s
12	Immerse in 90% (v/v) IMS	5 s
13	Immerse in 100% (v/v) IMS	5 s
14	Immerse in 100% (v/v) IMS	5 s
15	Immerse in 1:1 IMS/Citroclear	5 s
16	Immerse in Citroclear	5 s
17	Immerse in Citroclear	5 s
18	Air dry, mount DPX and dry at 40°C	Overnight

Table 20.4 Preparation of special solutions for Schiffs–Mallory trichrome schedule

Step No Schiffs 200 ml	Procedure	Chemicals
1	Bring to boiling point	Distilled water (200 ml)
2	Add stain (water is just off boiling point)	1 g pararosaniline
3	Add chemical (temperature 50°C)	2 g $K_2O_2S_2$
4	Add acid (room temperature)	2 ml HCl (35%)
5	Add charcoal and leave overnight in stoppered flask	2 g activated charcoal
6	Filter through Whatman No. 1 filter paper. Stain can be used several times and is stable at 4°C for several weeks. Discharge when pink	
Mallory trichrome 200 ml		
1	Dissolve stain in distilled water	1 g aniline blue
2	Add stain	2 g orange G
3	Add stain	2 g oxalic acid
4	Mix and keep at 4°C until use	

used. From each fish, ten renal sections should be prepared, and the KEH of 40 secondary proximal convoluted tubules measured. The measurements should include all tubules of the second proximal convoluted segment until the pre-set number of 40 is reached.

ELISA for spiggin

In CEFAS, we have developed a high throughput ELISA for the androgen-induced protein spiggin, which has the capacity of measuring the protein titers in whole kidney homogenates. Both the production of antisera and the ELISA procedure have been described in detail previously.[21]

ELISA procedure

A large number of hypertrophied kidneys and urinary bladders from breeding males were pooled and dissolved in the strong urea solution described earlier (1:5 w/v). This was divided into 100-ml aliquots, which were frozen and given an arbitrary value of 10,000 units/ml. The coating material for the ELISA plates was prepared solely from nest glue. A large pool of nest-derived material was collected, treated with the strong urea buffer and frozen in aliquots of 100 µl. The procedure was finalized as follows. All kidneys to be assayed should be mixed with 200 µl of spiggin buffer and heated at 70°C for 30 min. The spiggin content of 20 kidneys can be determined, without replication, in one plate. Assays are carried out over 48 h. On day 1, the nest-derived material is diluted 1:1000 with coating buffer, and 100 µl is dispensed into every well of the appropriate number of high protein-binding plates. The plates are then sealed, wrapped in moistened paper and a plastic bag and stored at 4°C overnight. Also on day 1, 135 µl of assay buffer is added to all wells of an appropriate number of low protein-binding plates (i.e., 1 plate/20 samples). A vial of spiggin standard is thawed out and diluted 1:10 (1000 units/ml) and 1:50 (200 units/ml) in assay buffer. Each of the wells in columns 1 and 5 and wells A–D of column 9 receives 15 µl of each sample. Wells E and F of column 9 receive 15 µl of the high standard, and wells G and H receive 15 ml of the low standard. Using a

multipipette set to 15 μl, the contents of the wells in column 1 are mixed thoroughly by pipetting up and down 20 times; 15 μl are then transferred to column 2 and the procedure repeated up to column 4. The final 15 μl are discarded and the pipette tips changed. This complete procedure is then in turn carried out on the samples/standards that had been added to the wells in columns 5 and 9. The result is a set of 10-fold dilutions of all samples and standards.

Rabbit anti-spiggin serum is diluted 1:10,000 in assay buffer and 65 μl are added to all wells. The plates are then sealed, wrapped in moistened paper and a plastic bag, and stored at 4°C overnight.

On the morning of day 2, the high protein-binding plates are washed three times with 200 μl of wash buffer. A multipipette is used to transfer 150 μl of the contents of the wells of the low protein-binding plate into the corresponding wells of the high protein-binding plate. The transfer starts with column 12 and work backwards. Pipette tips are discarded after every four columns so as to avoid contamination between samples. The plates are resealed and wrapped and left to incubate for 4–6 h at room temperature. After this period, they are washed three times with wash buffer followed by addition of 150 μl of second antibody, diluted 1/15,000 in assay buffer. The plates are resealed, wrapped, and incubated overnight at 4°C.

On the morning of day 3, the plates are washed three times with distilled water followed by the addition of 150 μl of *p*-NPP. Color development is measured within an hour with the microplate reader set at a wavelength of 405 nm. The number of spiggin units/kidney is then divided by the weight of fish to give spiggin units/g of body weight.

The validation procedure of the developed ELISA for spiggin in CEFAS involved bisection of 160 kidneys in approximately two equal parts (which were weighed to the nearest mg), one of which was used for the immunoassay and the other half for light microscopy. Estimates of intra- and inter-assay variations, which measure the precision and reproducibility of the assay, respectively, were determined by repeatedly measuring the same kidney extracts and calculated as coefficients of variation (CV). Values were analyzed by ANOVA with a *post-hoc* Duncan's test during statistical analysis. Data were logarithmically transformed prior to analysis.

At the moment there is no available commercial kit for an immunoassay. However, it is anticipated that due to the recent advances in the molecular characterization of the protein, recombinant spiggin and antibodies will soon become commercially available.

Results and discussion

Besides the fact that the numbers of tests that measure estrogenic action are increasing,[3–6,24–28] to date there has been no reliable *in vivo* screening test system for environmental androgens and/or antiandrogens in the aquatic environment.

The experiments that took place in CEFAS were the first to demonstrate kidney hypertrophy and spiggin production in intact males (not gonadectomized) and females exposed to a number of androgens added to the water. A series of both natural and synthetic androgens have been tested in our lab and successfully induced spiggin production in female sticklebacks. However, the majority of the data we have produced (about 15 experiments) are on 17α-MT and DHT.

17α-MT was proved to be very efficient at inducing kidney hypertrophy in female sticklebacks in a dose response manner (Figure 20.1). The increase in the kidney epithelium

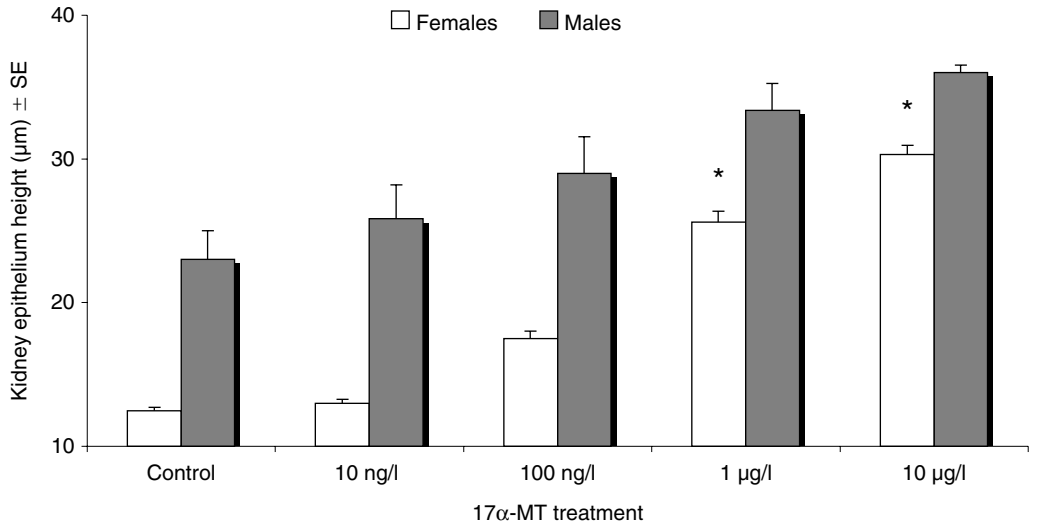

Figure 20.1 KEH of sticklebacks treated with 17α-MT. Asterisk indicates significant difference from control group ($p < 0.01$).

height (on average about 13 µm in female fish) between the different treatment groups was statistically significant ($p < 0.01$) for the highest 17α-MT doses used (1 and 10 µg/l).

The novel structural glycoprotein, spiggin, which was first characterized by Jakobsson et al. (1999),[13] is localized in the tubules of the secondary proximal segment, as shown by staining of androgen-treated female kidney sections with PAS (Figure 20.2, Plates (b)–(f)). A few females demonstrated small amounts of a PAS-positive material in their kidney tubules upon treatment with 17α-MT at 100 ng l/l. The increase in the KEH in this group, however, was not statistically significant. No PAS-positive material was detected when using lower concentrations of 17α-MT. Therefore, the lowest observed effect concentration (LOEC) for 17α-MT based on histological sections after a 3-week exposure was determined as 1 µg/l of ambient water and the no observed effect concentration (NOEC) as 100 ng/l.

Although the histological changes in the kidney provided a dose–response curve for 17α-MT (based on the height of the kidney cells) and proved to be a valid bioassay, the processing of a large number of samples for microscopy was time consuming. The development of an immunoassay based on the androgen-induced protein, spiggin, was expected to provide a much faster and specific bioassay. Our validation method, which consisted of comparing the KEH of 160 fish that received different doses of 17α-MT and/or FL (as measured on histological sections of half of the kidney) with the amount of spiggin units (as measured in the other half of the kidney), resulted in an excellent correlation ($r^2 = 0.93$, Figure 20.3). We observed no false positive or negative samples and parallelism in the ELISA assay were good. The spiggin assay was completed within 3 days of sacrificing the fish, while the histological method took a further 4 weeks. Spiggin concentrations showed a 10^5-fold variation — as opposed to a 4-fold difference in KEHs. This highlighted the differences between the different doses of 17α-MT much more clearly than the histological method. The excellent correlation between the histological assay and the developed ELISA for spiggin provided solid evidence of a fully functional novel bioassay for androgens. The precision of the assay, determined by calculating the intra-assay CV of repeated measures of samples within the same assay, was less than 9%.

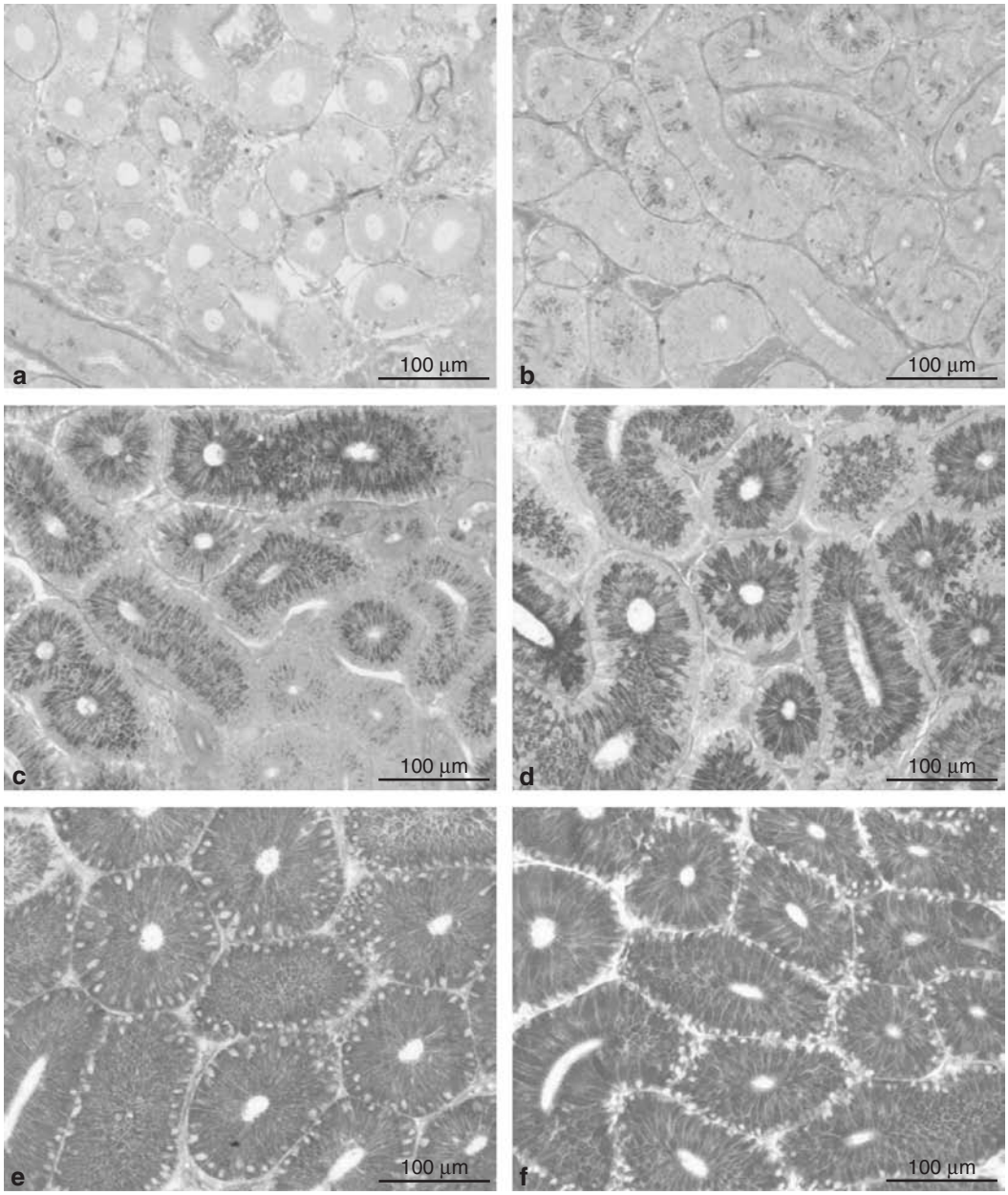

***Figure 20.2* (see color insert following page 464)** Renal histology of 17α-MT-treated female sticklebacks (a–e) and a naturally breeding male (f). Plates are at the same power and stained with periodic acid-Schiff (PAS). (a) control fish (KEH = 13.5 µm); (b) 100 ng/l 17α-MT (KEH = 18 µm); (c) 1 µg/l 17α-MT (KEH = 24.2 µm); (d) 10 µg/l 17α-MT (KEH = 29.7 µm); (e) 500 µg/l 17α-MT (KEH = 36.2 µm); (f) Breeding male (KEH = 34.5 µm).

The reproducibility of the assay, calculated as the inter-assay CV, was determined by measuring the same samples in four separate assays. The average inter-assay CVs of samples were less than 13%.

With the aim of applying the assay in the field of ecotoxicology, investigations into the potency of other model androgens, such as DHT, on the kidney hypertrophy in the three-spined stickleback started. Figure 20.4 displays the results of a time course of action experiment (1–5 weeks) using DHT to induce spiggin in female kidneys. Unfortunately, the fish present in the tanks with a DHT concentration at 5 μg/l developed a bacterial infection and died after day 14. This dose had nevertheless caused significant ($p < 0.01$) induction of spiggin at 7 and 14 days. One female fish showed spiggin induction at the

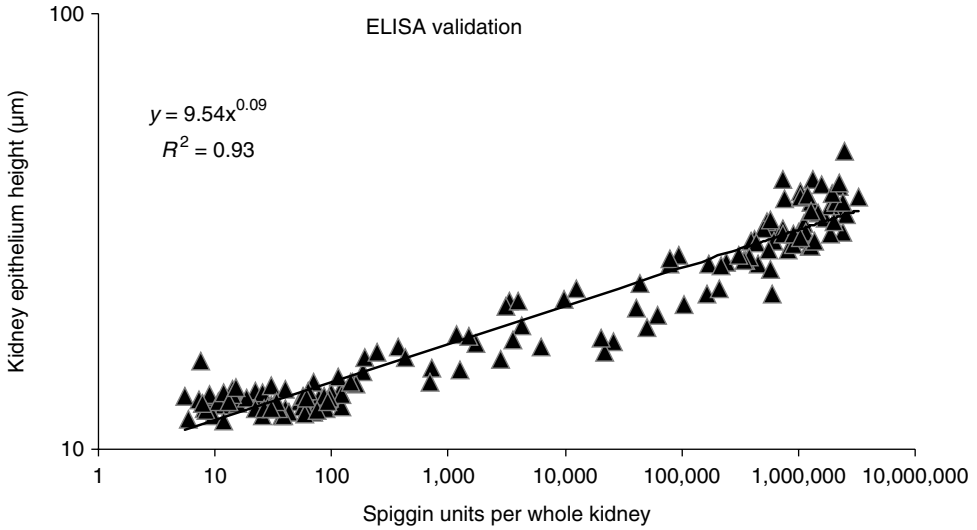

Figure 20.3 Comparison of the developed ELISA for spiggin with the KEHs obtained with histological examination.

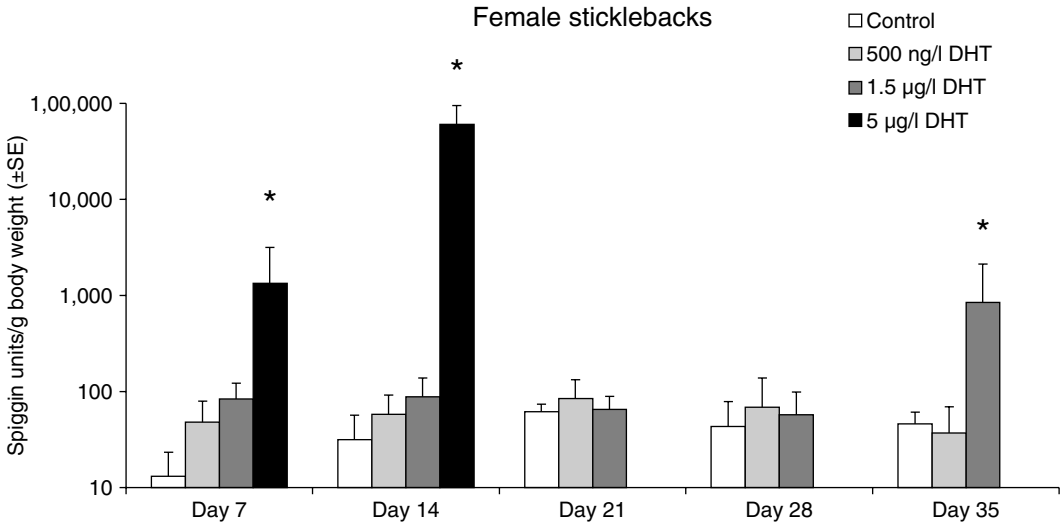

Figure 20.4 Time course of action of DHT as a model androgen. The asterisk denotes significant difference from control ($p < 0.01$).

dose of 1.5 μg/l, but only after 35 days exposure. No spiggin-positive females were observed at the dose of 500 ng/l.

The dose response experiment with DHT used the model androgens at the range 1–5 μg/l. Fish were exposed for either 3 or 5 weeks (Figure 20.5). Upon statistical analysis of the results, the NOEC and LOEC for a 3-week exposure to DHT were 2 and 3 μg/l, respectively ($p < 0.05$). However, after 5 weeks of exposure, statistical analysis ($p < 0.05$) indicated that the 5-week NOEC and LOEC for DHT were 1 and 2 μg/l, respectively.

Our results suggested that 17α-MT is slightly more potent than DHT. A differential potency of androgens, however, is not always related to a higher affinity for the relevant receptor; it could reflect a different degree of binding to steroid serum binding proteins (not mediated via the receptor).

During the main dose response experiment with DHT, the determination of the NOEC and LOEC was very accurate, and the differences between the DHT doses were very narrow. The only paradox in the construction of a dose–response curve for DHT was the apparently higher spiggin levels at 2 μg/l in comparison to the 3 μg/l during the 5-week exposure test. This difference, however, was non-significant. Several biological factors (i.e., other than the androgen dose used) — for instance size, age, social interaction, and infection — may influence the total amounts of spiggin produced in the kidney upon androgen stimulation of very similar doses. Another possible explanation could lie in the presence of natural androgens excreted by the males into the aquaria water. Indeed, in the group treated with 2 μg/l, there were more males present (11 males) than in the 3-μg/l group (8 males).

A laboratory exposure of female sticklebacks to several dilutions of Swedish PME for a period of 3 weeks took place in collaboration with Stockholm University. After treatment with PME, the kidneys were excised and divided into two parts, one of which was studied with light microscopy and the other assayed with the spiggin immunoassay. Both assays indicated significant kidney stimulation, showing on average 83,000 spiggin units per kidney (Figure 20.6) and a KEH of 27 μm. Regression analysis of the data obtained

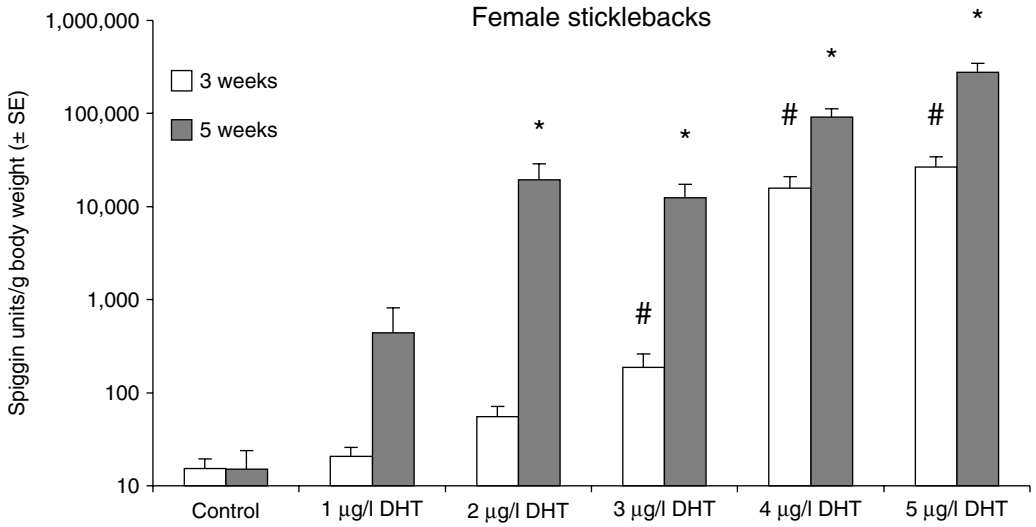

Figure 20.5 Dose–response curve for DHT after 3 and 5 weeks exposure. Significant inductions ($p < 0.05$) of spiggin in relation to the control groups is denoted by # (3 weeks) and * (5 weeks).

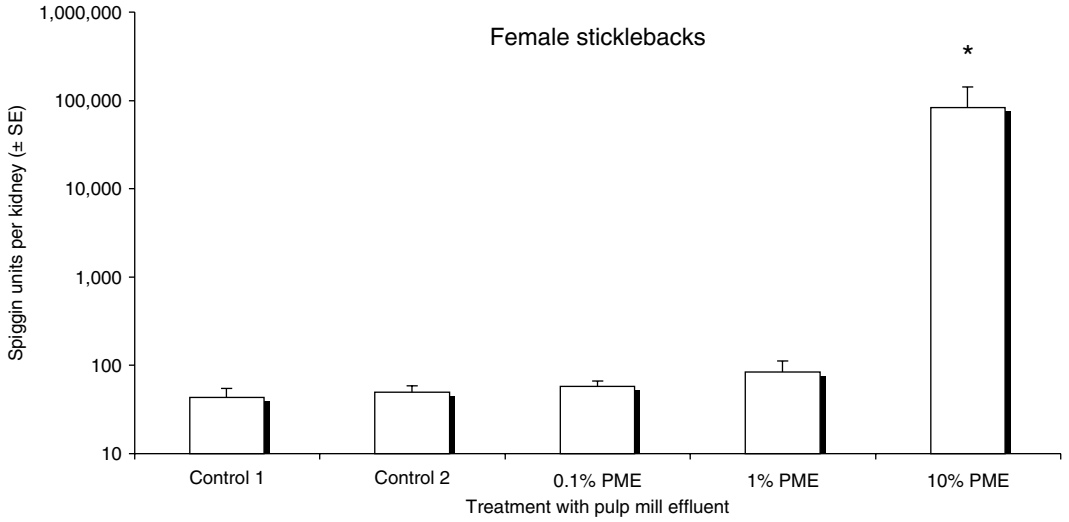

Figure 20.6 Female stickleback exposure to a well-known androgenic effluent. Significantly elevated spiggin denoted by asterisk ($p < 0.05$).

from the two assays revealed an excellent coefficient of correlation ($r^2 = 0.96$). The masculinizing effect of PME on female mosquito fish has been reported long ago,[8] but we have demonstrated for first time the androgenicity of this type of effluent in the stickleback.

Since the dissection of kidneys creates a potential bottleneck in what is an otherwise rapid method for assessing *in vivo* androgenicity of compounds, we investigated whether spiggin could be usefully measured in whole-body homogenates. The sticklebacks were first treated with different doses of 17α-MT for 3 weeks, then humanly killed, and their bodies digested with the same urea buffer used for preparing the kidney digest (Figure 20.7). It should be noted that the actual spiggin units induced by the 17α-MT concentrations, shown in Figure 20.7, seem low because the results are expressed in spiggin units per milliliter of homogenate, not in units per kidney. Although the range in response is not as good as when measured in kidneys alone, there is nevertheless a clear dose response effect in female fish. We have used this procedure to measure spiggin in juvenile sticklebacks with a very small body size (<0.1 g), where dissection is impossible, and have confirmed the validity of the method.

With the aim of addressing the problem of environmental anti-androgens, potentially a far more interesting group of xenobiotics than androgens themselves, we designed an exposure system where anti-androgenic activity could be tested. In this system, female sticklebacks are exposed simultaneously to an androgen and an anti-androgen, and the degree of kidney hypertrophy is measured. We have chosen FL at 500 μg/l of ambient water and demonstrated a very clear inhibitory effect (Figure 20.8). At both concentrations of 17α-MT tested (100 ng/l and 1 μg/l) the resulting spiggin units of fish treated with FL were statistically significantly lower ($p < 0.05$) than the protein units of the fish that were treated with 17α-MT only. In more recent experiments, we have used 17α-MT at 500 ng/l and found it to induce on average 90,000 spiggin units/g body weight in female sticklebacks. This lower concentration of 17α-MT allows the FL-induced inhibition of spiggin production to be become evident at lower concentrations of FL (Figure 20.9).

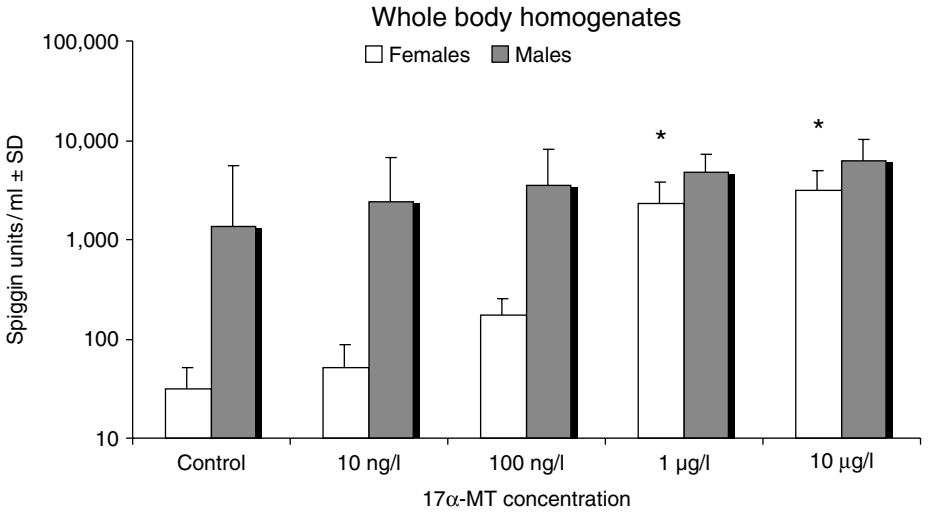

Figure 20.7 Application of the ELISA for spiggin on whole-body digests. Asterisks denote concentrations of 17α-MT that significantly induced spiggin ($p < 0.05$).

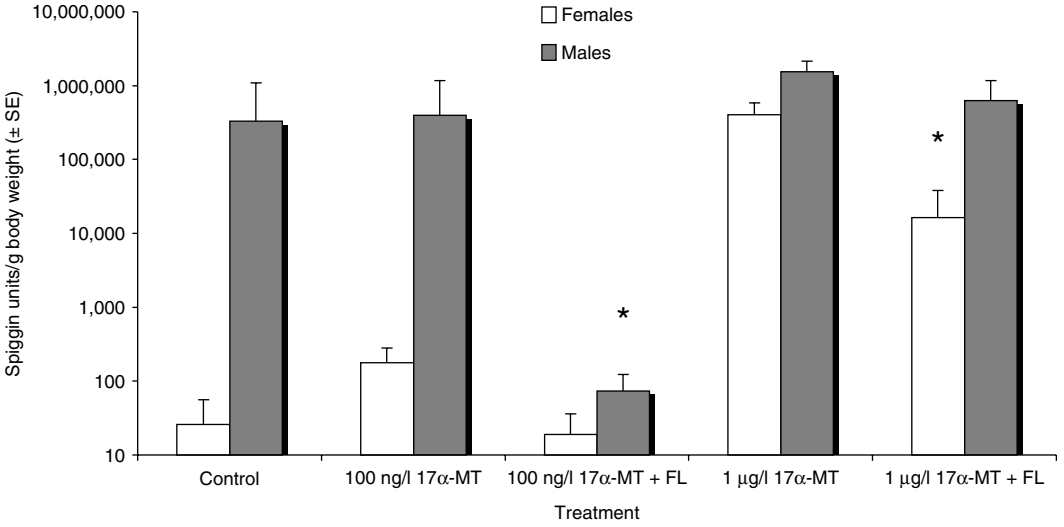

Figure 20.8 Spiggin units per kidney of sticklebacks treated with 17α-MT or 17α-MT and FL at 500 µg/l. Asterisks denote 17α-MT and FL treatments that were significantly different from the 17α-MT only group ($p < 0.05$).

In addition, the dose response experiment with DHT included EE_2 at 20 ng/l and FL at 500 µg/l. Figure 20.10 presents the results; which show that after 3 weeks of exposure, no spiggin-positive females were found in the tanks containing FL (along with 5 µg/l DHT), EE_2, or methanol only, and no spiggin-positive males were found in the FL-treated tanks, only 1 in the EE_2-treated tank (low levels) and 2 in the control tanks. After 5 weeks of exposure, no spiggin-positive females were found in the tanks containing FL (along with 5 µg/l DHT), EE_2, or methanol only, and no spiggin-positive males were found in the FL-treated tanks, only 1 in the EE_2-treated tank (low levels) whilst there were 6 in the

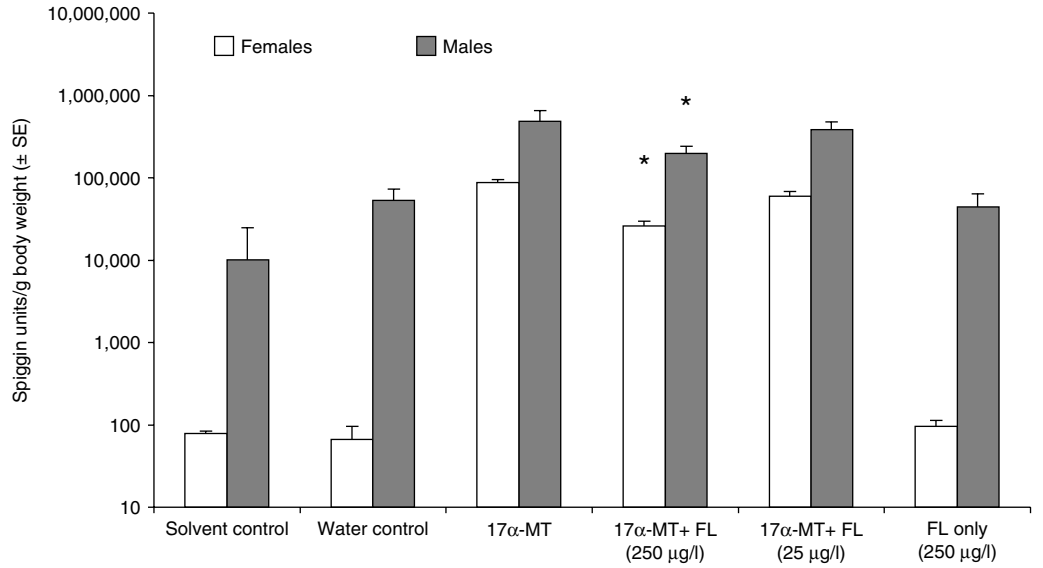

Figure 20.9 Spiggin units per kidney of sticklebacks treated with 17α-MT at 500 ng/l or 17α-MT and FL. Asterisks denote 17α-MT and FL treatments that were significantly different from the 17α-MT only group ($p < 0.05$).

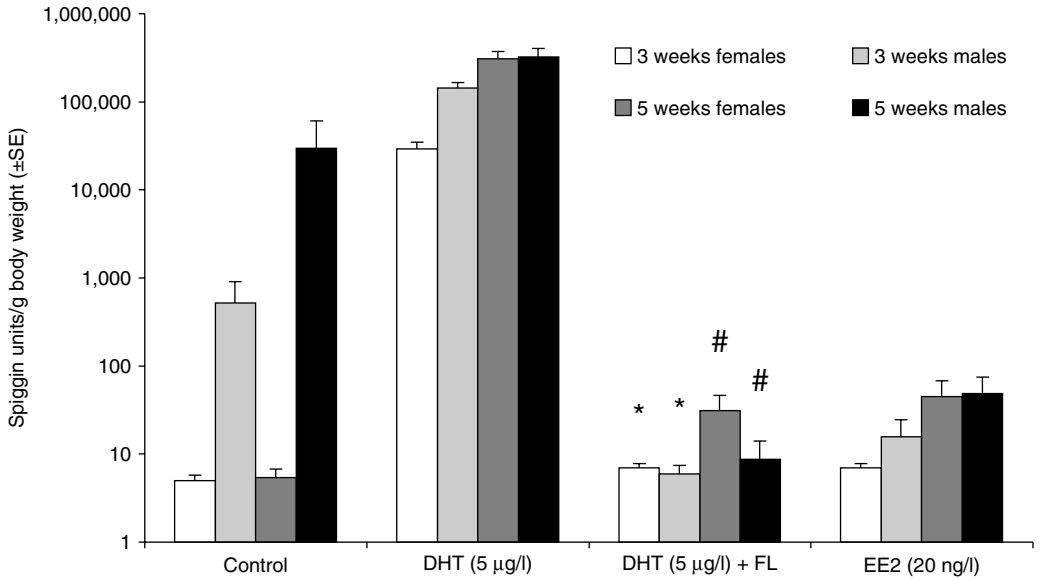

Figure 20.10 Spiggin units in different groups of sticklebacks. Significant inhibition ($p < 0.05$) of spiggin relative to the DHT-treated group is denoted by # (3 weeks) and * (5 weeks).

control tanks. The lack of kidney stimulation in the EE_2-treated groups in comparison to the controls suggests that estrogens antagonize natural androgens. Indeed, in all control male groups used throughout the whole study, kidney stimulation was observed in a significant number of males under laboratory conditions. More recent experiments have

confirmed that relatively low concentrations of estrogens (i.e., $EE_2 > 10\,ng/l$ and $E_2 > 100\,ng/l$) are able to inhibit spiggin production in male fish.

More recently, we have run a series of experiments using suspected environmental antiandrogens and have demonstrated that vinclozolin, fenitrothion, linuron, and DDE metabolites all inhibit or significantly reduced spiggin production in the androgen-stimulated female kidney.

The following list summarizes the advantages of the stickleback as a test and sentinel species for EDCs:

1. During the spawning season, the kidneys of male sticklebacks produce substantial amounts of a nest-building protein, spiggin, in response to androgenic stimulation, and this response is highly specific to androgens (i.e., progestogens and estrogens are ineffective).
2. The spiggin can be readily quantified using the validated ELISA (providing a 10^5-fold range in spiggin content of stickleback kidneys) and can be induced in female kidneys by androgen exposure, while this induction is antagonized by antiandrogens.
3. The stickleback is: a recognized Organisation of the Economic Co-operation of Developed countries (OECD) test species (OECD Guideline 210); an extremely ubiquitous species found in all aquatic habitats from full seawater to freshwater; endemic in Europe and North America, unlike other fish used as test-species, it is easily obtained in very large numbers in the field, allowing for *in situ* biomonitoring; easy to maintain in laboratory aquaria (both adults and larvae); has a short life cycle and is readily induced into breeding condition all-year-round by photothermal manipulation; its reproductive behavior is better known than any other fish and its reproductive physiology/endocrinology is well documented; it presents very high egg/fry survival rates (close to 100%).
4. A vitellogenin assay has also been developed at CEFAS, thus the stickleback has the potential to act as a simultaneous biomarker for androgens (spiggin induction in females) and estrogens (vitellogenin production in males), dramatically reducing the number of fish and the costs used for testing.

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chapter twenty-one

Simple methods for estimating exposure concentrations of pesticide resulting from non-point source applications in agricultural drainage networks

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Syngenta

Contents

Introduction

Refinement of quantitative assessment for pesticides and other agricultural chemicals in surface water networks (rivers, streams, lakes, and reservoirs) is being pursued with increased intensity. Recent passage of the Food Quality Protection Act (FQPA) by Congress and the conclusions reached by the US EPA Ecological Committee on FIFRA Risk Assessment Methods (ECOFRAM)[1] have particularly accelerated the effort, requiring that

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such assessment be conducted accurately with reasonable certainty. Pesticide occurrence in surface water systems is complicated and highly variable over time and geographical locations due to different use patterns (application timing, rate, and method), weather, and watershed characteristics. Monitoring to directly determine exposure with sufficient frequency on a site-by-site basis is almost prohibitively expensive and is impossible for new compounds. As a result, predictive models with measurable accuracy have become important assessment tools to address ecological, as well as drinking water, concerns.

There have been a large number of process-based models that have been developed to estimate pesticide concentrations in the environment.[2–5] These models are mostly mechanistic in nature, each representing a specific homogeneous environment with well-defined pesticide use patterns and physical processes of environmental fate, weather, and hydrology. For example, the Pesticide Root Zone Model (PRZM)[3] simulates surface runoff, soil erosion, and leaching from a specified homogeneous field, while the Exposure Analysis Modeling System (EXAMS) predicts the fate processes in an aquatic environment (such as a pond or lake).[4] Mechanistic models are most useful in parameter sensitivity analysis and comparative assessment where simulations of different chemicals are conducted under the same environmental conditions. Although mechanistic models adequately describe relatively homogeneous and small plot runoff and microcosm/mesocosm experiments (generally <1 ha), extrapolating these models to large-scale watersheds is not as successful.[6,7] Model performance can be further degraded, when compounding worst-case assumptions on use rate and environmental fate parameter values as required by current regulatory guidelines.[5,7] As a result, predictions may be many orders of magnitude different from the monitoring data.

Mechanistic models are developed and calibrated at a small field scale. However, very little is known as to how a physical or chemical process calibrated on a small field scale of given landscape characteristics may be extrapolated to a much larger-scale watershed, within which many variable small-scale or sub-watersheds/catchments are arranged in a particular pattern or with variable degrees of spatial correlation. Consider a pulse of an environmental chemical resulting from a runoff event in a relative homogeneous environmental segment. The fate and transport processes may be well described deterministically by a mechanistic model. However, as the pulse continues to move through the watershed, experiencing different segments of wide variability in surface hydrology, wetness, topography, soil, and vegetation, the resulting process, and interactions may be more complex than the original model would predict.

The response of the whole watershed is not only dependent on a set of parameter values (even they are distributed) but also on the spatial correlation of all segments (i.e., the physical segment layout and geometry) and their inter-segment linkages that are often poorly understood. It is arguable that such problem of spatial variability can be effectively dealt with probabilistically without considering spatial correlation. Spatial and temporal variability in farming practices, hydrological properties, and coincidence of agricultural applications and storm/runoff events at a given point in time and space can all easily lead to additional complexity in the development of a comprehensive and fully physically based watershed model.

To overcome the complexity and uncertainty of mechanistic models as above, several simple regression approaches have been proposed recently in the development of watershed scale models.[8–11] Larson and Gilliom[8] found that, regardless the time dimension, percentiles of the monitoring data of several major herbicides obtained from the US Geological Survey (USGS) National Water-Quality Assessment (NAWQA) Program could be explained in a significant regression by chemical use intensity and four

watershed hydrological characteristics (runoff factor, soil erodibility coefficient, Dunn overland flow, and drainage area). Larson et al.[9] later further extended the regression to include more percentiles derived from the atrazine monitoring data and named the regression as Watershed Regression for Pesticides (WARP) model.

Since the WARP model does not include pesticide environmental fate properties, different WARP models would need to be developed for different pesticides. In 2002, an independent regression approach was developed by Chen et al.[10] using the newly proposed pesticide surface water mobility index (SWMI) and watershed use intensity to explain pesticide percentiles.[10] As SWMI is determined by the organic carbon normalized soil/water sorption coefficient (K_{oc}, ml/g) and soil degradation half-life, the SWMI regression models would be able to differentiate pesticides of different properties. More recently, the SWMI concept was further incorporated into the WARP method to obtain ability of multi-compound predictions.[11] None of the approaches, however, have been able to predict the time series data required for accurate environmental exposure assessments.

Regression models are completely based on monitoring data. While not explicitly concerned with the exact mechanisms involved in the watershed transport process, the removal of *a priori* assumptions in regression models may offer the opportunity to simulate reality more closely as they are distilled past experiences (data). In the next few sections, the SWMI regression approach for distributional concentration (percentile) predictions is reviewed, and the SWMI approach is extended to establish a new regression method for predicting daily time series concentrations. Wherever it is deemed necessary, specific calculation procedures are also included.

Mobility of organic compounds to surface water

Any organic compound is more or less mobile in the environment. The term mobility can be defined as its inherent propensity susceptible to off-site transport by a hydrologic process, such as soil leaching or surface runoff. Traditionally, pesticide mobility has been quantified by the organic carbon normalized soil/water sorption coefficient (K_{oc}, ml/g). Compounds with higher K_{oc} values tend to bind more to soil particles than the lower K_{oc} compounds, thus movement by water is retarded.

The parameter K_{oc} alone, however, is not a full indicator of mobility, as the chance of a pesticide to be potentially transported off-site is also dependent on how quickly or slowly it is degraded, i.e., its persistence in soil. The concept of mobility was thus extended to include degradation half-life ($T_{1/2}$) by forming a combined mobility index to rank soil leaching potential of various chemicals.[12–14] However, only recently has the concept been further developed to benchmark pesticide mobility specifically relevant to surface water transport processes, such as surface runoff and soil erosion.[10]

Recognizing that surface water transport processes like storm-generated runoff events are more transient and distinct intermittent events compared to soil leaching (generally gradual, slow, and continuous), SWMI was derived from the basic equations of field runoff and erosion.[10] A standard environmental scenario was parameterized to benchmark off-site runoff potential of various chemicals with different pairs of K_{oc} and $T_{1/2}$. The resulting SWMI expression in terms of K_{oc} and $T_{1/2,}$ was simplified as[10]:

$$\mathrm{SWMI} = \frac{\exp(-3.466/T_{1/2})}{(1 + 0.00348K_{oc})}(1 + 0.00026K_{oc}) \tag{21.1}$$

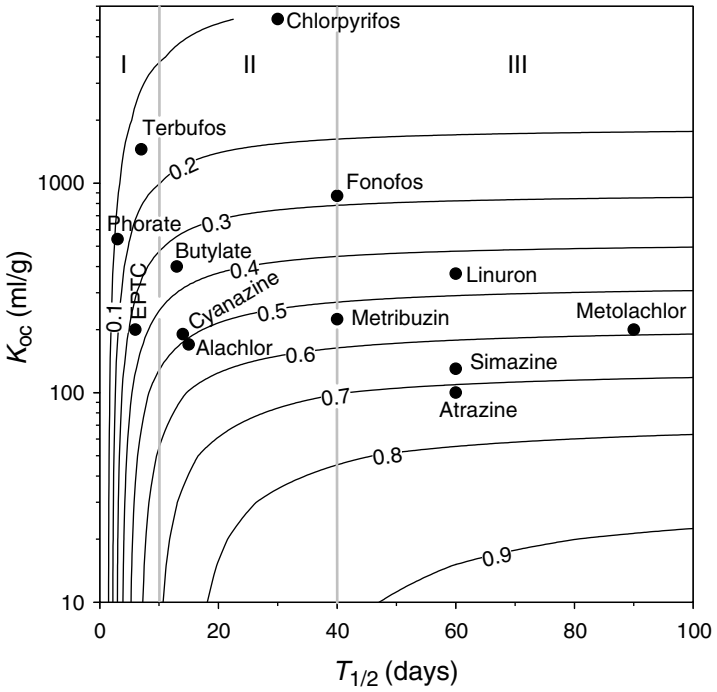

Figure 21.1 Contour plot of the pesticide SWMI as a function of the organic carbon-normalized soil/water sorption partition coefficient (K_{oc}) and degradation half-life ($T_{1/2}$). (From Chen, W. et al., *Environ. Toxicol. Chem*., 21, 300, 2002. With permission.)

SWMI, as calculated in Equation (21.1), provides a relative mobility scale, with value 0 being the asymptotically least mobile and 1 the asymptotically most mobile and susceptible to transport by the processes of surface water runoff and soil erosion.

Calculating SWMI is straightforward. Paired K_{oc} and $T_{1/2}$ values measured from the same soil in the laboratory or field studies are preferred whenever possible for a given compound. If multiple paired measurements are available for K_{oc} and $T_{1/2}$, calculating SWMI for each individual paired values and averaging should provide a more representative estimate for SWMI.

A contour plot of SWMI as a function of K_{oc} and $T_{1/2}$ is provided in Figure 21.1 for a number of commonly used pesticides. The lines in the plot represent equal values of SWMI. As discussed by Chen et al.,[10] three sensitivity regions in the SWMI plot can be identified to illustrate the differential roles of K_{oc} and $T_{1/2}$ in determining mobility. For Region I ($T_{1/2} < 10$ days), SWMI is predominantly a function of half-life, and it is much less sensitive to K_{oc}. Off-site movement of these short-living compounds is expected to be much less dependent on their soil adsorption but more correlated with the temporal and spatial coincidence of pesticide applications and runoff events during the life span. For region II, half-life is between 10 and 40 days, and the mobility index is sensitive to both parameters. For Region III, compounds are more persistent, and K_{oc} primarily determines SWMI. Such is expected, as runoff is more likely during a longer half-life period.

Predictive regression models using SWMI

With the pesticide mobility index defined in the previous section, one would expect that a regression relationship could be established between SWMI and the corresponding watershed observed pesticide concentrations. Depending on sorting patterns of the observed data, two general types of regression models can be classified. In this chapter, we refer to the first type as the distributional concentration model (DCM) and the second as the time series concentration model (TSCM).

DCM is developed on data points at a given percentile level, which is obtained from sorting a data set in ascending order according to the magnitude of the observed values. Note that a percentile is the rank of a value in a data set as a percentage of that entire data set (but see next section for time-weighted percentiles). For example, the 95th percentile value means that 95% of the data values in a data set are equal to or less than the given value. A 95th percentile DCM, therefore, is the regression based on the 95th percentile data and would predict the possible 95th percentile values.

Unlike DCM that disregards the time dimension, TSCM retains the temporal sequence in the same order of sampling. Time thus is always an independent variable in TSCM regressions. As such, TSCM is able to simulate the fluctuation, trend, and seasonality in the concentration dynamics of a surface water system.

Time-weighted sampling

Most surface water monitoring programs are designed to sample more frequently immediately after field application of pesticides (usually late spring to early summer) when higher runoff is expected. Less frequent samples are collected in the rest of the year. The uneven sample frequency within a calendar year requires the measured pesticide concentrations to be time-weighted to remove bias. By time weighting, the 95th percentile value would mean that over 95% of the total time during an observation period the observed data are equal to or less than the given value.

Assuming each sampling point represents half the time to the preceding sample and half to the following sample as described by Chen et al.,[10] a time weight for each sample can be calculated as:

$$w_i = [(t_{i+1} - t_{i-1})/2] \Big/ \sum_{i=1}^{n} t_i \tag{21.2}$$

where t_i is time point of sample i; n is number of samples during the total sampling period. The weights are then organized by their associated corresponding concentrations in an ascending order. The cumulative time-weighted percentile associated with each concentration is thus determined as:

$$P_i = 100 \sum_{j=1}^{i} w_j \tag{21.3}$$

where P_i corresponds to the time-weighted percentile at concentration C_i of sample i. An exact percentile, for example, the 95th, is estimated by linear interpolation between two adjacent samples. With Equation (21.2), the time-weighted mean concentration (TWMC) is then calculated:

$$\text{TWMC} = \sum_{i=1}^{n} w_i C_i \tag{21.4}$$

Distributional concentration model (percentiles)

Data

The data set used for the SWMI DCM development and evaluation was obtained from the pesticide monitoring program conducted in six drainage basins of Lake Erie tributaries by the Water Quality Laboratory at Heidelberg College, Ohio, USA.[10,15] Sampling locations in each of the six drainage basins are shown in Figure 21.2. We refer this data set as Heidelberg data.

The Heidelberg data set is one of the most comprehensive databases available in the U.S., with recorded pesticide riverine concentrations continuously since 1983. Sampling frequencies in the data set range from 3 samples/day during high runoff season (typically April 15 to August 15) to about 2 samples/month in the rest of each calendar year.[15] A list of the pesticide environmental fate properties, total use, and calculated SWMI values is provided in Table 21.1. Each drainage basin and its area are in Table 21.2. A more detailed description of the monitoring program can be found in Reference 15.

Model development

A power function of SWMI with a linear factor of pesticide watershed use intensity was found to describe the Heidelberg data well at various percentiles for each tributary or all six tributaries combined. Detailed descriptions of the model development are available in Reference 10. The SWMI DCM reads:

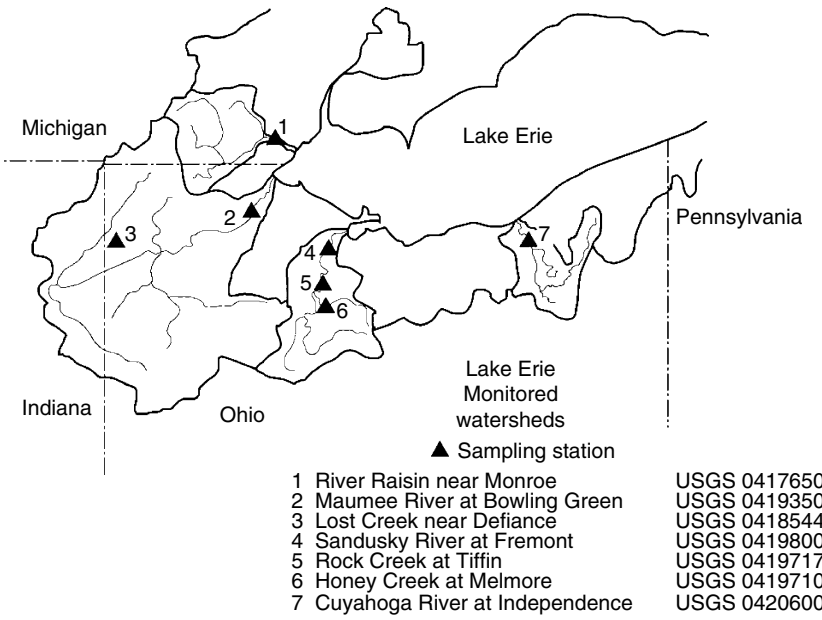

Figure 21.2 Pesticide sampling stations of the agricultural runoff-monitoring program in the Lake Erie tributary drainage basins, Heidelberg College, Ohio, USA. The U.S. Geological Survey (USGS) stream gauging station numbers are shown on the right side of the label. (From Chen, W. et al., *Environ. Toxicol. Chem.*, 21, 301, 2002. With permission.)

Table 21.1 Pesticide information used in the development of the DCM with the SWMI. (Modified from Chen, W. et al., *Environ. Toxicol. Chem.*, 21, 301, 2002. With permission.)

Pesticides	Type	K_{oc} (l/kg)	$T_{1/2}$ (days)	SWMI	Total use in 1986 (kg)	Use intensity[a] (kg/ha)
Alachlor	Herbicide	170	15	0.5207	1,058,896	0.4522
Metolachlor	Herbicide	200	90	0.5969	665,417	0.2842
Atrazine	Herbicide	100	60	0.7184	581,750	0.2485
Cyanazine	Herbicide	190	14	0.4932	206,463	0.0882
Metribuzin	Herbicide	224	40	0.5453	203,405	0.0869
Linuron	Herbicide	370	60	0.4523	110,931	0.0474
Terbufos	Insecticide	1540	7	0.1388	59,265	0.0253
Butylate	Herbicide	400	13	0.3536	50,558	0.0216
Chlorpyrifos	Insecticide	6070	30	0.1038	24,565	0.0105
EPTC	Herbicide	200	6	0.3482	30,757	0.0131
Phorate	Insecticide	540	3	0.1248	25,358	0.0108
Fonofos	Insecticide	870	40	0.2792	19,432	0.0083
Simazine	Herbicide	130	60	0.6718	14,132	0.0060

[a] Based on the total land areas of 2,341,468 ha where the total use data we obtained in the Lake Erie tributary region, 1986.

Table 21.2 Drainage areas of Lake Erie major tributaries, Ohio, and White River, Indiana

River name	Drainage area (ha)
Lost Creek	1,130
Rock Creek	8,800
Honey Creek	38,600
River Raisin	269,900
Sandusky River	324,000
Maumee River	1,639,500
White River	2,938,382

$$C_{P(x)} = \frac{M_{\text{use}}}{A_{\text{b}}} \alpha \text{SWMI}^{\beta} \tag{21.5}$$

where $C_{P(x)}$ is the xth percentile concentration, μg/l, or ppb; M_{use} is total annual use of a pesticide in a basin or watershed, kg; A_{b} is total area of a drainage basin or watershed, ha; α and β are regression constants. Defining $R_{\text{b}} = M_{\text{use}}/A_{\text{b}}$, R_{b} then becomes the annual watershed use intensity, kg/ha. As a power function, the SWMI DCM would predict 0 concentrations for any percentile levels as SWMI approaches 0 (the most immobile compound).

The SWMI DCM was calibrated initially based on data at three concentration levels (TWMC, the 95th percentile, and peak) for the entire monitoring period (1983–1991) and for year 1986 data only as the pesticide use information in the region was only surveyed in that year.[10] The model calibration was further extended to include the 80th, 90th, and 99th percentiles for the 1986 data. Lower percentile regressions are not available as most of the concentrations were non-detectable (recorded as 0). The calibrated 1986 model parameters are summarized in Table 21.3. Results of other calibrations, such as for each individual river or over the entire 9-year's data set (1983–1991), are available in Reference 10.

Model use

Calculation of the SWMI DCM [Equation (21.5)] is simple once the parameter values are determined. It can be easily implemented on spreadsheet programs, such as Excel® (Microsoft Corporation, Washington, USA). When a range of environmental fate (i.e., variable K_{oc} or $T_{1/2}$) or variability in use intensity is expected, the calculation can be done in a probabilistic manner. Three simple steps are summarized below:

1. Selection of a percentile model should be based on the assessment purpose. For example, if the TWMC is of interest, the regression constants for TWMC should be used (Table 21.3).
2. Calculate SWMI using Equation (21.1) if a pair of K_{oc} and $T_{1/2}$ determined from the same soil is given. If multiple paired K_{oc} and $T_{1/2}$ data are available, calculate SWMI per each pair of parameters, then average the SWMI estimates. For multiple unpaired K_{oc} and $T_{1/2}$ data, an independent random sampling from the distributions of the two parameters may be executed to obtain a distribution of DCM estimates (e.g., using Crystal Ball® software, Decisioneering, Denver, Colorado, USA).
3. Use intensity should be estimated always as accurately and as currently as possible. If the total use (M_{use}) in a watershed of interest is not known, it may be estimated by multiplying the label use rate (often available on the product package) by the total treated area expected in the watershed. If a distribution of M_{use} is available, such as through survey, a distribution of the model runs can be carried out accordingly.

Some example runs and cautions for interpreting the model results are provided in the section ''Results and discussion.''

Time series concentration model (daily)

The occurrence of pesticides in natural water systems is generally seasonal. In addition to seasonality, local transient fluctuations are inevitable, as agricultural practices, weather, and pesticide dissipation processes are all time-dependent, either deterministically or

Table 21.3 Calibrated model parameters for the DCM based on the single year (1986) data of the six Lake Erie agricultural tributary drainage basins, Ohio. (Modified from Chen, W. et al., *Environ. Toxicol. Chem.*, 21, 305, 2002. With permission.)

Parameters	Log(α)	β	R^2	n [a]	S^2 [b]
TWMC	1.253	2.854	0.67	72	0.24
80th percentile	1.558	2.745	0.74	54	0.11
90th percentile	1.937	3.131	0.81	63	0.13
95th percentile	2.093	2.825	0.87	67	0.07
99th percentile	2.293	2.442	0.65	71	0.19
Peak	2.492	2.267	0.53	72	0.26

[a] Number of data points. The different number of data points is due to no samples or values excluded when below limit of detection.

[b] Sample variance of regression residuals.

stochastically. Predictions of the detailed time sequence of pesticide concentrations are challenging and yet critical in assessing exposures of different time periods often important to address eco-toxicological or drinking water concerns. In this section, we extend the SWMI concept to a TSCM for predicting pesticide daily concentrations in rivers and streams. We first establish a specific atrazine TSCM as a base model. The specific TSCM is then generalized for other compounds through SWMI and product use rate in a relationship with atrazine.

Model development

The TSCM to be developed considers three components in a typical pesticide daily concentration sequence: seasonality, long-term trend, and local instant fluctuation (random or flow-caused). Its basic form of the equation, modified after Helsel and Hirsch,[16] reads:

$$\ln (C + 1) = (1 + b_0 \ \exp [b_1 \sin (6.2832t) + b_2 \cos (6.2832t) + b_3 t]) \, x^k \qquad (21.6)$$

where C is concentration, μg/l, or ppb; t is time, year; 6.2832 is constant for the periodic function expressed in years; x is the ratio of stream flow rate (m^3/day) to the watershed area (m^2), m/day; b_0, b_1, b_2, b_3, and k are regression constants. The left-hand side of Equation (21.6) is a transformation to ensure valid mathematical operations in case of non-detectable concentration records (i.e., 0's).

A meaningful interpretation for each of the regression constants in Equation (21.6) is: b_1, and b_2 in the sine and cosine terms determine the amplitude and the time of the cycle of the concentration peaks, b_3 represents a global trend (slope), k is a coefficient accounting for the transient fluctuation caused by short-term flow dynamics, and b_0 is an overall adjusting coefficient.

Equation (21.6) is calibrated using the atrazine concentrations of the Heidelberg data set observed at the Maumee River station in a 10-year time span from January 1, 1986 to December 31, 1995. The stream flow data was obtained from the corresponding USGS gauging station (Figure 21.2). Results of the regression are presented in Figure 21.3A (transformed scale) and Figure 21.3B (linear scale). Residual quantile–quantile, 1:1, and percentile plots of the regression are shown in Figures 21.4A, B, and C, respectively.

The resulting atrazine TSCM coefficients are $b_0 = 0.00957$, $b_1 = 1.393$, $b_2 = -6.109$, $b_3 = -0.0173$, and $k = 0.14$. Using the phase shift relationship with b_1 and b_2, one can easily calculate the peak day[16]:

$$\text{peak day} = 58.019\left[1.5708 - \tan^{-1}\left(\frac{b_2}{b_1}\right)\right] \approx 169 \text{ (or June 18)}$$

which indicates that over the 10 years from 1986 to 1995 there is a global seasonality of atrazine concentrations that peaked around June 18 each year in the Maumee River drainage watershed. Actual peak time may be influenced by the instant local fluctuations caused by stream flow ($k = 0.14$) and other random factors, such as storm events. The negative sign of b_3 suggests a slight long-term decline trend for atrazine in the watershed.

The specific atrazine TSCM can be generalized for other compounds by incorporating two key factors; the chemical environmental fate properties (as represented by SWMI) and annual product use intensity in a watershed (R_b). The assumption is that the calibrated atrazine TSCM reflects the basic structure of the pesticide occurrence patterns in a watershed that are primarily influenced by agricultural seasonality, long-term trend,

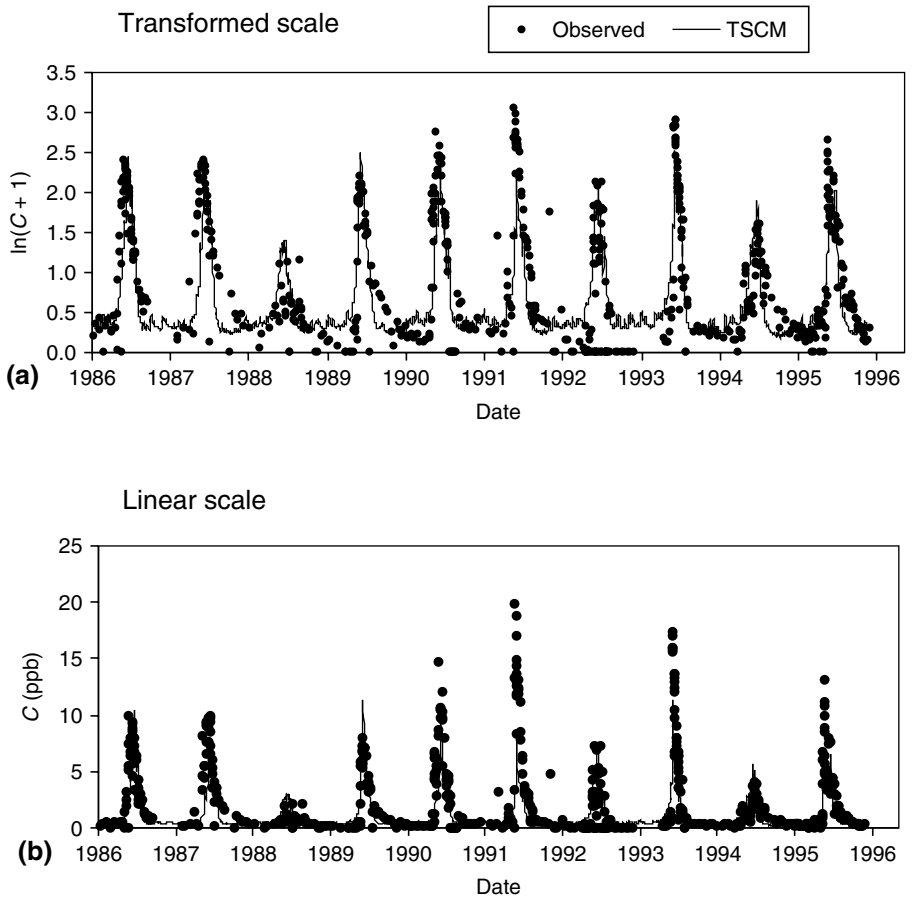

Figure 21. 3 The atrazine TSCM and the observed concentrations of the Heidelberg data set at the Maumee River station, Ohio, USA, in a 10-year time span between January 1, 1986 and December 31, 1995. The resulting model coefficients $b_0 = 0.00957$, $b_1 = 1.393$, $b_2 = -6.109$, $b_3 = -0.0173$, and $k = 0.14$.

and stream flow (an integrated parameter of weather and watershed hydrology). To achieve a generalized TSCM, atrazine is thus used as a standard to which other pesticide concentrations are compared. If the general use pattern and agricultural seasons are similar, the ratio of a pesticide's concentration to atrazine observed at the same time and location would be expected to correlate to the pesticide's environmental fate properties and its use rate in the watershed.

Atrazine as a comparison standard is primarily rationalized by its moderate mobility and persistence in the environment and the fact that it is one of the most popular, widely used, and longest established herbicides in the US agriculture. It can be shown that atrazine concentrations in the Heidelberg data set correlate well with many other compound's observed data. To seek for accuracy and representativeness, however, only the 1986 data from Maumee River, the largest Lake Erie tributary watershed, is processed as the pesticide use information was surveyed only specifically in that year.[10] As examples shown in Figure 21.5, a general good correlation exists between atrazine and

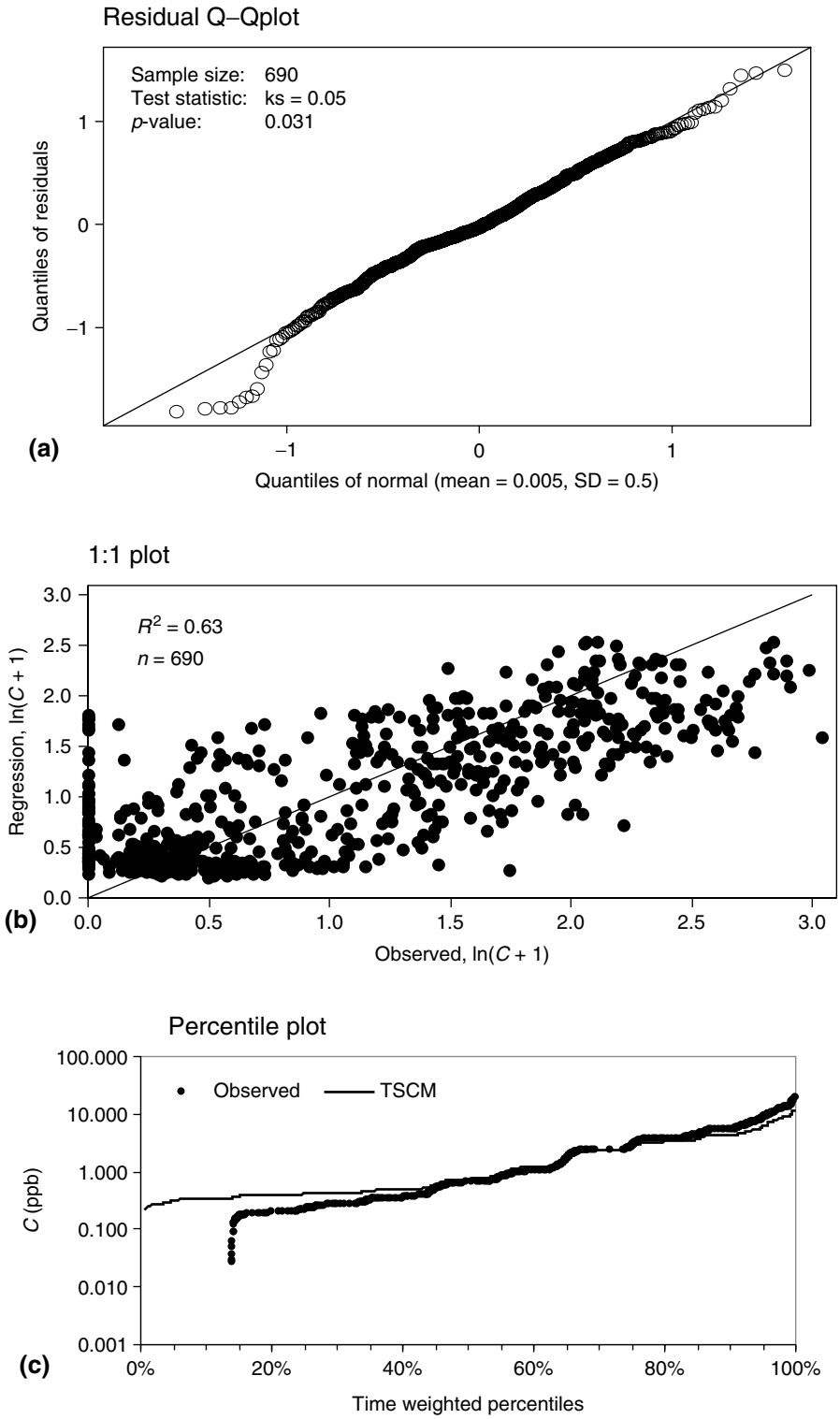

Figure 21.4 Residual quantile–quantile (a), 1:1 (b), and percentile (c) plots of the regression between the atrazine TSCM and the observed concentrations of the Heidelberg data set at the Maumee River station, Ohio, USA. Shown on the Plot A are also the results of the Kolmogorov–Smirnov (ks) goodness of fit test.

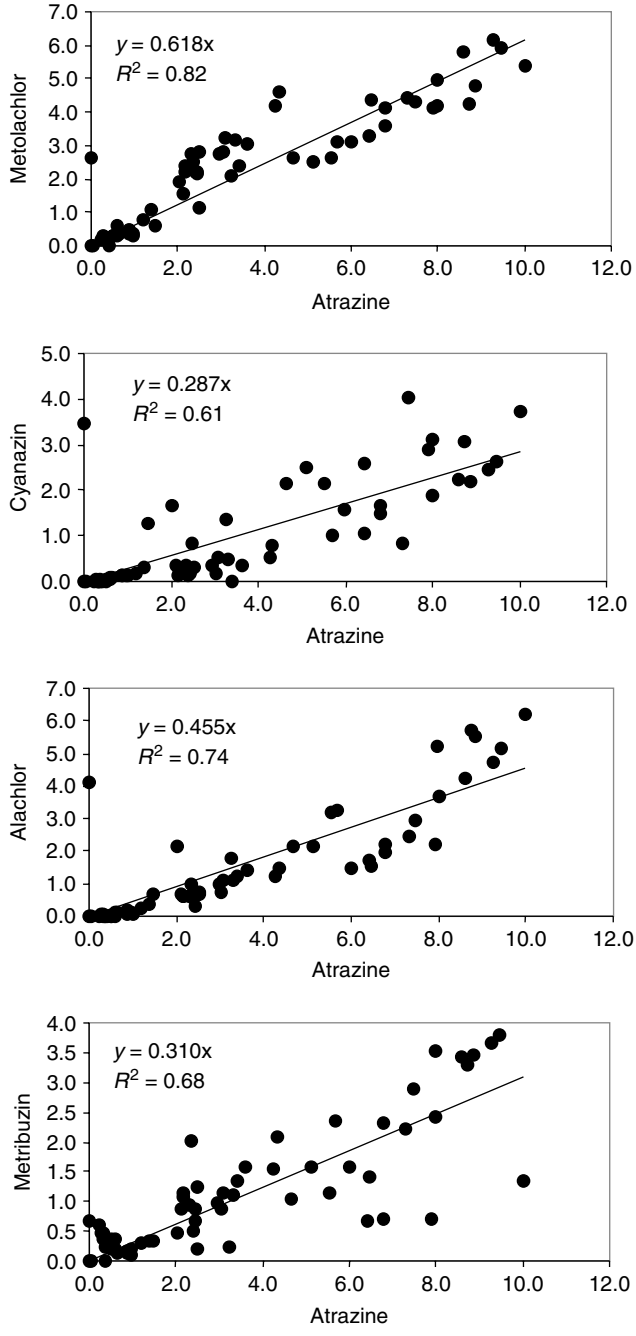

Figure 21.5 Examples of pesticide concentrations (ppb) in relation to atrazine observed in Maumee River, Ohio, 1986.

the concentrations of metribuzin, metolachlor, and alachlor. The resulting slopes of eight pesticide concentrations to atrazine in the Maumee River sub-data set are listed in Table 21.4, together with their SWMI and use intensity values. Pesticides with a majority of

Table 21.4 Ratio (slope) of pesticide concentrations (ppb) to atrazine observed in Maumee River, Ohio, 1986, in relationship with compound environmental fate properties (SWMI) and watershed use intensity (R_b, kg/ha). Example results are also plotted in Figure 21.6

	Simazine	Atrazine	Metribuzin	Alachlor	Metolachlor	Cyanazine	Terbufos	Linuron
SWMI	0.6718	0.7184	0.5453	0.5207	0.5969	0.4932	0.1388	0.4523
Watershed use intensity (Rb, kg/ha)	0.00604	0.248	0.0869	0.452	0.284	0.0882	0.0253	0.0474
Slope to atrazine	0.093	1.000	0.310	0.455	0.618	0.287	0.00106	0.0220

non-detectable concentrations (generally > 80% of the data) and showing little correlation with atrazine were not included in Table 21.4 (mostly insecticides, such as phorate).

As expected, the pesticide–atrazine slope was found to have a good correlation with SWMI and the watershed use intensity R_b, interestingly with a cubic power of SWMI [close to the β value in the DCM; Equation (21.5) and Table 21.3] and square root of R_b (Figure 21.6). This means that less mobile (smaller SWMI) and lower use compounds tend to have lower detectable concentrations. With this simple relation, a generalized SWMI TSCM, expressed in the linear scale, is readily derived from Equation (21.6):

$$C = 5.381\left(\exp\left\{\left(1 + b_0 \exp\begin{bmatrix} b_1 & \sin(6.2832t)+ \\ b_2 & \cos(6.2832t)+ \\ b_3 & t \end{bmatrix}\right)x^k\right\} - 1\right)\sqrt{R_b}\ \mathrm{SWMI}^3 \qquad (21.7)$$

The parameter values in the generalized TSCM [Equation (21.7)] are given previously in the atrazine TSCM calibration. With available compound-specific watershed use intensity, SWMI value, and stream flow data, daily estimates of the likely concentrations in a given year can be easily obtained from Equation (21.7). Validation of the generalized TSCM using independent data sets from different watersheds are presented in the section ''Results and discussion.''

Model use

Calculation of Equation (21.7) is easy as long as input parameters are prepared properly. A few points, however, are deemed prudent to put the model use into context:

1. Prior to a simulation, potential differences in agricultural use patterns of a test compound versus atrazine need to be understood thoroughly. For example, if a product is used primarily in fall or winter, use of the atrazine-based TSCM will result in wrong predictions of seasonality. However, if the application timing is similar to atrazine, a slight shift of seasonality may be tolerable in an assessment as long as the overall distributions of the estimates are reasonable.

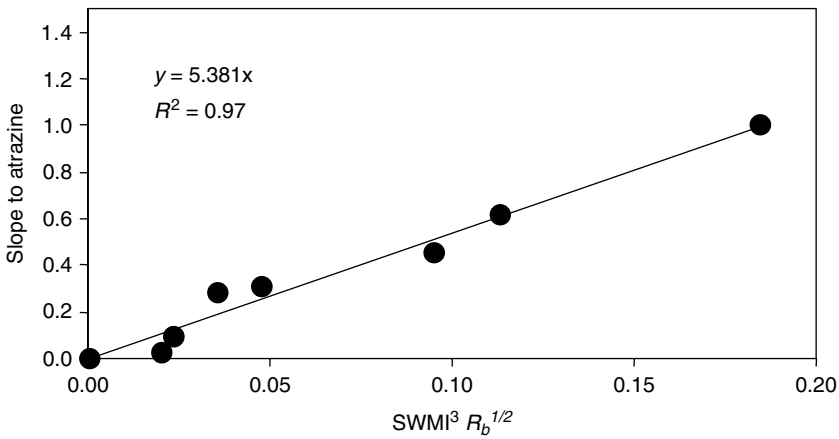

Figure 21.6 Ratio (slope) of pesticide concentrations (ppb) to atrazine observed in Maumee River, Ohio, 1986, in relationship with the compound SWMI and watershed use intensity (R_b, kg/ha).

2. The physical watershed, to which a simulation is relevant, needs to be clearly defined. The TSCM estimated concentrations are only referred to at the watershed exit point. It does not represent spatially averaged predictions of a watershed. As such, the stream flow data should come from the same watershed exit point and the estimate of the annual use intensity (R_b) should be watershed-based (not per political borders).
3. Calculate SWMI and R_b parameters as in the section ''Distributional concentration model.'' Use intensity should be estimated as accurately as possible. If multiple years are simulated, be aware of potential changes in annual use rate.
4. The time unit in TSCM is in years. That is, 1 day is approximately 0.00274 year.

Results and discussions

Distributional concentration model

The SWMI DCM (Equation (21.5]) predicts concentration percentiles in its overall distribution that are likely detected at the exit of a watershed. Limited test of the SMWI DCM on a drainage basin near 3,000,000 ha of a river system (White River, IN, USA) and a 1400 ha of a reservoir basin (Higginsville City Lake, MO, USA) indicates that the model was able to predict TWMC, peak and the 95th percentile concentrations within about 5-fold of the observed data.[10] Comparisons of the model to the observed data from both surface water systems are provided in Figures 21.7 and 21.8.

It should be noted that the response variable was log transformed in the DCM regression. As discussed in Reference 16, inverse transform of the regression would indicate that model predictions are related to the median trend (as oppose to the arithmetic mean) on the original linear scale. For predicting the arithmetic mean, an adjustment factor can be estimated as $\exp(0.5\,S^2)$, where S is the standard deviation of the regression residuals on the natural log scale. As in Table 21.3, the residual variance (S^2) ranges from 0.07 to 0.26 over the developed percentile models, indicating that the adjustment factor to Equation (21.5) will be from 1.04 (the 95th percentiles) to 1.14 (peak). In this chapter, the adjustment factor for the arithmetic mean is not considered.

Consistent with the regression results (Table 21.3), model predictions appear to closely match the observed data around the 95th percentile. Model deviation tends to increase generally as it moves to the lower or higher end in the distribution (Figures 21.7 and 21.8). An explanation could be that data near the 90–95th percentile are most likely generated by ''normal'' runoff events, while peaks or the higher percentiles are generally associated with extreme events that could not be explained by the chemical environmental fate properties or the use rate alone. Such rare events can be the coincidence of an application and a high runoff-generating storm or snowmelt. Lower percentile data are often dominated with non-detectable records (or 0's as in the Heidelberg data set). Thus, a decent regression at the lower percentiles is hard to obtain, such is the case for data below the 80th percentile. TWMC, however, is a weighted average of the entire distribution. Its ''quality'' of the model predictions would lie in between those of the peak and the lower portion of the distribution.

For a demonstration, the TWMC DCM in Table 21.3 is executed for a hypothetical compound, assuming a lognormal distribution for each of its three parameters without inter-correlation: $T_{1/2}$ (mean 30 days, SD 15 days), K_{oc} (mean 100 ml/g, SD 50 ml/g), and R_b (mean 0.2 kg/ ha, SD 0.1 kg/ha). The simple Monte Carlo simulation is easily

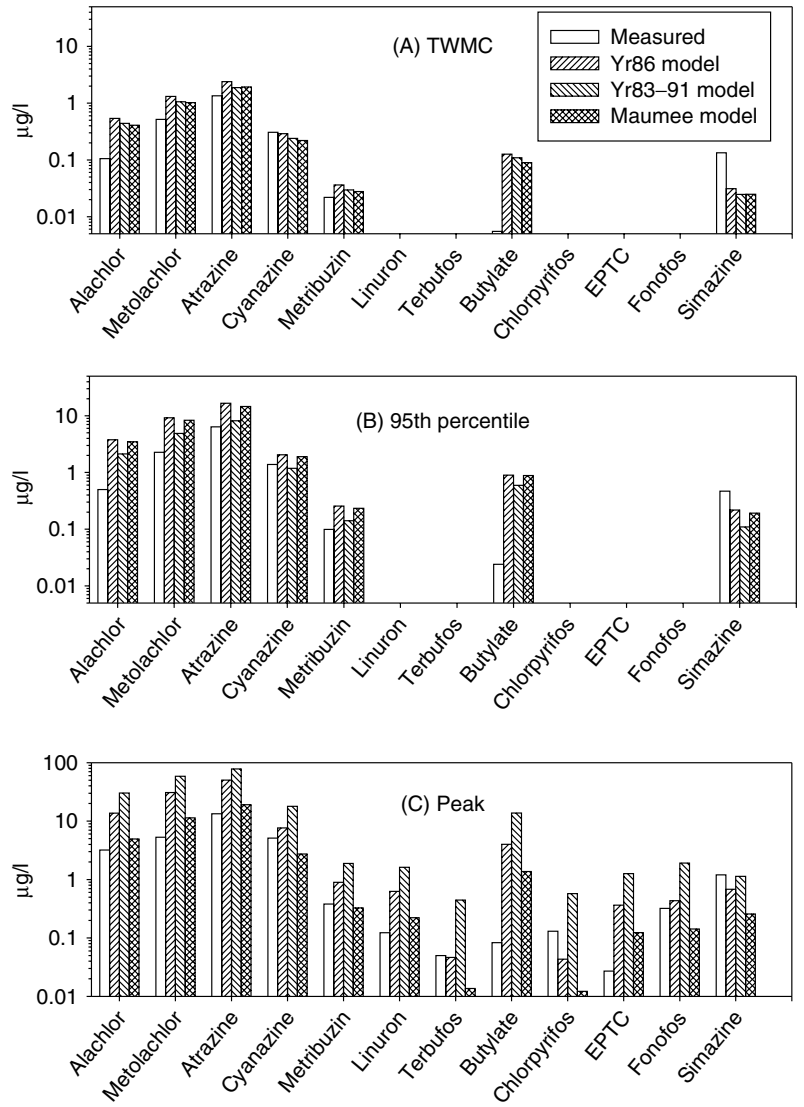

Figure 21.7 Comparison of the DCM predictions to the observed data of 12 pesticides in White River at Hazelton, Indiana, USA, 1991–1996. (From Chen, W. et al., *Environ. Toxicol. Chem.*, 21, 300, 2002. With permission.)

conducted using Excel® with the embedded Crystal Ball software. Results of the estimated TWMC distribution are presented in Figure 21.9 for 1000 runs of repeated random sampling from the three lognormal distributions of the input parameters. The estimated mean TWMC is 1.15 ppb with an SD of 0.7. The Crystal Ball routine also outputs a sensitivity chart that ranks contributions to the total simulated model variance from each parameter (Figure 21.10). For the SWMI DCM, sensitivity is in the order

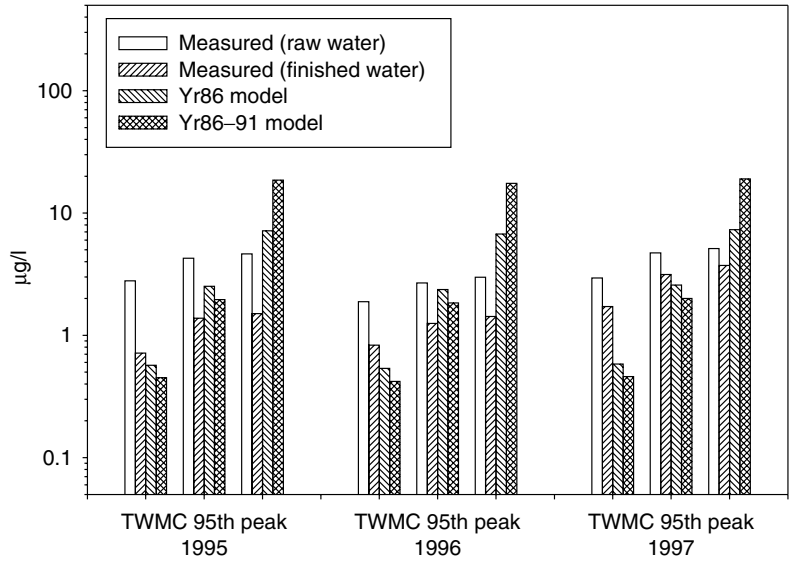

Figure 21.8 Comparison of the DCM predictions to the observed data of atrazine in raw and finish waters at the Higginsville City Lake, Missouri, USA, 1995–1997. (From Chen, W. et al., *Environ. Toxicol. Chem.*, 21, 300, 2002. With permission.)

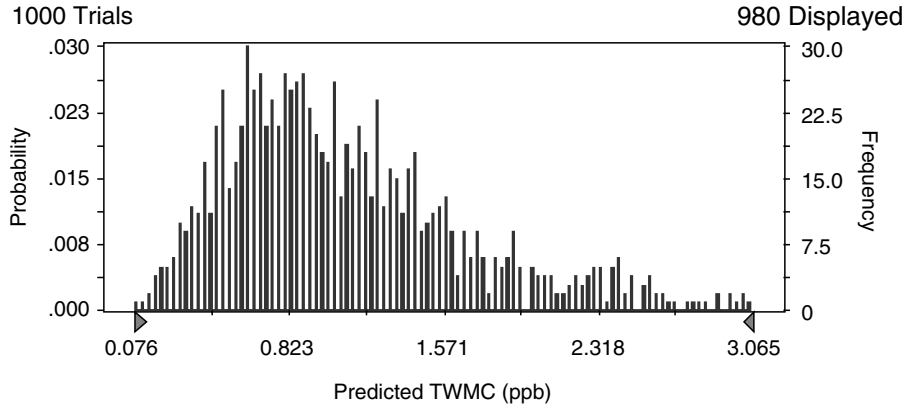

Figure 21.9 Frequency chart of the SWMI DCM for predicting the TWMC, assuming lognormal distribution for half-life (mean 30 days, SD 15 days), K_{oc} (mean 100 ml/g, SD 50 ml/g), and watershed use intensity (mean 0.2 kg/ha, SD 0.1 kg/ha). Simulated by Crystal Ball 2000.2.

$R_b > K_{oc} > T_{1/2}$. The K_{oc} sign is negative, indicating that smaller K_{oc} values mean higher model predictions.

The SWMI DCM is essentially determined by three parameters, the compound use intensity in a watershed (or basin, R_b), soil adsorption property (K_{oc}), and degradation half-life ($T_{1/2}$). The simple form and computational robustness of the model make it an ideal tool for relative risk assessment of different chemicals over large databases. Although some preliminary evaluations indicated no distinct differences in predicting

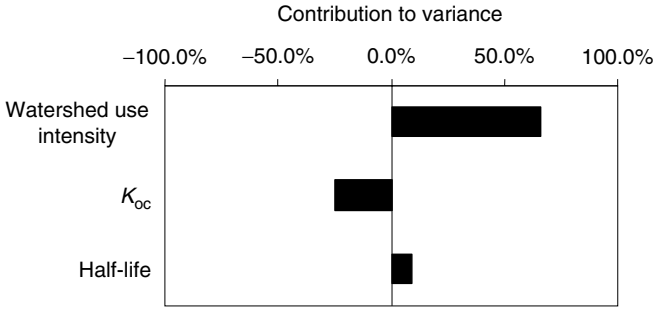

Figure 21.10 Sensitivity chart of the SWMI DCM for predicting the TWMC, assuming lognormal distribution for half-life (mean 30 days, SD 15 days), K_{oc} (mean 100 ml/g, SD 50 ml/g), and basin use intensity (mean 0.2 kg/ha, SD 0.1 kg/ha). Simulated by Crystal Ball 2000.2.

different surface water systems,[10] the model ability to differentiate inherent watershed runoff vulnerability is expected to be limited as no specific watershed hydrological characteristics are considered in the model. However, recognizing the high level of watershed complexity in almost every detailed configuration of the physical mass transport processes, the SWMI DCM is a useful and effective screening tool.

Time series concentration model

As shown in Figures 21.3A and B, a fairly close agreement was achieved between the base atrazine TSCM and the observed data with an R^2 of 0.63 over 690 data points. The regression residuals exhibit approximately a normal distribution with only a slight departure in the lower concentration end (too many non-detectable records) (Figure 21.4A). The residual SD is about 0.5, resulting in an adjustment factor of 1.13 if the regression is intended for predicting the arithmetic mean of $(C + 1)$ on the original linear scale (see the discussion in the section ''Distributional concentration model''). However, the adjustment factor was not adopted in the development of the extended TSCM [Equation (21.7)] to retain the lognormal nature in the data.

Figures 21.4B and C further illustrate the model-data agreement by a 1:1 and a percentile plot. The 1:1 plot compares the model to data on a 1-to-1 basis in the order of time, while the percentile plot compares the sorted data and model predictions in an ascending order based on the magnitude of the compared variables. Both comparisons show a reasonable model fit to the majority range of the observed data with some tendency to over-predict small concentrations, while under-estimate higher extremes (e.g., >95th percentile). Some autocorrelation structure of the regression residuals also exists (not shown), but this is not explored further here as the data set has variable sampling intervals.

The atrazine-specific TSCM is extended to a general form through the ratio of a given compound to atrazine that is further related to SWMI and its watershed use intensity [Equation (21.7)]. Model validation results are presented in Figures 21.11–21.17, where the generalized TSCM predictions are compared to eight chemicals with monitoring data available in four different watersheds (White River of Indiana, Sandusky River, Honey Creek, and Rock Creek of Ohio). These watersheds and monitoring data are independent of the Ohio Maumee River watershed, which was used in the model calibration. The eight

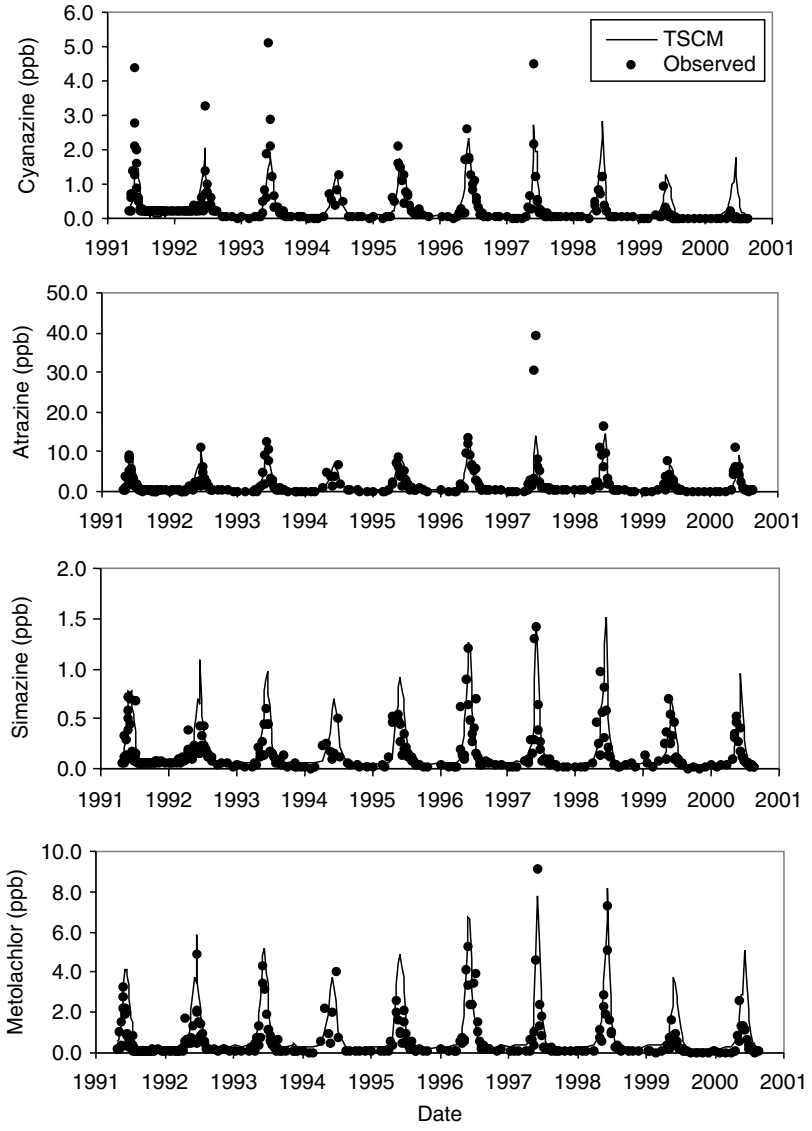

Figure 21.11 Independent predictions of the generalized TSCM compared to the observed herbicides, USGS-NAWQA White River Study Unit at Hazelton, Indiana (May 1, 1991 to August 29, 2000). *Data source*: http://www-dinind.er.usgs.gov/nawqa/wr06000.htm.

chemicals are distributed in the three SWMI regions as shown in Figure 21.1 (Region I: terbufos, phorate; Region II: butylate, cyanazine, fonofos; Region III: atrazine, simazine, metolachlor). Detailed descriptions of these data sets can be found in References 10, 15, and 17.

As shown in Figures 21.11–21.17, the generalized TSCM predicted well the overall trend, seasonality, and to a good extent the local transient fluctuations in all four watersheds for two 10-year time periods: 1986–1995 for the Ohio watersheds; and 1991–2000 for White River, Indiana. The model worked best for the most commonly used triazine and

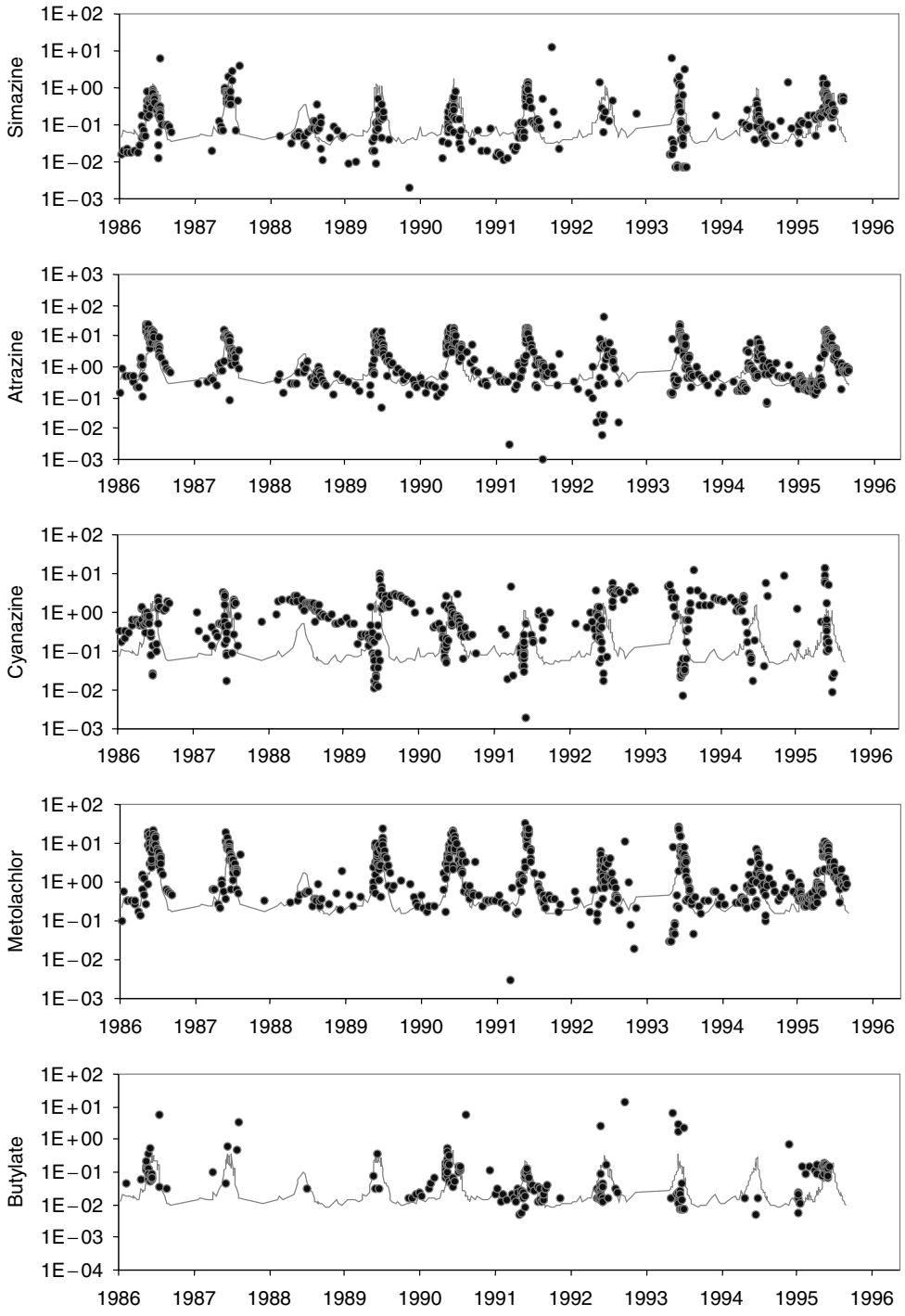

Figure 21.12 Independent predictions of the generalized TSCM compared to the observed herbicides in Sandusky River, Ohio (January 13, 1986 to December 4, 1995). Lines are TSCM predicted and points represent data. Unit: μg/l or ppb.

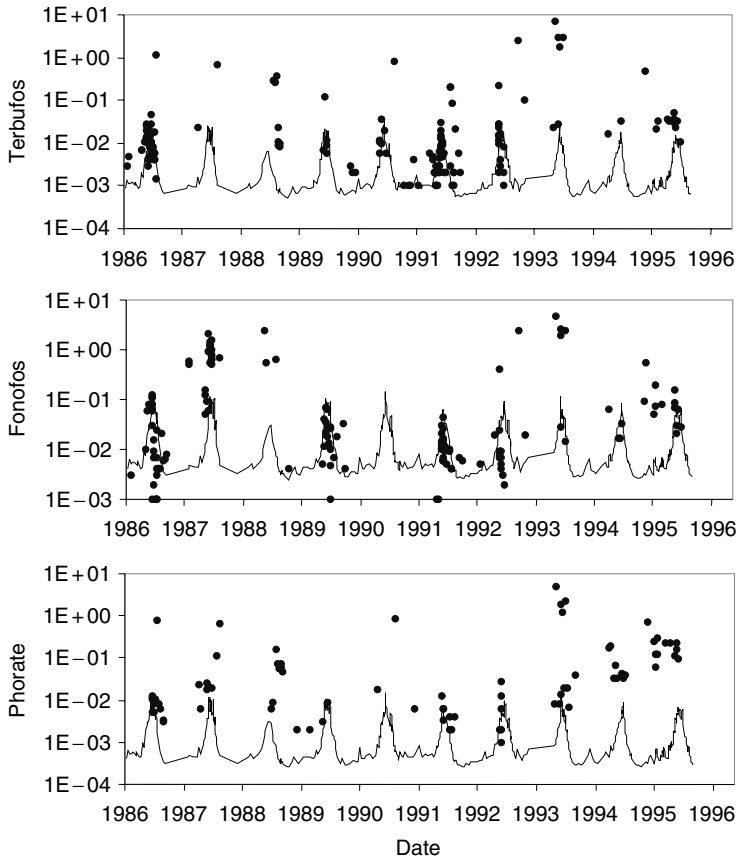

Figure 21.13 Independent predictions of the generalized TSCM compared to the observed insecticides in Sandusky River, Ohio (January 13, 1986 to December 4, 1995). Lines are TSCM predicted and points represent data. Unit: μg/l or ppb.

chloroacetanilide herbicides (atrazine, cyanazine, simazine, and metolachlor), while moderately well for butylate. Although predictions for the insecticides (fonofos, terbufos, and phorate) are not as good as for the herbicides, the model does provide a believable pattern of the occurrence of these chemicals. The less accurate insecticide results can be primarily due to scarce data with a few extreme values, potential inaccurate use rate, and application patterns (timing, frequency, areas of use, etc.) that are significantly different from atrazine.

Differences in model performance were not observed among different watersheds that range in drainage areas from 1130 ha (Rock Creek) to 2,938,382 ha (White River) in this model validation exercise. This is probably attributed to the effective incorporation of the non-linear stream flow term in the model [Equation (21.7)]. Influence of stream flow on chemical mass transport within a watershed is complicated and variable in timing. Straight correlation between daily flow and concentration is often hardly identifiable, at least for the watersheds studied in this work (results not shown). The reason is likely because of the dual roles of stream flow, i.e., mass delivery and dilution. During cropping seasons as new applications of chemicals are made to the watershed, runoff and stream

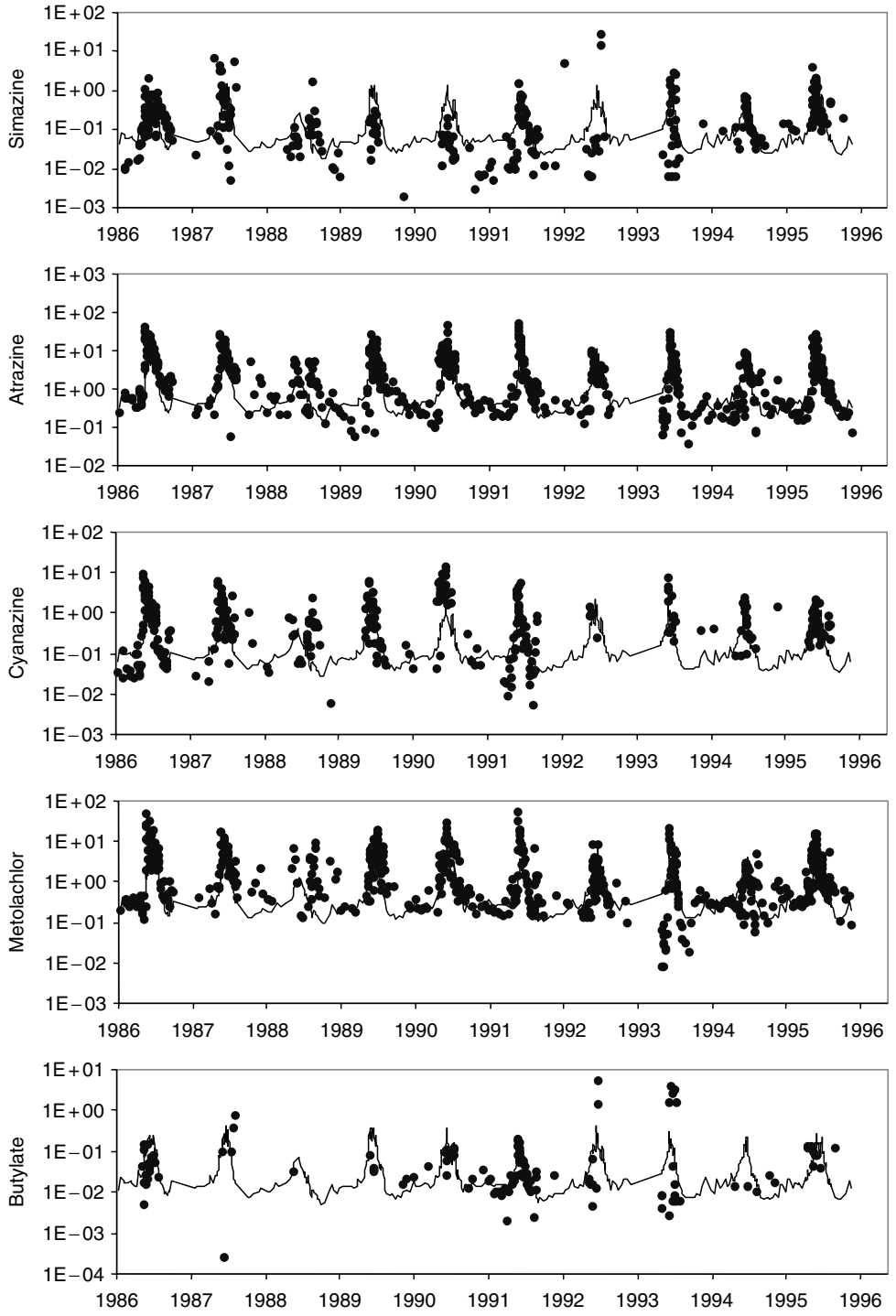

Figure 21.14 Independent predictions of the generalized TSCM compared to the observed herbicides in Honey Creek, Ohio (January 13, 1986 to November 27, 1995). Lines are TSCM predicted and points represent data. Unit: μg/l or ppb.

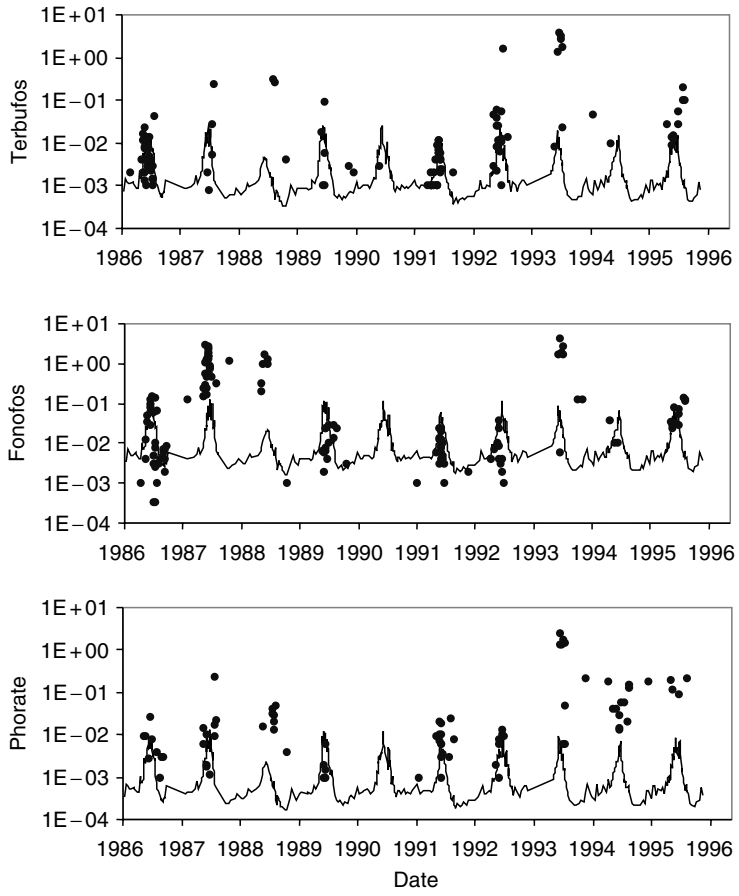

Figure 21.15 Independent predictions of the generalized TSCM compared to the observed insecticides in Honey Creek, Ohio (January 13, 1986 to November 27, 1995). Lines are TSCM predicted and points represent data. Unit: μg/l or ppb.

flow are more likely to play a role of mass delivery, thus they likely positively correlate (albeit noisily) with riverine concentrations. On the other hand, dilution role would be observed during non-cropping seasons, such as winter, when only a small amount of aged residues (if any) in soil is available to runoff.

Beyond the potential inefficiencies of the model inherent structure, a number of data quality factors deserve mentioning. The first is the watershed use intensity that needs to be accurate and watershed-specific for each year. As mentioned previously, only the 1986 pesticide annual use was available for the Ohio watersheds and the 1992–1994 annual average for White River. Assuming constant annual use for every year may not introduce much error for major and well-established herbicide, such as atrazine. However, substantial errors could be generated for herbicides with changing use patterns, and for compounds whose demands are primarily driven by presence of pests (insecticides) and diseases (fungicides). A close examination of the model validation results for the Ohio watersheds (Figures 21.12–21.17) reveals that the model tends to predict more accurately in the first year (i.e., 1986), with model-data correlation (R^2) as high as 0.77. This is

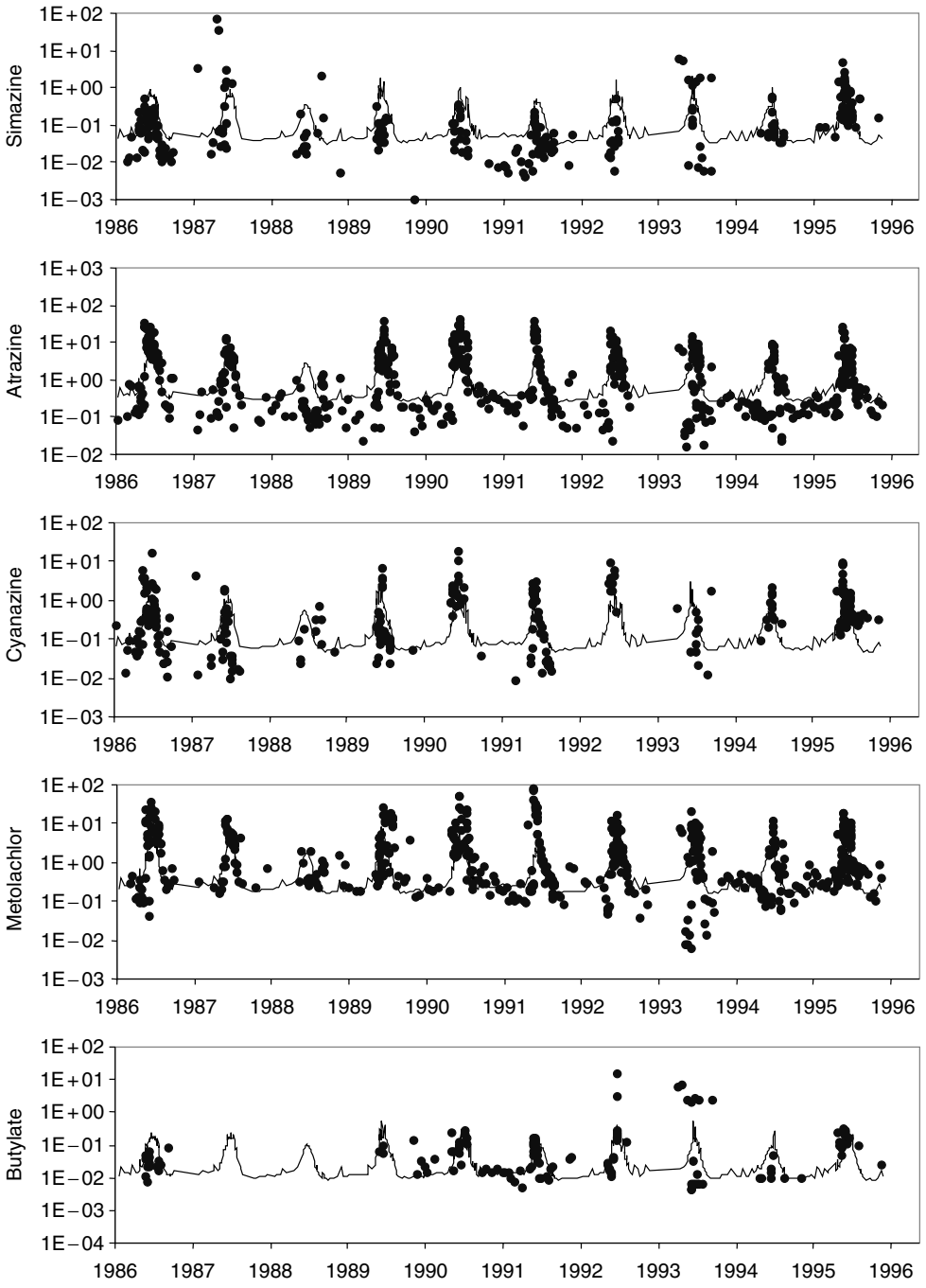

Figure 21.16 Independent predictions of the generalized TSCM compared to the observed herbicides in Rock Creek, Ohio (January 13, 1986 to November 27, 1995). Lines are TSCM predicted and points represent data. Unit: μg/l or ppb.

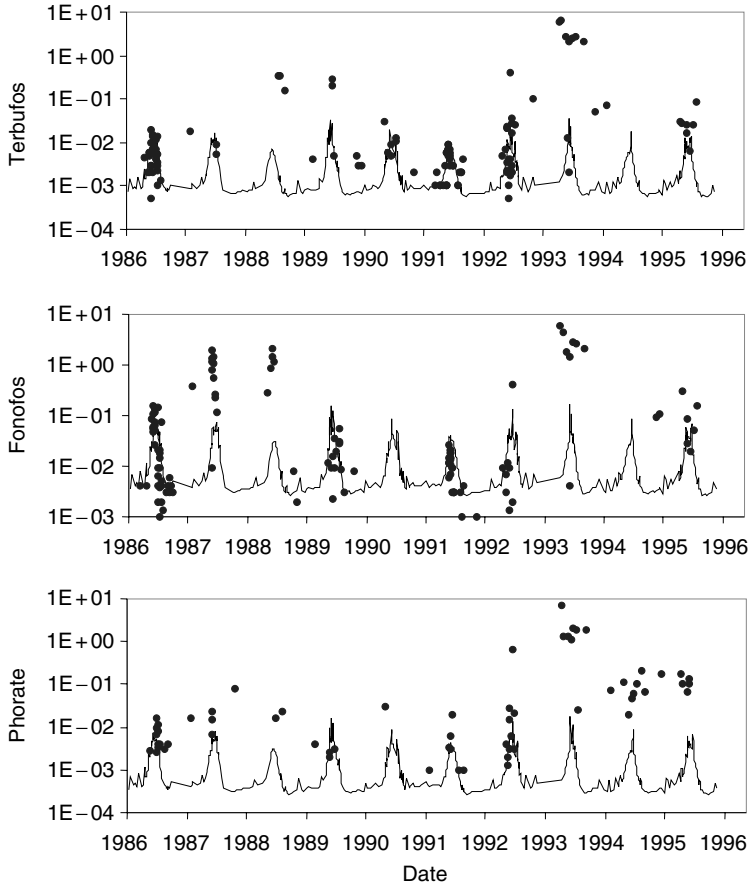

Figure 21.17 Independent predictions of the generalized TSCM compared to the observed insecticides in Rock Creek, Ohio (January 13, 1986 to November 27, 1995). Lines are TSCM predicted and points represent data. Unit: μg/l or ppb.

particularly true for the less used herbicide butylate and all three insecticides, which had no detectable concentrations in some subsequent years after 1986. Similar patterns are not obvious for the White River, as the four herbicides are all commonly used and well established in the region during the monitoring years (except cyanazine after year 2000 due to phase out of the product) (Figure 21.11).

The second data quality factor is the analytical accuracy of low concentrations for infrequently used compounds, such as the scarce insecticide data. This can affect the model calibration. Censorship or data reported only as below laboratory detection limit is another critical aspect that needs to be dealt with properly.[16] Data sets with a large portion that is censored may not be appropriate at all for accurate model calibration. The Heidelberg data set used in this report was not censored. However, the decreased accuracy is noticeable as concentrations approach to 0. While increasing analytical accuracy is a long-term goal, some specifically designed studies targeting at controllable mesoscale watersheds or catchments, where a known amount of such pesticide use is available, would provide accurate data for model calibration.

The environmental fate parameters K_{oc} and half-life are the last data quality factors that deserve attention. In general, short-term (usually 24 h) determined soil adsorption parameters are sufficient in relation to surface runoff processes. Thus, K_{oc} values determined from long-term aged residues may not be necessarily useful for the modeling purpose here. Half-life based on field studies may be more appropriate than the laboratory-determined values if pesticide dissipation due to runoff and leaching are insignificant in the field study. In case of available multiple parameter values, the ones determined from soils that are most similar to the major watershed characteristics should be used.

Summarizing the TSCM generalization from the atrazine-specific version [Equation (21.6)] to a multi-compound model [Equation (21.7)] is of considerable interest. The bridge is to use atrazine as a tracer or surrogate of other compounds so that the general global seasonality and stream flow effects are characterized. Local/specific behavior of an observation in a surface water system would be primarily caused by their specific use patterns and environmental fate properties. The approach is thus general in the sense that a surrogate compound different from atrazine may be selected to simulate different chemical classes in different regions, if the targeted class of chemistry or a geographical region has a distinct use pattern or seasonality.

Acknowledgments

P. Richards provided the Lake Erie drainage basin monitoring data. Beneficial discussions with C.G. Crawford and P. Richards were obtained during the preparation of this chapter, as well as in various previously related works. Comments from colleagues P. Hertl, W. Phelps, S. Chen, P. Hendley, and C. Breckenridge are appreciated.

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chapter twenty-two

Design and analysis of toxicity tests for the development and validation of biotic ligand models for predicting metal bioavailability and toxicity

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Contents

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Introduction

The biotic ligand model (BLM) is an integrative framework to evaluate and predict bioavailability and toxicity of metals to freshwater organisms.[1–3] It considers both metal complexation and speciation in the solution surrounding the organism and the interactions between metal ions and competing ions at binding sites on the organism–water interface (e.g., epithelial cells of gill tissue).

Some early studies (e.g., References 4–6) led to the formulation of the free-ion activity model[7] and the gill surface interaction model,[8] whose central hypothesis was that metal toxicity was related to the amount of metal bound to toxic action sites on this organism–water interface. Although conceptually those models contained almost all the features of the BLM, the use of the former models for regulatory purposes remained limited.[3] Perhaps, the reason of the current success of the BLM is that, for the first time, a model was able to integrate all state-of-the-science knowledge on complexation, speciation, and interactions at the toxic site of action into a generalized, visually attractive and easy-to-handle computerized framework (Figure 22.1).

Another reason for the increasing success of the BLM is the overwhelming body of evidence that supports the concepts formulated in it. For example, numerous detailed studies have demonstrated the effects of organic matter, pH, and hardness cations on zinc toxicity to fish, crustaceans, and algae.[9–14] Concurrently, increasing physiological evidence has been reported on the protective effects of cations against metal toxicity.[15,16] Additionally, it has been demonstrated that cations like Ca^{2+} and H^{+} decrease the binding of toxic metal cations to fish gills.[17] The derivation of stability constants for the toxic metal cation and competing cations,[17] together with the quantitative relation of metal bound to the fish gill and toxicity, independent of water chemistry,[18] coupled to a geochemical speciation model,[19] resulted in the BLM.[3]

For small invertebrates, the determination of gill-metal concentrations is experimentally difficult to obtain due to the size of the organisms. As a result, the derivation of stability constants for the toxic metal and the competing cations becomes compromised. In this chapter, we present an experimental design to determine those constants directly from toxicity data, and this specifically for the case of zinc toxicity to the crustacean *Daphnia magna*, based on data reported in Reference 9. Special emphasis is also given to the mathematical background of the proposed design, the analyses of the data, and the validation of the BLM with field-collected surface water samples.

Materials required

Supplies

- Polyethylene test cups, 50 ml (TEDECO, Belgium)
- Polyethylene vessels, 2 l, to store stock solutions (#kart614, VWR, Belgium)
- Polyethylene vessels, 5 l, to collect field water samples (#cove5020, VWR, Belgium)
- Volumetric flasks, 200 ml and 2 l (#hirs2800180 and #brnd37265, VWR, Belgium)

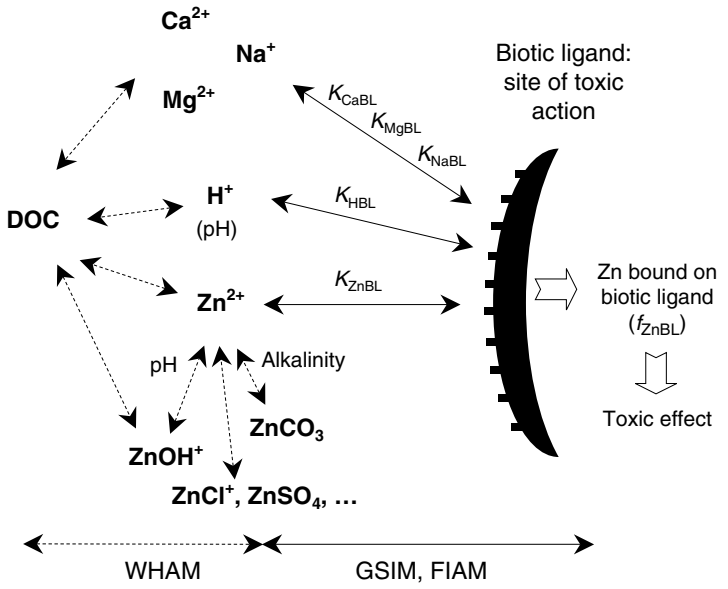

Figure 22.1 Schematic overview of the BLM for metal bioavailability and toxicity (exemplified for Zn, redrawn from Reference 3). Dashed lines represent speciation reactions (modeled by WHAM[19]); solid lines represent binding to the BL (which are assumed to be sites of toxic action). The free zinc ion, i.e., Zn^{2+}, forms complexes with inorganic ligands, such as OH^- (determined by pH), CO_3^{2-} (determined by alkalinity), SO_4^{2-}, and Cl^-. Zn^{2+} and $ZnOH^+$ also form complexes with DOC and Ca^{2+}, Mg^{2+}, and H^+ (also determined by pH) competes with Zn for binding sites on DOC. The speciation of Zn can be calculated with any speciation model (here WHAM[19]) when concentrations of all ligands present in solution and the stability constants of complexes (e.g., Reference 20) are known. Zn^{2+} binds to the BL and the concentration of zinc bound to the BL (or the fraction of BL sites occupied by zinc, f_{ZnBL}) determines the toxic effect. The latter concentration is assumed to be constant for a given effect size (e.g., $f_{ZnBL} = f_{ZnBL}^{50\%}$ at 50% mortality). The formation of complexes reduces Zn^{2+}, resulting in a reduced f_{ZnBL}, resulting in decrease of toxicity. Ca^{2+}, Mg^{2+}, Na^+, and H^+ can compete with copper for BL sites thus decreasing f_{CuBL} and toxicity. This concept was first formulated in the free ion activity model (FIAM[7]) and the gill surface interaction model (GSIM[8]). Binding affinities of Zn^{2+}, Ca^{2+}, Mg^{2+}, Na^+, and H^+ are defined by K_{ZnBL}, K_{CaBL}, K_{MgBL}, K_{NaBL}, and K_{HBL}, respectively.

- Glass beakers, 250 ml and 2 l (#kart1545 and #kart1549, VWR, Belgium)
- 0.45-μm filters, 32 mm diameter, Acrodisc®, polyethersulfon (#pall4655, VWR, Belgium)
- 0.45-μm filters, 142 mm diameter, Supor®, polyethersulfon (#pall60177, VWR, Belgium
- 10-ml syringes
- 10-ml vials for AAS sample storage

Reagents

- $CaCl_2 \cdot 7H_2O$, $MgCl_2$, $MgSO_4$, $NaHCO_3$, KCl, $ZnCl_2$ (pro analyse, Merck)
- pH 4 and pH 7 buffer solutions (Merck), for calibration of pH electrode

- HCl (>35%, w/v), HNO_3 (>69%, w/v) (pro analyse, Merck)
- Standard solution of $ZnCl_2$ in 1% (v/v) HNO_3, 1 g Zn/l (Sigma-Aldrich)
- Certified reference solutions for Zn (TM-25.2 and TMDA-62, Evironment Canada, NWRI)

Equipment model number, source

- Balance, precision 0.1 g (Mettler PJ3600 DeltaRange®)
- pH meter (Consort P407, Turnhout, Belgium)
- Flame Atomic Absorption Spectrophotometer (SpectrAA800, Varian, Mulgrave, Australia)
- Inductively coupled mass spectrometer (ICP, Spectro Analytical Instruments, Kleve, Germany)
- Ion chromatography apparatus (DIONEX2000i/SP, Dionex, Sunnyvale, CA, USA)
- TOC analyzer (TOC-5000, Shimadzu, Duisburg, Germany)
- Facilities for testing under controlled temperature and light cycle
- Automatic pipettes, volumes between 20 µl and 10 ml (Finnpipette® digital, Labsystems)

Software/databases

- Excel® (Microsoft)
- BLM windows version 1.0.0 (free download from www.hydroqual.com/winblm)
- Database of critically selected stability constants, according to Martell et al.[20] (NIST)

Procedure

Mathematical theory

This section gives the mathematical description of the BLM theory and the experimental design that results from it to derive the BLM parameters, as first proposed by De Schamphelaere and Janssen.[1] Although in theory it is applicable to any other toxic cationic metal, zinc is used as an example in all equations in the following. The model can be written for any other metal by replacing zinc by another metal cation in the equations.

The total number of zinc binding sites on the biotic ligand (BL) is called the complexation capacity of the BL (CC_{BL}), which is analogous to a total concentration of any other ligand in the test medium. In the BLM, they are treated as uniformly distributed in the exposure water, i.e., reacting with the entire water volume.[21] A mass balance equation on the BL can be written as:

$$CC_{BL} = [ZnBL] + [CaBL] + [MgBL] + [NaBL] + [HBL] + [BL] \quad (22.1)$$

where CC_{BL} is the complexation capacity of the BL (mol/l); [ZnBL], [CaBL], [MgBL], [NaBL], and [HBL] are concentrations of cation–BL complexes (mol/l); and [BL] is the concentration of unoccupied BL sites (mol/l). In this reaction, one cation is assumed to

react with one ligand site (monodentate binding), and for simplicity, charge balance is not taken into account.

Equilibrium equations for the binding of cations (e.g., Zn^{2+}) to the BL sites can be written as (conditional) stability constant expressions of the form:

$$K_{\mathrm{ZnBL}} = \frac{[\mathrm{ZnBL}]}{(\mathrm{Zn}^{2+}) \times [\mathrm{BL}]} \tag{22.2}$$

where K_{ZnBL} is the stability constant for Zn^{2+} binding to BL sites (l/mol) and (Zn^{2+}) is the chemical activity (round brackets) of the free zinc ion (mol/l). It is stressed at this point that this and subsequent equations make use of chemical activities of cations, and not of concentrations of cations. For simplicity, ionic strength corrections of the stability constants that describe cation binding to the BL are not carried out. Similar equations are valid for cations competing with zinc:

$$\begin{aligned} K_{\mathrm{CaBL}} &= \frac{[\mathrm{CaBL}]}{(\mathrm{Ca}^{2+}) \times [\mathrm{BL}]}; & K_{\mathrm{MgBL}} &= \frac{[\mathrm{MgBL}]}{(\mathrm{Mg}^{2+}) \times [\mathrm{BL}]}; \\ K_{\mathrm{NaBL}} &= \frac{[\mathrm{NaBL}]}{(\mathrm{Na}^{+}) \times [\mathrm{BL}]}; & K_{\mathrm{HBL}} &= \frac{[\mathrm{HBL}]}{(\mathrm{H}^{+}) \times [\mathrm{BL}]} \end{aligned} \tag{22.3}$$

It is stressed here that this list is not restricted to the cations mentioned here. Neither is it anticipated that all these cations would compete significantly with any toxic metal cation, including zinc. However, these cations are the ones most reported to have significant effects on metal toxicity.[1,9]

The concentration of zinc bound to the BL, which according to the BLM assumptions determines the magnitude of toxic effect, can be expressed as a function of (Zn^{2+}), (Ca^{2+}), (Mg^{2+}), (Na^{+}), and (H^{+}) by combining Equations (22.1)–(22.3):

$$[\mathrm{ZnBL}] = \frac{K_{\mathrm{ZnBL}} \cdot (\mathrm{Zn}^{2+})}{1 + K_{\mathrm{ZnBL}} \cdot (\mathrm{Zn}^{2+}) + K_{\mathrm{CaBL}} \cdot (\mathrm{Ca}^{2+}) + K_{\mathrm{MgBL}} \cdot (\mathrm{Mg}^{2+}) + K_{\mathrm{NaBL}} \cdot (\mathrm{Na}^{+}) + K_{\mathrm{HBL}} \cdot (\mathrm{H}^{+})} \cdot \mathrm{CC}_{\mathrm{BL}} \tag{22.4}$$

Assuming, in accordance with the BLM theory, that the complexation capacity of the BL is independent of the water quality characteristics, the fraction of the total number of zinc binding sites occupied by zinc (f_{CuBL}) equals:

$$\begin{aligned} f_{\mathrm{ZnBL}} &= \frac{[\mathrm{ZnBL}^{+}]}{CC_{\mathrm{BL}}} \\ &= \frac{K_{\mathrm{ZnBL}} \cdot (\mathrm{Zn}^{2+})}{1 + K_{\mathrm{ZnBL}} \cdot (\mathrm{Zn}^{2+}) + K_{\mathrm{CaBL}} \cdot (\mathrm{Ca}^{2+}) + K_{\mathrm{MgBL}} \cdot (\mathrm{Mg}^{2+}) + K_{\mathrm{NaBL}} \cdot (\mathrm{Na}^{+}) + K_{\mathrm{HBL}} \cdot (\mathrm{H}^{+})} \end{aligned} \tag{22.5}$$

This fraction can also be assumed, still in accordance with BLM theory, to determine the magnitude of toxic effect and, therefore, is constant at 50% effect ($f_{\mathrm{ZnBL}}{}^{50\%}$); i.e., independent of the water quality characteristics, as demonstrated recently for copper and nickel.[18,21] As such, Equation (22.5) can be rearranged as:

$$\begin{aligned} \mathrm{EC50}_{\mathrm{Zn2+}} = {} & \frac{f_{\mathrm{ZnBL}}^{50\%}}{(1 - f_{\mathrm{ZnBL}}^{50\%}) \cdot K_{\mathrm{ZnBL}}} \\ & \cdot \left\{1 + K_{\mathrm{CaBL}} \cdot (\mathrm{Ca}^{2+}) + K_{\mathrm{MgBL}} \cdot (\mathrm{Mg}^{2+}) + K_{\mathrm{NaBL}} \cdot (\mathrm{Na}^{+}) + K_{\mathrm{HBL}} \cdot (\mathrm{H}^{+})\right\} \end{aligned} \tag{22.6}$$

where $EC50_{Zn2+}$ is the free zinc ion activity resulting in 50% effect or the median effect concentration expressed as free zinc ion activity. The latter equation is not restricted to the EC50 but can be applied to any other point effect estimate (e.g., EC10). In theory, it is applicable to any endpoint (mortality, growth, reproduction) measured and for any exposure duration (short-term, long-term). In this study, the example of a 48-h acute immobilization assay with *D. magna* is presented.[9] Equation (22.6), however, has already been applied successfully to chronic exposures to copper and zinc.[11,22]

The absence of CC_{BL} in Equation (22.6) indicates that measured zinc concentrations on the BL are not essential for the development of a BLM. Consequently, we suggest that BLM parameters can be estimated from toxicity data alone. More importantly, Equation (22.6) clearly shows that, if the BLM concept applies, linear relationships should be observed between $EC50_{Zn2+}$ and the activity of one competing cation when the activity of other competing cations is constant. It thus allows for an explicit test of the BLM theory of cation competition.

How this finding dictates the experimental design to be followed can be understood as follows. If toxicity tests are performed at different concentrations of one competing cation and a constant concentration of all other cations, the linear relation between the activity of this one competing cation and the $EC50_{Zn2+}$ is characterized by a slope and an intercept. The example is given for Ca as competing cation, but similar equations can be written for other cations:

$$\text{intercept}_{Ca} = \frac{f_{ZnBL}^{50\%}}{\left(1 - f_{ZnBL}^{50\%} \cdot K_{ZnBL}\right)} \cdot \left\{1 + K_{MgBL} \cdot \left(Mg^{2+}\right)_{Ca} + K_{NaBL} \cdot \left(Na^{+}\right)_{Ca} + K_{HBL} \cdot \left(H^{+}\right)_{Ca}\right\} \tag{22.7}$$

and

$$\text{slope}_{Ca} = \frac{f_{ZnBL}^{50\%}}{\left(1 - f_{ZnBL}^{50\%}\right) \cdot K_{ZnBL}} \cdot K_{CaBL} \tag{22.8}$$

where $(Mg^{2+})_{Ca}$, $(Na^{+})_{Ca}$, and $(H^{+})_{Ca}$ are the mean activities of other cations in the tests performed at different Ca concentrations. Dividing slope by intercept gives the following ratio (R_{Ca}):

$$\frac{\text{slope}_{Ca}}{\text{intercept}_{Ca}} = \frac{K_{CaBL}}{\left\{1 + K_{MgBL} \cdot \left(Mg^{2+}\right)_{Ca} + K_{NaBL} \cdot \left(Na^{+}\right)_{Ca} + K_{HBL}\left(H^{+}\right)_{Ca}\right\}} = R_{Ca} \tag{22.9}$$

Similar equations can be derived when the $EC50_{Zn2+}$ is determined for varying Mg, Na, and H concentrations. Rearranging these equations into a matrix form, results in:

$$\begin{pmatrix} 1 & -R_{Ca} \cdot \left(Mg^{2+}\right)_{Ca} & -R_{Ca} \cdot \left(Na^{+}\right)_{Ca} & -R_{Ca} \cdot \left(H^{+}\right)_{Ca} \\ -R_{Mg} \cdot \left(Ca^{2+}\right)_{Mg} & 1 & -R_{Mg} \cdot \left(Na^{+}\right)_{Mg} & -R_{Mg} \cdot \left(H^{+}\right)_{Mg} \\ -R_{Na} \cdot \left(Ca^{2+}\right)_{Na} & -R_{Na} \cdot \left(Mg^{2+}\right)_{Na} & 1 & -R_{Na} \cdot \left(H^{+}\right)_{Na} \\ -R_{H} \cdot \left(Ca^{2+}\right)_{H} & -R_{H} \cdot \left(Mg^{2+}\right)_{H} & -R_{H} \cdot \left(Na^{+}\right)_{H} & 1 \end{pmatrix} \cdot \begin{pmatrix} K_{CaBL} \\ K_{MgBL} \\ K_{NaBL} \\ K_{HBL} \end{pmatrix} = \begin{pmatrix} R_{Ca} \\ R_{Mg} \\ R_{Na} \\ R_{H} \end{pmatrix} \tag{22.10}$$

The solution of this matrix results in the estimation of the stability constants K_{CaBL}, K_{MgBL}, K_{NaBL}, and K_{HBL}. In practice, the following experimental design is suggested. Complete

toxicity tests with Zn should be performed in a base medium, with low concentrations of all cations, and in several test media with increasing concentrations of one competing cation, while other cations are kept constant and as low as possible. The latter is necessary for obtaining reliable slope and intercept estimates. In this study, the individual effect of Ca^{2+}, Mg^{2+}, Na^{+}, and H^{+} activities on the acute toxicity of zinc to *D. magna* is reported.[9]

Steps in the development of a BLM

Figure 22.2 summarizes the different steps that need to be taken for the development and validation of a BLM predicting metal toxicity. The sequence of steps is described in brief in this paragraph, a more detailed explanation of each individual step is given in separate paragraphs in the following. Each step is assigned a number, referring to the different steps depicted in Figure 22.2 and to the separate paragraphs in the section ''Toxicity testing, model development and validation.''

1. *Experimental design*: Before starting the experiments one needs to decide on several ecotoxicological and bioavailability aspects, for example, which organisms will be used, are acute or chronic effects investigated, which endpoint needs to be modeled (e.g., mortality, growth, reproduction), which competing cations will be investigated (e.g., Ca, Mg, Na).
2. *Testing/measuring*: Once the above-mentioned aspects are addressed, ecotoxicity tests need to be performed to assess the effect of competing cations on the toxic effects of the metal on the chosen endpoint.
3. *Speciation calculation*: A next step is the calculation of speciation of the metal. Not all species are equally toxic, and in general, the free metal ion is considered most toxic. The free metal ion activities are the basis of most BLM and thus need to be determined before BLM parameters can be estimated. Next, the endpoint of interest (e.g., EC50 of mortality) is calculated as free metal ion activity for each test medium (each concentration of competing cation). Care should be taken in choosing the speciation model and the thermodynamic database of stability constants for metal complexes, and in correctly taking into account the complexation properties of dissolved organic carbon (DOC).
4. *Derivation of stability constants for competing cations*: The calculation of these constants is explained in detail in Reference 1 and is based on the solution of matrix equation (22.13).
5. *Estimation of K_{ZnBL} and $f_{ZnBL}^{50\%}$*: The calculation is based on the optimization of the logit-transformed effect versus f_{ZnBL} for varying K_{ZnBL}. Together with the stability constants for competing cations, these parameters can be introduced into the BLM model software (hydroqual). The development of the BLM is now complete.
6. *Validation testing with natural surface waters*: For regulatory application, it is of utmost importance that a BLM can not only predict metal toxicity in synthetic waters but also in natural surface waters. Water samples are taken from natural surface waters varying in important water chemistry characteristics (i.e., relevant for bioavailability and for BLM predictions). Those samples are then spiked and ecotoxicity tests are performed. The endpoint of interest (e.g., EC50) is again calculated.

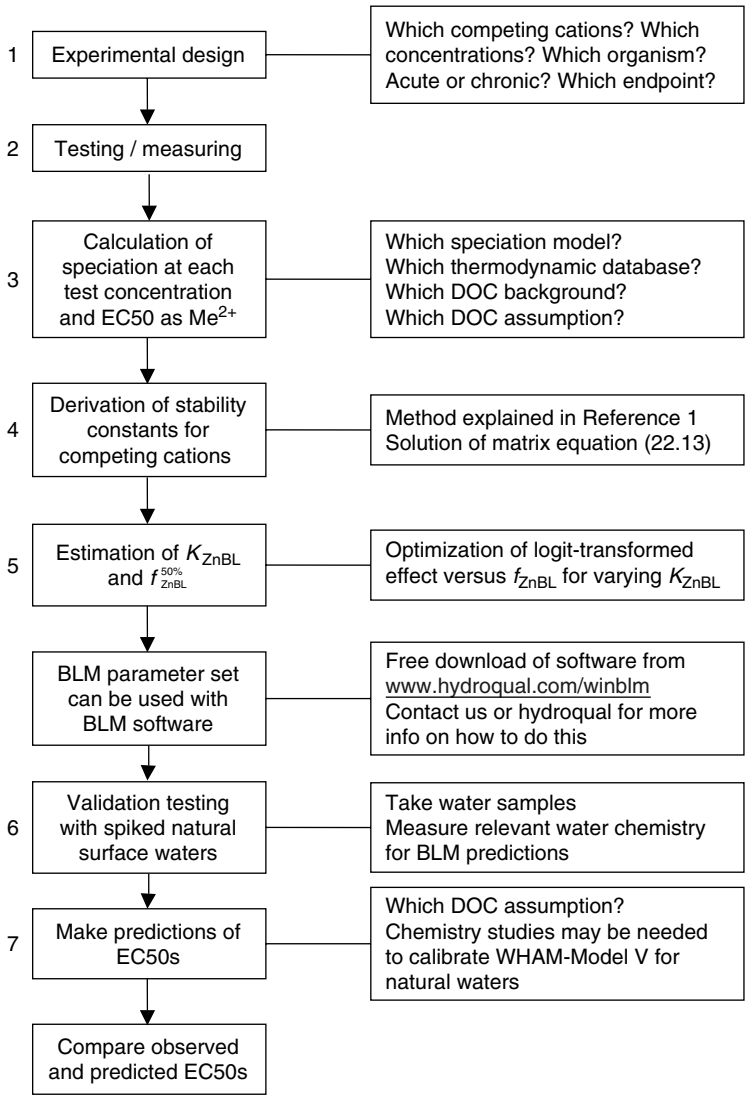

***Figure* 22.2** Summary of sequence of steps in the development of a BLM for predicting metal toxicity, along with questions/critical issues that need to be addressed with each step.

7. *Comparison observed and predicted EC50*: The developed BLM is used to make predictions of EC50 in the natural surface waters. Here it may first be necessary to calibrate the speciation model WHAM-Model V, which calculates complexation of metals to natural DOC, to independently generated chemical speciation data sets. When all this is done, observed and predicted EC50s are compared and the predictive potential of the BLM is evaluated.

The paragraphs below each describe in more detail the above explained procedure with acute zinc toxicity to *D. magna* as an example.

Toxicity testing, model development and validation

Step 1: experimental design

The experimental design is depicted in Figure 22.3. In each test series, the concentration of one cation was varied, while keeping all other cation concentrations as low and as constant as possible. Seven series of bioassays were performed: 2 Ca sets, 2 Mg sets,

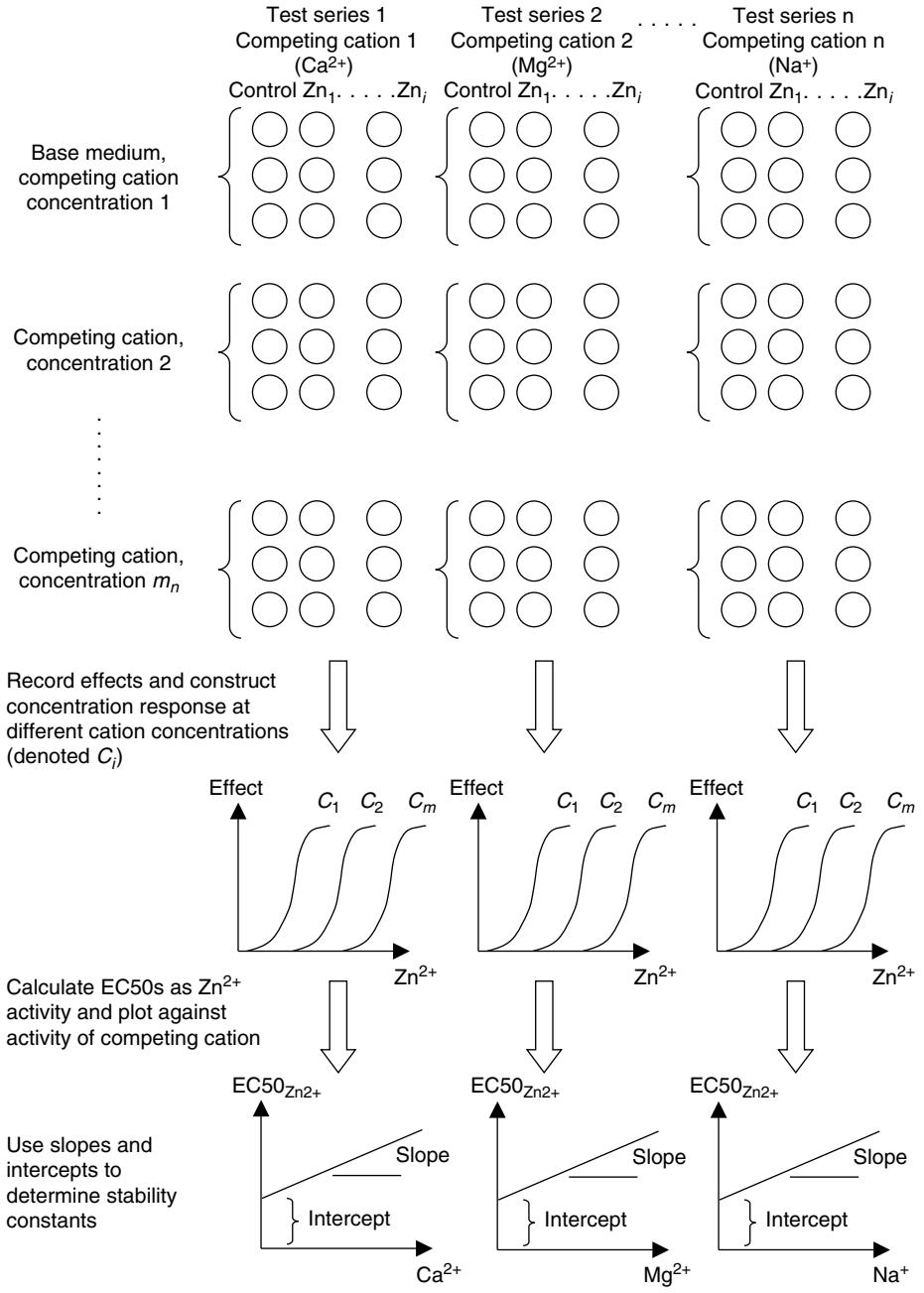

Figure 22.3 Experimental design for estimation of stability constants for competing cations.

1 Na set, 1 K set, and 1 pH set. All test series consisted of at least four bioassays (4 different cation concentrations). All bioassays of one test set were conducted simultaneously to minimize variability due to possible slight changes in *D. magna* culture conditions.

Step 2: toxicity testing/measuring

Preparation of test media

Preparation of test media is illustrated in Figure 22.4. All synthetic test media were prepared by adding appropriate volumes of stock solutions of $CaCl_2$, $MgCl_2$ or $MgSO_4$, NaCl, KCl, and $NaHCO_3$ to a base medium that contained 0.25 m*M* $CaCl_2$, 0.25 m*M* $MgCl_2$, and 0.078 m*M* KCl. Except for the pH sets, these media were adjusted to pH 6.8 by adding 0.078 m*M* $NaHCO_3$. The pH in the pH set was controlled by adding $NaHCO_3$ until the desired pH was reached. Sodium concentrations in all different pH tests were kept constant through addition of NaCl. All test media were prepared in volumetric flasks of 2 l using deionized water as dilution water. For each bioassay, the prepared test medium was then used as the dilution water to make a Zn-concentration series. The

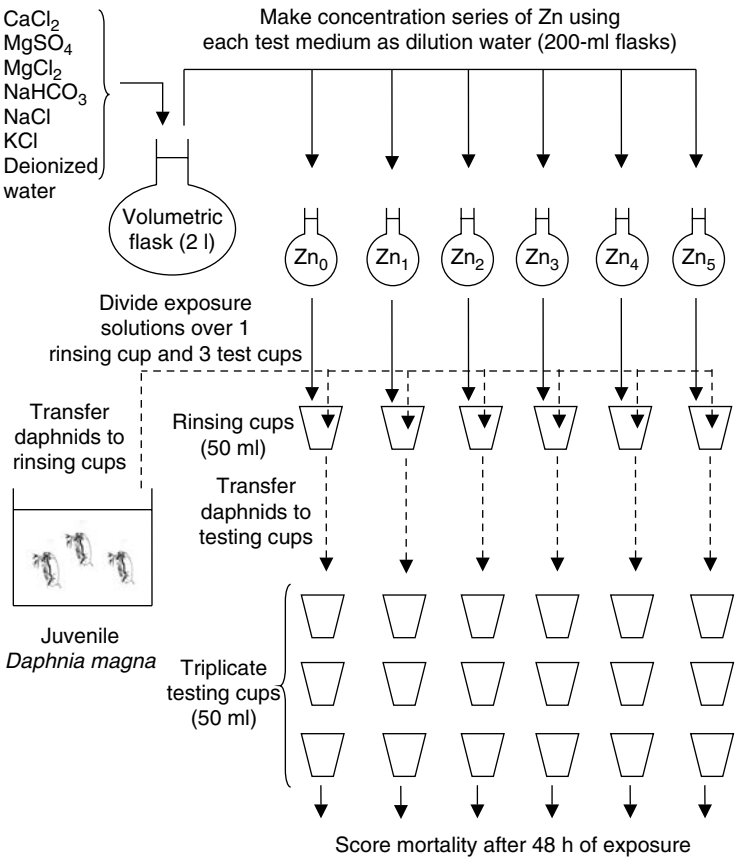

Figure 22.4 Preparation of synthetic test media and test setup.

difference between two consecutive (nominal) zinc concentrations was 0.25 log-units. For each zinc concentration, 200 ml of test solution were prepared in a volumetric flask and divided into 50-ml quantities over four polyethylene cups (three test cups and one rinsing cup). In order to obtain near-equilibrium situations, all media were stored in the test cups at 20°C for 1 day prior to being used in the toxicity tests.

Acute toxicity tests with D. magna

Acute 48-h immobilization assays with juvenile *D. magna* (<24-h old) were performed according to Test guideline 202 of the Organization for Economic Cooperation and Development (OECD, Paris, France).[23] The test organisms originated from a healthy *D. magna* clone (K6), which has been cultured under in M4-medium,[24] at a temperature of 20°C, under a 12-h light:12-h dark cycle, and which is fed a green algal mix of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio. Algae were obtained from the Culture Collection of Algae and Protozoa (CCAP, Windermere, United Kingdom). Juveniles from the first two broods were not be used for testing.

The main testing conditions are summarized in Table 22.1. For each test medium, an acute toxicity assay was conducted consisting of at least six treatments (1 control + 5 zinc concentrations) with a difference of 0.25 log-units between two consecutive zinc concentrations tested. Each treatment was performed with three replicates using 10 organisms per replicate. The number of immobilized juveniles in each cup was recorded after 48 h.

Approximately 40–50 organisms were first transferred to the rinsing cup with a glass pipette in order to dilute the culture medium, which is transferred along with the juvenile daphnids (usually <1 ml), with 50 ml of test medium (Figure 22.4). From this rinsing cup, ten juveniles were then transferred into each of the three replicates. This procedure was repeated for each test concentration.

Chemical measurements

Dissolved zinc levels (0.45 μm filtered) in each test concentration were determined at the beginning of the test using a flame-atomic absorption spectrophotometer (SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). Calibration standards (Sigma-Aldrich, Steinheim, Germany) and a reagent blank were analyzed with every ten samples. Two certified reference samples, TMDA-62 and TM-25.2 (National Water

Table 22.1 Test conditions of the acute toxicity test with *D. magna*

Temperature	20 ± 1°C in a temperature controlled cabinet
Light	12-h light:12-h dark; illumination not exceeding 800 lx
Number of concentrations	Control + 5 concentrations (logarithmically arranged, maximal 1 log-unit apart)
Number of replicates/concentration	3
Number of organisms/replicate	10
Test vessel	Polyethylene cups
Volume of test medium	50 ml
Feeding	None
Test medium	Variable, see Tables 22.4–22.7
Aeration	None

Research Institute, Burlington, ON, Canada) with certified Zn concentrations (mean $\pm$ 95% confidence interval) of $110 \pm 15.5\,\mu g/l$ and $24 \pm 4.6\,\mu g/l$, respectively, were analyzed at the beginning and end of each series of Zn measurements. Measured values were always within 10% of the certified value.

Since earlier measurements had shown that, for synthetic waters, concentrations of other cations (Ca, Mg, Na, K) and anions [Cl, SO_4, dissolved inorganic carbon (DIC)] are always within 10% of the nominal concentrations, nominal concentrations are reported here and are used as input for the speciation calculations.

The pH (pH meter P407, Consort, Turnhout, Belgium) of each test medium was checked before and after the test. pH of the natural water samples was adjusted to the pH measured in the field, just before the start of each test. The pH glass electrode was calibrated daily using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany).

Step 3: calculation of speciation and EC50s

Speciation calculations

For each test medium and for all zinc concentrations tested speciation was calculated using WHAM-V.[19] Before starting speciation calculations of metals in test media, some important issues must be addressed: (1) Which speciation model should be used? (2) Which stability constants for inorganic and/or organic complexes should be chosen? (3) What is the effect of background concentration of DOC in test water?

In principle, the choice of speciation model itself is less critical than the choice of stability constants for complexation reactions (especially those with the toxic metal cation). Using thermodynamic principles, most often-used speciation programs (e.g., MINTEQA2,[25] MINEQL+,[26] WHAM-V[19]) will compute very similar distributions of species provided that the same stability constants are used. Stability constants of metal complexing to chemically defined ligands (e.g., OH^-, CO_3^{2-}, SO_4^{2-}) are available in a number of databases. A particularly good source is the NIST Critical Stability Constants of Metal Complexes Database.[20] The use of standardized constants should definitely be encouraged. In the context of the BLM, WHAM has become an integral part of the BLM software, as it can not only compute inorganic metal speciation but also takes complexation to natural organic matter into account (i.e., humic and fulvic acids).

The advantage of WHAM, however, is that it has an in-built module for complexation to natural organic matter, such as humic and fulvic acids. This model's description of metal complexation with NOM is, compared to interactions with inorganic ligands, much more complicated. Indeed, NOM is an irresolvable mixture of a very large number of compounds varying in their properties, including their ability to bind metal ions. The WHAM-Model V[19,27] is a multiple site model that is able to relatively accurately predict metal and proton binding to NOM. More recently, WHAM-6, an update of WHAM-Model V has become available from the Centre for Ecology and Hydrology (Windermere, United Kingdom).[28] This version has not been included in the BLM software yet.

The advantage of using WHAM is that it can predict speciation both in absence and in the presence of organic matter. Although most often in test media based on deionized water no organic matter is added deliberately, a considerable amount of background DOC may be present. De Schamphelaere and Janssen[1] have measured a background DOC level of $\sim 280\,\mu g/l$, and they assumed that 50% reacted as fulvic acid and the other 50% was inert for chemical reactions. Since organic matter usually has a large affinity for metals, the significance of complexing of metals to background DOC should always be

considered in speciation calculations. Another important consideration may be the amount of DOC that the organisms themselves introduce release into the test vessels. Although Fish and Morel[29] have demonstrated that daphnids can release important amounts of DOC, De Schamphelaere and Janssen[1] demonstrated that no significant amounts of DOC were introduced in 48-h tests with 10 juveniles of *Daphnia* in 50 ml of test water, the setup that was also used in the present study.

The two issues above are illustrated with two examples for Zn speciation: effect of different inorganic stability constants on Zn speciation and effect of background DOC on zinc speciation. The results of these calculations are given in Table 22.3. Calculations were performed at 20°C, ionic strength of 10 m*M* (as NaCl), DIC = 1 m*M*, Zn = 10 and 100 μ*M*, and at pH levels ranging from 5.5 to 8.5. Calculations were performed for DOC levels of 0 μg/l, 140 μg/l, 500 μg/l, and 1000 mg/l (assumed 100% fulvic acid, performed with BLM windows version 1.0.0.).

The stability constants for inorganic zinc complexes are somewhat different in WHAM (see Reference 19) and in the NIST database (Table 22.2), especially the ones for the $ZnHCO_3$ and the $Zn(OH)_2$ complexes. From Table 22.3, it can be deduced that with the original WHAM constants the calculated Zn^{2+} activity is somewhat lower than with the NIST constants, and that this difference increases with increasing pH levels (up to factor 1.5 at pH 8.5). This clearly illustrates the importance of carefully considering the choice of stability constants whenever speciation calculations are to be carried out. The NIST constants are preferred because they are standardized and they were also used here.

Table 22.3 also demonstrates that for zinc the potential presence of background DOC is not very important at the zinc concentrations typical for toxic effects in the present study (10–100 μ*M*, 654–6540 μg/l). For DOC levels up to 1 mg/l, differences with 0 mg DOC/l assumed are less than factor 1.1 in all cases. Hence, for this study, we pursued with calculations assuming 0 mg DOC/l. However, for studies with other metals and/or with lower concentrations tested, such preliminary calculations should always be carried out before doing calculations for the tests performed.

Calculation of EC50s

Based on recorded immobility percentages at each tested zinc concentration, 48-h EC50s were calculated using the Trimmed Spearman–Karber method.[30] 48-h EC50s were also calculated based on calculated Zn^{2+} activities at each tested zinc concentration. Zn^{2+} activities (and activities of other cations in solution) were calculated using BLM software,

Table 22.2 Stability constants for inorganic zinc complexes according to the thermodynamic databases in NIST[19] and WHAM-Model V[20]

Species	Log *K* (NIST)	Log *K* (WHAM-V)
$ZnOH^+$	5.00	5.04
$Zn(OH)_2$	10.2	11.1
$ZnHCO_3^+$	11.83	13.12
$ZnCO_3$	4.76	4.76
$ZnSO_4$	2.34	2.38
$ZnCl^+$	0.4	0.4

Table 22.3 Effect of choice of thermodynamic database, NIST[20] or WHAM-Model V[19] (see Table 22.2) and of background DOC on Zn speciation at 20°C, ionic strength = 0.01 *M* (as NaCl) and total inorganic carbon = 1 m*M*. Results expressed as μ*M* Zn^{2+} activity. DOC input assumed 100% fulvic acid

pH	Effect of thermodynamic database			
	10 μ*M* total Zn, NIST	100 μ*M* total Zn, NIST	10 μ*M* total Zn, WHAM-V	100 μ*M* total Zn, WHAM-V
5.5	6.44	63.6	6.17	61.0
6	6.36	62.8	5.75	56.8
6.5	6.24	61.7	5.20	51.6
7	6.08	60.4	4.76	47.6
7.5	5.79	57.8	4.43	44.6
8	5.12	51.4	3.92	39.6
8.5	3.73	37.7	2.65	26.9
Effect of background DOC at 1 μM total Zn, calculated with NIST constants				
pH	0 mg DOC/l	0.14 mg DOC/l	0.5 mg DOC/l	1 mg DOC/l
5.5	6.44	6.36	6.16	5.89
6	6.36	6.26	6.01	5.68
6.5	6.24	6.13	5.83	5.44
7	6.08	5.95	5.62	5.19
7.5	5.79	5.65	5.30	4.83
8	5.12	4.99	4.65	4.20
8.5	3.73	3.63	3.37	3.04
Effect of background DOC at 10 μM total Zn, calculated with NIST constants				
pH	0 mg DOC/l	0.14 mg DOC/l	0.5 mg DOC/l	1 mg DOC/l
5.5	63.6	63.3	62.4	61.3
6	62.8	62.4	61.6	60.3
6.5	61.7	61.4	60.5	59.2
7	60.4	60.1	59.1	57.8
7.5	57.8	57.5	56.5	55.2
8	51.4	51.1	50.2	49.0
8.5	37.7	37.5	36.8	35.9

which has WHAM-V incorporated as a geochemical speciation module. In our case, speciation calculations were run assuming no background DOC (see the following) and using standard stability constants for inorganic complexes[20] (Table 22.2).

Effect of Ca, Mg, Na, and pH on zinc toxicity

In Tables 22.4–22.7, next to physico-chemistry of all test media, also the 48-h EC50s are given. An increase of Ca from 0.125 to 5 m*M* resulted in a 10-fold increase of 48-h EC50, i.e., from 4.88 to 45.7 μ*M* Zn (319–2990 μg/l). Mg and Na had a less protective effect than Ca. Mg reduced toxicity only 4-fold between concentrations of 0.21 and 3 m*M*, i.e., 48-h EC50s from 4.53 to 20.4 μ*M* Zn (296–1330 μg/l). Na reduced toxicity only 5-fold between concentrations of 0.077 and 15 m*M*, i.e., 48-h EC50s from 7.31 to 39.8 μ*M* Zn (478–2600 μg/l).

Table 22.4 Water chemistry[a] and speciation in the two Ca test series (separated by dashed lines) and 48-h median effective concentrations of Zn for *D. magna* as dissolved Zn and as Zn^{2+} activity

Ca (m*M*)	Cl^- (m*M*)	$EC50_{dissolved}$ (μ*M*)	Ca^{2+} (*M*)[b]	Mg^{2+} (*M*)[b]	Na^+ (*M*)[b]	$EC50_{Zn2+}$ (*M*)
0.25	0.577	8.69	2.01E – 04	2.01E – 04	7.34E – 05	6.51E – 06
0.5	1.077	11.8	3.92E – 04	1.96E – 04	7.28E – 05	8.72E – 06
1	2.077	21.7	7.50E – 04	1.87E – 04	7.18E – 05	1.44E – 05
3	6.077	33.4	2.03E – 03	1.66E – 04	6.93E – 05	1.98E – 05
4	8.077	39.6	2.56E – 03	1.59E – 04	6.83E – 05	2.12E – 05
0.125	0.327	4.88	1.02E – 04	2.04E – 04	7.37E – 05	3.77E – 06
0.25	0.577	5.79	2.01E – 04	2.01E – 04	7.34E – 05	4.49E – 06
0.5	1.077	9.56	3.92E – 04	1.95E – 04	7.27E – 05	7.23E – 06
1.25	2.577	13	9.19E – 04	1.84E – 04	7.15E – 05	9.65E – 06
2	4.077	22.6	1.32E – 03	1.76E – 04	7.05E – 05	1.49E – 05
2.5	5.077	26.6	1.71E – 03	1.68E – 04	6.95E – 05	1.82E – 05
3.75	7.577	43.4	2.42E – 03	1.61E – 04	6.85E – 05	2.69E – 05
5	10.077	45.7	3.08E – 03	1.54E – 04	6.75E – 05	2.84E – 05

[a] Other chemistry relevant for BLM: pH = 6.8; DOC = 0 mg/l; Mg = 0.25 m*M*; Na = 0.077 m*M*; *K* = 0.077 m*M*; SO_4^{2-} = 0.25 m*M*; DIC = 0.077 m*M*.

[b] Chemical activity of competing cations.

Table 22.5 Water chemistry[a] and speciation in the two Mg test series (separated by dashed lines) and 48-h median effective concentrations of Zn for *D. magna* as dissolved Zn and as Zn^{2+} activity

Mg (m*M*)	Cl^- (mM)	$EC50_{dissolved}$ (μ*M*)	Ca^{2+} (*M*)[b]	Mg^{2+} (*M*)[b]	Na^+ (M)[b]	$EC50_{Zn2+}$ (*M*)
0.25	0.577	8.48	2.02E – 04	2.01E – 04	7.34E – 05	6.31E – 06
0.5	1.077	9.81	1.96E – 04	3.91E – 04	7.28E – 05	7.70E – 06
1	2.077	12.3	1.87E – 04	7.49E – 04	7.18E – 05	8.54E – 06
2	4.077	16.2	1.75E – 04	1.40E – 03	7.04E – 05	1.05E – 05
3	6.077	20.4	1.66E – 04	2.00E – 03	6.92E – 05	1.31E – 05
0.21	0.577	4.53	2.04E – 04	1.71E – 04	7.35E – 05	3.56E – 06
0.41	0.977	4.65	1.98E – 04	3.25E – 04	7.30E – 05	5.68E – 06
0.82	1.797	8.61	1.90E – 04	6.26E – 04	7.21E – 05	6.19E – 06
1.23	2.617	13.3	1.84E – 04	9.07E – 04	7.14E – 05	9.63E – 06
1.65	3.457	15.1	1.79E – 04	1.18E – 03	7.08E – 05	1.07E – 05
2.47	5.097	15.6	1.71E – 04	1.69E – 03	6.98E – 05	1.16E – 05

[a] Other chemistry relevant for BLM: pH = 6.8; DOC = 0 mg/l; Ca = 0.25 m*M*; Na = 0.077 m*M*; *K* = 0.077 m*M*; SO_4^{2-} = 0.25 m*M*; DIC = 0.077 m*M*.

[b] Chemical activity of competing cations.

When pH was increased from 6 to 8, this resulted in a less than 3-fold reduction of toxicity, i.e., 48-h EC50s increased from 8.4 to 22.7 μ*M* Zn (549–1770 μg/l). Although the effect of water hardness (the sum of Ca and Mg effects) on zinc toxicity to freshwater organisms has been investigated by numerous authors (see Reference 31, for a review), the individual effects of Ca, Mg, and Na have not been investigated in detail. Alsop and Wood[14] found that whole body uptake of zinc by the rainbow trout *Oncorhynchus mykiss* was reduced by Ca, Mg, and Na, but that only Ca had a protective effect on acute zinc toxicity. The cation concentrations tested by these authors, was however, much lower (i.e., up to only 1 m*M*) than the levels that were tested in the present study. Perhaps, effects on uptake are detectable at lower cation concentrations than effect on toxicity. This indicates that, if one

Table 22.6 Water chemistry[a] and speciation in the Na test series and 48-h median effective concentrations of Zn for *D. magna* as dissolved Zn and as Zn^{2+} activity

Na (m*M*)	Cl^- (m*M*)	$EC50_{dissolved}$ (μM)	Ca^{2+} (*M*)[b]	Mg^{2+} (*M*)[b]	Na^+ (*M*)[b]	$EC50_{Zn2+}$ (*M*)
0.077	0.577	7.31	2.01E – 04	2.01E – 04	7.34E – 05	5.80E – 06
1	1.5	10.2	1.94E – 04	1.94E – 04	9.44E – 04	7.52E – 06
3	3.5	12.6	1.83E – 04	1.83E – 04	2.78E – 03	8.69E – 06
6	6.5	16.2	1.72E – 04	1.72E – 04	5.46E – 03	1.13E – 05
9	9.5	23.7	1.64E – 04	1.64E – 04	8.07E – 03	1.52E – 05
12	12.5	25.6	1.59E – 04	1.59E – 04	1.07E – 02	1.56E – 05
15	15.5	39.8	1.52E – 04	1.52E – 04	1.31E – 02	1.79E – 05

[a] Other chemistry relevant for BLM: pH = 6.8; DOC = 0 mg/l; Ca = 0.25 m*M*; Mg = 0.25 m*M*; *K* = 0.077 m*M*; SO_4^{2-} = 0.25 m*M*; DIC = 0.077 m*M*.

[b] Chemical activity of competing cations.

Table 22.7 Water chemistry[a] and speciation in the pH test series and 48-h median effective concentrations of Zn for *D. magna* as dissolved Zn and as Zn^{2+} activity

pH	Cl^- (m*M*)	DIC (m*M*)	$EC50_{dissolved}$ (μM)	$EC50_{Zn2+}$ (M)
6	1.737	0.04	8.4	6.40E – 06
6.5	1.687	0.09	11	8.49E – 06
7.5	1.277	0.5	11.4	7.01E – 06
8	0.577	1.2	22.7	9.23E – 06

[a] Other chemistry relevant for BLM: DOC = 0 mg/l; Ca = 0.25 m*M*; Mg = 0.25 m*M*; Na = 1.2 m*M*; *K* = 0.077 m*M*; SO_4^{2-} = 0.25 m*M*.

wants to use toxicity data to quantify protective effects of cations, high enough cation concentrations should be tested.

The strong effect of Ca^{2+}, compared to Mg^{2+} and Na^+, can be understood better when reported interactions between calcium and zinc are considered: studies with *O. mykiss* have shown that elevated calcium concentrations inhibited zinc influx competitively and consequently, it has been postulated that calcium and zinc share a common uptake pathway across the apical membrane of the chloride cells of the gills[15,16] The specific acute toxic mode of action of zinc is the disturbance of calcium uptake by the gills primarily through competitive inhibition, and hence it seems logic that Ca provides more competitive protection against zinc toxicity than Mg and Na.

Whereas the effects of Ca, Mg, and Na can be attributed to competition effects (see the following), the effect of pH is most likely a pure speciation effect. Indeed, at higher pH, more Zn^{2+} is complexed to carbonate and hydroxide complexes, and thus less Zn is left as the free Zn^{2+} ion. This is illustrated by the fact that, when EC50s are expressed as Zn^{2+} activity, no significant relation with pH is observed.

If H^+ would be an important competitor of Zn^{2+} for binding on the BL, an increase of the 48-h EC50 (as free Zn^{2+} activity) with increasing H^+ (decreasing pH) is expected. The absence of this trend indicates that the possible effect of H^+ competition on zinc toxicity to *D. magna* is negligible in the pH range tested and should therefore not be incorporated in the BLM (for this organism and metal). The fact that for other species, a quantitative H^+ competition has been demonstrated,[11,31] illustrates that species-specific differences in competitive interactions may exist. The here-described experimental design easily

allows testing competition effects of cations on metal toxicity for various organisms. The H^+ effect will not be considered further in this chapter.

Step 4: estimation of stability constants for competing cations

The relation between activities of Ca^{2+}, Mg^{2+}, and Na^+ and 48-h $EC50_{Zn2+}$ is presented in Figure 22.5. Significant linear relations were observed for Ca ($R^2 = 0.94$, $p < 0.05$), Mg ($R^2 = 0.89$, $p < 0.05$), and Na ($R^2 = 0.98$, $p < 0.05$), and this corroborates with the BLM concept of cation competition according to Equations (22.6)–(22.9). However, since the H^+ competition effect was not significant, the Equations (22.5), (22.6), and (22.10) can be simplified to:

$$f_{\mathrm{ZnBL}} = \frac{K_{\mathrm{ZnBL}} \cdot \left(\mathrm{Zn}^{2+}\right)}{\left\{1 + K_{\mathrm{ZnBL}} \cdot \left(\mathrm{Zn}^{2+}\right) + K_{\mathrm{CaBL}} \cdot \left(\mathrm{Ca}^{2+}\right) + K_{\mathrm{MgBL}} \cdot \left(\mathrm{Mg}^{2+}\right) + K_{\mathrm{NaBL}} \cdot \left(\mathrm{Na}^{+}\right)\right\}} \quad (22.11)$$

$$\mathrm{EC50}_{\mathrm{Zn}^{2+}} = \frac{f_{\mathrm{ZnBL}}^{50\%}}{\left(1 - f_{\mathrm{ZnBL}}^{50\%}\right) \cdot \mathrm{K}_{\mathrm{ZnBL}}} \cdot \left\{1 + \mathrm{K}_{\mathrm{CaBL}} \cdot \left(\mathrm{Ca}^{2+}\right) + \mathrm{K}_{\mathrm{MgBL}} \cdot \left(\mathrm{Mg}^{2+}\right) + \mathrm{K}_{\mathrm{NaBL}} \cdot \left(\mathrm{Na}^{+}\right)\right\} \quad (22.12)$$

$$\begin{pmatrix} 1 & -R_{\mathrm{Ca}} \cdot \left(\mathrm{Mg}^{2+}\right)_{\mathrm{Ca}} & -R_{\mathrm{Ca}} \cdot \left(\mathrm{Na}^{+}\right)_{\mathrm{Ca}} \\ -R_{\mathrm{Mg}} \cdot \left(\mathrm{Ca}^{2+}\right)_{\mathrm{Mg}} & 1 & -R_{\mathrm{Mg}} \cdot \left(\mathrm{Na}^{+}\right)_{\mathrm{Mg}} \\ -R_{\mathrm{Na}} \cdot \left(\mathrm{Ca}^{2+}\right)_{\mathrm{Na}} & -R_{\mathrm{Na}} \cdot \left(\mathrm{Mg}^{2+}\right)_{\mathrm{Na}} & 1 \end{pmatrix} \cdot \begin{pmatrix} K_{\mathrm{CaBL}} \\ K_{\mathrm{MgBL}} \\ K_{\mathrm{NaBL}} \end{pmatrix} = \begin{pmatrix} R_{\mathrm{Ca}} \\ R_{\mathrm{Mg}} \\ R_{\mathrm{Na}} \end{pmatrix} \quad (22.13)$$

It is noted that the equations can easily be adapted for any other case by omitting or adding competing cations to the equations. First, the data presented in Tables 22.4–22.7 were used to solve matrix equation (22.13) (Table 22.8). The matrix equation can be solved on a hand calculator or with the functions ''PRODUCTMAT'' and ''INVERSEMAT'' available in Excel (Microsoft). Estimated stability constants are also summarized in Table 22.9, where they are compared to stability constants found for acute zinc toxicity to fish species.[31] The acute BLM for fish did not contain constants for Mg and Na competition, but this is because there were simply no data available for the estimation of these constants. The authors did not explicitly exclude the possibility of Mg or Na competition. The log K_{CaBL} was ~1.5 log-units lower for *D. magna* than for fish and no H^+ competition constant was included for *D. magna*, as opposed to fish. This indicates that species-specific differences may exist in stability constants of competing cations.

Step 5: estimation of Zn binding parameters for the biotic ligand

For the final development of the Zn-BLM for *D. magna*, the K_{ZnBL} and $f_{\mathrm{ZnBL}}{}^{50\%}$ of Equation (22.12) can be optimized as follows, according to the method first proposed by De Schamphelaere and Janssen.[1] It must be noted that the approach presented here is

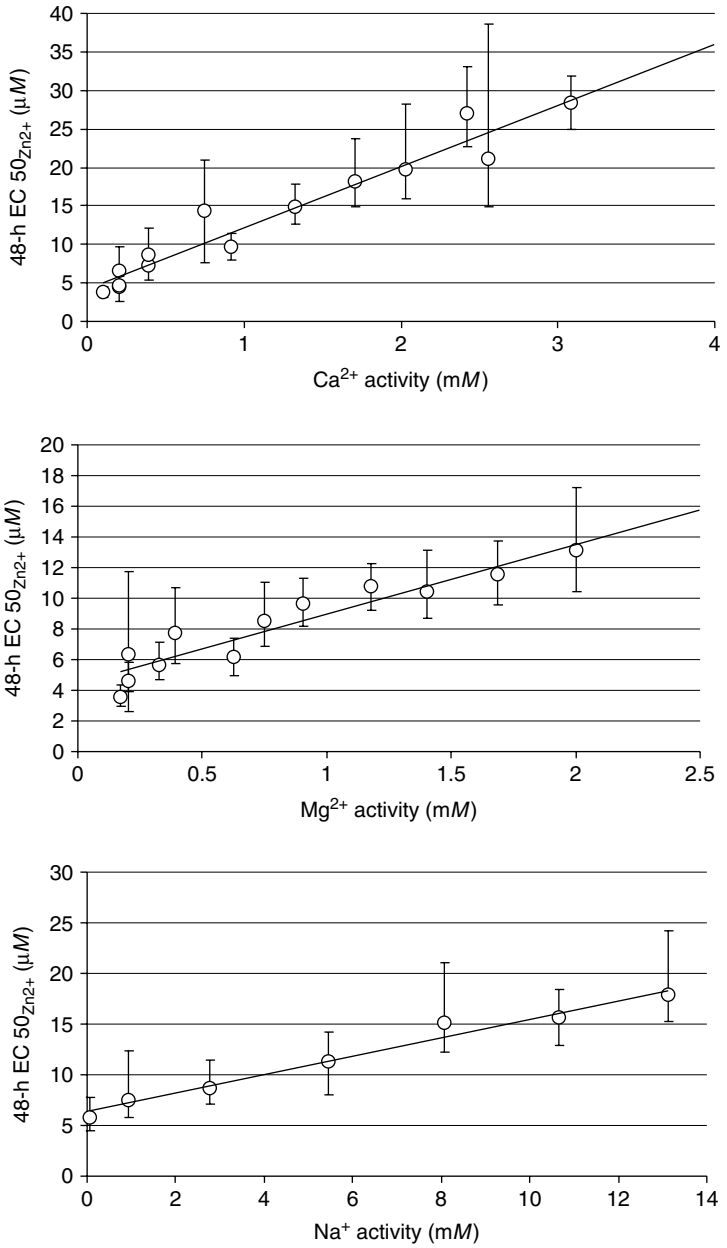

Figure 22.5 Effects of Ca^{2+}, Mg^{2+}, and Na^{+} on the 48-h EC50 (as Zn^{2+} activity) for *D. magna*. Error bars are 95% confidence limits.

an optimization of two parameters to a toxicity data set, and that perhaps other parameters might be found when real binding data of zinc to the BLM were available. Although the authors are aware that this separation of K_{ZnBL} and $f_{ZnBL}^{50\%}$ is predominantly a mathematical distinction, they would like to point out that this initial approximation does not influence toxicity predictions. Indeed, it is the ratio

Table 22.8 Estimation of stability constants (K, in M^{-1}) for the competing cations for the acute Zn-BLM for *D. magna* (48-h immobility) derived using data of Tables 22.4–22.7 and Equation (22.13) (NA, not applicable)

$Cation_X$	Slope	Intercept (M)	R_X (M^{-1})	$(Ca^{2+})_X$ (M)	$(Mg^{2+})_X$ (M)	$(Na^{+})_X$ (M)	K_{XBL} (M^{-1})	Log K_{XBL}
Ca^{2+}	7.81E – 03	4.49E – 06	1740	NA	1.81E – 04	7.10E – 05	2190	3.34
Mg^{2+}	4.83E – 03	4.66E – 06	940	1.87E – 04	NA	7.17E – 05	1320	3.12
Na^{+}	9.15E – 04	6.34E – 06	144	1.75e – 04	1.75E – 04	NA	234	2.37

$$\frac{f_{ZnBL}^{50\%}}{(1-f_{ZnBL}^{50\%})\cdot K_{ZnBL}}$$

in Equation (22.12) that determines the 48-h $EC50_{Zn2+}$ when competing cation activities are known. As stated by De Schamphelaere and Janssen,[1] this ratio can be regarded as the $EC50_{Zn2+}$ when no cation competition is observed in the test medium and is equal to 3.51 μM ($\pm$ 0.58 μM; 95% CL) ($n = 31$). Nevertheless, below a possible approach is presented to separate K_{ZnBL} and $f_{ZnBL}{}^{50\%}$.

For each treatment (all test media $\times$ 5 zinc concentrations per test medium) activities of Ca^{2+}, Mg^{2+}, Na^{+}, and Zn^{2+} had already been calculated. When a log K_{ZnBL} is now chosen, the f_{ZnBL} can easily be calculated for each treatment. Now, since one of the main assumptions of the BLM is that this f_{ZnBL} is directly related to the toxic effect, a good relation should be observed between f_{ZnBL} and the effect. In practice, the log K_{ZnBL} is varied and for each value, a linear regression is performed on logit (48-h immobility) versus the calculated f_{ZnBL} and the R^2 is determined. The point where the regression line crosses the X-axes is the $f_{ZnBL}{}^{50\%}$. The combination of log K_{ZnBL} and $f_{ZnBL}{}^{50\%}$ that results in the highest R^2 is retained as the final combination to be used in the BLM (see Figure 22.6 for illustration of this method). A log $K_{ZnBL} = 5.31$ with an associated $f_{ZnBL}{}^{50\%} = 0.417$ resulted in the best fit ($R^2 = 0.806$) (Figure 22.5). This means that, when 41.7% of the BL is

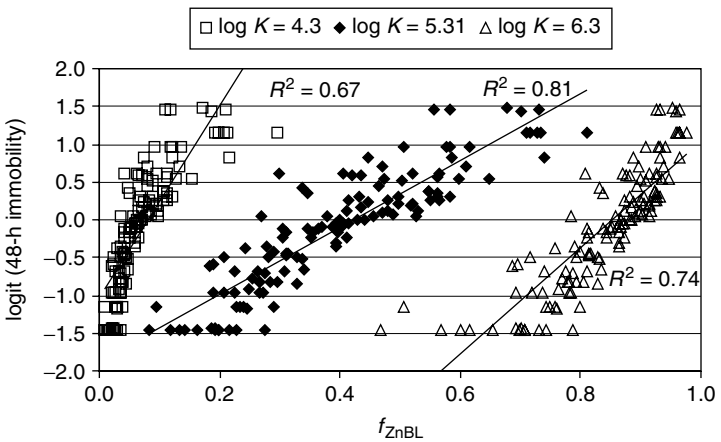

Figure 22.6 Relation between the logit-transformed effect and the fraction of BL sites occupied with Zn for different values of log K_{ZnBL}. For log K_{ZnBL} = 5.31, an optimal R^2 value was obtained. The $f_{ZnBL}{}^{50\%}$ is 0.417 (where logit = 0).

occupied by zinc, 50% effect (immobility) is expected to occur after 48 h of exposure. A log K_{ZnBL} of 5.31 is comparable with stability constants (K_{Zn}) for zinc binding to gills of juvenile rainbow trout.[32,33] Alsop and Wood[32] found a log K_{ZnBL} (as Zn^{2+}) ranging between 5.3 and 5.6. Galvez et al.[33] reported a log K_{ZnBL} of 5.1.

Finally, all BLM parameters for acute zinc toxicity to *D. magna* are reported in Table 22.9. Those values can easily be entered into the BLM modeling software (Hydroqual, Mahwah, NJ, USA), and at this point the model development is complete and the BLM can be used for making EC50 predictions in natural waters.

Step 6: validation testing with spiked natural surface waters

Natural water samples were collected in March 2003 in The Netherlands, Belgium, and France. pH was measured in the field using a portable pH meter. Samples of 5 l arrived in the laboratory the same day of collection and were filtered through a 0.45-μm filter (Gellman, Ann Arbor, MI, USA). Subsamples were taken for analysis of DOC, Ca, Mg, Na, K, SO_4, Cl, and DIC. These parameters were measured as they are required as input for the BLM. For toxicity testing, pH of the natural water samples was adjusted to the pH measured in the field. Samples were subsequently spiked with 5 different zinc concentrations and equilibrated for 2 days at 20°C. This equilibration is of utmost importance, since kinetics of metal complexation to dissolved organic matter may be slow in some cases.[34] Toxicity tests and zinc analysis procedures were similar as those described earlier.

For natural water samples, cations were measured using inductively coupled mass spectrometry (ICP, Spectro Analytical Instruments, Kleve, Germany) and chloride and sulphate concentrations using ion chromatography (DIONEX2000i/SP, Dionex, Sunnyvale, CA, USA). DOC (0.45 μm filtered) and inorganic carbon (IC) were measured using a TOC analyzer based on analytical combustion (TOC-5000, Shimadzu, Duisburg, Germany).

Step 7: comparison of observed and predicted EC50s

In a regulatory context, it is of utmost importance that a BLM can not only predict metal toxicity in synthetic lab waters but also in spiked natural surface waters. Preferably, the tested waters should have chemical characteristics that are representative of the area (stream catchment, region, country, continent, etc.) that needs to be protected.

The main difference between synthetic lab waters and natural surface waters is that the latter may contain considerable concentrations of DOC, which may bind metals

Table 22.9 Stability constants (log *K*) in the acute Zn-BLM for *D. magna* (this study) and for fish[32]

	Daphnia magna	Fish
Ca^{2+}	3.34	4.8
Mg^{2+}	3.12	—
Na^{+}	2.37	—
H^{+}	—	6.7
Zn^{2+}	5.31	5.5

like zinc and reduce their bioavailability. The effect of DOC is that it reduces the free metal ion activity, and if one can accurately estimate the extent to which this occurs, one can also predict the effect on toxicity. Although WHAM-V, the speciation module of the BLM-software, has demonstrated to be a robust tool for predicting metal binding to organic matter, it has been observed that the model may overestimate metal binding to natural organic matter in some cases.[35,36] This can, for example, be solved by assuming that only a fraction of the natural organic matter behaves as active fulvic acid. For copper, accurate toxicity predictions were made using the assumption of 50% active fulvic acid.[35,36] Within the BLM software this can be achieved by using the measured DOC concentration divided by 2 as the DOC input value and by setting the percentage of humic acid content to 0%. However, the default parameters for Zn binding to organic matter in WHAM-V are based on a very limited data set,[27] and until recently, no validation/refinement of these parameters was carried out based on Zn titration data sets with natural water samples. In such cases, before the predictive capacity of the BLM is assessed, a calibration of WHAM-V to organic-metal complexing data sets is mostly preferable. Recently, we have been able to demonstrate that WHAM-V yields the best Zn speciation estimates for natural waters if 61% active fulvic acid is assumed and 39% of the DOC is assumed to be inert.[37] The latter estimate was used for the predictions of 48-h EC50s in all natural waters tested.

Six water samples were taken in three different countries and both running and standing waters were sampled. Table 22.10 gives the water chemistry and the 48-h EC50s of Zn in the six tested waters. 48-h EC50s were between 315 and 3290 μg Zn/l, indicating a 10-fold difference. In Figure 22.6, the predicted 48-h EC50s were plotted against the observed 48-h EC50s. All EC50s were predicted with an error less than factor 2, which should be considered very good, given normal biological variability in replicated toxicity tests.

Table 22.10 Water chemistry and observed 48-h EC50s of zinc for *D. magna* in natural surface waters

Water, type,[a] location, country code	pH	DOC[b] (mg/l)	Ca (m*M*)	Mg (m*M*)	Na (m*M*)	*K* (m*M*)	Cl (m*M*)	SO_4 (m*M*)	DIC[c] (m*M*)	$EC50_{dissolved}$ (μ*M*)	$EC50_{dissolved}$ (μg/l)
Ankeveensche plas, S, Nederhorst-den-Berg, NL	6.77	17.3	0.956	0.269	0.757	0.156	1.322	1.410	0.340	33.3	2180
Ruisseau de St. Martin, R, Bihain, B	6.03	5.37	0.092	0.044	0.272	0.022	0.042	0.423	0.427	4.82	315
Ourthe Orientale, R, Brisy, B	7.34	2.53	0.125	0.139	0.384	0.053	0.010	0.649	0.299	5.41	354
Markermeer, S, Marken, NL	8.03	7.49	1.316	0.577	3.799	0.223	1.135	8.970	2.578	50.3	3290
Rhine, R, Lobith, NL	8.22	2.30	1.522	0.441	2.409	0.129	0.594	6.064	3.195	39.5	2580
Le Voyon, R, Trélon, FR	8.10	4.17	0.925	0.293	0.437	0.034	0.208	0.592	2.527	25.5	1670

[a] R = running water; S = standing water.

[b] Dissolved organic carbon; for input in BLM software, multiply with 0.61 and set humic acid percentage to 0% (set to 0.01% as software does not work with 0%).

[c] Dissolved inorganic carbon; BLM software needs alkalinity as input; DIC can be transformed to alkalinity as explained in the help menu of the BLM software (*www.hydroqual.com/winblm*).

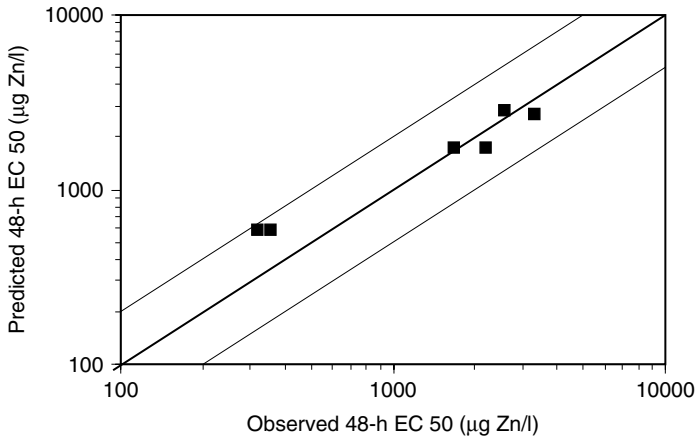

Figure 22.7 Observed versus BLM-predicted 48-h EC50 in six spiked natural surface waters. Bold line, perfect prediction; thin line, factor 2 prediction error.

Summary

The process of developing and validating a BLM for metal toxicity was explained in detail in this chapter. The approach has been originally developed for acute copper toxicity to *D. magna*[1,35] and has been shown a valid alternative of gill-metal binding studies (e.g., Reference 17) for deriving stability constants. The here-shown example for acute zinc toxicity to *D. magna* again indicates that this methodology is very sound and results in a model that yields very accurate toxicity predictions. The methodology can be applied to exposures of any organism to any metal for any exposure duration (acute and chronic). The methodology can yield a BLM in a relative short period of time (<1–2 years) and is thus interesting for the rapid incorporation of bioavailability in criteria setting and risk assessment of metals.

Acknowledgments

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chapter twenty-three

Rapid toxicity fingerprinting of polluted waters using lux-marked bacteria

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Contents

Introduction

The release of toxic chemicals to surface waters in the UK and EU has, in the past, been largely controlled by legislation pertaining to pollutant chemistry alone. Environmental quality standards (EQSs) for controlled substances in the UK, required under the 1976 Control of Dangerous Substances Act, are derived from discharge limits based on total pollutant load.[1] Pollutant bioavailability is accounted for to some extent with this approach (e.g., certain pollutants will have higher discharge limits in hard-water areas); however, actual environmental toxicity (and the resultant ecological implications) are not

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implicitly considered. With the adoption of the Water Framework Directive by the EU in 2000, this is now changing. Article 16 of the directive requires all member states to attain ''good'' ecological status in all surface waters by 2015 (this approach is comparable with the USEPA Toxicity Reduction Evaluation procedures).[1,2] This places increasing importance on defining the *in situ* toxicity of pollutants (and pollutant mixtures) in receiving waters, thus increasing the need for accurate, representative, and innovative methods of toxicity assessment.

Traditional environmental toxicity testing has focused on the use of higher organisms (i.e., macrophytes and animals) as biosensors. However, bacterial assays are being increasingly exploited due to their rapidity, practicality, and versatility.[3] In addition, as bacteria represent the ''lowest rung'' of the ecological ladder, it can be argued that their inclusion for toxicity testing is essential if ecological impacts on receiving waters are to be accurately determined. A wide range of bacterial assays currently exist; luminescent assays have become especially popular since the commercial introduction of Microtox® in 1981, which uses the naturally luminescent marine bacterium *Vibrio fischeri*.[3,4] Genes from this, and other luminescent species, have been inserted into numerous terrestrial bacteria to create a wide range of ''*lux*-marked biosensors.''[5]

lux Biosensors have been applied to toxicity testing for a wide range of environmental pollutants.[6–8] However, such biosensors (in common with toxicity assays in general) indicate sample toxicity (and bioavailability) alone, whilst revealing nothing of the chemical nature of the pollutants directly. In other words, there is little mutuality between toxicity biosensors and analytical chemistry. The purpose of the method described here is to bridge the gap between traditional biosensors and chemical analysis, by presenting a technique by which a luminescent biosensor may be used for both pollutant identification and toxicity assessment at the same time. Its primary application is as an environmental screening tool. A related approach has been developed through the use of a battery of catabolic *lux* biosensors, where luminescence is induced by the presence of target chemicals.[9] However, such an approach only works for certain toxicants, is less practical than using just a single biosensor and, most importantly, does not indicate toxicity.

The procedure described here involves a single constitutive *lux* bacterial biosensor, used to kinetically ''fingerprint'' individual toxicants and complex effluents. This is possible due to the fact that different toxicants elicit highly characteristic light response-curves when the biosensor is exposed to them. Such response-curves are derived by simply measuring luminescence continuously from the point of exposure for a 5-min assay. This technique is relatively inexpensive, rapid, simple to perform, and combines the qualitative potential of chemical analysis with the toxicological information of a biosensor. In addition, the technique can be applied to any pollutant or pollutant mixture that elicits a toxic response in the biosensor.

The methods presented here include a detailed description of the assay procedure, the way in which ''reference'' toxicant response-curves may be derived, and examples of its application using spiked environmental samples and industrial effluents. Mathematical procedures are demonstrated by which assay results may be interpreted.

Materials required

Biosensor

This research has focused on the use of *Escherichia coli* HB101, containing the full *lux* gene cassette (*lux* CDABE) as a multicopy plasmid insertion (pUCD607).[10] Insertion of the full

gene cassette results in the expression of luminescence without the requirement for any exogenous substrate addition. This biosensor was used for toxicity testing because it is robust, simple to use, and shows strong luminescent expression. The biosensor was lyophilized (freeze-dried) at late logarithmic growth stage in 1-ml aliquots and stored at −20°C prior to use.[11,12] These individual aliquots of lyophilized biosensor may be stored for at least 6 months before use. There is no reason why other luminescent constructs (or Microtox®) could not be used in the same way as described here, although minor modifications in protocol may be required depending on the biosensor used (especially with regard to resuscitation procedure). The source of the construct used in this research was the Department of Plant and Soil Science, University of Aberdeen, Scotland.

Equipment

- Temperature-controlled automatic luminometer; BioOrbit 1251 (Turku, Finland): This model uses a carousel pre-loaded with samples in plastic cuvettes. Luminescence of each sample in turn is measured for a pre-determined time (programmed via a computer interface: Multiuse software, version 1.01/April 1991/JN), with individual measurements taken every 2 s. Results are stored in data files for later analysis (they can be transferred into a spreadsheet program, such as Microsoft® Excel).
- Standard PC (with a 486 processor or above) for programming the luminometer.
- BioOrbit integrated injection system (Turku, Finland): enables pre-programmed injection of reagents directly into sample cuvettes during luminescence measurement. This should be fitted with a 100-μl syringe and comes with a programmable interface. The syringe is connected to a reagent source via a Teflon® tube, and the outlet (which leads into the luminometer light chamber) is via a second Teflon tube.
- Glass universal bottles (c. 30 ml): used for re-suspension of freeze-dried bacteria.
- Shaking incubator (at 25°C): used for resuscitation of freeze-dried bacteria.
- Two controlled-temperature chambers/refrigerators to hold biosensor aliquots at 15°C and 5°C, respectively.
- 1.5-ml plastic disposable cuvettes (Clinicon, Petworth, UK): used for holding samples in the luminometer.
- HI 8424 microcomputer pH meter.
- Baird Alpha 4 flame atomic absorption spectrometer (FAAS): used for quantification of heavy metals in environmental samples.
- ''Pollution and Process Monitoring'' Labtoc UV digestion and infrared TOC detector: for determination of total organic carbon (TOC) in environmental samples.
- Adjustable pipettes covering a range from 0.1 to 1 ml: these should use non-toxic disposable tips.

Chemicals

All chemicals used are analytical grade.

- Potassium chloride (KCl); Aldrich, Gillingham, UK; used for re-suspension of biosensor
- 2,4-dichlorophenol (2,4-DCP); Acros, Geel, Belgium; toxicant

- 3,5-dichlorophenol (3,5-DCP); Aldrich, Gillingham, UK; toxicant
- Sodium arsenite (As); BDH Chemicals, Poole, UK; toxicant
- 2-brom-2-nitro-1,3-propand (bronopol); Aldrich, Gillingham, UK; toxicant
- Copper (II) sulphate (Cu); Fisons, Loughborough, UK; toxicant
- Tetradecyltrimethylammonium bromide (TTAB), Aldrich, Gillingham, UK; toxicant (an anionic surfactant)
- Zinc (II) sulphate (Zn); Fisons, Loughborough, UK; toxicant

Environmental samples for spiking

Water samples were taken from three locations for subsequent spiking: these were kept refrigerated at 5°C in 1-l Duran bottles (without head-space) and were used within 1 week:

- River Don (Seaton Park, Aberdeen, Scotland)
- River Dee (Ballater, Deeside, Scotland)
- Tap water (Aberdeen University)

Toxic environmental effluents

Environmental effluent samples were taken from three locations: these were kept refrigerated at 5°C in 2-l Duran bottles (without head-space) and were used within 2 weeks. Samples were filtered (pore-size of 0.45 μm) prior to use.

- Sample 1: pre-treatment whiskey distillery effluent from the Glenfarclas whisky distillery, Speyside, Scotland
- Sample 2: untreated metal-rich effluent from a galvanizing and electroplating plant, Grampian, Scotland
- Sample 3: untreated effluent from a paper-processing plant, Grampian, Scotland

Procedures

A procedure is described herein by which *lux* bacteria may be used to fingerprint toxic samples by deriving characteristic light-inhibition curves for samples, thus combining toxicity assessment with chemical identification. This technique may be used to identify individual toxicants (or classes of toxicants) by comparing sample response-curves with those of reference toxicants. Because this technique is chemically non-specific, whole complex effluents may also be identified using the same technique (something difficult to achieve by chemical techniques). Moreover, this technique is extremely rapid, repeatable, and simple to perform, making it a potentially valuable tool for environmental screening purposes.

Procedures are described here for deriving series of reference luminescence-inhibition response-curves for specific toxicants (7 in this case). These act as a ''database'' of known responses against which ''unknown'' samples may be compared. Toxicant spiked water samples and complex effluents are also characterized in the same way, for comparison with the reference responses-curve series.

The procedures described here are divided into two main sections:

1. Assay protocol: the use of a cellular-injection method for deriving light-inhibition response-curves for a small data set of reference toxicants, spiked water samples, and environmental effluents.
2. Data interpretation:
   (i) the computer-based application of an algorithm for differentiating response-curves;
   (ii) analytical techniques for interpreting output from the algorithm program.

Assay protocol

The following procedure describes the derivation of a series of light-inhibition response-curves for a reference toxicant, in this example Zn, using a range of concentrations. This is done by injection of *lux*-marked bacteria into chemical standards inside a BioOrbit 1251 luminometer, whilst recording resultant luminescence inhibition. This assay series consists of 6 individual assays: 5 concentrations of Zn (from ''low'' to ''high'' light inhibition) and a control. This technique can be used to produce a set of reference response-curves for any chemical that elicits a toxic response to the biosensor: the specific reference toxicants characterized will depend upon the research carried out. In this chapter, reference response-curve series were derived for Cu, As, 2,4-DCP, 3,5-DCP, bronopol, and TTAB. These were chosen to represent a range of chemical classes and modes of toxicity, to form a database of characterized toxicity response-curves. Essentially, the same method is used to determine response-curves from experimental samples (spiked water samples and industrial effluents), which represent unknown samples to be tested against the reference database. All reference standards, spiked environmental samples, and industrial effluents were pH adjusted, by addition of HCl_{aq} and NaOH, to a value of 5.5 prior to use. All assays are performed in triplicate.

Resuscitation and control of the biosensor prior to use:

1. Remove a glass vial containing a freeze-dried aliquot of *E. coli* HB101 pUCD607 biosensor from frozen storage (−20°C) and allow to equilibrate with room temperature for half an hour.
2. Pipette 1 ml of a 10-ml, 0.1 *M*, KCl solution from a glass universal bottle into the vial and agitate for 10 s, then pipette the cell suspension back into the universal tube and agitate further using the pipette.
3. Place in a shaking incubator at 25°C for 30 min, then pipette 1-ml aliquots of cell suspension into 3-ml plastic cuvettes and place in a refrigerator at 5°C (repeatability of assays was found to be fairly insensitive to resuscitation time).[12] The cells can be stored at this temperature for up to 2 h prior to use without significantly altering the repeatability of the results (luminescence declines to very low levels at 5°C, indicating reduced metabolic activity).
4. A 1-ml aliquot of cell suspension is used for each assay run, i.e., 6 concentrations tested for a reference series. Therefore, for triplicate replication, 3 aliquots are used. Each aliquot is stored at 5°C prior to use, being raised to 15°C, 15 min prior to use (the incubator block of a Microbics Model 500 luminometer was used for this purpose).

Optimization experiments have shown that following this temperature regime significantly increases experimental repeatability.[12] This is due to the metabolic state of the

cells remaining more constant than they are at room temperature. For example, where triplicate cell suspensions were held at either 15°C or 25°C, after 2 h the mean luminescence of the 15°C suspensions had decreased slightly to 80% of the control (SD = 5%), whilst the 25°C suspensions had increased to 270% of the control (SD = 23%). Maintaining cells at 5°C effectively suspends metabolic activity for several hours, although luminescence is too low for experimentation, hence the need to raise the temperature prior to use.

Preparation of chemical standards: All chemical standards used should be made from pre-prepared top standards. Test standards are made in double-deionized water immediately prior to use to represent a toxicity range from low (light inhibition is minimal) to high (light inhibition is considerable). This involves preliminary range-finding experiments (using the techniques described here) to ascertain a desired concentration range for each chemical standard. As response-curves differ in shape between toxicants, it is not necessary (or practical) to produce exact degrees of inhibition for specific reference concentrations. Therefore, a certain degree of subjectivity may be used in deriving reference response-curves. The most important consideration is to ensure that mid-range toxicity is well described. The lowest concentration used for the series of Zn reference response-curves was 0.0065 m*M*, resulting in 17.2% light inhibition after 5 min; the highest was 0.036 m*M*, resulting in 96.8% inhibition.

Aliquots of each standard (900 μl) are aliquoted into individual plastic luminometer cuvettes in triplicate (i.e., $6 \times 3 = 18$ cuvettes in total). These are incubated at 15°C prior to use.

Use of the luminometer and injection system for derivation of light response-curves: The BioOrbit 1251 luminometer is pre-programmed, using the MultiUse software via a computer interface, to measure luminescence of each of the 18 cuvettes in turn as one ''assay run.'' General instructions for programming are given in the luminometer handbook. For each cuvette, luminescence is programmed to be measured ''continuously'' for 310 s (measurements are actually taken at 2-s intervals) with luminescent cells injected after 10 s, giving an effective assay time of 5 min. It takes around 110 min to process a full set of 18 cuvettes in this way. It was found that with longer assay run times than this repeatability became a problem due to the time that the cells were left standing. The cuvettes are continuously agitated by rotation, whilst in the light chamber of the luminometer.

The luminometer is cooled to 20°C by running tap water through an internal cooling system. This is the lowest temperature that could be reached with this cooling system, although running assays at 15°C would probably be preferable if possible. The injection system is connected to the luminometer via an electrical interface. This enables time of injection to be programmed into the assay run. The injector is programmed via a dedicated controller (which plugs into the injector) to inject a single 100-μl dose of cells, with a 1-s injection time, directly into each cuvette via a narrow Teflon tube fed into the top of the light chamber. This occurs when the cuvette is inside the luminometer light chamber, thus luminescence is recorded from the initial luminescence peak onwards. It has been demonstrated that the force of injection is sufficient to achieve good mixing within the cuvette by the injection of dyes.

Once the luminometer, injector, reagents, and cell suspensions are prepared, the assay series is executed as follows:

1. The 18 toxicant standards (including the three controls) are loaded into the luminometer carousel in random order.

2. The injector syringe is primed with 1 ml of cell suspension. This is loaded into the syringe, via a short Teflon tube, using a built-in piston that automatically re-loads the syringe following each injection.
3. Once everything is primed, the assay run is initiated via the computer interface and may be left to run unattended.
4. On finishing the run, the luminometer readings may be saved as data files to be loaded into a spreadsheet (such as Microsoft Excel).
5. The injection system should be cleaned by flushing with 70% ethanol.

Once the standards/effluents are prepared, this procedure (once set up) involves little actual work, enabling numerous individual samples to be run in a day without too much effort.

The above technique can be used to produce a set of reference curves for any chemical that elicits a toxic response to the biosensor. Reference curves were derived for 7 toxicants for the results presented here: Zn, Cu, As, 2,4-DCP, 3,5-DCP, bronopol, and TTAB. These were chosen to represent a range of chemical classes and modes of toxicity. Mathematical methods for making inter-comparisons between these response-curve series, in order to determine their relative ''distinctiveness,'' are described in the section ''Data interpretation.''

Derivation of spiked water response-curves: Aliquots of the three water samples described earlier were each spiked with single concentrations of 5 reference toxicants. Concentrations were selected to give mid-range responses based on the reference series as follows: Cu (0.15 m*M*), Zn (0.012 m*M*), As (0.016 m*M*), bronopol (0.016 m*M*), and 2,4-DCP (0.05 m*M*). Response-curves were derived in the same way as described earlier, with single concentrations used rather than a concentration series. For each spiked sample, the corresponding water sample was used as the control.

Derivation of effluent response-curves: Effluent response-curve series (5 concentrations plus 1 control) were derived as for the reference response-curves. This involved range-finding assays to establish a suitable dilution range in each case. Unpolluted controls were unavailable for the three samples, so deionized water was used for the control in each case and was used for making the serial dilutions.

Chemical characterization of effluents: The effluents were characterized with regard to pH, TOC, and heavy metal concentration (Cu, Zn, Cd, and Pb), as these were considered toxicologically relevant for the specific effluents. Toxic concentrations of Cu had previously been detected in whiskey distillery effluent from the same source,[13] and the other metals had previously been found in effluent from the galvanizing and electroplating plant (unpublished data). The paper processing plant effluent was not fully characterized, but previous research had shown it be a highly complex effluent containing significant concentrations of Hg and mixed phenolic compounds.[14]

Data interpretation

Fingerprinting algorithm: A simple algorithm was derived to compare an unknown response-curve with a reference response-curve series. This was implemented as a simple computer program written in Microsoft Visual C++, by which an unknown response-curve could be automatically compared against all available reference response-curves to identify a ''best-fit.'' The algorithm operates as follows:

1. Each 300-s response-curve is simplified by using just 6 time points (t_1, t_2, t_3, etc.) taken at 50-s intervals. This linear time series has been found to give the best identification success in general, although an exponential time series (where time points are biased towards the start of the assay) has also been used successfully.[15] The reason for using a linear series is that there is often a lot of ''noise'' at the start of the assay, resulting in greater error. However, the first 30 s of some toxicant response-curves (e.g., Cu) may show characteristic signatures that may not be captured by a linear series. In practice, it was found that there was greater ''initial'' variability with environmental samples than with laboratory standards, making a linear series preferable overall.
2. L_u is defined as the luminescence value of the unknown toxicant at the first time point. For each of the toxicants in the reference database, there is a luminescent value stored for each concentration (C_1, C_2, C_3, etc.), e.g., for database toxicant 1, these are denoted as: $L_{p1}(C_1)$ for concentration 1, $L_{p1}(C_2)$ for concentration 2, $L_{p1}(C_3)$ for concentration 3, etc.
3. The algorithm determines between which pair of concentrations L_u occurs, as shown in Figure 23.1.
4. The algorithm, based on the simple technique of proportionality, then determines the concentration C_u of the reference toxicant that would have the same toxic response (i.e., percentage luminescence). A linear relationship between concentration and luminescence between each pair of concentrations is assumed. Hence, C_u is given by:

$$C_u = \left(1 - \frac{L_u - L_{p1}(C_2)}{L_{p1}(C_1) - L_{p1}(C_2)}\right)(C_2 - C_1) + C_1$$

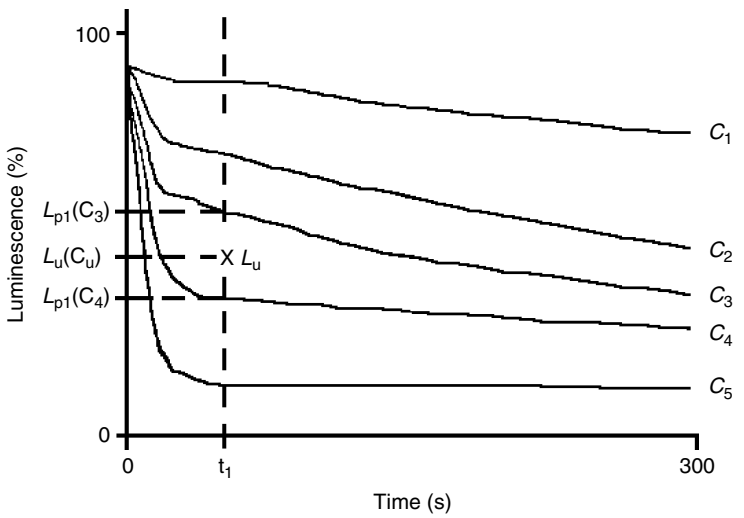

Figure 23.1 Method by which the concentration of an unknown toxicant is predicted: L_u is the luminescence of the unknown toxicant at time t_1. This falls between the luminescence values of concentrations C_3 and C_4 for the reference toxicant (L_{d1}). The predicted concentration (C_u) is determined by the model relative to these two values. (From Turner, N.L. Horsburgh, A., Paton, G.I., Killham, K., Meharg, A.A., Primrose, S., and Strachan N.J.C, *Environ. Toxicol. Chem.*, 20, 2456–2461, 2001. With permission.)

5. The 95% confidence interval for C_u is determined by propagating the confidence interval in L_u through the above equation.
6. These steps are repeated for each reference toxicant for the specific time point, and then the same process is repeated for each of the remaining 5 time points. The result is a temporal series of predicted concentrations and confidence intervals for each reference toxicant, based on the shape of the unknown response-curve. Therefore, if the predicted concentration of a reference toxicant remains consistent over the 6 time points, then it may be considered a likely match for the unknown toxicant.

Interpretation of results

Simply checking the output from the above method visually can indicate roughly which reference toxicants represent a good fit, and which do not. Two different methods for interpreting the computer output are presented, an implicit (yes/no) best-fit approach and a relative ''goodness-of-fit'' approach. The relative application of these two approaches is dependent on the type of analysis being done.

Best-fit approach: Where the aim of the analysis is to see whether an unknown toxicant response matches a reference toxicant response, one simply determines if the predicted concentration is consistent for each time point (i.e., falls within the 95% confidence intervals derived by applying the algorithm). In addition, the mean predicted concentration can be taken as a quantitative estimate of toxicant bioavailability. Figure 23.2 gives an illustration of this method. In this example, the 0.018-m*M* response for bronopol, when compared against the bronopol reference responses, gives the same predicted concentration within the 95% confidence interval for each time point (as would be expected). When compared against As, however, the predicted concentration is not consistent for all time points. This represents a positive-fit using the best-fit approach for bronopol, but not for As.

Best-fit analysis was used to make comparisons between the response-curves of the reference toxicants to determine how well the different toxicants can be differentiated. The highest and lowest concentrations were excluded in these inter-comparisons, as in many cases they are out of range, e.g., if observed luminescence inhibition is higher than

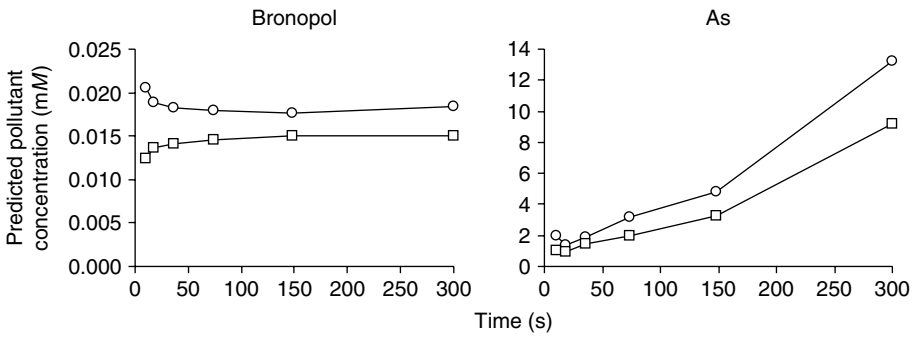

Figure 23.2 Example of model output: 0.018 m*M* bronopol response compared against reference responses for (a) bronopol and (b) As (○ upper 95% CI, □ lower 95% CI). This illustrates a typical positive identification (where predicted concentration is constant with time) versus a negative identification. (From Turner, N.L. Horsburgh, A., Paton, G.I., Killham, K., Meharg, A.A., Primrose, S., and Strachan N.J.C, *Environ. Toxicol. Chem.*, 20, 2456–2461, 2001. With permission.)

for the highest concentration of the reference toxicant, the highest concentration of the reference toxicant is given as the best-estimate, which will be an underestimation. This is because extrapolation beyond the range of the reference series was not done. Where it can be demonstrated that the dose response-curve for a reference toxicant is linear, extrapolation beyond the characterized range could be performed (although this approach is not used here). The best-fit technique was also applied to the identification of the toxicant spikes in environmental samples and to comparison of the industrial effluents.

Relative-fit approach: The above approach is useful when implicit identification is being looked for. However, as the fingerprinting technique is best used as a screening tool, it may often be more useful to determine the relative goodness-of-fit for an unknown toxicant compared against a range of reference toxicants. This may be done as follows:

1. Predicted concentrations (c_i) are obtained, using the algorithm, for comparison of an unknown toxicant to a reference toxicant for each time point. Unless the match is perfect, these 5 concentrations (one for each time point) will vary.
2. The overall predicted concentration (C) is defined as that which results in the smallest summed deviation (F) when subtracted from the individual predicted concentrations (c_1 to c_5). This is done via the following equation, which can be solved using the Microsoft Excel ''solver'' function to find the minimum value of F by changing C:

$$F = \text{absolute}(c_1 - C) + \text{absolute}(c_2 - C) + \cdots + \text{absolute}(c_5 - C)$$

3. In conclusion, two parameters are derived: the best-estimate concentration of the reference toxicant (C) and a measure of how consistent the predicted concentration is throughout the course of a 5-min assay (the deviation coefficient, F).
4. As the size of F is not independent of the size of C, an unbiased measure of goodness-of-fit is defined as F/C. This F/C value is used to compare goodness-of-fit between different reference toxicants.

This technique was used to demonstrate identification of toxicants in the industrial effluents.

Results and discussion

Reference response series

The temporal response-curves for the 7 reference toxicants are shown in Figure 23.3. Each concentration curve is plotted using the 6 time points required by the model. Clear differences can be seen between the response-curves of the different toxicants, with some more distinctiveness than others. Arsenate, Cu, Zn, bronopol, and TTAB all show responses related to exponential decay, with especially rapid inhibition at the start of the assay for arsenate and bronopol, with the most gradual inhibition for Zn. The response-curves of 2,4-DCP, and to a lesser extent 3,5-DCP, are markedly different from the other toxicants, in that varying degrees of luminescent recovery are seen following initial luminescence inhibition. This effect had been observed in a previous study, where low concentrations of phenolics were shown to cause luminescent stimulation in standard acute assays.[16] The overall shapes of the response-curves shown in Figure 23.3 show good

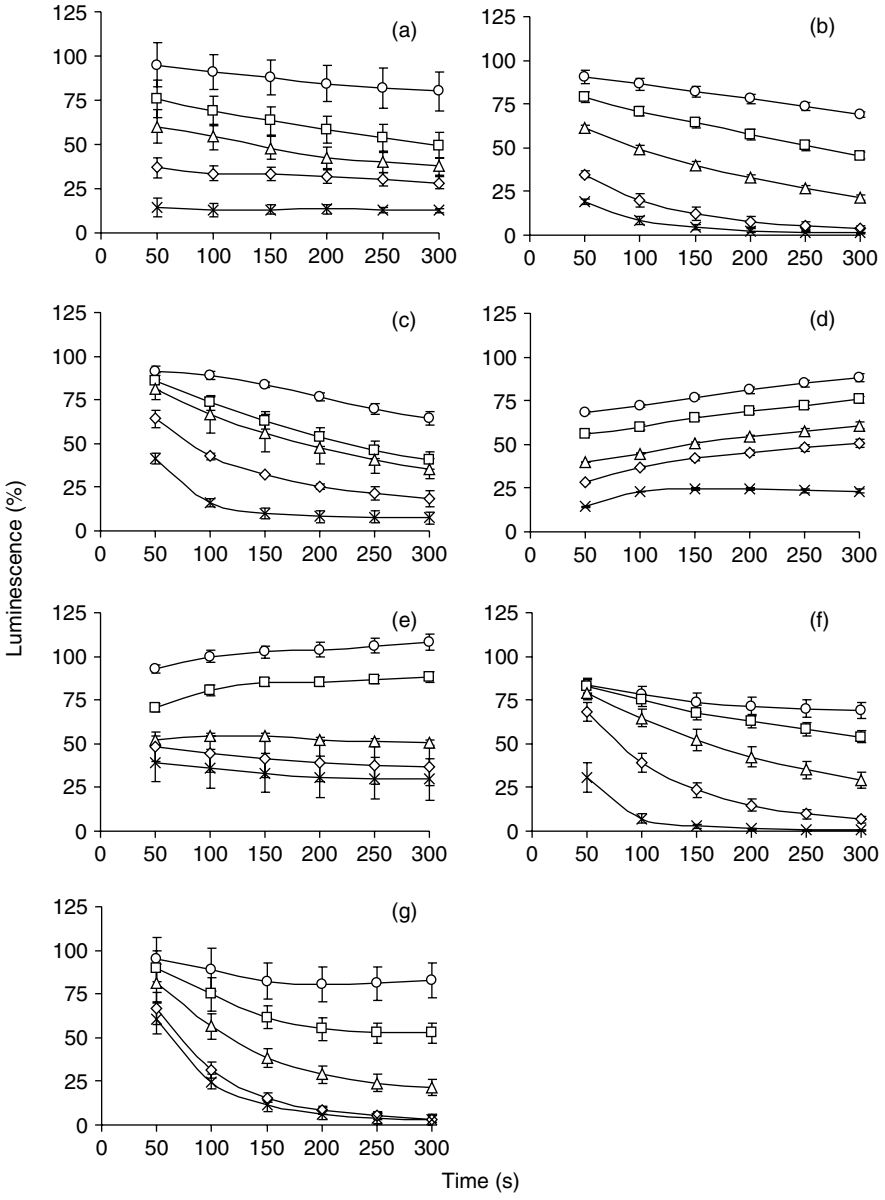

Figure 23.3 Reference toxicant temporal response-curves. Each point on a curve represents the mean value from triplicate data, with 95% confidence interval error bars:

(a) As	(○ 0.3 m*M*,	□ 1.0 m*M*,	△ 2.0 m*M*,	◇ 6.0 m*M*,	× 18.0 m*M*);
(b) Bronopol	(○ 0.004 m*M*,	□ 0.008 m*M*,	△ 0.016 m*M*,	◇ 0.032 m*M*,	× 0.048 m*M*);
(c) Cu	(○ 0.09 m*M*,	□ 0.15 m*M*,	△ 0.18 m*M*,	◇ 0.22 m*M*,	× 0.30 m*M*);
(d) 2,4-DCP	(○ 0.025 m*M*,	□ 0.05 m*M*,	△ 0.10 m*M*,	◇ 0.20 m*M*,	× 0.50 m*M*);
(e) 3,5-DCP	(○ 0.02 m*M*,	□ 0.08 m*M*,	△ 0.12 m*M*,	◇ 0.16 m*M*,	× 0.20 m*M*);
(f) TTAB	(○ 0.05 m*M*,	□ 0.07 m*M*,	△ 0.09 m*M*,	◇ 0.14 m*M*,	× 0.18 m*M*);
(g) Zn	(○ 0.0065 m*M*,	□ 0.009 m*M*,	△ 0.012 m*M*,	◇ 0.024 m*M*,	× 0.036 m*M*).

consistency with earlier assays using different stocks of chemical standards and batches of biosensor.[12]

Fingerprinting algorithm and use of best-fit identification technique

The assay technique and fingerprint algorithm were validated by comparing the reference toxicants to see whether they could be differentiated from each other using the algorithm. This was done by individually comparing the response-curves from each toxicant, as unknown responses, with the reference database responses. The highest and lowest concentrations for each toxicant were not tested as unknowns as many of these are out of range, so only the mid-range concentrations were compared, i.e., the computer program only extrapolates between known values, as an approximately linear dose response outside the measured concentration responses cannot be assumed. This is why each reference response series should cover as full a range of toxic responses as possible.

The results from the best-fit analysis are shown in Table 23.1. The value shown represents the number of ''false-positives'' for each pair of toxicants (where the concentration response of a given toxicant could not be differentiated from another reference toxicant). The maximum number is 6, as the middle 3 response-curves of ''toxicant 1'' were compared as unknowns against ''toxicant 2'' (as a reference toxicant), and vice versa. The model gives slightly different results depending on whether a given toxicant is treated as the unknown test response or the ''known'' reference response with respect to the comparative toxicant. The most false-positives occurred for TTAB. This is because the TTAB response series was somewhat intermediate between the response series of Cu, Zn, As, and bronopol. Cu differentiation was not as good as might be expected, as the 0.18-m*M* concentration had uncharacteristically high error. Similar results have been published previously, with the difference that an exponential time series was used to describe each curve.[15] This resulted in slightly better identification success, i.e., reduction of false-positives. However, a linear time series is used here as it gave better results overall when considering the spiked samples and the industrial effluents. This is because it puts less emphasis on the start of the assay, where there is greater variability, thus reducing the chance of false-negatives. However, for comparisons between the actual reference responses, where there is no chance of false-negatives arising (as toxicant

Table 23.1 Identification of reference pollutants using the fingerprint algorithm. Values given as the total number of false-positives for each pair of chemicals, where total comparisons for each pollutant pair = 6 (3 comparisons for the first pollutant as an unknown + 3 comparisons for the second pollutant as an unknown)

	Reference toxicants						
	As	Bronopol	Cu	2,4-DCP	3,5-DCP	TTAB	Zn
As	—						
Bronopol	1	—					
Cu	2	2	—				
2,4-DCP	0	0	0	—			
3,5-DCP	1	0	0	0	—		
TTAB	2	2	3	0	0	—	
Zn	0	0	2	0	0	2	—

Table 23.2 Quantification of pollutants spiked in environmental samples

	Predicted concentration (m*M*) in each environmental sample		
Pollutant spike	Tap water	Dee water	Don water
2.0 m*M* As	2.7 (± 0.9)	2.3 (± 0.6)	3.7 (± 0.9)
0.016 m*M* bronopol	0.014 (± 0.001)	0.012 (± 0.002)	0.014 (± 0.001)
0.15 m*M* Cu	0.09 (± 0.01)	0.11 (± 0.01)	0.10 (± 0.01)
0.05 m*M* 2,4-DCP	0.07 (± 0.01)	0.05 (± 0.01)	0.07 (± 0.01)

response-curves are compared against themselves), potential for differentiation is slightly reduced using a linear time series.

Identification of toxicant spikes

Single-concentration response-curves were derived for 2.0 m*M* As, 0.016 m*M* bronopol, 0.15 m*M* Cu, and 0.05 m*M* 2,4-DCP spikes in three environmental samples (tap water, River Don water and River Dee water). Figure 23.4 shows the response-curves for the three environmental samples; it can be seen that there is strong similarity between them. The results from the best-fit analysis showed that all the spikes were successfully identified (at the 95% test limit), in all three samples, with no false-positives. This demonstrates that in simple aquatic media, this technique is highly effective. However, as Cu, bronopol, and As responses are very similar, this degree of success may not necessarily always be repeatable. Table 23.2 shows that the predicted concentrations for the toxicant spikes were similar to the actual concentrations for all 4 toxicants, with

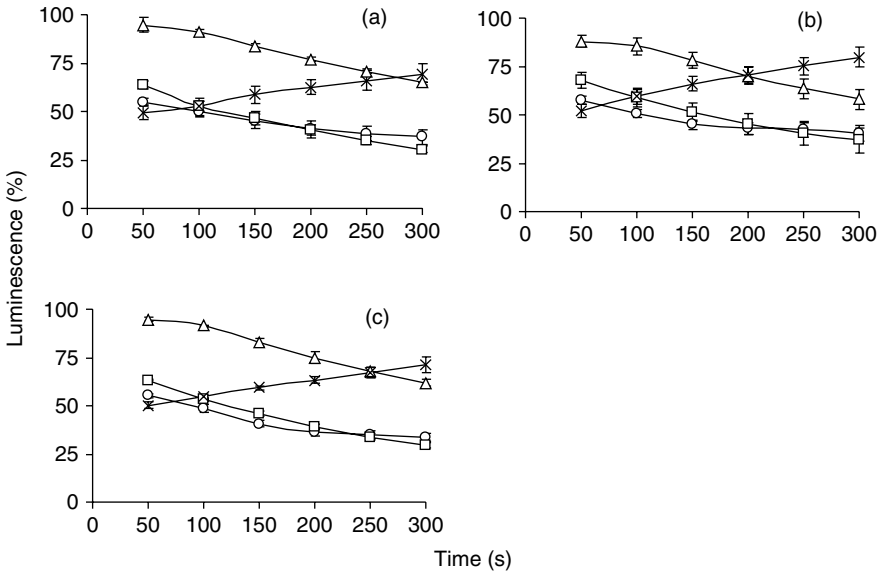

Figure 23.4 Temporal response-curves of spiked environmental samples: (a) tap water, (b) river Dee water, and (c) river Don water, spiked with single concentrations of toxicants: ○ 2.0 m*M* As; □ 0.016 m*M* bronopol; △ 0.15 m*M* Cu; × 0.05 m*M* 2,4-DCP. Each point on a curve represents the mean value from triplicate data, with 95% confidence interval error bars.

As and 2,4-DCP slightly overestimated, and Cu and bronopol slightly underestimated. This indicates that the bioavailability of the toxicants in the samples was approximately the same as for the reference standards. In the presence of significant concentrations of humic acids or dissolved matter, the predicted concentration would be expected to be significantly less as the bioavailable fraction would likely be reduced. The small differences in predicted concentrations, however, may be due in part to differences in water sample chemistry.

Characterization and differentiation of industrial effluents

Using FAAS, the concentrations of Cu, Zn, Cd, and Pb were quantified in the three effluents, as were TOC and pH. These results are shown in Table 23.3. No Cd or Pb was found in any of the effluents. These metals were considered to represent the likely main toxic constituents of the whiskey distillery and galvanizing plant effluents, although the toxic components of the paper processing plant were not identified.

All three effluents were found to be highly toxic to the biosensor and required considerable dilution to bring them ''in range.'' The highest concentrations of the three effluents (expressed as percentage dilution of the raw effluent) were 25% for the whiskey distillery, 0.5% for the galvanizing and electroplating plant, and 0.1% for the paper processing plant. Figure 23.5 shows the response-curve series for each of these effluents. The difference in response-curve shapes between all three effluents is clear, indicating that complex effluents may result in characteristic kinetic signatures. When the algorithm was used to make direct comparisons between the effluent response-curves, there were no false-positives, i.e., all three effluents were differentiated at the 95% test level. The approach used was the same as for the comparisons between the reference toxicants: the middle three response-curves from each effluent were compared against the response-curves of the other two effluents.

The effluent response-curves were next compared with the reference response-curves. As complex effluents were involved and not single toxicants, the ''relative-fit'' approach was used to compare the effluents with the reference toxicants. This was because the best-fit approach is suitable where an exact fit with a given toxicant is looked for. The relative-fit approach (using F/C values as a measure of goodness-of-fit) is suitable where the relative similarity of an unknown response with respect to a range of toxicants is required, and not implicit identification. F/C values were derived for each concentration of each effluent for comparison with all seven reference pollutants. The average F/C values for each effluent $\times$ reference toxicant comparison (being the average of the three effluent dilutions tested) are shown in Figure 23.6 (error bars = 95% confidence intervals). The F/C values for 2,4-DCP are not shown for any of the effluents, as

Table 23.3 Chemical characterization of environmental effluents

	Chemical parameter			
Effluent	pH	Cu concentration (m*M*)	Zn concentration (m*M*)	TOC (m*M*)
Whiskey distillery (pre-treatment)	6.1	1.05	0.03	3.0
Galvanizing and electroplating plant	6.3	0.66	1.65	2.2
Paper processing plant	3.3	None detectable	None detectable	3.6

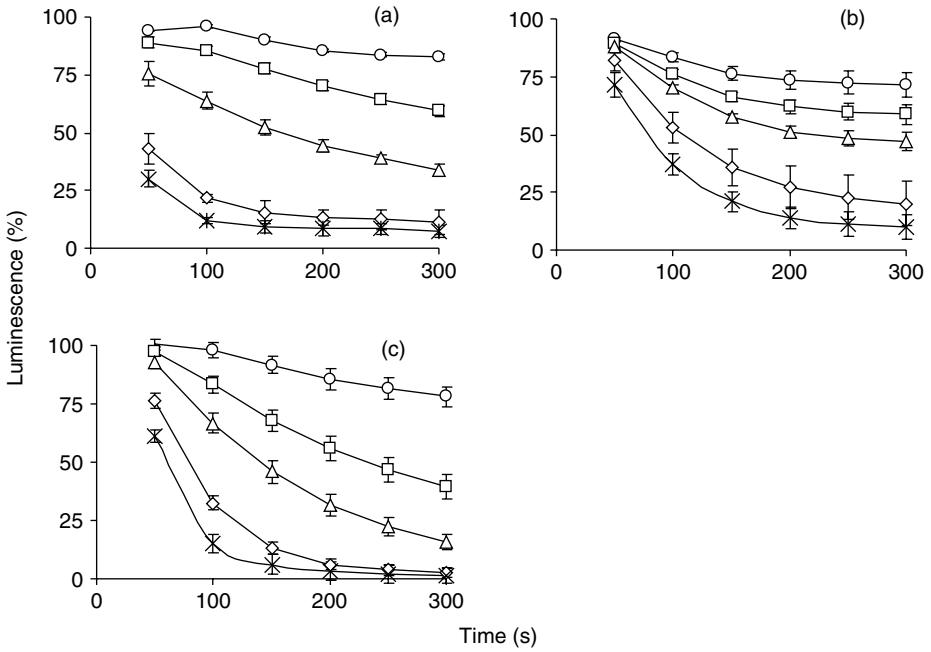

Figure 23.5 Temporal response-curves of environmental effluents. Each point on a curve represents the mean value from triplicate data, with 95% confidence interval error bars. Each curve represents a different percentage dilution of the raw effluent:
(a) Whiskey distillery (○ 5%, □ 10%, △ 18%, ◇ 25%, × 50%);
(b) galvanizing plant (○ 0.2%, □ 0.25%, △ 0.3%, ◇ 0.35%, × 0.5%);
(c) paper processing plant (○ 0.04%, □ 0.06%, △ 0.07%, ◇ 0.08%, × 0.1%).

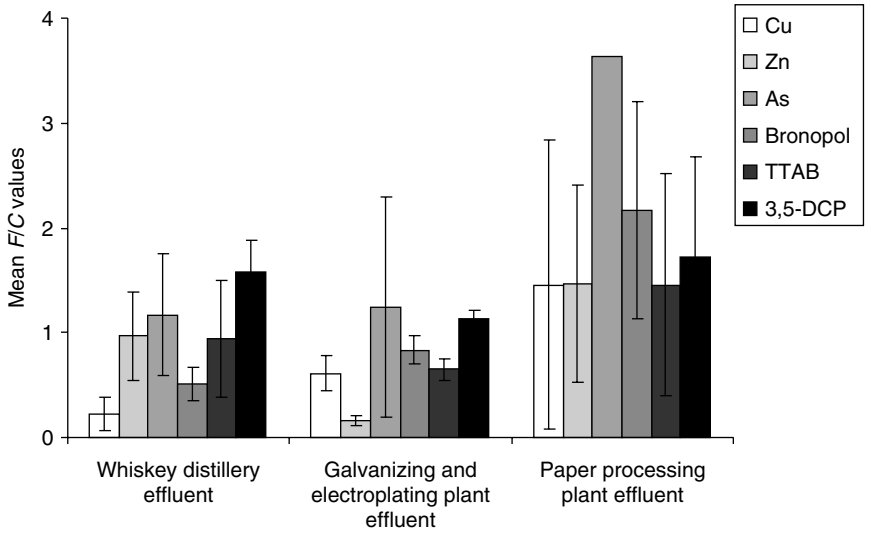

Figure 23.6 Relative similarity of reference toxicant response-curves to effluent response-curves for three industrial effluents. For each effluent, the mean F/C value (from the five effluent dilutions tested) is shown for each reference toxicant. The error bars represent the 95% confidence intervals of the F/C values for each of these dilutions. A small mean F/C value indicates a strong degree of similarity between the effluent and the reference response-curves (a possible identification). The 2,4-DCP comparisons are not included, as the mean F/C values were too great in all cases. The error bars for As are not included due to their size (95% CI $= \pm 2.65$).

they are an order of magnitude greater than the rest (i.e., 2,4-DCP responses were greatly dissimilar from all the effluent responses). It can be seen that for the whiskey distillery effluent Cu was identified as the closest fit; for the electroplating and galvanizing plant Zn was identified as the closest fit; for the paper processing plant there were no close fits.

It is not surprising that there were no close fits for the paper processing plant from the reference toxicants, as none of them were expected to contribute significantly to the toxicity. Cu was correctly identified as the main toxicant present in the whiskey distillery effluent. This indicates that the high TOC of the effluent did not significantly influence the signature of the toxicant. The fact that the Cu signature did not appear altered by the presence of Zn was possibly due to differences in speciation of the two metals, as Zn has been shown to be considerably more toxic to the biosensor than Cu (EC50 of Cu = 0.150 m*M*; EC50 of Zn = 0.07 m*M*).[12] The success in identifying the primary toxicant in a complex effluent was also demonstrated for the galvanizing and electroplating plant effluent, where Zn was identified as the primary toxicant (although Cu was present as well, Zn would be expected to contribute overwhelmingly to the overall toxicity at their respective concentrations). This validates the use of the technique as a screening tool for toxic ingredients in complex effluents. However, successful ''identification'' of a toxicant should not be taken as implicit proof of its presence; it is an indication of what is likely to be a cause of toxicity or, conversely, what is unlikely (i.e., a screening tool). This can guide subsequent actions, such as chemical analysis.

Overall, this technique represents a reasonably simple and inexpensive screening process using a single bacterial biosensor. Although it does not have the accuracy of chemical analysis, it represents a way in which a simple biosensor may be applied to both effluent characterization and toxicity assessment at the same time. Specific advantages of this technique are:

1. As with any standard biosensor, it reports on the bioavailable fraction of any toxicant that has a toxic effect on the bacterium.
2. Effluents may be screened to identify likely toxicants based on the shape of their toxicity response-curves, or to discard toxicants that are a poor match.
3. Where a single toxicant is present, and compared with reference response-curves, an accurate estimate of the bioavailable concentration may be derived.
4. Complex effluents may be fingerprinted by their response-curves. This can be used to identify effluents without the need to chemically characterize them.
5. This technique has the potential to be adapted for on-line use.[17] This would allow ephemeral toxicants (i.e., highly degradable or volatile organic compounds) to be characterized in flow-through systems.

It should be noted, however, that using a greater range of toxicants will invariably result in an increasing amount of false-positives as this technique does not have the discriminatory potential of chemical analysis. Toxicants can be clearly differentiated where different modes of toxicity result in distinct signatures. Therefore, if chemically distinct groups of toxicants with similar modes of toxicity are compared, differentiation potential may not be that strong. The main practical difficulty to overcome with this technique is controlling between-sample variability, although if the timing and temperature are closely controlled, variability can be kept acceptably low. An important consideration when performing a large number of assays is to ensure that all assays are performed using vials from the same freeze-dried batch. This is because subtle differences in biosensor response may be seen between batches. Quality control procedures ensure

that differences between batches are minimal.[12] Overall, this methodology is relatively simple to follow with no major pitfalls. As assays can be performed rapidly, and results obtained instantly (light inhibition is presented on the computer screen during the assay), any mistakes made can be easily identified and rectified.

In conclusion, this is a versatile and novel technique that may be adapted to a wide range of exploratory environmental problems, especially for screening prior to chemical analysis, and for identification of complex effluents.

Acknowledgment

This research was supported by the Natural Environment Research Council (NERC) and AZUR Environmental, and supervised by Dr. Graeme Paton and Prof. Ken Killham of the University of Aberdeen, Scotland. Dr. Norval Strachan (University of Aberdeen) wrote the algorithm program and helped formulate the mathematical techniques used for this research.

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chapter twenty-four

Aquatic in situ *bioassays to detect agricultural non-point source pesticide pollution: A link between laboratory and field*

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Contents

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Introduction

Active biomonitoring, which includes all methods that insert organisms under controlled conditions into the site to be monitored, has been developed as a link between laboratory, microcosm, and mesocosm studies on the one hand and passive biomonitoring in the field on the other hand. In ecotoxicology, active biomonitoring is often referred to as the use of *in situ* bioassays. Those *in situ* bioassays may be defined in the context of this book section as the experimental measurement of the responses of test organisms directly exposed to agricultural non-point source pesticide pollution in the aquatic environment over a defined time period. The results of *in situ* bioassays are thus considerably more relevant to the natural situation than those of laboratory experiments, especially with respect to the contamination scenario.[1] Whereas chemical analyses in the field provide abiotic data, the results of the *in situ* bioassay are based on a toxicological response[2] and, accordingly, substantially more informative regarding the protection of animal and plant communities.

The toxicological and ecological relevance are two important requirements that need to be fulfilled when bioassays should be used to assess non-point source pesticide pollution:

- There must be a clear relationship between the pesticide contamination and the response measured in the bioassay. To establish this toxicological relationship is particularly difficult, when runoff is the main route of pesticide entry, as various factors change at the same time (turbidity, flow, nutrients, pesticides). However, this is an intrinsic problem of any sort of ecotoxicological assessment of runoff-related pesticide pollution. The detection and assessment of spray drift is in comparison much easier, as no other environmental factors change at the same time.
- The response in the bioassay must directly or indirectly reflect responses of the same species (or the whole community) in the field. Otherwise, *in situ* bioassays rather serve as a measurement tool to detect unfavorable environmental conditions, which may eventually be identified as pesticide pollution but not necessarily as an indicator of ecologically relevant effects on the population or community level.

The responses of the test organisms used in *in situ* bioassays may be various lethal and sublethal ecotoxicological endpoints measured at suborganismic, organismic, or even the population level, see the section ''Procedures'' for further details. There are several important considerations for the selection of suitable test organisms, such as reproducibility, sensitivity, and ecological relevance, see the section ''Material required'' for further details. The time period used for testing are among other factors dependent on the expected exposure scenario and the test organisms used, see the section ''Procedures'' for further details. The subsequent section ''Results and discussion'' summarizes the outcome of the case study. The case study results are finally interpreted focusing on the two already mentioned and crucial aspects toxicological and ecological relevance.

Material required

Exposure system

As can be seen from Table 24.1, most *in situ* exposure systems are fairly simple self-made pieces of equipment. Plastic or PVC tubes with the ends closed off either by mesh or by lids have often been used. If lids are used to close off the ends, additional mesh covered

Table 24.1 Examples for characteristics of *in situ* exposure systems

Test organism	Exposure boxes			Openings for water exchange		Access to sediment	Reference
	Type/material	Size	Fixation	Number and size	Mesh size; material		
Mussel (*Mytilus trossulus*)	Bags of flexible vexar mesh tubing	1.5 m long, divided in 10 compartments	Nylon lines, buoyed weights	Completely mesh-covered	Not clear	No	8
Cladoceran (*C. dubia*)	Clear cellulose acetate butyrate sediment core liner tubes	12.7 cm, Ø 5.1 cm, ends closed with lids	In 1-cm nylon mesh holding bags fixed with stakes	2 side openings, 2.5 × 6 cm	149-μm polypropylene microporous filter mesh	No	9
Cladoceran (*D. magna*), zooplankton	Polypropylene beakers	50 ml	Plastic frame and weight	2 side and 1 bottom, Ø 0.2 cm	50 μm; nylon, thermal glue from Elis-Taiwan	Yes, through bottom opening	4
Amphipod (*Chaetocorophium* cf. *lucasi*)	PVC pipe	10 cm, Ø 10 cm	Placed in 2 cm deep depression in sediment	2 side, 8 × 10 cm	440 μm; plastic screen, epoxy glued	Yes, through bottom opening	10
Amphipod (*P. nigroculus*)	Polyethylene jars	10 cm, Ø 9 cm	Metal stakes	2 side, 6 × 8 cm	1 mm stainless steel mesh	No	11
Amphipod (*G. pulex*)	PVC cages	5 cm, Ø 5 cm	In 2.5-cm mesh holding baskets fixed with bricks	Both ends	1 mm	No	6
Amphipod (*H. azteca*), dipteran (*Chironomus tentans*)	Clear plastic tubes	15.2 cm, Ø 6.35 cm, ends closed with lids	In PE mesh dunk holding bags fixed with stakes	2 side openings, 4.5 × 9 cm	149-μm polypropylene woven screen cloth, silicone glued	Yes, through one opening	1
Amphipod (*H. azteca*), dipteran (*C. tentans*)	Clear cellulose acetate butyrate sediment core liner tubes	12.7 cm, Ø 5.1 cm, ends closed with lids	In PE mesh holding bags fixed with bricks	2 side openings, 3 × 5 cm	Polypropylene screen	Yes, through one opening	12
Amphipod (*G. pulex*), caddisfly (*L. lunatus*)	Plastic boxes	40 × 17 × 16 cm	Metal stakes	1 front and 1 rear	1 mm, polypropylene screen, thermal glued	No	7
Crayfish (*Procambarus* spp.)	Conical cages	50.8 × 50.8 × 15.2 cm	Not clear	Completely mesh-covered	Not clear	Yes, embedded into sediment	13

Continues

Table 24.1 Continued

Test organism	Exposure boxes			Openings for water exchange		Access to sediment	Reference
	Type/material	Size	Fixation	Number and size	Mesh size; material		
Dipteran (*C. riparius*)	PVC pipe	Whole water column, Ø 6.8 cm	Pipe driven 10–15 cm into sediment + metal stake	No	Sediment exposure only, uncontaminated water used	Yes, pipe driven into sediment	5
Dipteran (*C. tentans*), oligochaet (*Lumbriculus variegatus*)	Plastic core tube	46 cm, Ø 8 cm	Tube driven 15 cm into sediment	Several side holes, Ø 2.5 cm	224 µm; Nitex®, silicon glued	Yes, tube driven into sediment	3
Leopard frog (*Rana pipiens*), green frog (*R. clamitans*)	Nylon mesh cage	20 cm, Ø 15 cm	Wooden dowels	Completely mesh-covered	500 µm; Nitex Nylon	No	14
Rainbow trout (*Oncorhynchus mykiss*)	Cylindrical nylon mesh cages	38 cm, Ø 38 cm	Three fiberglass rods	Completely mesh-covered	6.3 mm; Nylon	No	15

openings have been placed into the side of the tube. There are no general guidelines with regard to the size of the bioassays or the material provided within the bioassays as food, shelter, or substrate. Both depend greatly on the ecological requirements of the test organisms used. Their density in the bioassay should not be higher than densities commonly found in the field. For frequently used *in situ* test organisms, such as amphipods, cladocerans, or chironomids, several papers focusing on methodological aspects do exist.[1,3–6] For case-bearing caddis flies, it is important to add case-building material to the bioassays.[7] If the emergence of semiaquatic insects is considered as an endpoint, it is required to make sure that the exposure containers do extent over the water surface even at varying water levels (see the section ''Case study'' for further details).

The mesh size used to cover the openings of the bioassay containers also depends mainly on the size of the test organisms; however, there are some other considerations that need to be taken into account. Too large mesh sizes might allow other organisms (e.g., predators or individuals of the same species) to enter the containers and affect the test organisms' survival or the assessment of results. Too small mesh sizes may reduce the flow within the containers, which, once again, may affect the test organisms, specifically if typical stream organisms are to be used. Moreover, the water exchange within the system or the access of potentially contaminated suspended particles may be reduced, and thus the exposure within the container may be different from the surrounding, which would be a crucial disadvantage of any bioassay design.

As the turbidity of agricultural surface waters varies greatly, clogging of the mesh is another problem that may arise over time and that has the potential to greatly affect the overall-performance of the bioassays. Specifically for studies in running waters, it is thus recommended to ensure sufficient flow of water through the bioassay containers over the duration of the exposure. Many studies have used nylon or polypropylene screens (Table 24.1); however, stainless steel mesh, which is available in various mesh sizes, has been proven to be a very robust and UV-resistant alternative.[11]

The fixation of the bioassay containers needs to be sufficient to ensure flood resistance, e.g., during runoff events. Depending on the purpose of the study, it is also important to make sure that the test organisms are in close contact with contaminated sediments. Various studies have suggested design options, such as exposure to the sediment through one of the mesh-covered openings[12] or through an open end of the bioassay tube directly inserted into the sediment.[3] The latter option in combination with the use of an uncontaminated water layer placed on top of the contaminated sediment may even serve as a sediment-only exposure system.[5]

Type, number, and source of organisms

Generally, the test organisms should be sensitive enough to indicate any potential pesticide effect, but tough enough to survive the exposure procedure at high rates under control conditions. As can also be seen from Table 24.2, the number of individuals used per bioassay ranges from 2 for fish[15] to 100 for mussels[8] or amphipods.[16] The main criteria affecting the decision about numbers of test organisms might be availability, ethical considerations, statistical, and methodological requirements, i.e., if a certain amount of tissue is necessary to measure specific biomarkers.

Many authors have used lab-cultured organisms during their studies (Table 24.2) as they offer the advantage of better reproducibility of the method in other labs and better comparability of results. However, for crayfish and fish, commercial suppliers may be

Table 24.2 Examples for the design of *in situ* exposure studies

Test organism	Site selection	Origin of test organisms	Number of replicates per site	Individuals per replicate size or age	Test duration/ feeding	Endpoints	Statistics	Reference
Mussel (*M. trossulus*)	1 reference + 12 contaminated	Mussel farm, farm holding 1 day	2 or 3	100, 5–6 cm	82 days	Bioaccumulation	Toxicity-normalization and ranking	8
Cladoceran (*C. dubia*)	1 reference + 1 contaminated	Lab culture	4	10, <24-h old	48 h/No	Survival	Not clear	9
Cladoceran (*D. magna*), Zooplankton	2 reference + 6 contaminated	Lab cultures	4–6	5	48 h/No	Survival	Kruskal-Wallis; Mann-Whitney	4
Amphipod (*Chaetocorophium* cf. *lucasi*)	1 contaminated	Field/lab holding 4 days	3 (per treatment)	20, 2–4 mm	10 days	Survival	ANOVA, Dunnett	10
Amphipod (*P. nigroculus*)	2 contaminated, internal control	Field, lab holding 2 days	4	10, juvenile	3–7 days/ butterspoon leaf	Survival, 6 exposure periods	ANOVA-Fisher PLSD	11
Amphipod (*G. pulex*)	24 reference + 15 contaminated	Field/lab holding 10 days	17–18	30, adult	6 days/alder leaf	Feeding rate, survival	ANOVA, *t* test	6
Amphipod (*G. pulex*)	1 contaminated	Field	Not clear	100, adult	11 weeks	Feeding rate and survival, weekly control	No testing	16
Amphipod (*H. azteca*)	3 contaminated	Lab cultures	4	10, <2 weeks old	7 or 18 days, ground rabbit chow	Survival	*T* test	1

Dipteran (*C. tentans*)	1 reference + 2 contaminated	Lab cultures	>4	10, 2nd instar	TetraFin®	Survival	Not clear	12
Amphipod (*G. pulex*), caddisfly (*L. lunatus*)	1 reference + 3 contaminated, internal control	Field, control samples	4	30, adults 30, 4th instar	11 weeks	Survival, weekly control	ANOVA-Fisher PLSD	7
Crayfish (*Procambarus* spp.)	1 reference + 1 contaminated	Commercial supplier, lab holding 1 week	3	10, 6–9 cm	4 days	Survival	Fishers exact test	13
Oligochaet (*L. variegatus*)	1 reference + 2 contaminated	Lab culture	5 or 6	40, adults	10 days/No	Survival. growth	ANOVA-Tukey	3
Leopard frog (*R. pipiens*), green frog (*R. clamitans*)	2 reference + 4 contaminated	Lab culture	4	10 fertilized eggs	2–3 weeks/ boiled lettuce	Hatching success, growth	Nested ANOVA	14
Rainbow trout (*O. mykiss*)	1 reference + 1 contaminated or 1 reference + 2 contaminated	Fish hatchery/ lab holding >4 weeks	1	2–5, 50–150 g weight	2–8 days/No	Biomarker gene expression	ANOVA-Tukey, *t* test	15

indicated; and for species not easy to culture in the lab, such as *Gammarus pulex* (Amphipoda), catches from the field were commonly used (Table 24.2). However, in these cases, a pre-holding period in the lab is often performed in order to adapt the organisms to experimental conditions and to account, as good as possible, for any effects of unwanted and unknown pre-exposure of the organisms at the site, from which they were obtained. Differences in sensitivity of lab-cultures and field catches to the same toxicant levels have been shown, e.g., for amphipods[17] or chironomids.[18] However, the reaction of test organisms present in the field and obtained directly from there may be more relevant for extrapolations of results to the target habitat under study (ecological relevance). If field samples of test organisms are the preferred option, they are ideally collected from uncontaminated control sites within the study catchment, which reflect similar environmental conditions.

Additional measurements

Electronic meters, photometric or spectrometric methods are commonly used to monitor general water-quality parameters (see also Reference 19). Suitable sampling methods for parameters that are directly related to runoff and thus need to be measured using an event-triggered design, such as turbidity, flow, and pesticide levels, have been described by Liess and Schulz.[20] The analysis of pesticide compounds generally follows standardized methods described in detail elsewhere.[19] These methods, however, depend on the equipment available and may be modified to account for matrix effects, e.g., from organic sediment fractions.[21]

Case study

Adult *Gammarus pulex* L. (Crustacea: Amphipoda; carapace length > 6 mm) were used for the case study on *in situ* exposure. The amphipods were obtained from the control sites used during this study. Prior to use, *G. pulex* specimens were kept for 7–9 days in the laboratory at $20 \pm 1^{\circ}$C in an aerated 50-l tank and fed with alder (*Alnus glutinosa*) leaves.

Plant containers ($40 \times 17 \times 15$ cm with an open top) made from green PVC were used as exposure boxes. The front and rear walls of the boxes were made of white nylon netting (1-mm mesh) to allow water flow through the boxes. The top of each container was also covered by 1-mm nylon mesh attached with Velcro tap to allow easy access. The boxes were supported by Styrofoam swimmers ($30 \times 5 \times 5$ cm) attached to the outside of each sidewall; they thus floated with the upper third above the water surface. Two nylon ropes (1 m long; 2 mm diameter) were connected to the front end of each box and the other end of the ropes was fixed to two metal stakes (1 m long; 10 mm diameter) hammered into the sediment. As the boxes were flexibly connected to the metal stakes via the rope, they were always floating at the water surface independent of changes in the actual water level during runoff events. In smaller and shallower water bodies, these exposure boxes may be directly attached to the sediment using metal stakes.

An initial experiment carried out during periods with high turbidity due to rainfall-related edge-of-field runoff indicated that the sediment accumulation rates in the boxes may be quite high and thus affect the survival of the amphipods. To overcome this problem, the design of the boxes was modified by replacing the basement of the boxes also with 1-mm nylon mesh. The aim was to reduce the sediment accumulation rates within the boxes by allowing the sediment to percolate through the bottom mesh. Both

box types were compared during exposure periods with runoff (see the section ''Results and discussion'' for details). The modified exposure box design was used in all subsequent experiments.

Sampling of amphipod populations occurring in the stream itself was conducted using conventional Surber sampling (area: 0.062 m^2; 1-mm mesh). At each site 4 independent samples were taken over a stretch of 50 m.

Total suspended solids (TSS) were measured using a HTS1 turbidity meter (Dr. Lange, Duesseldorf, Germany). To calibrate the turbidity measurements as described by Gippel,[22] certain samples were filtered through pre-weighed Whatman (Springfield Mill, UK) GF/F (0.45-μm pore size) glass microfiber filters and dried at 60°C for 48 h. The filter paper was re-weighed to determine TSS. Physico-chemical water parameters were measured with test kits from Macherey & Nagel, Dueren, Germany or electronic meters from Wissenschaftliche Technische Werkstaetten, Weilheim, Germany.

Analysis for the persistent organochlorine insecticide lindane and the currently used insecticides Fenvalerate (pyrethroid) and parathion-ethyl (organophosphate) was done at the Institute for Ecological Chemistry and Waste Analysis, TU Braunschweig. All water samples were filtered through pre-weighed Whatman GF/F (0.45-μm pore size) glass microfiber filters prior solid-phase extraction of 1000-ml samples with C_{18}-columns (J.T. Baker, Griesheim, Germany). Pesticides from solid particles were extracted with acetone. The measurements were made with GC/ECD and confirmed with GC/MS, with the following quantification limits: lindane and parathion-ethyl 0.01 μg/l for water and 1 μg/kg for particles; Fenvalerate, 0.05 μg/l for water and 5 μg/kg for particles. Details of the sampling methods and pesticide analysis are given by Liess et al.[21]

Procedures

Study objectives

A clear definition of the objectives of a planned *in situ* bioassay study and the data set to be generated are strongly recommended. This will help to identify implications for the test organism, exposure system and site selection, the experimental design, endpoints used, additional measurements required, as well as for the assessment of the toxicological and ecological validity and the statistical data analysis. Some of these aspects have already been discussed in the previous section, the others will follow below.

Site selection

Most importantly, the site or sites used for a particular study should be suitable to reflect the environmental stressor of interest. If agricultural non-point source pesticide pollution is concerned, sites will be most likely situated in upstream headwater sections of running water catchments or in ponds situated in areas of arable land use. Further downstream sections have the disadvantage of representing areas with multiple exposures, specifically if urban runoff, treated wastewater from urban settlements or industry enters the water bodies as well. It may thus be very difficult to separate the effect of non-point source pesticide pollution from other contaminants. If possible, it is also recommended to make sure that non-point source exposure is likely to happen in the area under study, either via spray drift during application or as a result of rainfall-induced runoff events. The

presence of erosion rills leading from the agricultural fields into surface waters may be an indicator of edge-of-field runoff.[23]

Generally, it is of great importance to carefully select control or reference sites for each study (Table 24.2). Ideally, the control site is situated in the same catchment in order to show similar environmental conditions. With regard to non-point source pollution, and more specific to runoff, an ideal control site would receive also edge of field runoff, including flow changes, turbidity, and nutrients, but no pesticide contamination. It is quite obvious that such a combination of characteristics of a control site is almost impossible to find. Some investigations have therefore used the same study site as an internal control site over time.[7] Runoff events occurring before commencement of pesticide application in the area may be used as a control to represent the effect of runoff without pesticide exposure, and subsequent events are then used to represent the effects of pesticide-contaminated runoff. For this type of study it is, however, suggested to monitor real exposure in samples taken during all runoff events in order to verify the assumptions.[24] Another option to try and understand or rule out some of the parameters always associated with runoff is to test, e.g., the potential effects of turbidity in the laboratory, as it was done with the South African freshwater amphipod *Paramelita nigroculus*.[11]

Finally, two further aspects need to be considered in the site selection process. On the one hand, it is necessary to regain the exposure systems to be able to retrieve the organisms, which may sometimes be a problem in large uniform or rapidly changing habitats, such as estuaries. Geographical positioning systems (GPS) may be a helpful tool here. On the other hand, the sites need to be sufficiently remote to avoid damage due to vandalism, which has been considered as an important threat by various authors.[10]

Experimental design

Information from other studies about the number of replicate exposure boxes and the number of organisms used per replicate are summarized in Table 24.2. As mentioned before, the density in the exposure boxes should not exceed densities observed for the same species in the field. DeWitt et al.[10] used slightly less than half of the average amphipod population density at the field site in their *in situ* bioassays. There are examples of using two or more species together in the same exposure boxes,[7,25] which may be a valuable to gain more information, given that interference or competition between the species can be ruled out as a factor. As can be seen from Table 24.2, many studies used at least three replicate containers at each site, which may be considered as the very minimum from a statistical point of view.

It is worth to note that inter-laboratory comparisons that have been undertaken with *in situ* bioassays using *G. pulex* and metalliferous effluents indicated striking differences in the results.[2] Unfortunately, there is not much data available to address this aspect further.

The exposure time used depends on the entry routes, i.e., the number and timing of the expected exposure events (runoff or spray drift), on the life cycle of the test organism, and on other aspects, such as the expected control mortality (ideally $\geq$80%). Survival of *G. pulex* over exposure periods of 1 week usually was greater than 80%.[7] For *Hyalella azteca*, survival rates of more than 80% have been observed over time periods of up to 4 weeks.[1]

However, other environmental factors may also influence the survival of the test organisms. Matthiesen et al.[16] reported that transient ice cover killed 53% of *G. pulex*

exposed over a 7-day period. Differences between exposure in sun and shade may have an effect,[26] specifically if the photo-induced toxicity, e.g., of polycyclic aromatic hydrocarbons (PAHs) is concerned.[9] As will be pointed out in the case study, suspended particles that accumulate in the exposure boxes may have a great impact on the test organism survival. Tucker and Burton[12] attributed mortalities detected using *H. azteca* exposed *in situ* to agricultural runoff to increased turbidities.

If tube exposure chambers are used that contain sediment-dwelling organisms and that are open at the bottom in order to allow exposure to contaminated sediment, the retrieval of these system needs to follow a procedure that ensures recovery of the test organisms.[3,5] The tube may be first gently loosened from the surrounding sediment by pushing it to a steep angle (relative to the sediment) and rotating it slowly in a circular fashion. With the tube tilted at approximately 45°, a thin plastic or metal disc may be slid over the bottom, while the tube is still in the sediment. With the disc held in place, the test chamber may then be pulled from the sediment.[3]

As a general advice, it is suggested to consult existing guidelines for conducting toxicity tests with the test species considered for *in situ* testing or to have a look at guidelines for similar species, prior to *in situ* testing. Some examples for general guidance are, e.g., References 27–30.

Toxicological endpoints

Survival is the most frequently used test endpoint used in *in situ* exposure studies (Table 24.2). A great advantage is that survival is an endpoint that can be measured in a rather objective way even by different investigators, although the disappearance of test organisms may cause problems if cannibalistic behavior cannot be ruled out. Most small invertebrates will also decay quickly, after they died, and they may thus not be found some days later. On the other hand, mortality is a relatively insensitive test endpoint, and other sub-lethal endpoints, such as behavior, reproduction, development, or sub-organismic biomarkers, may be more relevant, specifically as they do also have potential to indicate effects of endocrine disrupting chemicals.[31] Feeding rate (scope for growth) on pre-conditioned leaf discs has been successfully established in ecotoxicology as a functional sub-lethal biomarker for *in situ* exposed *G. pulex*.[6]

Most studies measure the response of the test organisms only once at the end of the *in situ* exposure period (Table 24.2). Amphipods exposed over a period of 11 weeks in an agricultural headwater stream in south England were controlled weekly and dead animals were replaced.[16] Exposure over the same time period was performed in a stream system receiving insecticide-contaminated runoff in Germany, during which the decrease in the number of test organisms was observed without replacement of dead animals.[7]

Additional measurements

Depending on the objectives of the study, a variety of additional variables are necessary to monitor. A general site characterization aiming at all potential factors affecting the survival of the test organisms and the performance of the bioassay exposure experiment thus needs to be performed. This characterization should include the upstream areas and their catchment, if running waters are concerned. Data on the historic use of the site may be very helpful in the interpretation of results from stagnant water bodies. General

water-quality parameters, such as nutrient levels, hardness, temperature, pH, and oxygen levels, will complete the characterization of the study sites.

In the context of studies on non-point source pesticide pollution, information about the timing and location of pesticide application, the application rates, and compounds used will greatly facilitate the study design and interpretation of results. This information may be obtained from the farmers. In order to assess the probability and occurrence of edge-of-field runoff events, rainfall data are required. Data about wind direction and wind speed are relevant for spray drift studies.

As many other factors, such as flow rate, turbidity, other contaminant or nutrient concentrations, may vary during runoff and may also affect the test organism response, these factors need to be monitored. Sampling for this purpose should be triggered by the runoff event, and automatic samplers are thus often used for this purpose.[20] However, passive runoff samplers,[23] suspended particle samplers,[32] and high-water level samplers[33] have been developed and used as cheaper and simpler alternatives. All those devices were specifically designed to detect transient pesticide peaks during edge-of-field runoff conditions. Being the main factor of interest for the *in situ* studies discussed in this chapter, the pesticide exposure needs to be characterized, as detailed as possible, with regard to both peak concentrations and exposure duration. Suitable sampling methods have been described by Liess and Schulz,[20] and the analysis of pesticide compounds generally follows standardized methods.[19] Contaminant concentrations may also be measured in the exposed test organisms in order to assess bioaccumulation.[34]

Data analysis and reporting

Analysis of variance (ANOVA) has been most commonly used to evaluate results from *in situ* exposure studies (Table 24.2). In cases where only pairwise comparisons are needed, *t* tests are frequently performed. However, all these methods may require transformation of the data in order to fulfill the criteria of normal distribution, or non-parametric alternatives may be indicated. If multiple comparisons are performed, a Bonferroni correction should be applied to control for type I statistical errors. Comparisons of various treatments with the same control may be undertaken using Dunnett's *post-hoc* test as part of the ANOVA procedure. There are various standard books that cover statistical procedures in great detail (e.g., Reference 35).

In order to account for the toxicological relevance of the bioassay response, a clear reflection of the cause and effect relationship is of key importance in evaluating *in situ* studies. It should include the temporal, and if relevant, also the spatial relation of exposure and response. Moreover, it should assess the likelihood of the response to be a true result of the observed exposure in the light of known toxicity data and other confounding parameters present. Finally, the ecological relevance of the results should be addressed through comparison of the bioassay response with effects on the population, community, or ecosystem level in the field.

Case study

The present pilot study was designed to answer (1) as to what extent *in situ* bioassays with *G. pulex* are suitable for the detection of short-term (about 1 h) runoff-related pesticide inputs, and (2) whether the response in bioassays is related to the responses of the same species in the stream. The first question was addressed by comparing the bioassay

response at two potentially contaminated sites with two uncontaminated control sites. The second question was approached by comparing bioassay responses with in-stream abundance data at the same site. In addition, some methodological aspects of confounding factors, such as elevated sediment accumulation in the exposure boxes or extensive macrophyte growth, were addressed. The assessment and interpretation of the results are finally presented in the section ''Results and discussion.''

The case study was carried out in 1997 at the Fuhse river system about 20 km west of Braunschweig in northern Germany (N 52° 1′; E 10° 27′; 150 m above sea level). An upstream (region 1; headwater) and a downstream (region 2) regions of the river system were chosen for the study, making sure that arable land was the main type of land-use (Table 24.3). Smaller headwater streams in the upstream region have agricultural non-point source pollution as the main anthropogenic impact. Some of the downstream reaches receive high nutrient loads, which in combination with lower water levels and higher light intensities in summer lead to very dense coverages with submerged aquatic macrophytes. In each region (regions 1 and 2), a control site (Controls 1 and 2), and an exposed site (Exposed 1 and 2) were selected. Sites protected from direct entry of edge-of-field runoff, e.g., by vegetated buffer strips, were assumed to serve as control sites, while sites with a high abundance of erosion rills were considered as exposed sites. Contamination with pesticides was, however, confirmed with event-triggered sampling and analysis of residues. The slopes in the catchment area vary between 1% and 2%. It is intensively cultivated (sugar beets, winter barley, and winter wheat). The most common soil types in the area are loess loam and clayey marl.

At each site, four boxes containing 30 adult *G. pulex* L. (Amphipoda; carapace length > 6 mm) were installed in the stream. The density of test organisms in the boxes corresponded to the densities commonly observed on plant structures in the field.[24] In each box, 100 g sand, two stones (*ca.* $5 \times 5 \times 3$ cm), four water-parsnip plants (*Berula erecta*), and four alder (*A. glutinosa*) leaves provided food and shelter. Amphipods pre-kept in the laboratory (see the section ''Material required'') were introduced into the boxes and retrieved after 7 days of exposure during all experiments. Survival of exposed amphipods was used as the toxicological endpoint. Three experiments were conducted in total:

- An initial experiment conducted during two subsequent exposure periods (7 days each) in April compared the survival of amphipods in differently designed boxes in order to assess the effects of accumulation of suspended particles in the boxes during runoff events with elevated turbidity. The site Exposed 1 was used for this experiment; however, no insecticide contamination occurred, as the exposure periods finished before commencement of insecticide spraying.

Table 24.3 Mean ($\pm$ SE; $n = 3$ measurements taken in May) of morphological and structural parameters at the four study sites

Parameter	Upstream sites Control 1	Downstream sites Exposed 1	Control 2	Exposed 2
Width (m)	1.5 ± 0.3	1.3 ± 0.4	4.1 ± 0.8	4.9 ± 1.0
Depth (m)	0.23 ± 0.04	0.25 ± 0.09	0.77 ± 0.32	0.72 ± 0.19
Current velocity (m/s)	0.21 ± 0.01	0.26 ± 0.02	0.42 ± 0.08	0.51 ± 0.12
Arable land (%)	95 ± 6	97 ± 5	83 ± 14	89 ± 15

- A second experiment (the main experiment) aimed to exemplarily assess potential pesticide effects. It consisted of two subsequent exposure periods (7 days each) in May and all four sites (Control 1, Exposed 1, Control 2, and Exposed 2) were used during this experiment. During the first week, no runoff occurred, while a 17-mm storm rainfall event led to edge-of-field runoff from arable land, previously treated with insecticides (the pyrethroid Fenvalerate and the organophosphate parathion-ethyl) as part of the usual farming program.
- The third experiment was conducted in August only at the two downstream sites (Control 2 and Exposed 2). This experiment aimed to exemplify the influence of aquatic macrophytes and related low oxygen concentrations in summer on the bioassay results.

In order to address the ecological validity of the bioassay approach, the densities of *G. pulex* populations at the four study sites were quantified using conventional Surber sampling at monthly intervals between April and August. This survey was done to complement the second exposure experiment. The results of this survey are not the main subject of this chapter; however, they were included, where it is necessary to assess the ecological validity of the *in situ* bioassays.

Various additional measurements of water-quality parameters, pesticide exposure, and site characteristics were undertaken. The deposition rates of sediments in the exposure boxes are based on measurements of volume of sediment layer before its removal at the end of the exposure period. The modified exposure box design that was used during all subsequent exposure experiments lead to reduced and similar sediment accumulation rates in the boxes at all sites.

Water samples for pesticide analysis were taken manually as 1-h composite samples at all four sites using 1-l amber glass bottles. Samples were obtained during elevated water levels resulting from the runoff event that occurred during the second exposure period in May. The particles suspended in the water were accumulated continuously at all four sites using suspended particle samplers,[32] from which they were collected and analyzed at 7-day intervals.

For a description of the abundances in the field, both species were collected at the four study sites at monthly intervals, with a Surber sampler (area: 0.062 m^2; 1-mm mesh). At each site, four independent samples were taken over a stream length of 50 m.

Differences between the mean mortality rates (*in situ* bioassays) or mean densities (field samples) were tested for significance ($p \leq 0.05$) using one-way ANOVA for multiple comparisons or t test for pairwise comparisons. Data were transformed using $\ln(x + 1)$ to satisfy the assumptions of the tests. A Bonferroni correction was applied to control for type I statistical errors, which assessed statistical significance with $\alpha = 0.012$.

Results and discussion

General

As shown in Table 24.4, important water-quality parameters, measured in May and August, are comparable between sites within acceptable limits. Orthophosphate levels showed a tendency to increase from May to August; however, this increase was most pronounced at the generally nutrient-richer downstream sites Control 2 and Exposed 2. At both the sites, the nitrite and nitrate levels were also higher in summer. As a likely

Table 24.4 Mean (± SE; $n = 3$) of water-quality parameters measured during the day and aquatic macrophyte coverage at the four study sites in May (week interval without runoff) and in August

	Control 1		Exposed 1		Control 2		Exposed 2	
Parameter	May	August	May	August	May	August	May	August
Nitrite (mg/l)	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Ammonium (mg/l)	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.7 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	1.9 ± 0.5
Nitrate (mg/l)	15 ± 8	14 ± 7	19 ± 4	17 ± 8	9 ± 3	15 ± 4	16 ± 5	35 ± 5
Orthophosphate (mg/l)	0.04 ± 0.03	0.08 ± 0.04	0.05 ± 0.03	0.05 ± 0.05	0.12 ± 0.03	0.17 ± 0.11	0.09 ± 0.07	0.28 ± 0.13
Hardness (mg $CaCO_3$/l)	491 ± 16	484 ± 25	460 ± 21	481 ± 24	369 ± 49	446 ± 25	490 ± 17	417 ± 65
pH	7.9 ± 0.6	7.8 ± 0.5	8.0 ± 0.1	8.1 ± 0.4	8.2 ± 0.2	8.0 ± 0.6	7.9 ± 0.4	8.4 ± 0.7
Oxygen (mg/l)	9.1 ± 1.2	8.4 ± 1.1	9.4 ± 1.5	8.5 ± 1.2	10.2 ± 1.3	7.5 ± 1.0	9.8 ± 0.5	4.8 ± 0.7
Temperature (°C)	10.5 ± 2.2	19.7 ± 3.1	11.1 ± 2.4	20.1 ± 1.7	13.7 ± 2.6	23.4 ± 2.6	13.8 ± 1.1	23.8 ± 1.9
Macrophyte coverage (%)	28 ± 12	25 ± 9	37 ± 15	45 ± 14	21 ± 15	27 ± 19	28 ± 17	89 ± 11[a]

[a] Mainly fennel pondweed (*P. pectinatus*).

result of the higher nutrient levels,[36] the macrophyte coverage of the streambed with funnel pondweed (*Potamogeton pectinatus*) strongly increased at the site Exposed 2 from 28 ± 17% in May to 89 ± 11% in August. Moreover, the oxygen content measured during the day decreased from 9.8 ± 0.5 to 4.8 ± 0.7 mg/l. Low oxygen levels associated with high macrophyte densities are a known feature of nutrient-rich surface waters in summer.[37] Decreased oxygen contents in August were also observed at the other sites; however, they were generally at least as high as 7.5 ± 1.0 mg/l.

First experiment on exposure box design

The initial methodological exposure experiment conducted in April compared two different design options: exposure boxes with a closed PVC bottom (box design 1) and a modified design with the bottom of the box replaced by 1-mm nylon mesh (box design 2). As expected, there is no difference in the survival of *G. pulex* between the two box design options during a no-runoff exposure period with clear water at TSS levels below 20 mg/l (Figure 24.1). The total accumulation of sediment in individual boxes averaged at 0.73 ± 0.22 l/week ($n = 4$) during no-runoff conditions. During the following week interval a rainfall-related edge-of-field runoff event occurred leading to an increased TSS level of 856 ± 49 mg/l ($n = 3$). As a result, the survival of *G. pulex* in box design 1 was significantly reduced ($p < 0.01$) in comparison with box design 2. Although the survival in the modified box design 2 with a mesh bottom was lower during runoff (about 80%) than during no-runoff time intervals (>90%), the differences were not significant (Figure 24.1). The sediment accumulation rate during runoff was 3.76 ± 0.71 l/week in box design

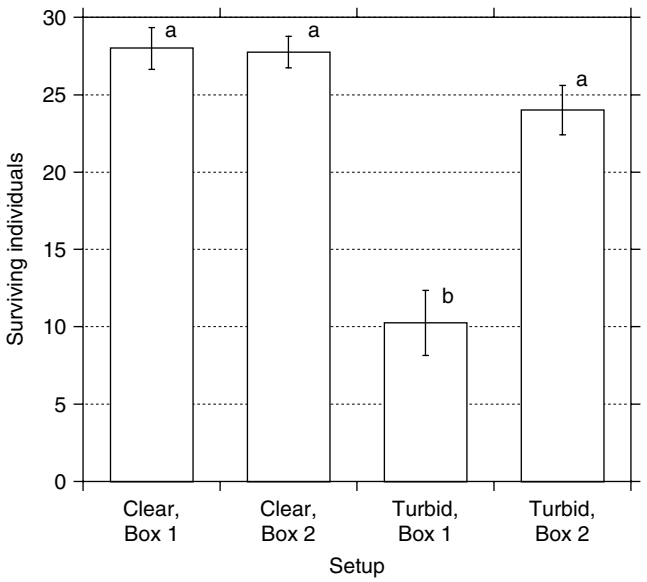

Figure 24.1 Mean (± SE; $n = 4$) survival of *G. pulex* exposed *in situ* in two different exposure box versions at the upstream site Exposed 1 during a no-runoff week interval with clear water and during a subsequent week interval with runoff-related increases in turbidity and sediment accumulation rates in April. Box 1 had a non-pervious PVC bottom, while the modified box 2 had a pervious 1-mm nylon mesh as the bottom. Different letters indicate significant (ANOVA; $p < 0.05$) differences in survival rate between the setups.

1 and 1.32 ± 0.69 l/week in box design 2 ($n = 4$). It is concluded that the modified box design 2 with a mesh bottom is more suitable during runoff studies with potential for high TSS levels. These boxes were thus used for the rest of the study, during which relatively low and similar sediment accumulation rates were observed in the boxes (<1.41 ± 0.95 l/week) at all sites. However, the results also suggest that elevated turbidity levels may affect the survival rates of *G. pulex*, which has already been reported from another study.[38] In contrast, a recent study using the amphipod *P. nigroculus*[11] demonstrated that the 7-day laboratory exposure to TSS levels as high as 1500 mg/l did not affect survival. *In situ* exposed *Ceriodaphnia dubia* showed a lower mortality during elevated turbidity rates in a stream where photo-induced PHAs were the main source of toxicity[9]; however, turbidity levels in this study were generally lower than during agricultural storm runoff in the present study.

Second experiment on pesticide effects: toxicological and ecological evaluation

Pesticide and TSS levels greatly increased during the second exposure interval in May, during which a rainfall-related edge-of-field runoff event occurred. Parathion-ethyl and Fenvalerate were detected at 47.5 and 35.8 μg/kg, respectively, in suspended particles, and at 2.1 and 0.3 μg/l, respectively in water samples taken from the upstream site Exposed 1 (Table 24.5). A much lower parathion-ethyl level of 2.5 μg/kg was detected at the downstream site Exposed 2 during runoff. Slightly elevated parathion-ethyl and background lindane levels were observed during no runoff at site Exposed 1 and during runoff at site Control 2. Compared with other studies the observed particle-associated pesticide levels were rather low, while the water-associated pesticide levels were comparably high.[39] Baughman et al.,[40] e.g., detected effects on *in situ* exposed estuarine shrimps *Palaemonetes pugio* during transient Fenvalerate peaks of 0.11 μg/l.

The survival of *in situ* exposed *G. pulex* was significantly decreased ($p < 0.0001$) to about 4 individuals (*ca*. 12%) during the week interval with runoff at the site Exposed 1 (Figure 24.2), at which elevated insecticide levels were observed (Table 24.5). No significant differences in survival were observed between no-runoff and runoff conditions at both control sites. Survival was also reduced to about 55% during runoff at the downstream site Exposed 2; however, differences were not significant ($p = 0.073$), most likely

Table 24.5 Pesticide levels detected in suspended particles and TSS at the four study sites in May during a week interval without runoff and during a subsequent week interval with edge-of-field runoff due to a 17-mm rainfall event (ND = not detected)

	Control 1		Exposed 1		Control 2		Exposed 2	
Parameter	No runoff	Runoff	No runoff	Runoff[a]	No runoff	Runoff	No runoff	Runoff
Lindane (μg/kg)	ND	ND	0.1	ND	ND	0.2	ND	ND
Parathion-ethyl (μg/kg)	ND	ND	ND	47.5	ND	0.3	ND	2.5
Fenvalerate (μg/kg)	ND	ND	ND	35.8	ND	ND	ND	ND
TSS (mg/l)	15	933	19	818	145	441	166	397

[a] Water samples taken during runoff contained 2.1 μg/l parathion-ethyl and 0.3 μg/l Fenvalerate.

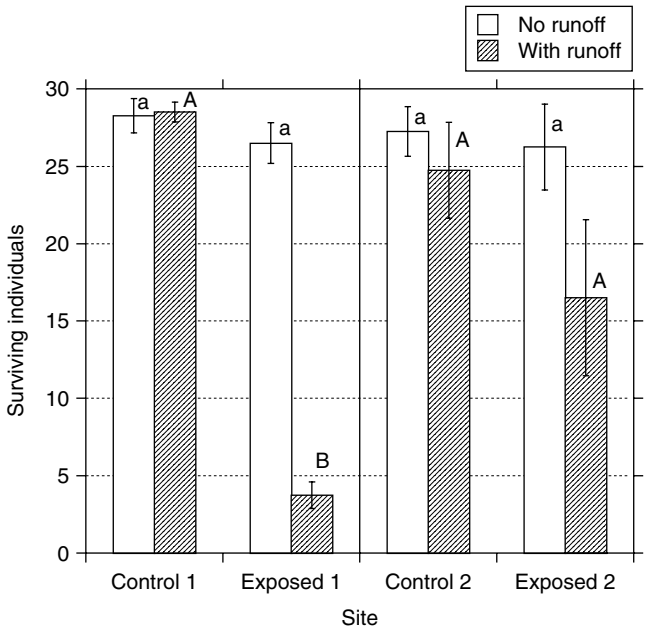

Figure 24.2 Mean ($\pm$ SE; $n = 4$) survival of *G. pulex* exposed *in situ* during a no-runoff week interval and during a subsequent week interval with runoff in May at all four study sites. Different letters indicate significant (*t* test; $p < 0.05$) differences in survival rate between separate pairwise comparisons of treatments at the control and the respective exposed site for each time interval.

because of the generally higher variation in survival rates that was observed at this site. An increased number of replicates at both downstream sites might have been an option to overcome this problem of a lack in statistical power.

For a toxicological evaluation of the results, the stressor pesticide contamination should be evaluated against other environmental variables. Water-quality parameters did not differ between sites (Table 24.4). A higher rate of sediment deposition resulting from increased TSS levels during runoff (Table 24.5) can sometimes be a cause of increased invertebrate mortality,[41,42] but in the present study, increased TSS levels were observed at the exposed and the respective control sites, which had neither strongly increased insecticide contamination (Table 24.5) nor greater mortality (Figure 24.2). This indicates that in the present study the pesticide contamination and not the water-quality parameters or sediment caused the observed mortalities. This conclusion is corroborated by other *in situ* and survey-based investigations from another agricultural catchment nearby, in which hydraulic and water-quality parameters were tested separately from insecticides and excluded as the responsible factor for effects on macroinvertebrates.[7,24]

As part of the toxicological evaluation, it can be concluded that the *in situ* bioassay with *G. pulex* used in this study serves as a valuable tool for a toxicology-based detection of short-term insecticide input events.

In order to evaluate the presented bioassay results in the ecological context, they need to be compared with the abundance dynamic of the same species in the field. In the bioassays, *G. pulex* exhibited a clear decrease in the number of adult individuals when insecticides were present at the contaminated sites, while no or only a minor decrease

occurred at the control site. In the field samples, however, the number of individuals of *G. pulex* present at these times remained almost the same. The abundance before and after the runoff event in May was 674 ± 443 individuals/m^2 and 649 ± 488 individuals/m^2. Overall increased the abundance of *G. pulex* from April to August at all four study sites.

While the bioassay indicated a significant decline of adult *G. pulex* at the contaminated sites, there was a general increase in the density of *G. pulex* in the field. The adult individuals confined to the exposure boxes are thus presumably exposed to higher concentrations and, accordingly, exhibit higher mortalities. In evaluating pesticide contaminations, and in extrapolating the results of *G. pulex* bioassays to the field, the possibility of an overestimation of toxicity must be considered. Even if the results of the *G. pulex* bioassay were not directly transferable to field populations of *G. pulex*, they can be used as a very helpful (measurement) tool to confirm that insecticide input events have occurred and to estimate their effects on other macroinvertebrate species. On the other hand, as shown in a previous study using *G. pulex* and the caddis fly *Limnephilus lunatus*,[7] the difficulty to observe an exposure–effect relationship in the field samples might be due to the elevated coefficients of variance. However, for *L. lunatus* even slightly higher differences in variance had not resulted in differences in measurable mortality.

Third experiment on the influence of extensive macrophyte coverage

As mentioned earlier, extensive coverage of funnel pondweed (*P. pectinatus*) was observed as a result of the combination of high light intensity, temperature, and strongly elevated nutrient levels at the downstream site Exposed 2 in summer. The extensive macrophyte coverage is a well-known phenomenon in the downstream stretches of the Fuhse river system, and mechanical weed control is usually performed during the summer months to avoid flooding of areas adjacent to the river during extensive rainfall events.

The survival of *G. pulex* at the site Exposed 2 in summer, which had an almost complete macrophyte coverage of 89 ± 11%, was significantly ($p < 0.01$) reduced (Figure 24.3). This is most likely due to the low oxygen levels (Table 24.4) that were observed as a result of the high macrophyte coverage. Considering the similarity of all other measured water-quality parameters, the oxygen level and reduced flow rates are assumed to be responsible for the observed increased mortality. It has been reported that *G. pulex* reacts susceptible to reduced oxygen levels.[43] Furthermore, *G. pulex* may be replaced in the field by the isopod *Asellus aquaticus*, which is less sensitive to low oxygen levels,[44] a phenomenon that can also be observed in downstream stretches of the river Fuhse.

Conclusion

Overall, these results emphasize that it is of high importance during *in situ* exposure studies to understand and/or eliminate confounding factors, such as macrophyte coverage or turbidity. Otherwise, a direct cause–effect relationship between contamination and bioassay response might be difficult to establish. However, the results also show that bioassays are a powerful tool for the detection and ecotoxicological assessment of agricultural non-point source pesticide pollution, given that a sufficient understanding of the ecological validity of the bioassay responses is available.

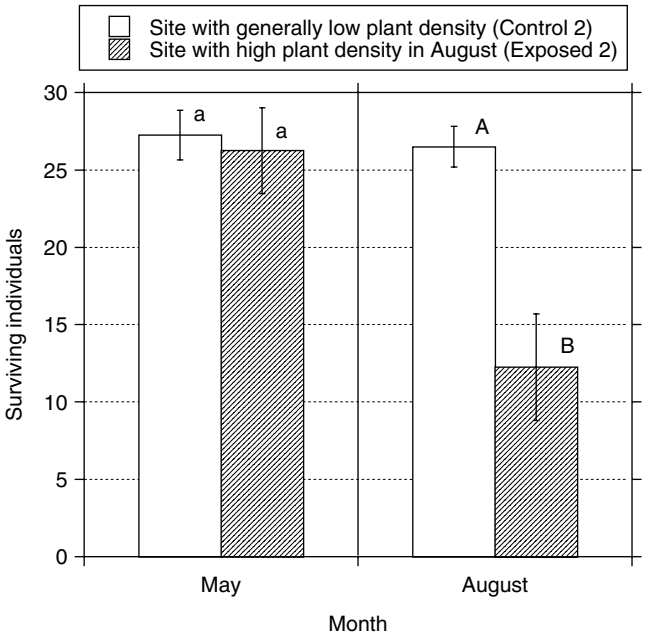

Figure 24.3 Mean ($\pm$ SE; $n = 4$) survival of *G. pulex* exposed *in situ* during a week interval each in May and August at site Control 2 with low macrophyte coverage and at site Exposed 2 with high macrophyte coverage in August. Different letters indicate significant (t test; $p < 0.05$) differences in survival rate between separate pairwise comparisons of sites in each May and August.

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chapter twenty-five

Improvements to high-performance liquid chromatography/photodiode array detection (HPLC/PDA) method that measures dioxin-like polychlorinated biphenyls and other selected organochlorines in marine biota

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Contents

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Introduction

Organochlorines (OCs) are widespread chemical contaminants that frequently occur in the marine environment. Many of these compounds [e.g., polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDTs), chlordane] possess certain chemical properties (e.g., stable, low flammability, lipophilic) that not only make them effective pesticides and industrial compounds but also make them highly persistent environmental contaminants.[1–3] Accumulation of certain OCs in marine organisms is linked to various deleterious biological and physiological effects, including reproductive impairment, immune suppression, and pathological lesions.[4–9] Measuring for toxic OCs, such as PCBs and DDTs, in marine organisms is therefore necessary to evaluate the risk to their health, as well as provide a measure of ecosystem quality.

Several methods have been developed to accurately determine trace levels of OCs.[10–18] Because numerous steps are needed to remove interfering compounds and to separate the various OCs before analyses by gas chromatography with electron capture detection (GC/ECD), gas chromatography/mass spectrometry (GC/MS), or high resolution GC/mass spectrometry (HRGC/MS), many of these methods are expensive, labor-intensive, and time-consuming. Due to these limitations, only a small number of samples can be analyzed in a short time span. Analytical methods that could rapidly and accurately measure toxic OCs in marine organisms are valuable, especially methods that could measure a suite of OCs at trace levels.

We have developed a rapid and inexpensive method to measure dioxin-like PCBs and other selected OCs using high-performance liquid chromatography with photodiode array detection (HPLC/PDA).[19] Total PCBs, summed DDTs and summed PCB toxic equivalents (TEQs) were calculated using these concentration data. Although the TEQ concentrations determined by HPLC/PDA are more conservative values compared to those measured by HRGC/MS because they include only dioxin-like PCBs and not other compounds (e.g., polychlorinated dibenzodioxins, polychlorinated dibenzofurans) that could contribute to the TEQ values, this method rapidly provided PCB TEQ concentration data for a wide range of marine biota. Recent modifications have been made to this HPLC/PDA method, including a more rapid extraction technique using an accelerated solvent extractor (ASE) with dichloromethane, as well as further analyses of HPLC/PDA extracts by GC/MS for a larger suite of OCs (e.g., additional PCB congeners, *o,p'*-DDE, oxychlordane, mirex, β-hexachlorocyclohexane). In this chapter, we describe the modifications made to the HPLC/PDA method and report some preliminary OC data for reference materials and killer whale samples that were analyzed using the expanded HPLC/PDA method. The OC levels were measured by the modified HPLC/PDA method in National Institute of Standards and Technology (NIST). Standard reference materials (SRMs) were then compared to the values determined by a more comprehensive method (GC/MS),[20] as well as to the certified values reported by NIST.

Materials required

Tissue extraction

See Reference 20 (Chapter 35 of this volume) for description of the ASE extraction method.

Tissue sample cleanup

Equipment:

- Auto desiccator, Sanpla Dry Keeper (Sanplatec Corp., Osaka, Japan)
- Rotovap with water bath (Buchi Switzerland, Krvr 65/45)
- Vacuum pump, model #0523-VHF-GS82DX (Gast Mfg. Corp., Benton Harbor, MI)
- Concentrator tube heaters with glass-cylinder shroud, wire tube holder, and aluminum inserts bored out to fit 50-ml conical tubes (Kontes, Vineland, NJ, catalog #720000-0000)

Supplies:

- Glass wool, Corning® 3950 (VWR Scientific; West Chester, PA; catalog #32848-003)
- Pyrex® glass funnel (Fisher Scientific; Pittsburgh, PA; catalog #10-346C)
- 16 in. Teflon® rod (Fisher Scientific; Pittsburgh, PA; catalog #14-518-10F)
- Glass cleanup columns, 305 mm long and 9 mm i.d., 20 mm i.d. for solvent reservoir (DJ Glass Factory, San Jose, CA; special-ordered glassware)
- 10-ml conical glass tubes (Fisher Scientific; Pittsburgh, PA; catalog #05-569-2)
- Kontes® 2-l one-neck round bottom flask (Fisher Scientific; Pittsburgh, PA; catalog #K601000-0824)
- 9-in. glass Pasteur pipettes (All World; Lynnwood, WA; catalog #020-03609)
- 50-ml concentrator tubes with caps (Fisher Scientific; Pittsburgh, PA; catalog #0553841a)
- 4-ml amber glass vials with self-sealing Teflon-coated caps and 250 μl glass inserts with springs (Sun SRI; Wilmington, NC; catalog #200-596)

Reagents:

- Acetone, HPLC grade (Fisher Scientific; Pittsburgh, PA; catalog #A929-4)
- Dichloromethane, high purity (Burdick & Jackson; Muskegon, MI; catalog #300-4)
- Concentrated hydrochloric acid, reagent grade (Fisher Scientific; Pittsburgh, PA; catalog #A144-212)
- Hexane, HPLC grade (VWR Scientific; West Chester, PA; catalog #BJGC217-4)
- Methanol, nanograde (VWR Scientific; West Chester, PA; catalog #MK516008)
- Concentrated sulfuric acid, trace metal grade (Fisher Scientific; Pittsburgh, PA; catalog #A510-500)
- Davisil® (WR Grace & Co.) 100–200 mesh silica, grade 634 (Fischer Scientific; Pittsburgh, PA; catalog #S734-1)
- Potassium hydroxide (Fisher Scientific; Pittsburgh, PA; catalog #P250-500)
- Ultra-pure nitrogen gas, Grade 4.8 (PraxAir, Danbury, CT)

Analyses of OCs by HPLC/PDA

Equipment:

- Two Cosmosil® 5-PYE analytical column (4.6 mm × 250 mm; 5 μm particles; Nacalai Tesque, Kyoto Japan; purchased through Phenomenex, Torrance, CA)

- Cosmosil 10-PYE guard column (4.6 mm × 50 mm; 10 μm particles; Nacalai Tesque, Kyoto Japan; purchased through Phenomenex, Torrance, CA)
- Two 6-port Rheodyne® electrically actuated valves (Cotati, CA)
- Model 7950 column chiller (Jones Chromatography, Lakewood, CO)
- Waters 515 isocratic pump (Milford, MA)
- Waters 715 Ultra WISP® autosampler (Milford, MA)
- Waters 996 PDA detector (Milford, MA)
- Empower® data acquisition software (Waters, Milford, MA)
- A Dell Dimension 8300 computer or equivalent computer that can run Microsoft Windows XP Pro®, has at least 2GB hard disk space, with at least 384 MB of RAM and is not a hard disk configured to be FAT 16

Reagents:

- Stock solution of 1,7,8-trichlorodibenzo-*p*-dioxin (1,7,8-Tri CDD), 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD), individual PCB congeners, individual DDTs, and hexachlorobenzene; all at 100 ng/μl in isooctane (AccuStandard; New Haven, CT; see AccuStandard catalog for numbers)
- Ultra-high purity helium gas, grade 5, 99.999% (PraxAir; Danbury, CT)
- Copper, reagent grade, granular, 20–30 mesh (JT Baker; catalog #1720-05)

SRMs

- SRM® 1974b, a blue mussel homogenate (NIST; Gaithersburg, MD)
- SRM 1945, a whale blubber homogenate (NIST; Gaithersburg, MD)
- SRM 1946, a fish tissue homogenate (NIST; Gaithersburg, MD)

Analyses of OCs by GC/MS

Equipment:

- Agilent 7683 autosampler with an on-column injection syringe and needle guide (Agilent Technologies, Wilmington, DE)
- Agilent 6890N gas chromatograph with a cool on-column injection port
- Deactivated fused-silica guard column, 10-m × 0.53-mm (460-2535-10, Agilent Technologies)
- DB-5® (J & W Scientific, Folsom, CA) GC column (60-m × 0.25-mm, 0.25-μm film thickness, 122-5062, Agilent Technologies)
- Agilent 5973N Mass Selective Detector® (Agilent Technologies)
- Computer system with ChemStation® software (Version DA, Agilent Technologies)

Supplies:

- Indicating moisture trap (GMT-4-HP, Agilent Technologies)
- Disposable oxygen trap (803088, Supelco)
- Indicating oxygen trap (4004, Alltech Associates, Deerfield, IL)
- Glass, universal column union (705-0825, Agilent Technologies) sealed with polyimide sealing resin (500-1200, Agilent Technologies)

Reagents:

- Ultra-high purity helium gas, grade 5, 99.999% (PraxAir, Danbury, CT)
- Isooctane, Optima (Fisher Scientific; Pittsburgh, PA; catalog #0301-4)
- Stock solutions of individual PCB congeners for GC calibration standards and GC internal standard (PCB 103), 100 ng/μl in isooctane (AccuStandard; New Haven, CT; see AccuStandard catalog for numbers)
- Stock solutions of individual OC pesticides for GC calibration standards, 100 ng/μl in isooctane (AccuStandard; New Haven, CT; see AccuStandard catalog for numbers)

Procedures

HPLC/PDA standards

A standard solution (10 ng/μl) of each internal standard (1,2,3,4-TCDD and 1,7,8-TriCDD) was prepared in hexane. A single HPLC/PDA calibration solution (0.322 ng/μl except where noted) was prepared in hexane and contained the following compounds (in order of HPLC elution): PCB 200, *o,p′*-DDT, *p,p′*-DDE, 101, 153, 110, 138, 180, 128, 118, 114, *p,p′*-DDT, 170, 105, *o,p′*-DDD, 190, 156, 157, 189, 77, HCB, 1,7,8-TriCDD (HPLC internal standard, 0.800 ng/μl), 126, *p,p′*-DDD, 169, and 1,2,3,4-TriCDD (surrogate standard, 0.800 ng/μl). A second HPLC/PDA calibration solution was prepared in hexane as a 1/4 serial dilution of the above calibration solution.

GC/MS standards

A solution of the GC internal standard (PCB 103, 10 ng/μl) was prepared in isooctane. A series of 11 GC calibration standards (Level 1–Level 11) containing the OCs listed in Table 35.1, Chapter 35,[20] were used for calibration.

Sample extraction and cleanup

Prior to use, all glassware were rinsed three times with acetone to remove any potential contaminants. All glassware rinsing, tissue extraction, and cleanup procedures were performed in fume hoods by personnel wearing nitrile gloves and safety glasses. Because PCBs and other OCs may adhere to glass, all glassware once used were discarded.

OCs and lipids were extracted from tissues using an ASE (see Chapter 35 for description).[20] For HPLC/PDA analyses, the amount of tissue extracted ranged from 0.20 to 5.0 g, depending on the expected lipid and water content. In all cases, the sample mass was adjusted such that the total amount of lipid loaded onto the cleanup column was less than 0.3 g. Each tissue sample was weighed to the nearest 0.01 g in a tared 10-oz jar. Sodium sulfate (15 cc) was added and the sample contents were mixed thoroughly using a solvent-rinsed stainless steel spatula. Next, magnesium sulfate (15 cc) was added to each sample jar and mixed thoroughly using the spatula. Each sample mixture was transferred to a 33-ml ASE extraction cell and the OC surrogate standard (1,2,3,4-TCDD, 10 ng/μl) was added to the top of each sample cell. The OCs and lipids were sequentially extracted at 2000 psi and 100°C with two cell volumes using

dichloromethane and the combined extract (~ 50 ml) was collected in a 60-ml collection tube. The extract was thoroughly mixed using a Vortex mixer. Prior to cleanup, a 1-ml aliquot of each sample extract was transferred to a 2-ml GC vial for lipid analysis by thin layer chromatography/flame ionization detection (TLC/FID) (see Chapter 12).[21] The remaining volume of each HPLC/PDA extract was then transferred to a 50-ml concentrator tube and reduced to <1 ml by evaporation with heat.

Sample extracts were cleaned up on gravity flow cleanup columns described by Krahn *et al*.[19] Briefly, each cleanup column was prepared with the following media (listed in order of addition to column): a glass wool plug, 0.4 g neutral silica, 0.5 g KOH-impregnated silica (basic silica),[22] and 2.5 g of 40% (w/w) sulfuric acid-impregnated silica gel. Because preparation of the basic silica gel is time consuming due to extensive glassware setup, it was advantageous to prepare several large batches (approximately 500 g each) at one time and store them in an autodesiccator until needed. Conversely, the acidic silica gel was prepared just prior to use by weighing 40 g of activated silica gel in a tared pre-cleaned 4-oz glass jar and then adding 14.5 ml of sulfuric acid. The jar was shaken by hand for 30 min to ensure that the silica gel was impregnated evenly with the sulfuric acid. The cleanup columns were washed with 20 ml of hexane/dichloromethane (1:1, v/v), and the total sample extract (< 1 ml) was added to the top of each column using a 9 in. glass pipette. The volume of sample extract added to the cleanup column must be <1 ml in order for the surrogate standard (1,2,3,4-TCDD) to be eluted properly. To remove any remaining OCs in the concentrator tube, each tube was rinsed with 1 ml dichloromethane and the rinsate was immediately added to the top of the column. After the sample extract was completely loaded onto the column, the OCs were eluted with 22 ml of hexane/dichloromethane (1:1, v/v) and were collected in a clean, pre-weighed 50-ml concentrator tube. The HPLC internal standard (1,7,8-TriCDD; 10 ng/μl) was then added to each tube and mixed thoroughly. Each sample tube was capped, weighed and the weights recorded. For selected samples, an 8-ml aliquot of the sample extract was transferred to 10-ml tapered glass tube to analyze for a larger suite of OCs by GC/MS. The 50-ml concentrator tube was capped and reweighed. Copper was activated by covering it with concentrated hydrochloric acid, stirring the mixture with a glass rod, and allowing it to stand for 5 min. The copper was then sequentially washed three times each with methanol and dichloromethane and was covered with dichloromethane until use. Approximately 0.50 g activated copper was added to each of the sample tubes to remove any elemental sulfur introduced by the sulfuric acid silica gel and the extract was concentrated to ~1 ml by evaporation with heat. The sample extract was transferred to a GC vial and the solvent volume was further reduced to 100–150 μl, depending on expected OC concentrations, under a stream of ultra-pure nitrogen gas. Using a 9-in. glass pipette, the sample extract was transferred to a 250-μl insert in a 4-ml amber vial.

OC analyses by HPLC/PDA

A detailed description of the HPLC/PDA method can be found in Reference 19. Briefly, the dioxin-like congeners (PCBs 77, 105, 118, 126, 156, 157, 169, 189) were resolved from other selected PCBs (PCBs 110, 128, 138, 153, 170/194, 180, 190, 200) and pesticides (*o,p′*-DDD, *p,p′*-DDD, *p,p′*-DDE, *o,p′*-DDT, *p,p′*-DDT, HCB) by HPLC on two Cosmosil PYE analytical columns, connected in series and cooled to 18°C using a column chiller. The flow rate of the eluent was 0.94 ml/min throughout the analysis. A 50-μl portion of each

sample extract was injected onto the PYE columns. For optimum separation of PCB congeners on the HPLC system, we have found that the column temperature and HPLC flow must be adjusted for each new set of PYE columns used.

The OCs were measured by an ultraviolet (UV) PDA detector using the following parameters: start wavelength, 200 nm; end wavelength, 310 nm; spectra/s, 2.0; resolution, 4.8 nm; as described previously in Reference 19. The OCs were identified by comparing their UV spectra (200–310 nm) and retention times to those of reference standards in a library. The analyte purity was confirmed by comparing spectra within a peak to the apex spectrum (see Reference 19 for description). The PDA measurements were performed by integrating peaks recorded at 220 nm (wavelength that is near the maximum UV absorbance for most PCB congeners) for all OCs except *p*,*p*′-DDE. For this analyte, the peak at 266 nm was integrated. Although the total run time for each sample analysis was 55 min, data were collected for only 40 min to minimize the size of the data files.

OC analysis by GC/MS

Each 8-ml aliquot of sample extract was reduced in volume to approximately 250 μl under a stream of ultra-pure nitrogen gas. The extract was then quantitatively transferred to a 1-ml GC vial containing a 450-μl glass insert to which 15 μl of GC internal standard (PCB 103, 2 ng/μl in isooctane) was previously added. The 10-ml tapered tube was rinsed with an additional 150 μl dichloromethane and the rinsate added to the insert and mixed thoroughly. The combined extract (~415 μl) was mixed thoroughly and exchanged into isooctane by first reducing its volume to approximately 100 μl under nitrogen gas and then adding 120 μl of isooctane to the sample insert. The sample extract was then reduced to a final volume of 100 μl.

The analytes of interest were separated on a 60-m DB-5 capillary column (250 μm film thickness) and analyzed by GC/MS (model Agilent 5972/3) operated in the electron impact (EI) single ion monitoring (SIM) mode (see the section ''GC/MS for Quantitating CHs'' in Chapter 35, for a comprehensive description of the GC/MS method).[20] The instrument was calibrated using a series of 11 multi-level calibrations standard solutions containing known amounts of 69 different OC compounds (PCBs, pesticides) from which response factors relative to PCB 103 were computed. The relative recovery of the surrogate standard, 1,2,3,4-TCDD was then computed for each sample and used to correct computed sample extract concentration results for losses incurred during sample processing.

Quality assurance

Each extraction sample set consisted of 11–14 field samples and various quality assurance samples. To determine if the extraction reagents, glassware, or other supplies were free of OC contaminants, a method blank was analyzed with every sample set. To evaluate the precision of the method, approximately 10% of the tissue samples were analyzed in duplicate. A NIST SRM (e.g., SRM 1945, SRM 1946, SRM 1974b) was analyzed with each sample set to measure the accuracy of our method set and to show that the laboratory quality assurance criteria were met for all analytes detected in the tissue samples.[23]

Results and discussion

In the mid-1990s, we developed a HPLC/PDA method[19] to rapidly measure concentrations of dioxin-like PCBs and other selected OCs in tissues of marine organisms for various studies.[8,24–28] With this older HPLC/PDA method, OCs were extracted from various marine tissue samples using a rather cumbersome and time-consuming technique — homogenization with sodium sulfate using a Tissuemizer and extraction with pentane/hexane (50:50, v/v). Recently, an ASE was set up in our laboratory, and OCs were extracted with dichloromethane from a wide range of marine tissues. Similar to the old method, the sample extracts obtained by the new method (ASE with dichloromethane) were then cleaned up using gravity flow acidic silica cleanup columns and were analyzed rapidly by HPLC/PDA for dioxin-like PCBs and other selected OCs.[19]

Replicate samples of three NIST SRMs were analyzed by HPLC/PDA, and the OC results were compared to NIST certified or recommended concentrations of these analytes, as well as to those measured by a GC/MS method, in which samples were extracted similarly by ASE with dichloromethane but were cleaned up using HPLC/size exclusion chromatography.[20] In general, the OC data (based on wet weight) obtained by our HPLC/PDA were in good agreement with the NIST concentrations, as well as the GC/MS results (Table 25.1). However, some PCB congeners coeluted on the HPLC system (e.g., PCB 170 coeluted with PCB 194; PCB 87 coeluted with PCB 153). In addition, we found coeluting PCBs present at the retention times for PCBs 118 and 128 in certain SRMs (SRM 1945 and SRM 1946). As a result of these interferences, the concentrations of these analytes measured by HPLC/PDA were higher than those measured by GC/MS.[19] For the blue mussel homogenate (SRM 1974b), neither *p,p′*-DDE nor *o,p′*-DDT could be quantitated by HPLC/PDA due to coelution of these pesticides with non-planar PCBs. Therefore, the ΣDDTs determined for SRM 1974b by HPLC/PDA did not include these values and, consequently, led to underestimated values compared to those determined by GC/MS (see Table 25.1).

We compared the HPLC/PDA-measured concentrations of various OCs in all three NIST SRMs using the two extraction methods, specifically homogenization with Tissuemizers (old method) or with ASE using dichloromethane (new method). The OC results are presented in Figure 25.1. In general, comparable levels of OCs were measured by HPLC/PDA in these homogenate samples extracted by either extraction method, with the largest differences occurring for the more polar DDTs. From a more practical viewpoint, the main difference between these two methods was the amount of time needed to extract a sample set. The old method typically required 12 h to prepare the Tissuemizer probes and extract samples, whereas only 8 h is usually needed to extract a set of 14 samples using the new ASE-based method. In addition, the amount of solvent needed to prepare and extract a set of samples was reduced approximately 50% when using the new ASE extraction method compared to the solvent amount used with the old method. Fewer contaminant peaks have been measured in method blanks extracted by ASE with dichloromethane compared to the method blanks extracted using Tissuemizers with pentane/hexane. Furthermore, because the ASE is an automated instrument that can extract a set of samples unattended, other laboratory tasks can concurrently be completed as the samples are being extracted — saving both time and labor costs.

As a test to determine if there were differences in OC values determined by HPLC/PDA and GC/MS, we analyzed the same killer whale sample extracts that had gone through acidic silica cleanup by both quantitation methods. The OC results for tissues collected from three killer whale tissue samples are shown in Table 25.2. Concentrations

Table 25.1 Mean concentrations (± SD) of selected PCBs and other OCs measured in three standard reference materials (SRMs) determined by HPLC/PDA and GC/MS

		NIST SRM 1974b, blue mussel homogenate			NIST SRM 1945, whale blubber homogenate			NIST SRM 1946, fish muscle homogenate		
		HPLC/PDA (n = 6)	GC/MS[a] (n = 15)	NIST (certified)	HPLC/PDA (n = 5)	GC/MS[a] (n = 15)	NIST (certified)	HPLC/PDA (n = 5)	GC/MS[a] (n = 8)	NIST (certified)
Dioxin-like PCBs	77	0.57 ± 0.034	[b]	**0.56 ± 0.023**[c]	[d]	[b]	[e]	0.39 ± 0.027	[b]	0.327 ± 0.025
	105	4.8 ± 0.29	4.3 ± 0.30	4.00 ± 0.18	33 ± 5.2	29 ± 1.8	30.1 ± 2.3	22 ± 1.3	21 ± 1.7	19.9 ± 0.9
	118	12 ± 1.0	11 ± 0.31	10.3 ± 0.4	110 ± 5.5[f]	85 ± 4.0	74.6 ± 5.1	66 ± 1.3[f]	52 ± 3.4	52.1 ± 1.0
	126	[d]	[b]	[e]	[d]	[b]	[e]	0.36 ± 0.038	[b]	0.380 ± 0.017
	156	0.50 ± 0.088	0.80 ± 0.07	0.718 ± 0.080	9.1 ± 0.92	14 ± 1.9	10.3 ± 1.1	7.0 ± 0.38	9.9 ± 0.63	9.52 ± 0.51
	157	[d]	[b]	**0.236 ± 0.024**[c]	[d]	[b]	[e]	2.0 ± 0.31	[b]	[e]
	169	[d]	[b]	[e]	[d]	[b]	[e]	[d]	[b]	0.106 ± 0.014
	189	[d]	[b]	[e]	[d]	[b]	[e]	1.3 ± 0.089	[b]	[e]
Other PCBs	110	9.2 ± 0.10	9.9 ± 0.30	10.0 ± 0.7	37 ± 14	38 ± 3.9	23.3 ± 4.0	32 ± 0.84	25 ± 1.2	22.8 ± 2.0
	128	2.3 ± 0.32	2.1 ± 0.10	1.79 ± 0.12	56 ± 9.6[f]	27 ± 3.5	23.7 ± 1.7	49 ± 2.3[f]	25 ± 1.4	22.8 ± 1.9
	138	8.4 ± 0.28	13 ± 0.42[g]	9.2 ± 1.4	150 ± 16	190 ± 13[g]	131.5 ± 7.4	110 ± 4.5	170 ± 9.1[g]	115 ± 13
	153	17 ± 1.0	15 ± 0.39[h]	12.3 ± 0.8	290 ± 15	250 ± 15[h]	213 ± 19	180 ± 4.5	200 ± 14[h]	170 ± 9
	170/190	[d]	0.20 ± 0.18	0.269 ± 0.034	92 ± 3.8[i]	54 ± 5.1	40.6 ± 2.6	34 ± 0.89[i]	34 ± 2.0	25.2 ± 2.2
	180	0.88 ± 0.14	1.3 ± 0.11	1.17 ± 0.10	140 ± 8.4	160 ± 14	106.7 ± 5.3	74 ± 1.2	81 ± 4.1	74.4 ± 4.0
Pesticides	*o,p′*-DDD	0.70 ± 0.10	1.3 ± 0.39	1.09 ± 0.16	17 ± 2.8	24 ± 3.3	18.1 ± 2.8	[j]	1.1 ± 0.64	2.2 ± 0.25
	p,p′-DDD	2.6 ± 0.17	4.5 ± 0.48	3.34 ± 0.22	120 ± 8.4	150 ± 14	133 ± 10	7.2 ± 0.19	13 ± 1.5	17.7 ± 2.8
	p,p′-DDE	[j]	4.5 ± 0.28	4.15 ± 0.38	500 ± 37	530 ± 40	445 ± 37	290 ± 4.0	380 ± 27	373 ± 48
	o,p′-DDT	[j]	[d]	**0.894 ± 0.057**[c]	83 ± 19	88 ± 6.1	106 ± 14	26 ± 2.6	18 ± 1.4	**22.3 ± 3.2**[c]

Continues

Table 25.1 Continued

	NIST SRM 1974b, blue mussel homogenate			NIST SRM 1945, whale blubber homogenate			NIST SRM 1946, fish muscle homogenate		
	HPLC/PDA ($n = 6$)	GC/MS[a] ($n = 15$)	NIST (certified)	HPLC/PDA ($n = 5$)	GC/MS[a] ($n = 15$)	NIST (certified)	HPLC/PDA ($n = 5$)	GC/MS[a] ($n = 8$)	NIST (certified)
p,p′-DDT	[d]	0.63 ± 0.26	**0.396 ± 0.096**[c]	220 ± 15	270 ± 33	245 ± 15	33 ± 1.8	41 ± 4.7	37.2 ± 3.5
HCB	[d]	[d]	[e]	31 ± 1.6	27 ± 1.3	32.9 ± 1.7	6.3 ± 0.10	7.5 ± 0.28	7.25 ± 0.83
Summed PCBs (ΣPCBs)	120 ± 5.2[k]	160 ± 4.1[l]	**205 ± 42**[c]	$1{,}900 \pm 160$[k]	$1{,}800 \pm 140$[l]	[e]	920 ± 24[k]	930 ± 45[l]	[e]
SummedDDTs (ΣDDTs)	3.3 ± 0.14[m]	11 ± 1.1[n]	[e]	930 ± 58[m]	$1{,}100 \pm 76$[n]	[e]	360 ± 6.7[m]	460 ± 31[n]	[e]

[a] GC/MS method described in Chapter 35.[20]

[b] Cannot be measured.

[c] Values in bold are NIST reference concentrations.

[d] Not detected.

[e] Not reported.

[f] There were coeluting PCBs that affected the quantitation (increased the value).

[g] Known to coelute with PCBs 166, 163, and 164.

[h] Known to coelute with PCB 132.

[i] On HPLC/PDA system, PCBs 170/194 coelute.

[j] Not quantitated because coeluting PCBs were present.

[k] Summed PCBs (ΣPCBs) were calculated by summing the concentrations of 14 reported congeners, then adding the concentrations of the unreported congeners, which are calculated using the response factor of PCB101 when present, of PCB138 if congener 101 is not present, or the average response factor over all congeners if neither PCB is present.

[l] Summed PCBs (ΣPCBs) were calculated by summing the concentrations of 40 reported congeners (PCBs 17, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 82, 87, 95, 99,101/90, 105, 110, 118, 128, 138, 149, 151, 153/132, 156, 158, 170/190, 171, 177, 180, 183, 187, 191, 194, 195, 199, 205, 206, 208, 209).

[m] Summed DDTs (ΣDDTs) were calculated by summing the concentrations of *o,p′*-DDD, *o,p′*-DDT, *p,p′*-DDE (at 266 nm), *p,p′*-DDD, and *p,p′*-DDT.

[n] Summed DDTs (ΣDDTs) were calculated by summing the concentrations of *p,p′*-DDT, *p,p′*-DDE, *p,p′*-DDD, *o,p′*-DDD, *o,p′*-DDE, and *o,p′*-DDT.

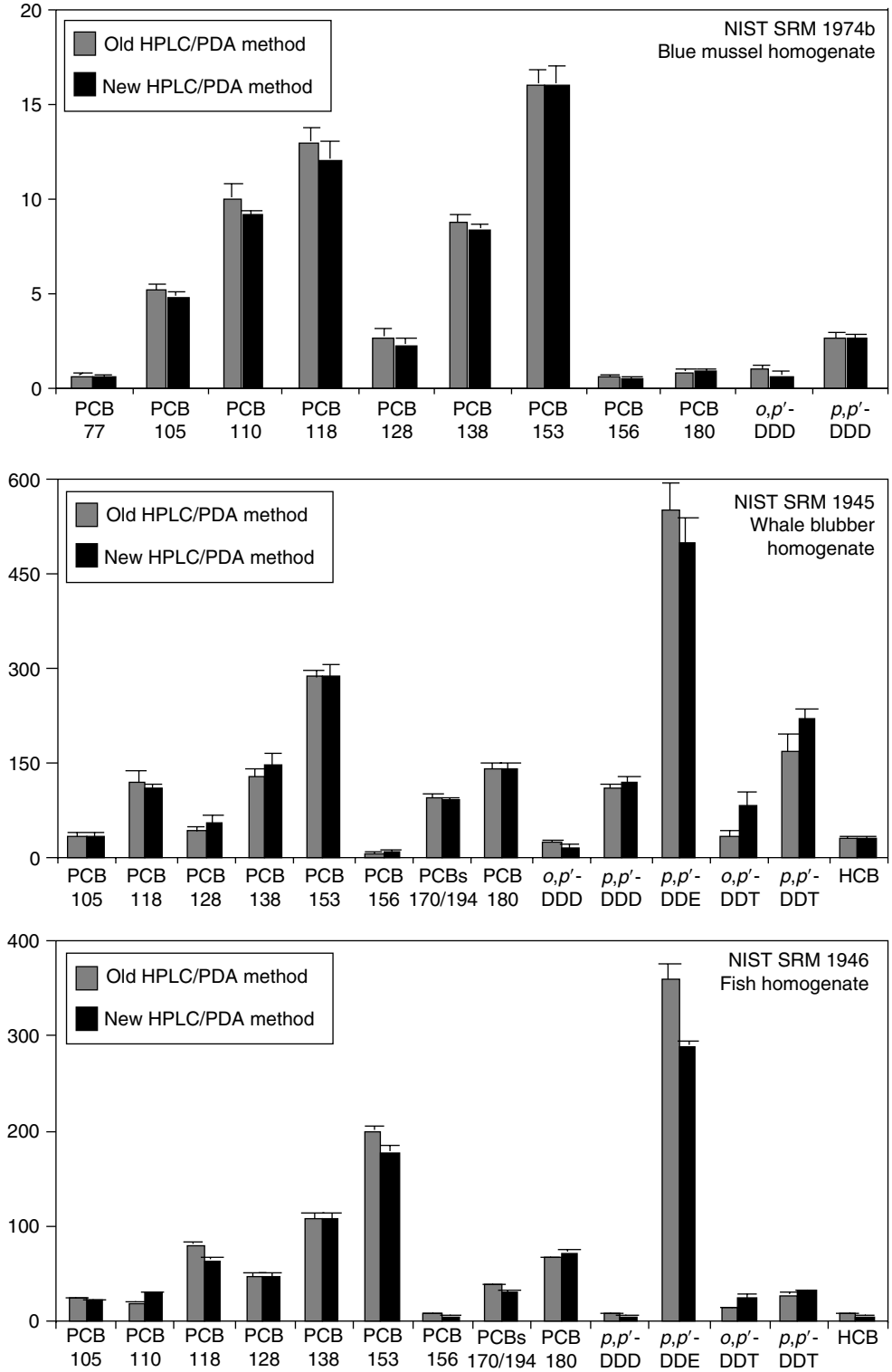

Figure 25.1 OC concentrations for NIST 1974b (a blue mussel homogenate), NIST SRM 1945 (a whale blubber homogenate), and NIST SRM 1946 (a fish muscle homogenate) extracted by two different extraction methods (old method: homogenization with pentane/hexane; new method: ASE with dichloromethane).

Table 25.2 Concentrations (ng/g, wet weight) of selected PCBs and other OCs in the same killer whale sample extracts that had gone through acidic silica cleanup and were measured by HPLC/PDA and GC/MS

		Northern resident, juvenile killer whale whole blood ($n = 1$)		Gulf of Alaska transient, female killer whale blubber biopsy ($n = 1$)		Western Alaska, offshore male killer whale blubber biopsy ($n = 1$)	
		HPLC/PDA	GC/MS	HPLC/PDA	GC/MS	HPLC/PDA	GC/MS
Dioxin-like PCBs	77	<0.056	[a]	<1.9	[a]	<1.1	[a]
	105	1.6	1.3	190	190	44	39
	118	7.7[b]	4.0	1,100[b]	870	190	180
	126	<0.053	[a]	<1.7	[a]	<0.98	[a]
	156	<0.036	0.52	26	57	13	22
	157	<0.033	[a]	20	[a]	5.1	[a]
	169	<0.071	[a]	<2.3	[a]	<1.4	[a]
	189	<0.035	[a]	[c]	[a]	<0.75	[a]
Other PCBs	52	[a]	4.5	[a]	720	[a]	53
	99	[a]	6.5	[a]	2,100	[a]	210
	101	[a]	4.7	[a]	500	[a]	240
	110	[a]	0.64	[a]	31	[a]	21
	128	2.6[b]	1.8	600[b]	430	110[b]	69
	138	8.6	12	3,600	4,200	370	530
	149	[a]	5.7	[a]	1,000	[a]	270
	153	16	17	8,100[b]	6,700	760	700
	170/190[d]	1.3	1.4	900	740	72	70
	180	2.4	3.7	2,300	2,000	160	200
	187	[a]	3.0	[a]	1,100	[a]	180
	194	[d]	0.19	[d]	310	[d]	20

Pesticides	*o,p′*-DDD	<0.098	0.48	230	260	26	23
	p,p′-DDD	2.0	1.8	1,300	1,500	110	150
	o,p′-DDE	[a]	0.80	[a]	240	[a]	46
	p,p′-DDE	110	99	32,000	34,000	4,400	4,600
	o,p′-DDT	<0.12	1.8	1,400	1,400	160	280
	p,p′-DDT	<0.14	0.56	310	840	120	200
	HCB	5.0	4.2	340	240	18	16
	β-HCH	[a]	2.4	[a]	1,500	[a]	17
	Mirex	[a]	[c]	[a]	420	[a]	45
	Oxychlordane	[a]	1.6	[a]	1,500	[a]	44
	Summed PCBs (ΣPCBs)	86[e]	82[f]	34,000[e]	25,000[f]	3,400[e]	3,100[f]
	Summed DDTs (ΣDDTs)	110[g]	100[h]	35,000[g]	38,000[h]	4,800[g]	5,300[h]

[a] Cannot be measured.

[b] There were coeluting PCBs that affected the quantitation (increased the value).

[c] Not quantitated because an interfering compound was present.

[d] On HPLC/PDA system, PCBs 170/194 coelute.

[e] Summed PCBs (ΣPCBs) = sum of concentrations of 14 reported congeners, then adding the concentrations of the unreported congeners, which are calculated using the response factor of PCB 101 when present, of PCB 138 if congener 101 is not present, or the average response factor over all congeners if neither PCB is present.

[f] Summed PCBs (ΣPCBs) = sum of concentrations of 40 reported congeners (PCBs 17, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 82, 87, 95, 99, 101/90, 105, 110, 118, 128, 138, 149, 151, 153/132, 156, 158, 170/190, 171, 177, 180, 183, 187, 191, 194, 195, 199, 205, 206, 208, 209).

[g] Summed DDTs (ΣDDTs) = sum of concentrations of *o,p′*-DDD, *o,p′*-DDT, *p,p′*-DDE (at 266 nm), *p,p′*-DDD, and *p,p′*-DDT.

[h] Summed DDTs (ΣDDTs) = sum of concentrations of *p,p′*-DDT, *p,p′*-DDE, *p,p′*-DDD, *o,p′*-DDD, *o,p′*-DDE, and *o,p′*-DDT.

of several OCs measured by HPLC/PDA were, in general, comparable to the GC/MS values. Again, we found that the PCB 128 values determined by HPLC/PDA were higher than those measured by GC/MS and is likely due to coelution with PCB 123 on the Cosmosil PYE column. Conversely, we found that the concentrations of PCB 156 determined by GC/MS in all three killer whale samples were higher than the HPLC/PDA values and is likely due to interference by PCB 171 and/or PCB 202 on the DB-5 GC column. A previous study reported that, although several types of contaminants (e.g., dieldrin, lindane, endosulfan A, endosulfan B, organophosphates) were completely or partially destroyed using acidic silica gel cleanup, many other environmental contaminants (e.g., all PCB congeners, DDTs, HCB) survived this rigorous cleanup technique.[29] Consequently, by analyzing a portion of the HPLC/PDA sample extract using GC/MS, we could successfully acquire OC concentration data for ten additional OC pesticides plus several additional PCB congeners that cannot be measured by the HPLC/PDA method. Because a much larger suite of OCs can be measured in the same sample extracts when combining both the HPLC and GC results, these additional OC data can be used for a wider range of statistical analyses (e.g., principal component analysis).

Dioxin-like PCB congeners and other selected OCs were readily extracted from tissues of marine organisms using the modified HPLC/PDA method. The advantages of the modified method are:

1. the time required to prepare and extract a sample set was reduced by 33%;
2. the amount of solvent used to prepare and extract a set of samples was reduced by approximately 50% compared to the amount needed using the old extraction method;
3. concentrations of dioxin-like PCBs, other PCBs, and selected OCs can be determined on the same sample extract by HPLC/PDA and/or GC/MS.

The only major limitation of the new ASE-based method is the initial cost of the instrument relative to the cost of the Tissuemizer motors and probes.

A performance-based quality assurance program for OC analyses is highly recommended. For that purpose, various marine tissues have been prepared by NIST and other agencies for which there are certified or recommended concentrations of many OCs, including dioxin-like PCB congeners. Analyses of a suitable reference material, method blank and replicate samples provide information on how well the extraction, cleanup, and quantitation methods are operating.

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chapter twenty-six

Estrogenic activity measurement in wastewater using in vitro *and* in vivo *methods*

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Contents

Introduction

Throughout the world wastewater from municipal treatment facilities has been evaluated for the occurrence of estrogenic chemicals. Estrogens have been reported to cause

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widespread reproductive dysfunction in aquatic fish species.[1,2] Several studies have shown physiological changes in male fish exposed to estrogens, including intersex conditions.[3,4] Studies initially in the UK have reported that male fish exposed to sewage effluents had increased concentrations of vitellogenin (VTG), a biomarker for environmental estrogens.[5–7]

To date only compounds binding and/or activating the estrogen receptor have been identified from wastewater.[8,9] Since many compounds may elicit estrogenic activity throughout the modulation of endogenous estrogens within organisms, it is likely *in vitro* receptor-driven assays significantly underestimate the estrogenic activity of environmental samples. To gain a better understanding of total estrogenic activity *in vivo* fish VTG and *in vitro* [yeast estrogen screen (YES)] assays were utilized for detecting estrogenic activity in wastewater from New York city. Through a series of chromatographic separations, isolated fractions of varied polarity were evaluated by *in vitro* and *in vivo* assays. Chromatographic steps included Empore SDB-XC extraction disks, Sep Pac Plus C-18 cartridges, and finally C-18 HPLC. All fractions were tested for estrogenic activity by both *in vivo* and *in vitro* assays, and the most active fraction was finally analyzed for unknown compounds using GC/MS/MS and LC/MS/MS.

Estradiol equivalent concentrations (EEQs) were measured in effluent extracts subjected to both assays utilizing standard curves constructed from exposing animals and cells to 17β-estradiol (E2). Identity and, in some cases, concentrations of estrogenic chemicals were determined with GC/MS/MS and LC/MS/MS analysis.

Material required

Animals:

Sexually mature male Japanese medaka (*Oryzias latipes*) (local supplier or other researcher stock).

Equipment:

- +30°C incubator (Precision, Model 815) with orbital platform shaker
- Cell culture cabinet (Precision Scientific Model 52200047)
- −80°C freezer (Revco Legaci Refrigeration system)
- −20°C freezer (Kenmore)
- +4°C Isotemp Chromatography Refrigerator (Fisher Scientific)
- Vortex (Fisher Genie2)
- Plate Reader (Molecular Device, Umax Kinetic microplate)
- HPLC with UV detector (Shimadzu)
- Fraction Collector (Spectra/Chrom CF-1)
- Microcentrifuge (Fisher Scientific, Micro12)
- Rotary Evaporator (Buchi No 14201, Switzerland)
- PH-meter (Orion, model 550A)
- Analytical balance (Mettler Toledo, AB54-S)
- Autoclave (Amsco Eagle, SV-3033)
- Stirrer (Fisher, Model 120S)
- Pipet Dispenser (Gilson)

(See Tables 26.1 and 26.2 for the details about supplies and reagents.)

Table 26.1 Supplies for *in vitro* and *in vivo* assay-guided fractionation

Item	Source	Catalog #
Sterile plates, 100 × 15 mm	Fisher	08–757–12
Disposable sterile centrifuge tubes, 50 ml	Fisher	05–5398
Disposable sterile centrifuge tubes, 15 ml	Fisher	12–565–286A
HPLC column, 25 cm × 4.6 mm × 4 μm Ultrasphere C-18	Beckman	235333
Drierite absorbent	Fisher	07–578–4A
Vacuum manifold Visiprep™ DL	Supelco	57044
Valve Liners to Visiprep™ DL	Supelco	57059
Glass vials 2 ml	Fisher	03–339
Round bottom flasks, 50 ml	Fisher	10–060–4B
Desiccator	Fisher	08–615B
High performance extraction disks SDB-XC, 47 mm, Empore	Empore[3M]	2240
Assay plate, 96–well, flat bottom	Fisher	12–565–502
Weighing paper	VWR	12578–165
Microcentrifuge tubes, 1.5 ml	Fisher	02–681–320
Disposable inoculation loops/needles	Fisher	13–075–3
5 3/4″ Disposable Pasteur Pipets	Fisher	13–678–20A
Sterile glass pipets, 10 ml	Fisher	13–675–71
Disposable sterile filter system, 1 l	Fisher	09–761–42
Glass Microfibre filters GF/C, 1.2 μm, 55 cm	Whatman	1822055
0.45 μm filter	Millipore	GNWPO4700
Brine shrimp	Local supplier	
ELISA Biosense Japanese medaka kit	Biosense	V01013402–096
Microfiltration Assemblies, 90 mm	Fisher	K953755–0090
Flasks, 1 l	Fisher	K953760–000
Flasks, 125 ml	Fisher	K953710–000
Cylinder, 50 ml	Fisher	08–549–17C
Syringe Hamilton, 100 μl	Fisher	14–824–6
Syringe Hamilton, 250 μl	Fisher	14–824–2
Culture tubes	Fisher	14–961–25
Beakers, 1 l	Fisher	02–539P

Procedures

EEQs were calculated for effluent extracts and fractions subjected to YES and VTG assays using constructed dose–response curves. For the YES assay E2 standards in a concentration range from 10^{-4} to 10^{-14} *M* was incubated with yeast suspension for 5 days as described in the following. Each measurement was made in duplicate and a dose–response curve was constructed as E2 concentration versus optical density (OD). E2 recovery was determined using spiked water. The recovery from the YES assay was 69 ± 8%, and the method detection limit (MDL) was 1 ng/l.

For the VTG assay, five replicates per each E2 concentration (from 1 ng/l to 100 μg/l) were applied to construct a concentration–response curve. The control treatment group received only ethanol carrier. Hepatic VTG was measured using the commercially available ELISA Kit for medaka (Biosense, Norway). EEQs were calculated from the linear portion of dose–response curve as previously described.[10–13]

Table 26.2 Reagents for *in vitro* and *in vivo* assay-guided fractionation

Item	Source	Catalog #
Yeast strain (*Saccharomyces cerevisae*)	Provided by Prof. J. Sumpter of Brunel University (United Kingdom)	
Yeast nitrogen base (YNB) without amino acids and ammonium sulfate	Difco	2084620
Ammonium sulfate	Fisher	A702–500
Peptone	Fisher	BP1420–100
Adenine, min 99%	Sigma	A3159
Dextrose anhydrous	Sigma	G-7021
Casamino acids	Bacto™	2007–04–30
Sucrose	IBI Shelton Scientific	IB 37160
Dihydrogen sodium phosphate	Fisher	424390025
Disodium hydrogen phosphate heptahydrate	Fisher	424395000
Potassium chloride	Merck KGaA	PX1405–5
Magnesium sulfate	Merck KGaA	MX0075–1
2-Mercaptoethanol	Merck KGaA	6010
Sodium dodecyl sulfate, 99% min	Fisher	BP 166–100
Agar granulated	Difco™	214530
Copper (II) sulfate	Fisher	197711000
17β-estradiol (E2)	Sigma	E-8875
O-nitro phenyl B-D-galactopyranoside (ONPG) (store in −20°C)	Sigma	N-1127
Reagent alcohol (ethanol), HPLC grade	Fisher	A995–4
Sodium carbonate	Merck KGaA	SX 0395–1
Methanol, HPLC grade	Fisher	2304/CS
Acetone, HPLC grade	B&J Brand	BJAH0104
Ethyl 3-aminobenzoate methane sulfonic acid salt, 98% (MS222)	Sigma	E1052–50G
Nitrogen gas, 99% pure	Local supplier	

Sample collection

Samples were collected in methanol-rinsed brown glass bottles. One liter sample of wastewater is required for the procedure. Wastewater samples should be maintained at 4°C until filtration within 1–2 days of collection. Portable pumps may be necessary with appropriate tubing length to transport wastewater to the collection bottle.

Wastewater extraction protocol

Removing particles: It is best if water samples are filtered with 1.2 and 0.45 μm filters together within 1–2 days of receiving the samples.

1. Set up a microfiltration apparatus and rinse with methanol. Use one 0.45 μm filter and one 1.2 μm filter to remove the particles from the water.
2. Begin to filter the water through the apparatus by turning on the vacuum. The flow rate does not matter when removing particles. (*Note*: Water containing many particles may clog the filters, causing the water flow to become very slow. Change the filters if needed.)
3. Once all of the water sample has passed through, turn off the vacuum, and disassemble the apparatus. Filters may be thrown away.

Empore disk extraction:

1. Use Empore filter disks to extract estrogens from water samples. (Store Empore filter disks in the desiccator with Drierite absorbents.)
2. Rinse funnel and flask with methanol. Set up microfiltration apparatus as before, using an Empore filter disc. The first vacuum flask will be used as a waste flask to condition the filter disc.
3. Condition the filter disc with 15 ml of acetone. Allow a few drops to pass through the disc, then shut off vacuum and allow the filter to soak for 1 min.
4. After 1 min pass the acetone through by applying the vacuum until the filter disc is dry.
5. Condition the filter with 15 ml of methanol. Allow a few drops to pass through the disc, then shut off the vacuum and allow the disc to soak for 1 min.
6. After 1 min pass the methanol through slowly until the level of methanol is just above the filter disc. It is important that the disc does not dry out. If it does, then repeat the conditioning process.
7. Once the filter disc is conditioned, the flasks must be changed. Carefully pull the stopper out of the waste flask and place it on a clean vacuum flask.
8. Begin to filter the water sample through the apparatus at a rate of 5 ml/min (approximately 1 drop/s). It will take approximately 3 h to filter a 1-l sample. It is important to keep the filter disc wet until all water has passed through.
9. After the water sample has passed though, turn vacuum on and dry filter for 5 min.
10. Remove the filter disc and elute it with methanol (see the following). If it is not possible to elute it right away, store it in a Zip lock bag, and label the bag with the water sample number/name and date of Empore filtration. Store in the -20°C freezer.

Empore disc elution

1. If the Empore filter disc was stored in the freezer, remove it and let it thaw for 15 min.
2. Set up microfiltration apparatus as previously described using a 125-ml vacuum flask.
3. Elute the disc with 2×15 ml methanol at a rate of 1 drop/s. It is important to keep the flow rate constant.
4. After methanol has passed through, allow the filter disc to dry with vacuum for a few seconds.
5. Transfer the methanol extract to a round bottom flask or pear-shaped flask for vaporization with a rotary evaporator.
6. Vaporize the extract with rotary evaporation at 65°C until dry. Reconstitute the dry extract in 300–500 μl ethanol, rinsing the walls of the flask, and transfer to a labeled 2-ml vial.
7. Evaporate the extract to 100 μl in a nitrogen stream. The extract is ready now for the YES assay.

Table 26.3 Recipes for preparing media and buffer (1 l)

Ura-Tryp media	YPS media
6.7 g yeast nitrogen base (YNB) w/o amino acids and ammonium sulfate	1% yeast extract (10 g)
5 g ammonium sulfate	0.5% peptone (5 g)
2 ml adenine sulfate (10 mg/ml)[a]	10% sucrose (100 g)
100 ml dextrose (20%)	16.1 g $Na_2HPO_4 \times 7\,H_2O$ (60 m*M* final)
100 ml casamino acids (5%)	Keep in +4°C refrigerator
800 ml DDI water	
Keep in +4°C refrigerator	*Z-buffer*:
	5.5 g $NaH_2PO_4 \times H_2O$ (40 m*M* final)
Solid media plates:	0.75 g KCl (10 m*M* final)
Add 16 g of agar to 1 l of Ura-Tryp	0.246 g $MgSO_4 \times H_2O$ (1 m*M* final)
Media, autoclave for 20 min, pour on the plates to create 2 mm layer, keep in +4°C refrigerator	2.7 ml B-mercaptoethanol (50 m*M* final)
	Adjust to pH 7.0
	Keep at room temperature
	Filter Sterilize after dissolving

[a] To dissolve adenine sulfate use water bath at 45–50°C.

In vitro *yeast estrogen screen assay*

Day 1

Cell growth on solid media (Table 26.3): Streak yeast cells from previous plate (or glycerol stock, keep yeast stock in −80°C freezer, thaw slowly on ice) on Ura-Tryp media plates and incubate at 30°C for 48 h. (When streaking from the stock, cells require at least 4 cycles of growth on the plates before growing in liquid media.) Single colonies may be observed after 24 h but require 48 h before they can be picked for growth in liquid media.

Day 3

Cell growth in liquid media: Pick one independent colony and inoculate in 50 ml sterile Falcon tube containing 5 ml Ura-Tryp media. Incubate at 30°C in a shaking incubator at 300 rpm for 24 h.

Day 4

Determination of cell density:

1. Vortex the tube containing the yeast.
2. Place 100 μl of the yeast solution on 96-well flat bottom microplate, YPS media in another well on the same plate. Read the plate at 650 nm in a plate reader.
3. Calculate the amount of yeast solution to prepare yeast suspension for YES assay:

$$\text{plate reading} = \text{yeast reading} - \text{media reading}$$

$$\text{special OD(SOD)} = [(\text{plate reading}) + 0.343]/0.3918$$

4. To prepare yeast suspension, use the following equation:

$$\text{volume } (V) \text{ of yeast solution to add (ml)} = 0.057^{*}10 \text{ ml/SOD}$$

Add this amount of yeast solution to $(10 - V)$ ml of YPS media.

5. Vortex, add 100 µl of 1.25 m*M* $CuSO_4$.

Assay

1. Add 100 µl of E2 of different concentration (10^{-4} to 10^{-14} *M*), sample extracts and ethanol to 1.5-ml microcentrifuge tubes.
2. Add 700 µl of yeast suspension to each tube.
3. Incubate at 30°C for 5 days with caps open but covered with sterile paper towel.

Day 10

1. After 5 days, centrifuge tubes for 3 min and aspirate supernatant (under hood) leaving pellet intact.
2. Add 200 µl of Z-buffer to each tube. Mix by gentle vortexing.
3. Prepare assay buffer:
   - 10 mg ONPG dissolve in 9.75 ml of Z-buffer, add 125 µl of B-mercaptoethanol,
   - 250 µl 10% SDS, vortex.
4. Add 400 µl of ONPG solution (assay buffer) to the tubes.
5. Incubate (caps closed) at 30°C until color develops (1 h).
6. Add 250 µl of 1 *M* sodium carbonate to stop the reaction.
7. Centrifuge for 3 min.
8. Withdraw 150 µl from each tube and place on 96-well plate.
9. Measure OD at 405 nm.
10. Subtract blank (ethanol OD from sample OD).
11. Create dose–response curve placing E2 molar concentrations (10^{-4} to 10^{-14} M) on *X*-axis, and corresponding ODs on *Y*-axis.
12. Calculate sample EEQs using constructed dose–response curve by linear extrapolation from linear portion of concentration–response curve ($R^2 = 0.96$).

In vivo *medaka screening bioassay*

Fish cultured as described previously.[12]

1. One-liter glass beakers are filled with 0.8 l water.
2. Add 1 ml of effluent extract at day 1 and day 3 with a complete water renewal at day 3.
3. Spike the control sample with 1 ml of solvent (ethanol).
4. At $t = 0$, add 3 sexually mature male Japanese medaka (*O. latipes*) to each treatment beaker.
5. After day 6, euthanize the animals in 1 g/l MS222 and remove the liver. Analyze for VTG using the Biosense Japanese medaka ELISA Kit following manufacture's instructions.
6. For the E2 concentration–response curve, carry out exposure with five concentrations of E2 (10 ng/l to 100 µg/l) in ethanol. EEQs are calculated as for the YES assay.

Solid phase extraction fractionation

To differentiate estrogenic compounds by their polarity, fractionation with an ethanol/water gradient on a solid phase extraction (SPE) cartridge can be applied.

1. Set up Sep Pac C-18 plus cartridges in Supelco vacuum manifold.
2. Condition cartridges with methanol allowing a few drops of methanol to pass through to waste vial below the column.
3. Close the valve then condition for 1 min. Pass methanol through, but leave a 1-mm layer on top of the cartridge.
4. Transfer the extract onto the cartridge using a disposable glass pipet.
5. Rinse the pipet with a small amount of ethanol and add it into cartridge.
6. Allow the sample to pass through cartridge.
7. Once the sample level is just above the cartridge surface, close the valve and remove the waste vial from beneath the column. Place a clean vial labeled with the sample number beneath the column.
8. Collect 5 elution fractions of 10 ml: 10%, 25%, 50%, 75%, 100% ethanol/water.
9. Elute the C-18 cartridge with the 5 fractions listed above, placing a clean vial beneath for each fraction.
10. Vaporize fractions to dryness using nitrogen gas.
11. Add 100 μl ethanol to reconstitute.
12. Perform YES and medaka assays.

Fractionation by HPLC

Estrogenic compounds can be further separated by reverse phase HPLC with a shallow methanol/water gradient.

1. Inject 200 μl (injection loop 250 μl) of effluent extract/active fraction onto an HPLC system using a C-18 column (25 cm × 4.6 mm × 4 μm) with flow rate of 1 ml/min with a methanol/water gradient. HPLC elution program:
   - methanol 40% 0–3 min,
   - methanol 40–100% 3–30 min.
2. Collect fractions every 3 min.
3. Assess estrogenic activity of fractions with *in vivo* and *in vitro* assays.

Results and discussion

The technique, described in this chapter, was developed to identify causative agents responsible for estrogenic activity in wastewater collected from two New York city sewage treatment plants (STPs). Wastewater samples were collected in September 2002 from two STPs in Long Island, NY: the Red Hook (RH) Treatment Plant, which is a full secondary treatment plant with 60 million gallons per day (MGD) average capacity; and the New Town Creek (NTC) STP (Advanced Primary, 310 MGD average capacity).

Sample preparation was conducted as shown in Figure 26.1.

The *in vitro* EEQ for Red Hook STP effluent was 22.2 ng/l, whereas it was only 2.4 ng/l for the effluent from New Town Creek STP (Figure 26.2). Therefore, Red Hook

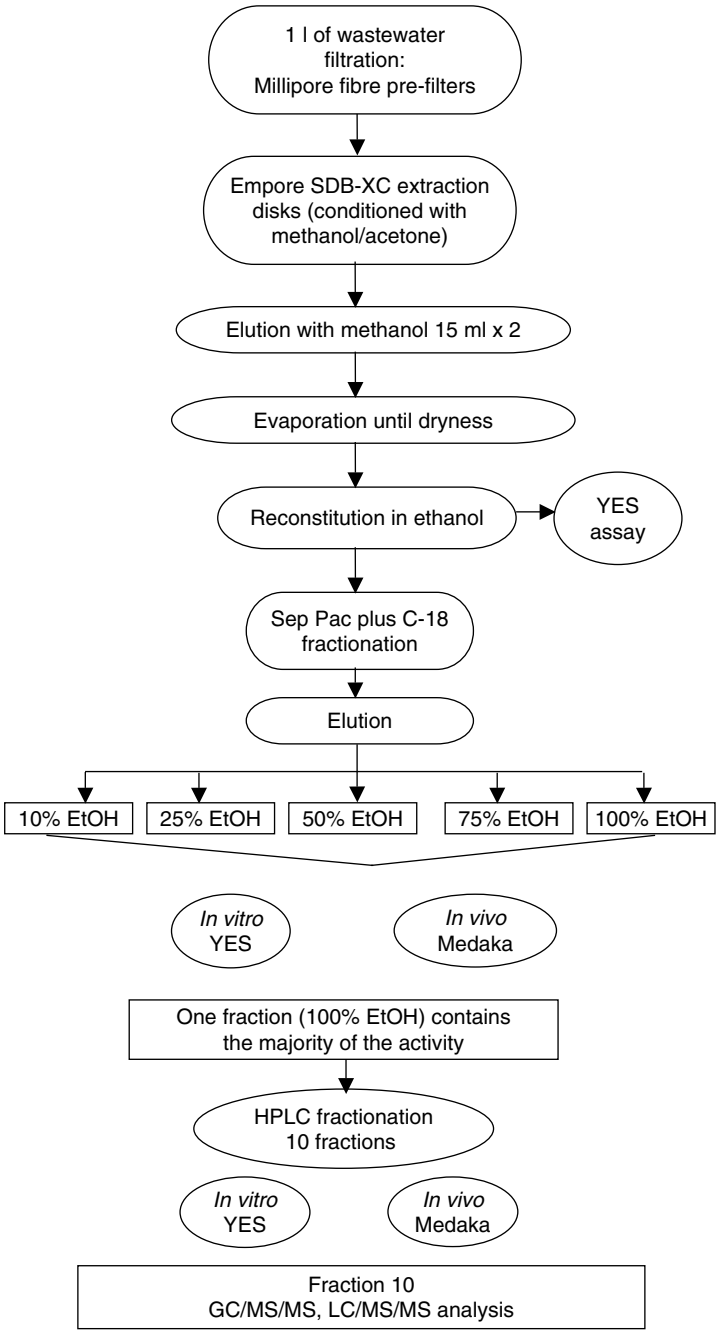

Figure 26.1 Sample preparation scheme.

effluent was used for further study using an ethanol/water fractionation scheme with an SPE C-18 Sep Pac cartridge. All 5 fractions (10%, 25%, 50%, 75%, and 100% ethanol) were assessed for estrogenic activity both by *in vivo* and *in vitro* assays. Both assays showed the highest EEQ in the 100% ethanol fraction (Figure 26.3). The YES assay showed 19.3 ng/l EEQ in the 100% ethanol fraction, but the EEQ measured with the medaka assay was

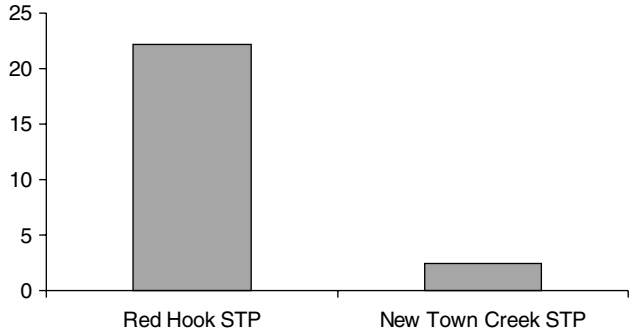

Figure 26.2 EEQs for Red Hook and New Town Creek STPs, ng/l.

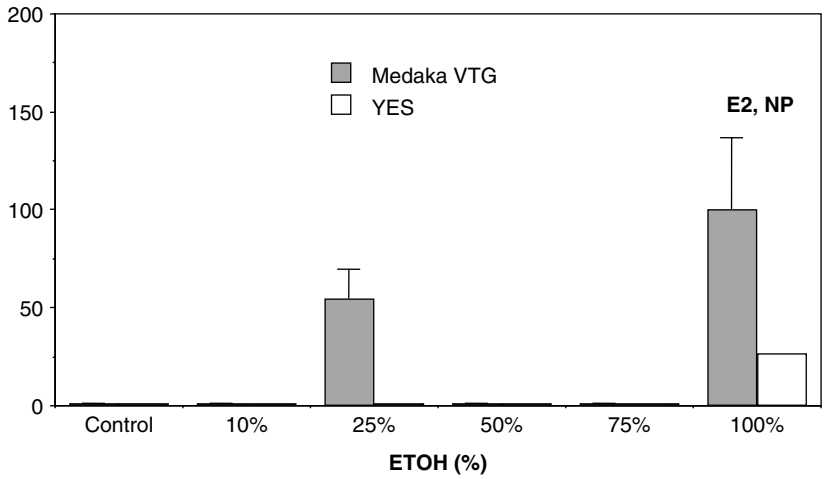

Figure 26.3 EEQs for ethanol fractions from Red Hook effluent, ng/l.

100 ng/l. The *in vivo* assay also detected estrogenic activity of 50 ng/l EEQ in the 25% ethanol fraction, where no YES activity was observed.

With further HPLC fractionation of the 100% ethanol fraction, 10 HPLC fractions of 3-min intervals were collected and measured for estrogenic activity. The YES assay showed 4.3 ng/l in two fractions — 6 and 9 (collected at 15–18 and 24–27 min, respectively) (Figure 26.4). In contrast, *in vivo* assays measured the highest EEQ in fraction 10 (27–30 min) — with 140 ng/l of activity. *In vivo* activity was also observed in fractions 1, 2, and 3 (0–3, 3–6, and 6–9 min, respectively) with activities as high as 80 ng/l EEQ. Fraction 8 (21–24 min) where E2 elutes did not show *in vitro* nor *in vivo* activity. 4-Nonylphenol (0.2 μg/l) was observed in fraction 10.

To identify other estrogenic agents, HPLC fraction 10 was analyzed for unknown compounds using GC/MS/MS and LC/MS/MS as described elsewhere.[14–16] (Table 26.4). Oxybenzone, a chemical used as a sunscreen agent and UV adsorber, and galaxolide used as musk odorants were found at concentrations 19 ng/l and 6 μg/l, respectively. Among other endocrine disruptors, determined in this fraction, triclosan, the antibacterial chemicals, was found in a concentration of 26 ng/l. Triclosan is included in detergents,

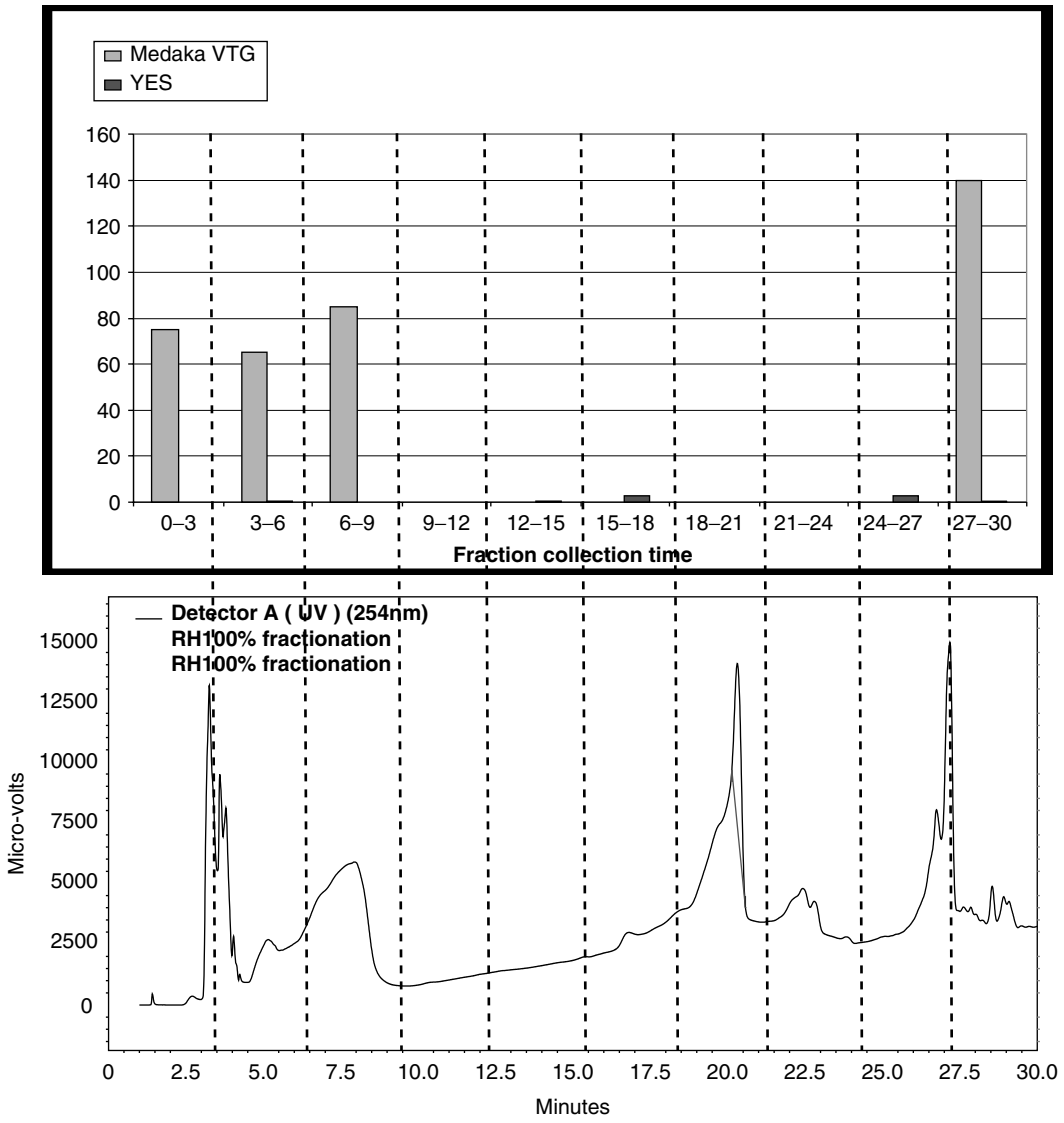

Figure 26.4 *In vitro* and *in vivo* estrogenic activity for 100% ethanol fraction, Red Hook, ng/l.

dish soaps, laundry soaps, deodorants, cosmetics, lotions, creams, and toothpastes and mouthwashes. Oxybenzone and triclosan are shown to be weakly estrogenic.[17,18] Two polyaromatic hydrocarbons (PAHs) pyrene and phenanthrene were detected in concentrations of 5 and 7.5 μg/l, respectively. Other compounds identified but not quantified were: butylated hydroxytoluene (BHT), plasticizers (bisphenol A like), diethyl phthalate, benzophenone, dibutyl phthalate, a TCEP-like fire retardant, triphenylphosphate, formylmethylenetriphenylphosphorane, triphenylphosphine sulfide, nonylphenol isomers, and squalene.

Summary

Wastewater effluents from two New York treatment facilities, Red Hook and New Town Creek, were evaluated for estrogens using *in vitro* and *in vivo* bioassays. EEQs for Red Hook STP showed higher results compared to New Town Creek STP for September 2002. Therefore, the Red Hook effluent extract was further fractionated to identify causative agents. *In vivo* activity was consistently higher than *in vitro*-based calculations indicating the presence of estrogenic agents that were not potent ER-ligands.

Chemical analysis provided with GC/MS/MS and LC/MS/MS showed nonylphenol, oxybenzone, triclosan, galaxolide, phenanthrene, and pyrene in concentrations ranging from 19 ng/l to 7.5 μg/l, and other agents, including plasticizers fire retardant nonylphenol isomers, and squalene.

These results indicate a mixture of estrogenic compounds with varied polarity, which are not potent estrogen receptor ligands within wastewater effluent from a New York treatment facility. Further study is necessary evaluating the amounts of these unknowns and determining the individual contributions of each compounds toward the *in vivo* activity observed in the extract or water sample.

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chapter twenty-seven

A toxicity assessment approach for evaluation of in-situ *bioremediation of PAH contaminated sediments*

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Contents

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a group of organic contaminants known for their prevalence and persistence in petroleum-impacted environment, such as groundwater, soils, and sediments. Many high molecular weight PAHs are suspected carcinogens, and the existence of these PAHs in dredged sediments can result in the classification of the sediment as hazardous waste, for which appropriate treatments may be necessary prior to disposal. Unfortunately, *ex-situ* PAH treatment is not cost effective, and natural attenuation/recovery has uncertainties, including possible resuspension, transport, and distribution over wide areas, that can produce substantial risk to human health and to the ecosystems. The widespread contamination of sediments by PAHs has thus created a need for a cost-effective remediation process for restoration of these sediments, and bioremediation is one method that may reduce the risk of sediment associated PAHs.[1–3]

Bioremediation is complicated by three factors: (1) the need to biodegrade a complex mixture of PAHs with varying ring numbers and molecular weight in aged sediments; (2) PAHs in sorbed and aqueous phase sediment must be made available to the degrading microbial consortia; (3) the need to determine whether the cause of toxicity in PAH contaminated sediments is eliminated by the use of selected bioremediation processes.

In recent years, considerable efforts have been made to characterize the impact of PAH bioavailability and the biodegradation of PAH mixtures on bioremediation process and recent advances in our understanding of the first two factors as they relate to sediment bioremediation have been adequately reviewed.[4] Recent studies on sequestration and bioavailability of PAHs in sediments/soils[5–11] indicate the importance of considering the impact of bioavailability in bioremediation strategies for sediments contaminated with PAHs. Studies on sorption and desorption characteristics of PAHs and other hydrophobic compounds in sediments have also been documented in the literature.[12–16] Aerobic biodegradation has been well documented, and PAH degraders have been isolated from geographically diverse PAH contaminated sediments. Recent studies have shown the degradation of lower-ring PAHs to occur under varied redox conditions and sulfate reducing conditions.[17–24] Most of these studies have focused on individual PAH compounds, which were added to the sediment and degraded by enrichments with pure cultures.

Recently, studies on biodegradability, bioavailability, and toxicity of PAHs in aged contaminated marine and estuarine sediments, on the influence of each of these factors on one another and on their impact on bioremediation[25] have helped to clarify these three important factors that complicate *in-situ* bioremediation of PAH contaminated sediments. These studies emphasized the need for determining the baseline and residual toxicity of these sediments before and after biological treatments and physico-chemical sediment manipulation tests in order to optimize the bioremediation strategy for rendering the PAH contaminated sediment sites significantly risk free to human and ecological health.

Recently, advances have been made in the development of toxicity identification and evaluation (TIE) methods for determining the cause(s) of observed acute and sublethal toxicity in contaminated freshwater, interstitial, and marine sediments.[26–32] The TIE methods selectively manipulate the bioavailability of the potential toxicants in sediments. Comparison of the manipulated samples versus non-manipulated samples (baseline) permits a determination of active toxicant(s). These TIE methods serve as a guide for site remediation and are a part of risk assessment for the contaminated sediment sites. The TIE approach comprises of three phases: phase 1, characterization (what classes of

toxicants are active? i.e., metals, organic toxicants); phase 2, identification (what specific toxicant is active? i.e., copper, PAHs); phase 3, confirmation (validate findings of phases 1 and 2). The manipulations reported in the literature are comprised of aeration (volatile toxicants), filtration, chelation (metals), solid phase extraction (organic toxicants), graduated pH tests (ammonia), oxidant reduction (chlorine), cation exchange resin addition, acid volatile sulfide addition and base metal reaction (metals), coconut charcoal addition and Ambersorb resin addition (organic toxicants), and zeolite addition (ammonia). Whole sediment manipulations include: (1) addition of toxicants (toxicants interact with materials added to sediment which reduces bioavailability); (2) removal of toxicants (toxicants are removed from the sediment, interstitial water and overlying water); (3) alteration of toxicants (toxicant is altered chemically to a form that is not toxic). The marine toxicity tests utilize:

- amphipods (*Ampelisca abdita*) — mortality, tubular dwelling in sediment;
- mysids (*Americamysis bahia*) — mortality, epibenthic;
- bivalves (*Mercenaria mercenaria*) — mortality and growth, infaunal.

The freshwater toxicity tests utilize:

- amphipods (*Hyalella azteca*) — mortality and growth, epibenthic;
- midges (*Chironomas tentants*) — mortality and growth, surface burrower.

In order to develop an effective strategy for bioremediation of contaminated sediments, it is essential to conduct toxicity tests to measure baseline toxicity, to determine the cause of toxicity, and to evaluate the effectiveness of sediment biotreatment methods in reducing the ecotoxicity. The overall objective of the present research effort was to develop a toxicity assessment approach for the evaluation of *in-situ* bioremediation of PAH contaminated sediments. The studies incorporated the use of: (1) varied biodegradation procedures (under aerobic and sulfate reducing conditions) on sediment samples collected from New York/New Jersey Harbor (NY/NJ H) (Figure 27.1) containing trace quantities of PAHs and from the East River (ER), New York, characterized by high PAH concentration levels; (2) toxicity tests to determine baseline toxicity of these sediment samples before any biological treatment; (3) toxicity tests to determine the residual toxicity of the sediment following the biotreatment procedures and the physico-chemical manipulation tests on those sediment samples to determine the cause of toxicity, the extent of detoxification by the above methods and the effectiveness of the developed biotreatment procedures and sediment manipulation tests in reducing the ecotoxicity and

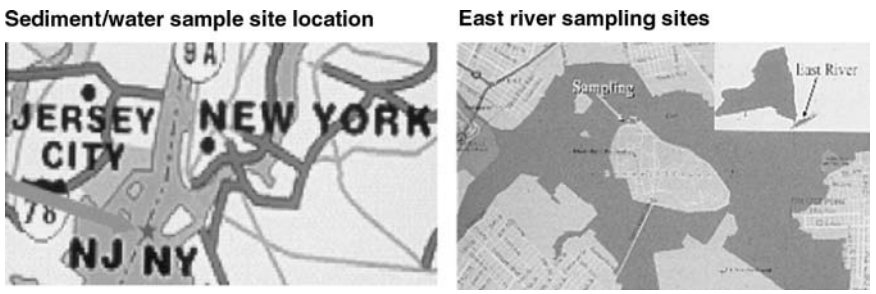

Figure 27.1 Sources of sediment samples.

plant toxicity. The recently published TIE methods served as a background in the development of a toxicity approach for the evaluation of the *in-situ* bioremediation of the PAH contaminated sediments.

The sediment toxicity testing is to provide a measure of biotreatment efficiency based on ecotoxicity values and to relate the reduction of contaminant concentration in sediments to the reduction of ecotoxicity based on biological assay methods. The toxicity methods are to be used to assess how much each biotreatment and sediment manipulation procedure reduces the lethal, sublethal, and bioaccumulative levels of PAH contaminants in sediments.

This chapter emphasizes studies on: (1) biodegradability of PAHs in East River aged sediments under aerobic and sulfate reducing conditions, with and without added co-substrates; (2) toxicity of the untreated and biotreated NY/NJH and East River sediments to aquatic ecosystems and benthic organisms and development of methods to reduce toxicity of untreated sediments. The information gained from the above studies should be useful in developing strategies for the biotreatment of PAH contaminated sediment.

Materials and methods

Characterization of sediment samples

Samples of the sediment from the NY/NJH and from the East River and the respective overlaying water were used in the adsorption/desorption, biodegradation, and toxicity studies. Pebbles, shells, and vegetable matter were removed before storing the sediments and natural water in sealed 20 gal plastic carboys at 4°C. The NY/NJH sediment was air dried in covered shallow pans for 2 weeks, followed by powdering and sieving. The East River sediment was taken directly from the sediment container. Sediment characterization data for the NY/NJH and the East River sediments are presented in Table 27.1. Initial PAH concentration analysis in the NY/NJH and the East River sediments were obtained by extraction with 1:1 methanol/methylene chloride mixture and analyzed by gas chromatography using a HP 5890 Series II GC (Hewlett Packard, Palo Alto, CA) equipped with flame ionization detector, a 7673 automatic sampler, and a PTE-5 fused silica capillary column (J&W. Folsom, CA; 30 m, 0.25 m film thickness). The column temperature started at 100°C for 1 min, increased to 280°C at 4°C/min, then increased to 300°C at 24°C/min, and was held at 300°C for 12 min. The GC/FlD method is similar to the US EPA SW 8100 method for PAH analysis. The 2-fluorobiphenyl was used as the surrogate compound and showed a recovery of 70–80%. The external standard method was used in the analysis. Mass spectroscopic analysis of the chromatography peaks was also performed to confirm the GC results on the individual PAH contaminants. Only trace quantities of PAHs were observed to be present in the NY/NJH sediment, which are shown in Table 27.1. The NY/NJH and East River sediment samples were found to contain 4.18% and 6.0% w/w organic carbon, respectively. Table 27.1 provides characterization of both sediments.

Sources of sediment samples

Figure 27.1 provides the sources of the NY/NJ H and East River sediments used in the toxicity assessment approach. The source of the East River sediment was near the Rikers Island area.

Table 27.1 Sediment characterization

NY/NJH sediment		East river sediment	
Cation exchange capacity	42.9 mEq/100 g	Cation exchange capacity	37.9 mEq/100 g
Organic carbon	4.16% (w/w)	Organic carbon	6.0% (w/w)
pH	7.6	pH	8.3
Total nitrogen	0.279%	Total nitrogen	0.289%
Total sulfur	0.60%	Total sulfur	1.02%
Sulfate	371 ppm	Sulfate	264 ppm
Olsen phosphorus	47 ppm	Olsen phosphorus	15 ppm
Soluble salts	4.25 mmhos/cm	Soluble salts	4.88 mmhos/cm
Metals (ppm)		Metals (ppm)	
Total iron	39,820	Total iron	34,200
Total manganese	894	Total manganese	381
Total copper	86.9	Total copper	321
Total lead	99.1	Total lead	477
Total chromium	59.6	Total chromium	71.3
Total mercury	15.6	Total mercury	11.9
PAHs[c] (ppm)		PAHs (ppm)	
Naphthalene	0.1	Naphthalene (2-ring)	46.7
Phenanthrene	0.2	2-Methyl naphthalene (2-ring)	27.7
Pyrene	0.6	Acenaphthylene (3-ring)	19.9
Benzo(*a*)pyrene	0.2	Acenaphthene (3-ring)	81.1
		Dibenzofuran (3-ring)	5.31
		Fluorene (3-ring)	34.6
		Phenanthrene (3-ring)	190.6
		Anthracene (3-ring)	79.1
		Fluoranthene (4-ring)	97.3
		Pyrene (4-ring)	141.8
		Benzo[*a*]anthracene (4-ring)	70.1
		Chrysene (4-ring)	64.1
		Benzo[*b* + *k*]fluoranthene (5- ring)	67.7
		Benzo[*e*] pyrene (5-ring)	30.9
		Benzo[*a*] pyrene (5-ring)	52.9
		Dibenzo [*a,h*] anthracene (5-ring)	4.4
		Indeno [*1,2,3-c,d*] pyrene (6-ring)	29.8
		Benzo [*g,h,i*] perylene (6-ring)	25.9
Particle size distribution (mm)	Percent	Particle size distribution (mm)	Percent
<2	21	<2	5.0
2–3	7	2–3	1.0
3–5	4	3–5	1.0
5–10	9	5–10	0.5
10–20	8	10–20	1.5
20–45	29.7	20–45	54.2
45–106	15.7	45–106	9.4
106–180	1.8	106–180	10.0
180–250	0.7	180–250	5.7
250–500	1.9	250–500	6.1
0.5–1.00	0.9	0.5–1.00	5.5
>1.00	0.3	>1.00	0.1

Aerobic biodegradation slurry system studies

Experiments were conducted to determine if supplemental nutrients (NH_4Cl, K_2HPO_4) were needed for enhancement of biodegradation of PAHs in East River sediment. In these studies, 963 mg/l NH_4Cl and 280.6 mg/l K_2HPO_4 were added to a set of serum bottles resulting in an N/P ratio of 5:1. A second set of serum bottles were set up as controls without those nutrient supplements. A series of experiments were also conducted to assess possible enhancement of PAH biodegradation by surfactants and co-substrates. Either 0.12 mg/l Triton X-100, 36.4 mg/l ethanol or 48.1 mg/l salicylic acid were added to three sets of serum bottles in these studies, to evaluate their ability to stimulate biodegradative activity of the indigenous microbiota. The 10-g samples of East River sediment and 50 ml of natural overlaying water collected from the same site were added to 100-ml capacity glass serum bottles. The system was buffered by the addition of crushed limestone (2 g) to the liquid. The crushed limestone shells were added to provide pH buffer against the production of sulfuric acid resulting from the oxidation of hydrogen sulfide present in East River sediment. The headspace in the bottles contained an atmosphere of ~75% O_2/~25% N_2 to provide adequate oxygen (DO) in the liquid phase for the duration of the study. The serum bottles sealed with Teflon-faced butyl stoppers and aluminum crimp-seals were placed on the rotating tumblers. In control set of serum bottles, a solution of sodium azide and sodium molybdate (20 m*M* Na_2MoO_4 and 1 mg/l NaN_3) was added to prevent biological activity and growth of microbiota. Samples were analyzed for the 19 contaminant PAHs, pH, and DO at the following time periods: 0, 1, 2, 4, 8, 17, and 24 weeks. Triplicate samples were sacrificed at each sampling event and subjected to analysis. The dissolved oxygen and the oxygen content in the headspace were checked regularly to confirm the availability of oxygen in the aerobic setups throughout the experimental run.

Sulfate reducing conditions slurry system studies

The NY/NJH and East River sediments were shown to have significant concentration of sulfate and hydrogen sulfide, and microbial analyses of the samples illustrated presence and significant activity of the sulfate reducing bacteria (SRB). A series of experiments were conducted to assess the biodegradation of PAHs in East River sediments under sulfate reducing conditions with and without the addition of supplemental nutrients. Studies were undertaken to determine the effect of added organic nutrients and co-substrates (volatile fatty acids, alcohols) and metals (Fe) as electron donors on enhancing the rate of biodegradation of PAHs in sediments slurry systems under sulfate reducing conditions. Experiments reported in this chapter are those without the use of additives and those with either acetate or ethanol addition to the serum bottle setups. For the sulfate reducing conditions, 30 m*M* Na_2SO_4 was added as the electron acceptor and 0.963 g/l NH_4Cl and 0.4209 g/l K_2HPO_4 were added as inorganic N and P supplements. A BOD_5/N/P ratio of 200:5:1 was established in the sediment slurry systems. The natural sediment overlaying water was purged with nitrogen, and the sediment slurry was prepared in an anaerobic hood (Forma Scientific, Marietta, OH) to obtain a pure N_2 headspace. In these studies with co-substrates, either 230 mg/l acetate or 150 mg/l ethanol were added to the serum bottles. The co-substrates were added again two times during the course of the experiment when the substrate was depleted. DO and oxygen in the headspace were measured at each sampling event, and O_2 free condition prevailed throughout the

experimental runs. Samples were analyzed for the 19 contaminant PAHs, sulfate, sulfides, DO, and CO_2 at the following time periods: 0, 1, 2, 4, 9, 14, 21, and 29 weeks. Triplicate samples were sacrificed at each sampling event and subjected to analysis. The slurry reactor bottles were provided with hydrocarbon traps and traps to collect CO_2 and H_2S gases when there was an indication of significant production of these gases.

In the aerobic and sulfate reducing studies, whole bottles were sacrificed for PAH analysis at each sampling time. Sediment was separated by centrifugation and extracted for 24 h, using a 50-ml mixture of 50% methanol and 50% dichloromethane. The extract was obtained by filtering the mixture through a 0.1-μm pore nylon membrane and then analyzed using a Hewlett Packard 5890 series II GC/FID for 19 contaminant PAH compounds. Sulfate analysis was performed using a Dionex DX 500 Ion Chromatograph. The O_2, N_2, and CO_2 contents in the headspace were measured using a GC/TCD system. Additives, ethanol, and acetate were measured with GC/FID system, while salicylic acid was measured with HPLC system. DO and pH were measured using an Orion model with 720A pH meter and Corning model 90 DO meter, respectively.

Sediment toxicity tests

Sediment toxicity tests were conducted on the NY/NJH and East River sediments to measure the baseline and residual toxicity of the sediment samples. The tests were undertaken to determine how effective were the developed biotreatment strategies on reducing the ecotoxicity of the PAH contaminated sediments and to provide a measure of biotreatment efficiency based on ecotoxicity values. The objective of running the tests was to relate the reduction of the contaminant concentration in sediments to the reduction of ecotoxicity (lethal, sublethal, or bioaccumulative endpoints) based on biological assay methods.

Four freshwater toxicity tests were as follows:

1. Epibenthic amphipod (*H. azteca*) — mortality and growth tests: a standard 10-day USEPA method,[33] using 100 ml sediment and 175 ml overlaying water, and two 7-day exposure methods (the EMAP method, using 50 ml sediment and 160 ml overlaying water[34]; and a modified volume method, developed by us, that uses 17 ml sediment and 30 ml of overlaying water).
2. A 70-day burrowing worm (*Lumbriculus variegatus*) — mortality and budding test.[35–37]
3. A 7–8-day fish embryo larval survival and teratogenic test with *Pimephales promelas* (fathead minnow embryo-larva, FHM-EL): USEPA[38] method that uses 40 ml sediment and 60 ml overlaying water.
4. A 4-day vascular aquatic plant, *L. minor* (duckweed): frond number and chlorophyl (*a*) test[39–41] that uses 15 ml sediment and 2 ml overlaying water.

Two marine toxicity tests were also used: (1) a marine amphipod *A. abdida*, 10-day mortality test[42] that uses 200 ml sediment and 600 ml overlaying water; (2) a sheepshead minnow, *C. variegatus*, embryo-larval (SHM-EL) sediment mortality test.[43]

The overlaying water for the amphipod and FHM-EL freshwater tests was reformulated using moderately hard water,[33,34] and moderately hard reconstituted water was used in the duckweed test. The reduced volume freshwater amphipod test was developed and used in this study, since existing larger volume requirements of

USEPA standard method exceeded the amounts available from the enhanced biotreatment studies.

To determine the cause of toxicity in these sediments, five sediment manipulations were performed:

1. Two sediment purge procedure (a), where 2–4 volumes of laboratory water were replaced over the sediment in a 24-h period and (b) a thin layer purging method.[44]
2. A sediment dilution procedure, where grade 40 silica sand was mixed with PAH contaminated sediments on a weight/weight basis.
3. A sediment aeration procedure, where sediment samples were aerated by adding 80 ml of sediment (140 g) to a 250-ml glass graduated cylinder and 120 ml of overlaying water followed by aeration for 24–48 h.
4. An Ambersorb treatment procedure, where PAH contaminated sediment samples were treated with two types of organics removal resins, Ambersorb 563 (AS 563) and Ambersorb 572 (AS 572).[45,46]
5. An Amberlite treatment procedure, where Amberlite IRC-718, an inorganic (metal) removal resin was mixed with the metal bearing and PAH contaminated sediments.[27]

Standard operating procedures for sediment toxicity tests

The standard operating procedures (SOPs) of the sediment toxicity tests for the amphipod, *H. azteca*, the worm, *L. variegatus*, the embryo-larva, *P. promelas*, and the plant, *L. minor* (duckweed) are provided in Table 27.2. The SOPs provide the test criteria and the corresponding specifications for each of the criteria. Comparisons of SOPs for *H. azteca* sediment toxicity tests using the standard volume/EMAP method/reduced volume methods are outlined in Table 27.3. Differences in the standard and the miniaturized procedures of sediment toxicity tests for the freshwater amphipod *H. azteca* are shown in Table 27.4. Differences in standard and miniaturized procedures the sediment toxicity tests for freshwater FHM-EL survival (FHM-ELS) and the marine SHM-EL survival SHM-ELS are shown in Table 27.5.

Sediment manipulation methods

To determine the cause of toxicity in the sediments, five sediment manipulations were performed as follows.

Sediment purge procedure

Purging of the sediment consisted of two methods: (1) replacing overlying water in the first 24 h with 4–6 volumes; (2) a thin-layer purging method, where 1.5 l of sediment is placed in a 16-in. wide × 12-in. long × 7-in. deep pan, with 15 l of overlying water added. The overlying water was changed every 24 h for 5 days, and a sediment sample collected for pore water unionized ammonia analysis. The pore water was collected by centrifuging the sediment sample at 2000 rpm for 20 min. Unionized ammonia was measured each day.

Table 27.2 Standard Operating procedures for the Sediment Toxicity Tests

Standard operating procedures for amophipad *H. azfeca* and aqualic worm sediment toxicity tests samples		Standard operating procedures for aquatic worm *L. variegates* sediment toxicity tests	
Test criteria	Specifications	Test criteria	Specifications
Test type	Static-reneval	Test type	Static-renewal
Test duration	10 or 7 days	Test duration	7 days
Temperature	23°C + 1°C or 25°C + 1°C	Temperature	25°C ± 1°C
Phelaperiod	16 h light/8 h dark	Photoperiod	16 h light/8 h dark
Test chamber size	400 or 200 ml	Test chamber size	200 ml
Sediment volume	100 or 40 ml	Sediment volume	40 ml
Overlying water volume	175 or 160 ml daily	Overlying water volume	160 ml
Removal of test solution	*H. azleca* — 7-days old	Renewal of test solution	Daily
Age of test organisms	*L variegalus* — adults	Age of test organisms	Adults
	10 or 20 each species	No. organisms/chamber	10 or 20
No. organisms/chamber	40 or 50 each species	No. replicate/conc.	4
No. replicates/conc.	1.5 or 2.0 ml FFAY*	Organisms/conc.	40 or 80
No. organisms/conc.	Reformulated	Feeding 1.5 or 2.0 ml FFAY*	
Feeding	Moderately hand	Overlying water	Reformulated moderately hard reconstituted water**
	Grade 40 milica sand	Control sediment	grade 40 silica sand
Control sediment	Mortality and/or growth	Endpoint mortality	and for Growth
Endpoint	>80% survival in the control	Test acceptability	>80% survival in controls
Test acceptability		* = Digested fish flakes/walfar yeast	
* Digested fish flakes walfar yeast			
** = Hardness = 80 to 100 mg/l 80–100 mgl Alkalinity = 60–80 mg/l			

Standard operating procedures for *P. propreties* and *C. veginatious* embryo-larval sediment toxicity test samples. (FHM-ELS) (SEM-ELS)		Standard operating procedures for duckweed *Lerravia minor* sediment toxicity tests	
Test criteria	Specifications	Test criteria	Specifications
Test type	Static-renewal	Test type	Static-renewal
Test duration	10 days or 7 days	Test duration	10 or 7 days
Temperature	25°C + 1°C	Temperature	25°C + 1°C
Photoperiod	14 h light/10 h dark	Photoperiod	14 h light/10 h dark
Test chamber size	30 ml	Test chamber size	30 ml
Sediment volume	15 ml	Sediment volume	15 ml

Continues

Table 27.2 Continued

Standard operating procedures for amophipad *H. azfeca* and aqualic worm sediment toxicity tests samples		Standard operating procedures for aquatic worm *L. variegates* sediment toxicity tests	
Test criteria	Specifications	Test criteria	Specifications
Overlying water volume	2 ml	Overlying water volume	2 ml
Removal of test solution	nt 48 h	Removal of test solution	nt 48 h
Age of plants	2 frond plants	Age of plants	2 frond plants
No. 2 frond plants chamber	6	No. 2 frond plants chamber	6
No. replicates chambers conc.	4	No. Replicates chambers/conc.	4
No. plants/conc.	48	No. Plants/conc.	48
Feeding	0.1 ml of 3 nutrient stocks	Feeding	0.1 ml of 3 nutrient stocks
Overlaying water	Moderately hard	Overlaying water	Moderately hard
Control sediment	Reconstituted water*	Control sediment	Reconstituted water*
Endpoint	Grade 40 milica sand + Alfalifa	Endpoint	Grade 40 milica sand + alfalifa
Test acceptability	Frond number		
Hardness = 80–100 mg/l			
Alkalinity = 60–80 mg/l	Growth as wet wt	Test acceptability	Frond number
			Growth as wet wt. Chlorophyll-*a*
	Number of control fronda		
		Test acceptability	Frond number
	Doubles controls		Growth as wet wt. Chlorophyll-*a*
		*Hardness = 80–100 mg/l Alkalinity = 60–80 mg/l	Number of control fronds doubles controls

Sediment dilution procedure

Grade 40 silica sand (wetted by mixing 100 ml of sand with 50 ml overlying water) was used as a dilution substrate. Sediment dilutions were made on a weight/weight basis. For example, 1% East River sediment was prepared by weighing out 1 g of sediment and 99 g of control sand and mixing them. A spoon or spatula was used to completely mix the materials and add them to the appropriate test container, after which the overlying water was added. The diluted sediments were then treated as a standard sediment sample.

Table 27.3 Comparisons of SOPs for *H. azteca* sediment toxicity tests using standard volumes/EMAP methods/reduced volumes[a]

Test criteria	Specifications
Test type	Static-renewal
Test duration	10 days or 7 days/7 days/7 days
Temperature	23 ± 1°C/25 ± 1°C
Photoperiod	25 ± 1°C
Test chamber size	16 h light/8 h dark
Sediment volume	400 ml/200 ml/60 ml
Overlying water volume	100 ml/40 ml/17 ml
Removal of test solution	175 ml/160 ml/30 ml
Age of test organisms	Daily
No. organisms/chamber	7-days old, 24 h age range
No. replicates chambers/conc.	10/20/5
No. organisms/conc.	4/4/4
Feeding	40/80/20
Overlaying Water	2 ml algae/alfalfa Reformulated
Control sediment	Moderately hard
Endpoint	Reconstituted water*
Test Acceptability	Grade 40 milica sand

[a] Mortality and/or growth: 80% survival in the controls.

Table 27.4 Differences in Standard and Miniaturized Procedures for *Hyalella azteca*, freshwater amphipod

	Specifications	
Test criteria	Standard test	Miniaturized test
Test temperature	23 ± 1°C	25 ± 1°C
Test chamber size	300 ml	60 ml
Sediment volume	100 ml	17 ml
Overlying water volume	175 ml	30 ml
Organisms/chamber	10	10
Replicates	8	4
Test duration	10 days	7 days

Table 27.5 Differences in standard and miniaturized procedures for freshwater FHM-ELS and marine SHM-ELS sediment toxicity tests

	Specifications	
Test criteria	Standard test (ml)	Miniaturized test (ml)
Test chamber size	125	60
Sediment volume	40	17
Overlying water volume	60	30

Sediment aeration procedure

An aeration procedure was developed, to ''blow-off'' the volatile sulfides and oxidize the remaining sulfides, thus reducing the overall toxicity of these samples. The sediment

samples were aerated by adding 80 ml of sediment (140 g) to a 250-ml glass graduated cylinder and then adding 120 ml of overlying water. An air tube was then inserted into this mixture, and a mild aeration started (~100 bubbles/min). The cylinders were placed into the hood, covered, and allowed to aerate overnight. After aeration, the slurry was removed from the cylinder, placed into centrifuge tubes and centrifuged at 2000 rpm for 20 min. After centrifuging, the excess overlying water was discarded, and the sediment samples were collected for use in the sediment toxicity tests. These aerated sediments were used as the 100% samples or diluted with sand as described above. If desired, the overlying water samples can be saved for aqueous testing with any species.

Ambersorb/Amberlite treatment procedures

East River sediment samples were treated with two types of organic contaminants (PAHs) removal resins, AS 563, and AS 572. The resins were mixed in the sediments at the rate of 4% or 8% AS 563 or AS 572 by weight. Each sample was treated as described above in the procedure for sediment aeration.

Amberlite IRC-718 is an inorganic contaminant (metals) removal resin. Data from other researchers indicate that the use of this type of resin can potentially reduce the toxicity associated with a sediment sample. IRC-718 procedure was the same as the Ambersorb except we used 8% by weight IRC-718 only.

Results and discussion

Aerobic biodegradation slurry system studies

A critical factor in the aerobic PAH biodegradation in East River sediment is the availability of a sufficient supply of oxygen to overcome the initial oxygen depletion due to the high content of sulfide in the sediment. Respirometric experimental data indicated that the largest O_2 uptake rate occurred during the first 24 h, and that the O_2 uptake rate was ~3.5 O_2/g sediment/day. In the slurry systems used in the aerobic studies, 10 g sediment would require ~175 ml air to meet the oxygen demand of the first 24-h period. Based on this observation, an O_2 supply protocol was adopted in the aerobic studies to provide an atmosphere ~75% O_2/25% N_2 in the headspace of the serum bottles to provide adequate DO in the liquid phase for the duration of the study. Others have reported similar effect of sulfides in sediment on BOD.[47]

Biodegradation studies with N/P amendments

In the experiments with and without nutrients (NH_4Cl and K_2HPO_4), with sufficient oxygen present and continuous oxygen resupply to avoid oxygen depletion during the course of the experiment, considerable biodegradation of 2-, 3-, 4-, and 5-ring PAHs was observed in sediment slurry bottle systems. Figure 27.2 shows changes in concentration of the 19 contaminant PAHs present in East River sediment under aerobic conditions without inorganic N and P addition over a period of 24 weeks. DO and oxygen content in the headspace were monitored at each sampling event. The existence of aerobic conditions throughout the experiment was confirmed by a final oxygen content of 14% and a DO above 4 mg/l. During a period of 24 weeks, considerable degradation of 2-, 3-, 4-, and 5-ring PAHs was achieved, while PAH concentration in the abiotic controls remained basically unchanged.

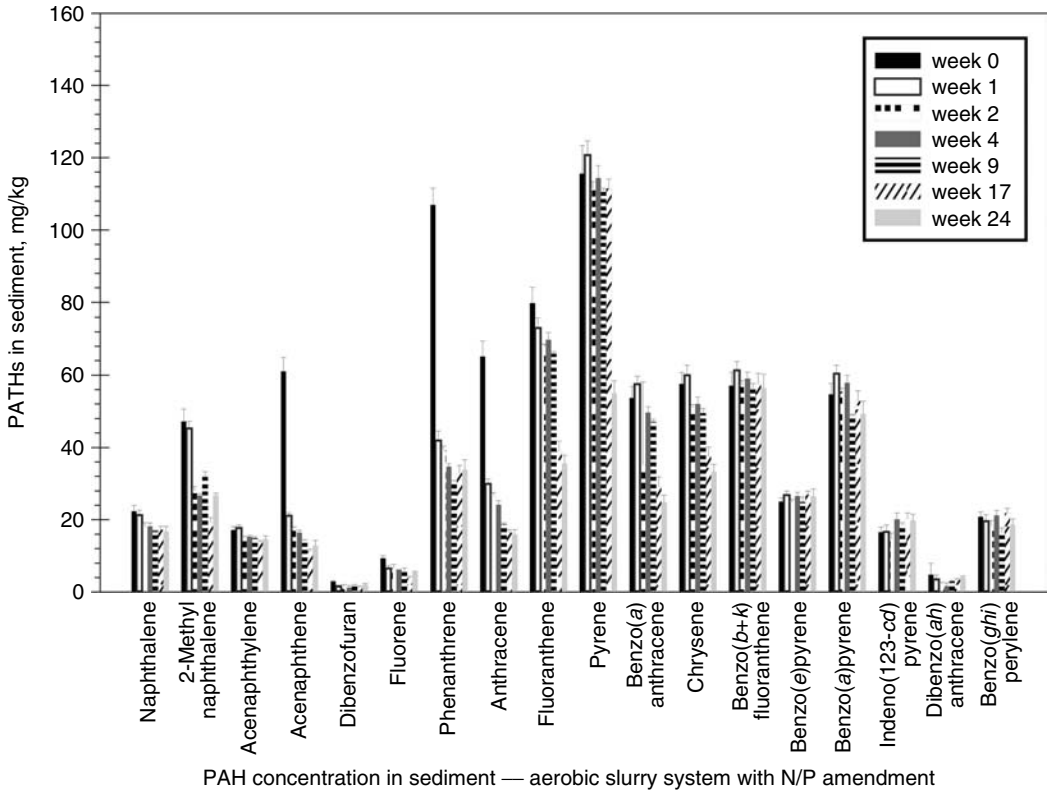

Figure 27.2 PAH concentration in sediment — aerobic, without nutrients.

As seen in Figures 27.2 and 27.3 compounds with lower molecular weight reached lower residual levels in the sediment. The removal of 2-, 3-, and 4-ring compounds was up to 70–80%, 5-ring compounds were removed by just 10–20%, while 6-ring compounds did not exhibit appreciable degradation. The removal of naphthalene, acenaphthylene, dibenzofuran, and fluorene was not as high as that of other lower molecular weight compounds. This may be due to their low initial concentrations in the sediment.

The changes in the concentrations of PAHs over time in the aerobic slurry system with N/P nutrient supplement were very close to those observed in the system without any nutrient addition. No appreciable increase of PAH removal was observed, which suggests that supplying additional N and P to the system did not accelerate biodegradation in the sediment tested. This is probably due to the presence of nitrogen and phosphorus in sufficient quantities in the sediment to support biodegradation.

Biodegradation studies with organic amendments

The experiments with the co-substrates, salicylic acid, and ethanol provided data that showed no considerable improvement of PAHs in the East River sediment over the 27-week incubation period. The addition of the surfactant, Triton X-100 did not appreciably enhanced the biodegradation of those PAHs over the same period. Under all conditions, the residual PAH concentrations were somewhat lower than those observed

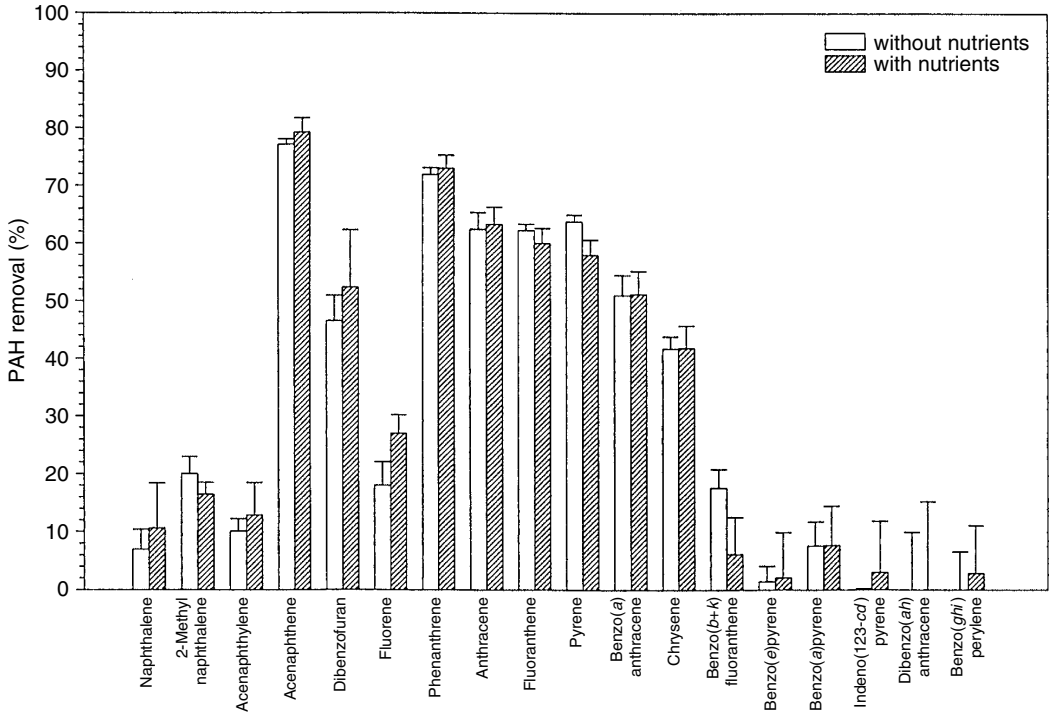

Figure 27.3 PAH Removals — aerobic, without and with nutrients.

in the non-amended systems. Figure 27.4 provides data on biodegradation of anthracene, acenaphthene, fluoranthene, pyrene, and benzo(b + k)fluoranthene in aerobic slurry systems with no additives and with the co-substrates, ethanol, salicylic acid, and surfactant Triton X-100, over a period of 27 weeks. More appreciable losses in PAH concentrations in amended systems were observed, however, after incubation periods longer than 27 weeks as compared to non-amended systems. Aerobic bioslurry biodegradation data for the 19 contaminant PAHs, showing the concentration levels at week 0 and residual concentrations of these PAHs after 54 weeks in milligrams per kilogram of sediment are shown in Tables 27.6–27.9 and in corresponding Figures 27.5–27.8, for systems amended with ethanol, salicylic acid, Triton X-100, and for the non-amended systems, respectively.

Biodegradation studies under sulfate reducing conditions

In the experiments under sulfate reducing conditions, with no co-substrates (ethanol, acetate) added to serum bottles, significant biodegradation of 2- and 3-ring PAHs was observed in sediment slurry bottle systems. In presence of sulfate, PAHs degraded simultaneously with sulfate reduction activity of the SRB, though the extent and rate of biodegradation were much lower then those observed under aerobic conditions. After a lag period of about 2 months, lower-ring PAH compounds up to 3-ring structure exhibited some degree of biodegradation with simultaneous loss of sulfate and significant production of hydrogen sulfide. The generation of biogenic H_2S suggested the activity of SRB in reducing sulfate, as well as SRB population possibly being involved in PAH biodegradation. Figure 27.9 provides a profile of PAH concentrations in sediments at the

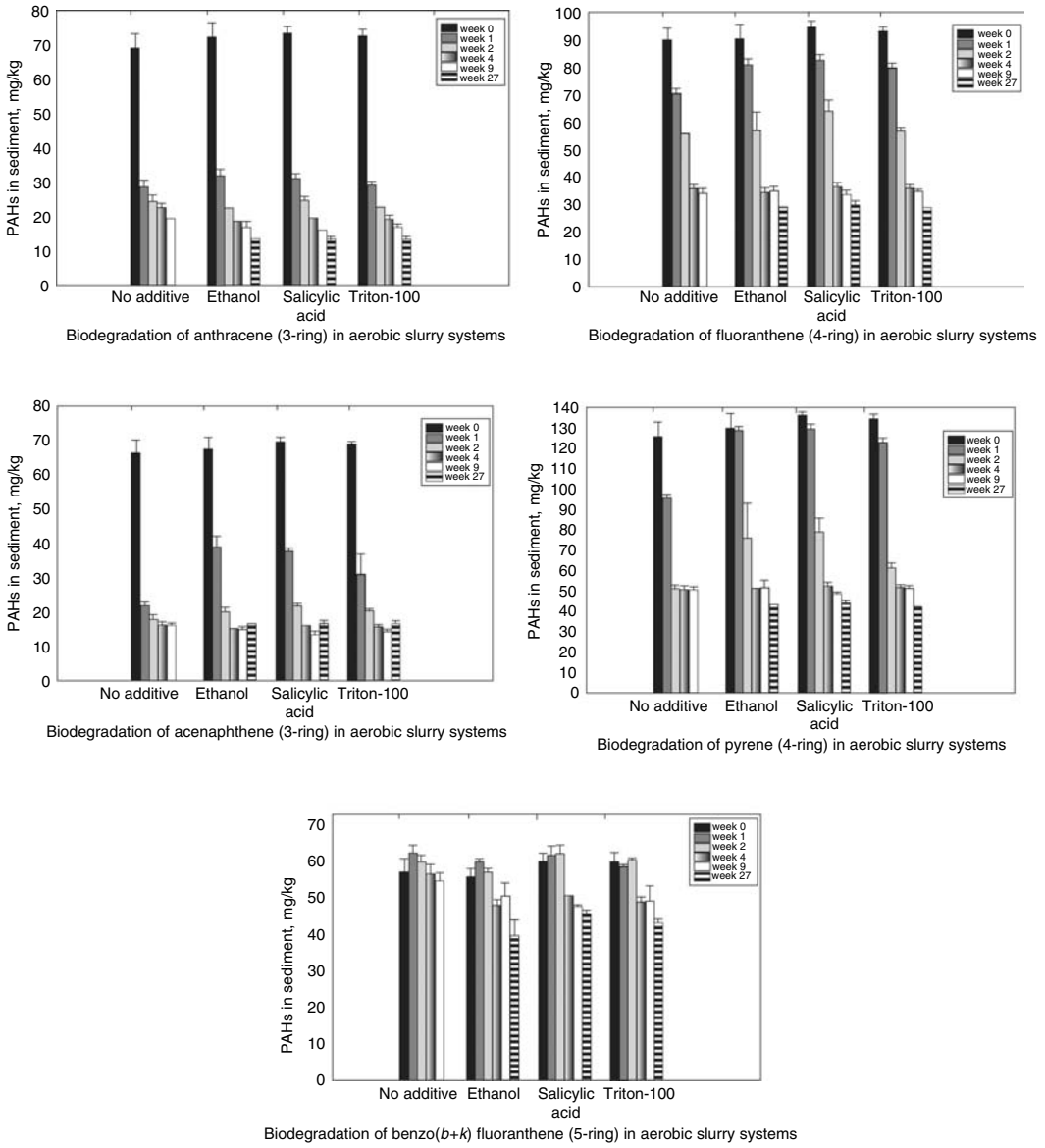

Figure 27.4 Aerobic biodegradation studies — with organic biostimulants.

end of 1, 2, 4, 9, 14, and 19 weeks of incubation. The percentage of removal of PAHs was much lower than under aerobic conditions. PAH levels in control systems with biocides remain the same. Sulfate concentrations were also observed to decrease in the experimental setups. Figure 27.10 provides data on biodegradation of phenanthrene, anthracene, acenaphthene, fluororanthene, pyrene, and benzo($b + k$)fluoranthene in sulfate reducing slurry systems with no additives and with the co-substrates, ethanol, and acetate over a period of 21 weeks.

After a lag phase of 2 months, phenanthrene biodegradation was observed to be significant in the slurry systems without co-substrates added. Phenanthrene decreased significantly from 160 to 95 mg/kg in 4 weeks, while biodegradation rate of other

Table 27.6 Aerobic bioslurry biodegradation data in ethanol amended system 3

Aerobic with ethanol	Week 0 (mg/kg)	Week 54 (mg/kg)
Naphthalene	25.456	22.17022
2-Methylnaphthalene	34.54472	37.3621
Acenaphthylene	17.90362	18.84999
Acenaphthene	67.05873	15.33721
Dibenzofuran	4.677455	2.283759
Fluorene	18.46644	4.03039
Phenanthrene	113.5975	30.37519
Anthracene	71.6221	13.37814
Fluoranthene	89.67024	30.36731
Pyrene	130.4363	43.72649
Benzo(*a*)anthracene	53.55876	22.03127
Chrysene	57.38439	31.89784
Benzo($b + k$)fluoranthene	55.74918	44.86067
Benzo(*e*)pyrene	25.78305	23.93589
Benzo(*a*)pyrene	59.2934	42.12915
Indeno(123-*cd*)pyrene	21.49348	17.74094
Dibenzo(*ah*)anthracene	5.088158	6.159342
Benzo(*ghi*)perylene	19.74418	22.4568

Table 27.7 Aerobic bioslurry biodegration data in salicylic amended systems

Aerobic with salicylic acid	Week 0 (mg/kg)	Week 54 (mg/kg)
Naphthalene	26.79728	21.02046
2-Methylnaphthalene	36.41523	36.7819
Acenaphthylene	18.82613	18.11814
Acenaphthene	69.26625	15.57402
Dibenzofuran	5.280595	1.888199
Fluorene	18.64831	3.451252
Phenanthrene	114.8897	31.08614
Anthracene	73.75823	13.27477
Fluoranthene	94.6796	31.38728
Pyrene	136.7311	44.95758
Benzo(*a*)anthracene	57.06599	22.68479
Chrysene	60.7539	32.67509
Benzo($b + k$)fluoranthene	59.82612	45.31577
Benzo(*e*)pyrene	26.21742	24.69259
Benzo(*a*)pyrene	60.08126	42.67976
Indeno(123-*cd*)pyrene	22.86969	18.06649
Dibenzo(*ah*)anthracene	4.337355	5.807288
Benzo(*ghi*)perylene	21.0528	20.1208

molecular PAHs was lower than that of phenanthrene. Loss of sulfate was accompanied by biodegradation of phenanthrene, while in control systems the phenanthrene degradation and sulfate concentration remained unchanged. The data suggest that phenanthrene degradation can be attributed to the activities of SRB existing in the sediment. In the experiments with added co-substrates, acetate, and ethanol, the extent of biodegradation

Table 27.8 Aerobic bioslurry biodegradation data in triton amended systems

Aerobic with triton	Week 0 (mg/kg)	Week 54 (mg/kg)
Naphthalene	25.45247	21.33433
2-Methylnaphthalene	36.29946	37.1525
Acenaphthylene	18.85257	18.29402
Acenaphthene	68.23156	15.26969
Dibenzofuran	4.367361	1.814585
Fluorene	17.87536	3.218282
Phenanthrene	111.7924	30.3522
Anthracene	72.9447	13.48011
Fluoranthene	93.21046	31.25655
Pyrene	134.7868	43.92004
Benzo(*a*)anthracene	56.92604	22.47102
Chrysene	60.33874	31.37765
Benzo($b + k$)fluoranthene	59.79	44.36316
Benzo(*e*)pyrene	27.33923	23.62958
Benzo(*a*)pyrene	60.81231	41.42859
Indeno(123-*cd*)pyrene	22.42689	18.243
Dibenzo(*ah*)anthracene	4.126818	5.820103
Benzo(*ghi*)perylene	19.80722	21.423

Table 27.9 Aerobic bioslurry biodegradation data in non-amended systems

Aerobic without other treatment	Week 0 (mg/kg)	Week 54 (mg/kg)
Naphthalene	27.6845	23.22133
2-Methylnaphthalene	35.95138	26.12385
Acenaphthylene	15.82853	19.24812
Acenaphthene	67.65496	21.92844
Dibenzofuran	3.441659	1.296103
Fluorene	12.91397	4.411246
Phenanthrene	98.38959	29.9047
Anthracene	68.60064	14.64701
Fluoranthene	90.88615	29.45856
Pyrene	133.031	42.16269
Benzo(*a*)anthracene	54.68672	21.8395
Chrysene	58.96554	28.66355
Benzo($b + k$)fluoranthene	57.423	43.88569
Benzo(*e*)pyrene	29.44014	28.22046
Benzo(*a*)pyrene	54.67897	44.81036
Indeno(123-*cd*)pyrene	24.89002	19.42264
Dibenzo(*ah*)anthracene	4.410595	2.527917
Benzo(*ghi*)perylene	20.3554	22.25528

of 2- and 3-ring PAHs was significantly improved, particularly in the later stages of incubation. In the same studies with the addition of co-substrates the biodegradation of phenanthrene was enhanced up to a level comparable to that under aerobic conditions. In experiments with longer incubation times, when ethanol or acetate was added to the system, phenanthrene biodegraded from 120 mg/kg to as low as 20 mg/kg in a period of 4 months, while no obvious lag phase was observed. Higher rate of biodegradation and

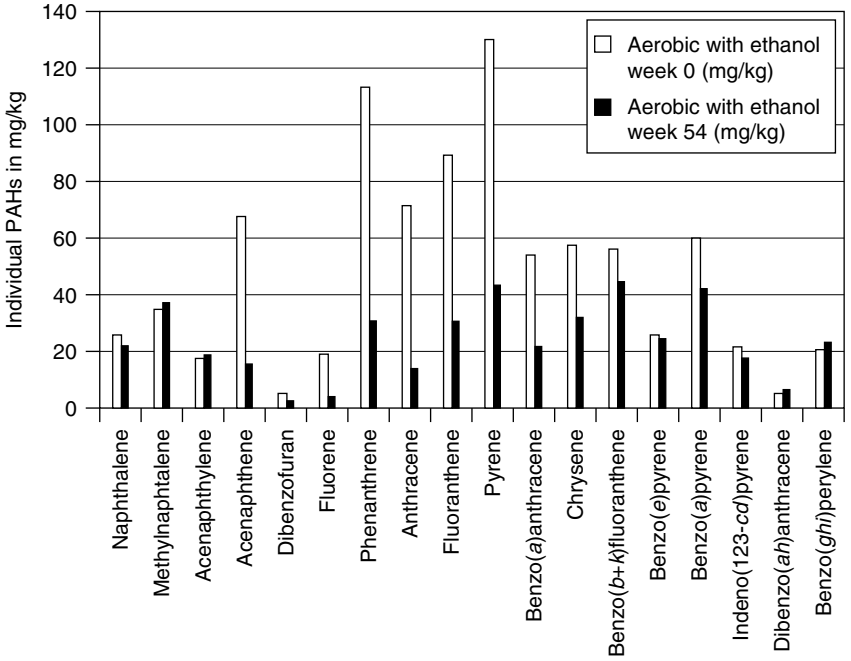

Figure 27.5 Loss of PAHs in aerobic systems amended with ethanol.

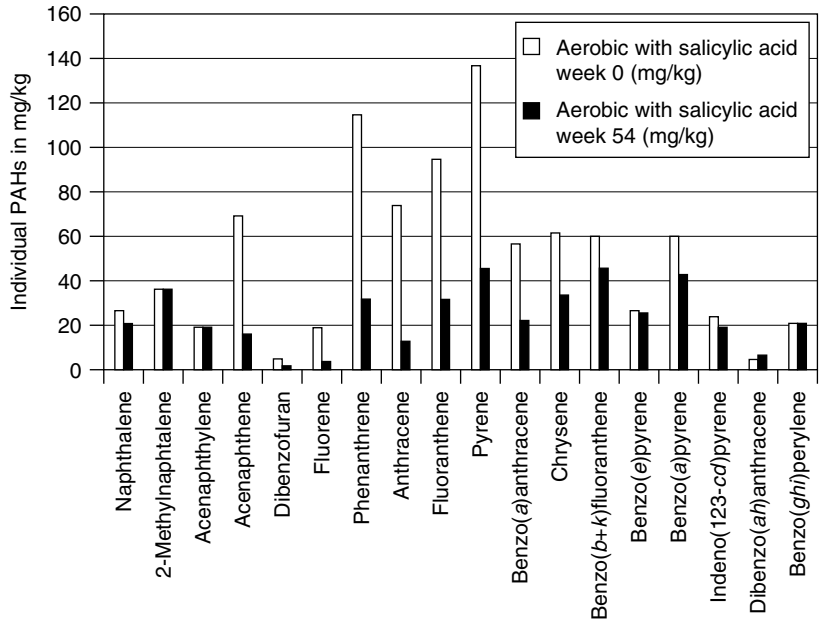

Figure 27.6 Loss of PAHs in aerobic systems amended with salicylic acid.

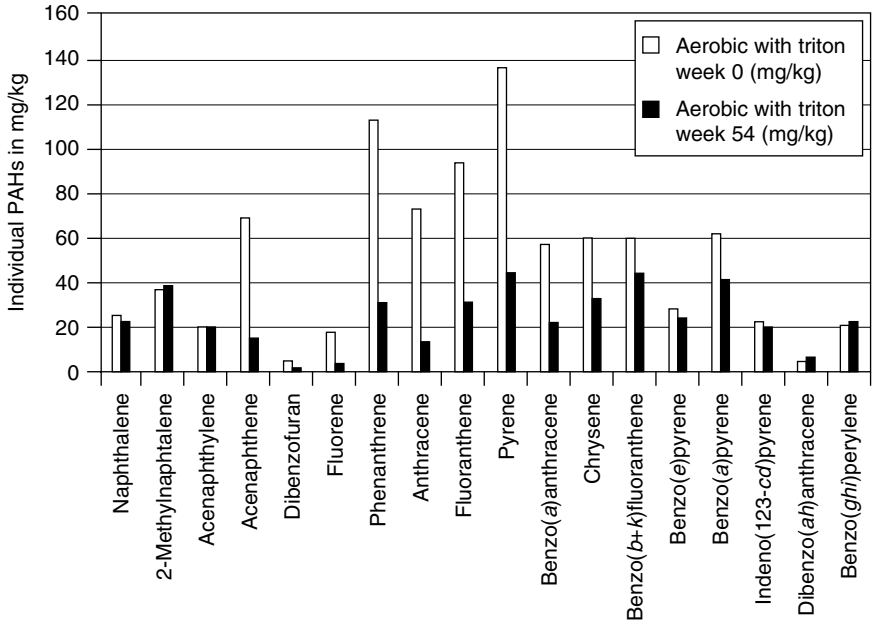

Figure 27.7 Loss of PAHs in aerobic systems amended with triton.

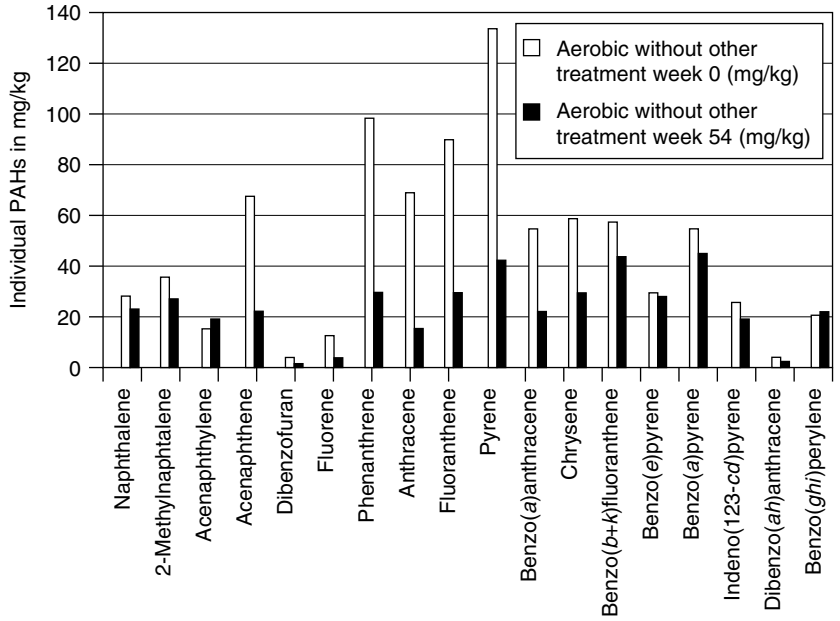

Figure 27.8 Loss of PAHs in non-amended aerobic systems.

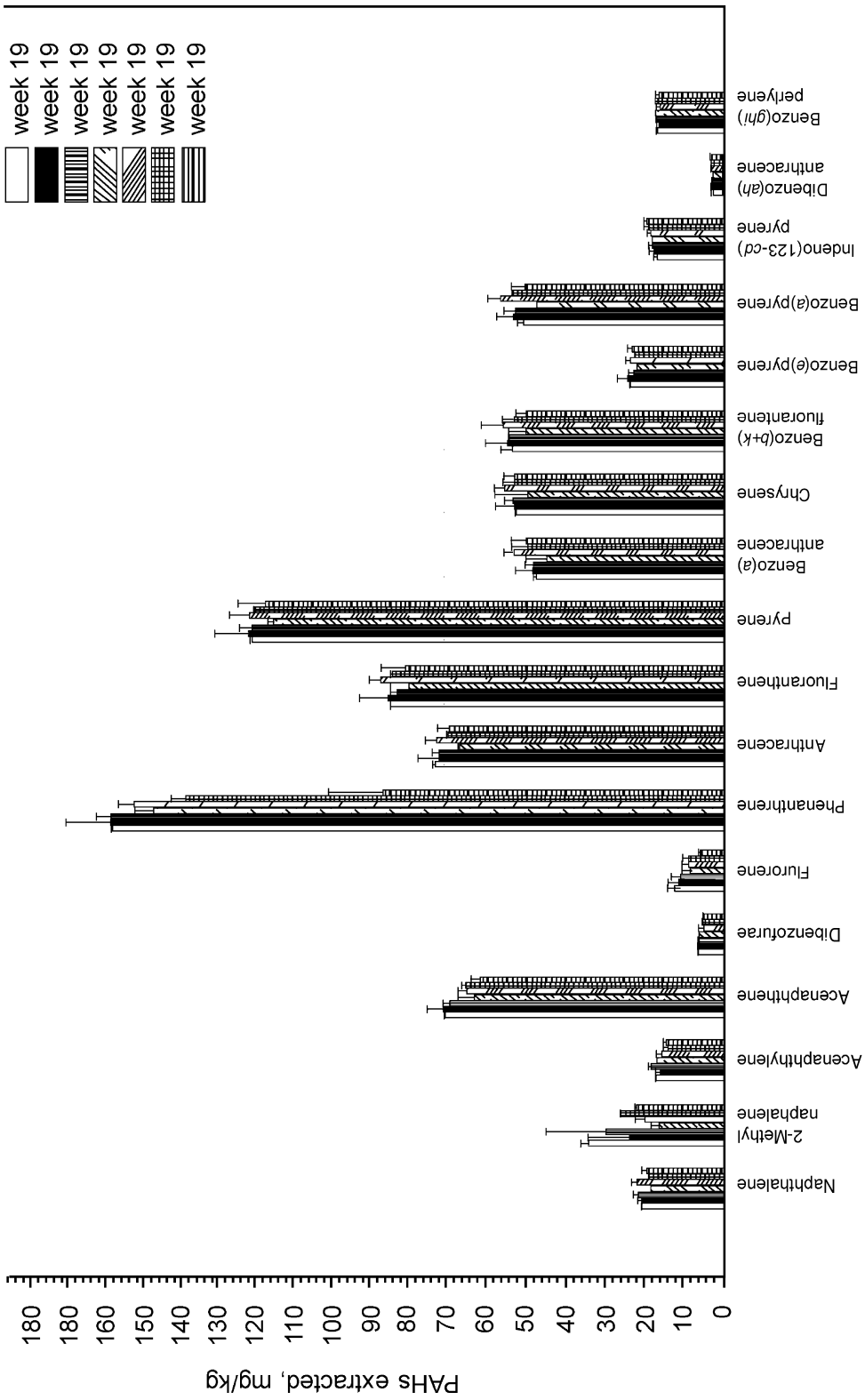

Figure 27.9 PAH concentrations in sediment in sulfate reducing slurry system.

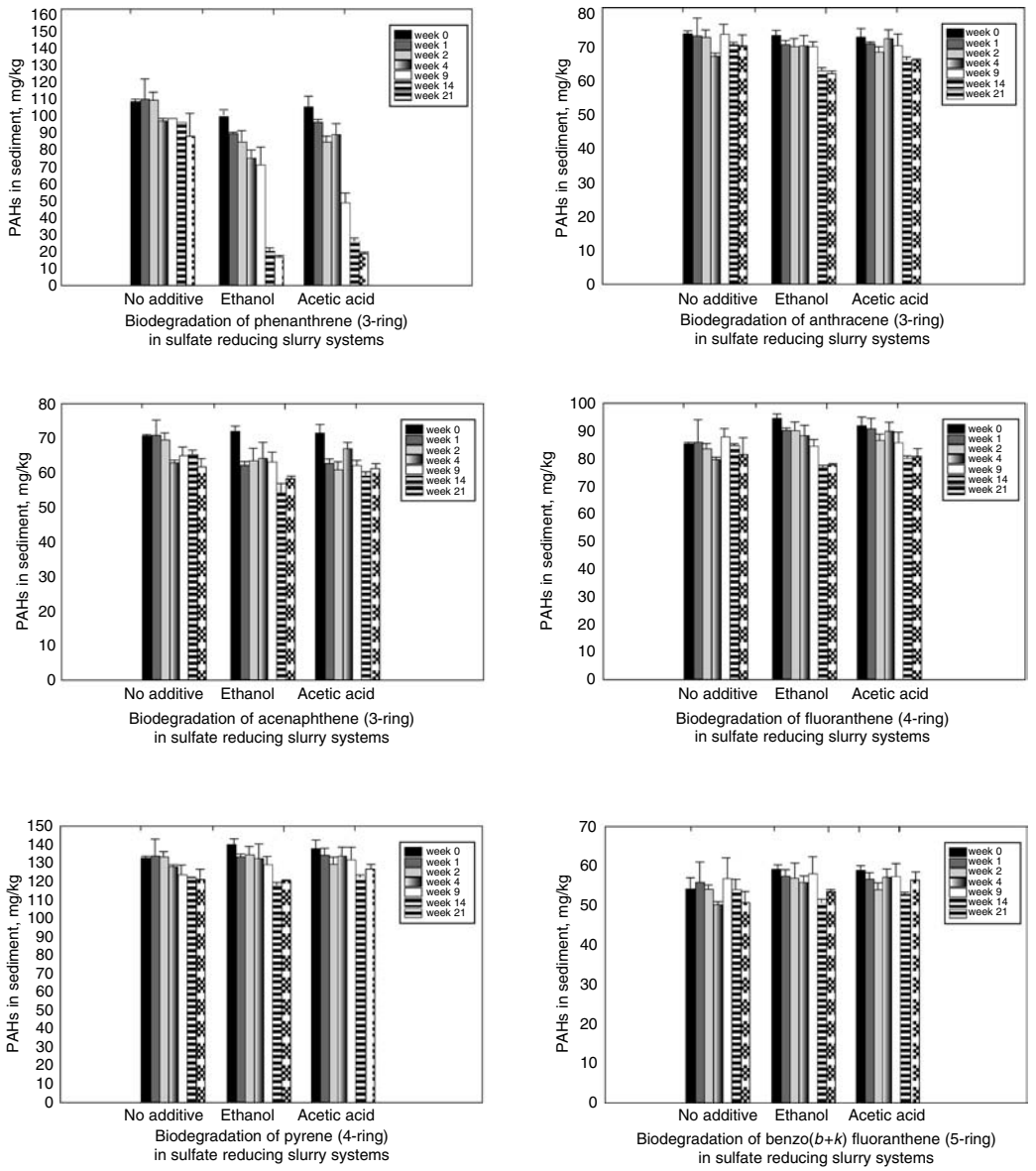

Figure 27.10 Anaerobic biodegradation studies — under sulfate reducing conditions — with organic biostimulants.

lower residuals of phenanthrene were thus achieved in presence of ethanol and acetate. The decrease of sulfate concentration in the systems with co-substrates was greater than that without co-substrates. The enhancement of phenanthrene degradation by ethanol or acetate may be attributed to the amplification of the phenanthrene degrading SRB through the utilization of readily available degradable co-substrates.

Our data on biodegradation of 2- and 3-ring PAHs and East River sediments under sulfate reducing conditions corroborate the findings of others,[21–24] who showed that low molecular weight PAHs can be degraded under these conditions in sediments from heavily contaminated sites.

Sediment toxicity testing

Sediment toxicity tests were conducted with the NY/NJH and East River sediments to measure the baseline and residual toxicity of the sediment samples, determine the source of toxicity, and to reduce the ecotoxicity in these sediments with the use of biotreatment and a series of manipulations, consisting of dilution, purging, aeration, and resin (Ambersorb and Amberlite) treatment procedures.

Table 27.10 summarizes the results of all tests performed on NY/NJH and East River sediments with three freshwater organisms, one freshwater plant, and one marine amphipod. The NY/NJH sediment samples were tested with the freshwater amphipod, *H. azteca*, three times over the course of the study. The marine amphipod, *A. abdita*, when tested with this sediment showed a high level of survival, 95%. Tests performed with the aquatic worm, *L. variegatus*, indicated that this test organism would not survive in the NY/NJH sediment unless the sediment was purged with 2–4 volumes of dilution water. Tests performed with NY/NJH sediment showed significant reduction in survival of the aquatic worms. When sediments were purged with dilution water, no significant reduction in survival was found. FHM-EL tests were performed with NY/NJH sediment and none of the larvae survived. It appears that the aquatic worms and the FHM-EL were very sensitive to salinity and organic and inorganic contaminants in this sediment. The freshwater and marine amphipods gave similar results with respect to survival.

Tests performed with East River sediment (Table 27.10) showed that the sediment was acutely toxic to freshwater and marine organisms, as well as to freshwater plants. Toxicity was found when freshwater aquatic worms and FHM-EL were exposed to NY/NYH sediment in one of three tests performed with the freshwater amphipod. No toxicity was found in the NY/NJH sediments when the marine amphipod was used. This may indicate that the freshwater organisms were more sensitive to the source of toxicity in the sediment. The East River sediment significantly reduced frond (leaves) production (∼58.3%) and chlorophyl *a* concentration (∼35.4%) in the freshwater duckweed test.

In preparation for running toxicity tests on treated sediment samples, a reduced volume method was developed for the freshwater methods. Because bench scale tests for natural attenuation and enhanced biodegradation studies do not contain large amounts of sediment, a toxicity method that uses less volume or weight was needed.

Table 27.10 Survival data results for *H. azteca* (freshwater amphipods 10-day), *L. variegatus* (freshwater worm), FHM-ElS, SHM-ELS, *L. minor* (duckweed), and *A. abdita* marine amphipod[a]

Sample	*H. azteca* (% survival)	*L. variegatus* (% survival)	FHM-ELS (% survival)	SHM-ELS (% survival)	*L. minor* (% reduction, Frond #/% reduction Chla, mg/g	*A. abdita* (% survival)
NY/NJH	50–100	5–100	0	0–0	# −96%; Chla −41%	95
1% NY/NJH	0–100		80	95	#0; Chla 0	
East River	0–55	0–0	0–0	0–0	# −94 to −58%; Chla −25% to −35.4%	0
1% East River	0–73		0–10	20–80	#0; Chla 0	

[a] NY/NJH, New York/New Jersey Harbor sediments; Chla, chlorophyl *a* in mg/g of dry weight of duckweed.

Table 27.11 shows the results of side-by-side tests performed using a 7-day EMAP[34] that was used in assessing toxicity in sediments collected in a U.S. EPA 1994–1995 Rocky Mountain Regional Environmental Monitoring and Assessment Program (REMAP) and a U.S. EPA Environmental Monitoring and Assessment Program (EMAP) in the mid-Atlantic region in 1994–1998. Table 27.11 shows the results of a number of side-by-side freshwater amphipod tests performed using both of these methods on one East River sediment, two laboratory sediment sand controls, and eight sediments collected from a local project on the Little Miami River, Ohio. All survival results were statistically similar. The miniaturized (or reduced volume) method appears to be very promising, since survivals were not statistically different from existing methods. There were some differences in the growth data. The reduced volume tests showed lower growth results between the two methods. Additional method modifications are currently underway, looking at the feeding regime to see if comparable results can be obtained.

H. azteca toxicity tests with East River sediments using sediment manipulations consisting of sediment dilution, aeration purging, and Ambersorb and Amberlite resin treatment disclosed presence of specific organic and inorganic toxic contaminants in the sediments and provided information on how to significantly reduce the specific contaminant toxicity to the amphipod.

Table 27.12 summarizes the percentage survival results from *H. azteca* sediment toxicity tests with East River contaminated sediments. The East River sediment was

Table 27.11 Comparison tests with *H. azteca* in both control and natural sediments, using the EMAP method and the reduced volume method[a]

	Reduced volume method				7-day EMAP method			
Sample	Survival (%)	C.V. (%)	Grw (μg/sur)	C.V. (%)	Survival (%)	C.V. (%)	Grw (μg/sur)	C.V. (%)
Cnt	100	0	18–42	12.3–45.3	92.5–100	0–6.98	49–89	3.4–29.8
ER	0	0	0	0	0	0	0	0
99-01-99-19	95–100	0–12.8	16–41	22.4–61.3	93.8–97.5	2.6–6.7	48–70	5.1–14.6

[a] All values represent ranges. ''Cnt'' is the sand control sediment, spiked with 0.5% alfalfa. ER is the East River contaminated sediment. The 99-01-99-19 sediments are sediments collected as part of the Little Miami River project. ''Grw'' (ug/sur) is the growth in dry weight per surviving individual. C.V. (%) is the percentage of coefficient of variation.

Table 27.12 Results from *H. azteca* sediment toxicity test with East River contaminated sediment. The East River sediment was diluted to 0.1%, 1%, 10%, and 100% for all treatments. The treatments included aeration, and two purging procedures

Sample purged 1 or 2 procedures	Sample aerated	*H. azteca* (% sur)	C.V. (%)
Sand control	24 h	100	0
1% ER P1	24 h	0	0
1% ER P1	24 h	25	40
1% ER P1	24 h	45	22.2
1% ER P1	48 h	25	76.6
Sand control	24 h	100	0
1% ER P2	24 h	35	54.7
1% ER P2	Not aerated	20	141.4

diluted to 1% for all treatments. The treatments performed on different dates, included aeration, no aeration, and two purging procedures. The percentage of survival of *H. azteca* ranged from 0% to 45% as contrasted to 100% survival in the sand controls. Table 27.13 shows the percentage of survival results from *H. azteca* sediment toxicity tests with East River sediment, which was undiluted and diluted to 10%, 1%, and 0.1% for all treatments. The treatment included aeration only procedure and aeration with subsequent addition of 8% AS 572. When only aeration was used, the percentage of survival of *H. azteca* in undiluted, 10% and 1% sediment was shown to be 0%, whereas in the 0.1% diluted sediment, 100% survival was exhibited. The use of aeration combined with the addition of AS 572 resulted in 0%, 20%, 40%, and 95% survival of *H. azteca* in undiluted, 10%, 1%, and 0.1% sediment samples, respectively, as compared to 100% survival in the sand controls. Table 27.14 shows the percentage of survival results from *H. azteca* sediment toxicity tests with East River sediment, which was diluted to 1%, 5%, and 10%. The treatment included aeration and aeration with subsequent addition of 8% Ambarsorb 572 and a mixture of AS 572 and Amberlite IRC-718. When only aeration was used, the percentage of survival of *H. azteca* was shown to be 35%, 25%, and 25% in the 10%, 5%,

Table 27.13 Results from *H. azteca* sediment toxicity tests with East River contaminated sediment. The East River sediment was diluted to 0.1%, 1%, 10%, and 100% for all treatments. The treatments included aeration, and aeration with the subsequent addition of 8% AS 572

Sample	Sample aerated	*H. azteca* (% sur)	C.V. (%)
Sand control	Yes	100	0
0.1% ER	Yes	100	0
1% ER	Yes	0	0
10% ER	Yes	0	0
100% ER	Yes	0	0
0.1% ER + 8% AS 572	Yes	95	10.5
1% ER + 8% AS 572	Yes	40	40.8
10% ER + 8% AS 572	Yes	20	0
100% ER + 8% AS 572	Yes	0	141.4

Table 27.14 Results from *H. azteca* sediment toxicity tests with East River contaminated sediment. The East River sediment was diluted to 1%, 5%, and 10% for all treatments. The treatments included aeration and aeration with subsequent addition of 8% AS 572 and a mixture of 8% AS 572 and 8% Amberlite IRC-718

Sample	Sample aerated	*H. azteca* (% sur)	C.V. (%)
Sand control	Yes	100	0
1% ER	Yes	25	40
5% ER	Yes	25	100.7
10% ER	Yes	35	71.9
Sand control	Yes	100	0
1% ER + 8% AS 572	Yes	90	12.8
5% ER + 8% AS 572	Yes	65	100.7
10% ER + 8% AS 572	Yes	25	64.4
Sand control + 8% IRC 718	Yes	95	10.5
1% ER + 8% AS 572 + 8% IRC-718	Yes	45	42.6
5% ER + 8% AS 572 + 8% IRC-718	Yes	40	57.7

and 1% sediment samples, respectively, as compared to 100% survival in sand controls. When aeration was combined with the addition of AS 572, the percentage of survival of *H. azteca* was 25%, 65%, and 90% in the 10%, 5%, and 1% sediment samples, respectively, as compared to 100% survival in sand controls. When aeration was combined with the addition of a mixture of 8% AS 572 and 8% Amberlite IRC-718, the percentage of survival was 40% and 45% in the 5% and 1% sediment samples, respectively, as compared to 95% survival in the sand control. Table 27.15 shows percentage of survival results of *H. azteca* sediments toxicity tests with East River contaminated sediment diluted to 10% for all treatments. The treatment included aeration with subsequent addition of 8% AS 563 or AS 572, addition of 8% AS 563 with no aeration, and addition of a mixture of 8% AS 572 and 8% Amberlite IRC-718 with aeration. With aeration alone, there was no survival in sediment samples. There were 90% and 85% survival in aerated sediment samples containing 8% AS 563 or AS 5782, respectively, and 0% survival in non-aerated sediment samples containing 8% AS 563. In aerated sediment samples containing a mixture of 8% AS 572 and 8% Amberlite IRC-718, 90% survival was exhibited. Table 27.16 summarizes the results on the percentage of survival of *H. azteca* in aerated or non-aerated, 0.1% and 1% diluted sediments, which were treated with varied combinations of Ambersorb and Amberlite resins and compares these results to a range of 90–100% survival. in sand controls.

Grade 40 silica sand was used as a control in these manipulation toxicity tests. The aeration treatments significantly reduced the concentration levels of dissolved and gaseous hydrogen sulfide in the sediments samples. The treatment of aerated and/or purged sediment samples with AS 563 and AS 552 reduced the concentration of organics, including PAHs in the aqueous phase through formation of bonded organics (PAH)/resin/sediment particle aggregates. Amberlite IRC-718 has significantly adsorbed the metals in the sediment to form metal/resin/sediment particle aggregates. The results on the survival of *H azteca* in sediment samples following the various sediment treatment manipulations illustrate a significant increase in the percentage of survival of the amphipod due to reduction of hydrogen sulfide, metals, and organics, including PAHs in sediment samples and thus implicate them as specific contaminants causing toxicity. Figures 27.11 and 27.12 illustrate percentage of survival of *H. azteca* in the East River sediment samples following aeration, dilution, purging, and Ambersorb and Amberlite resin treatments. The sediment treatment manipulations are listed in an increasing order of effectiveness to raise the percentage of survival of the amphipod, *H. azteca*, in the

Table 27.15 Results of *H. azetca* sediment toxicity tests with East River contaminated sediment. The treatments included aeration, aeration with subsequent addition of 8% AS 563 and AS 572, and addition of 8% AS 563 with no aeration and a mixture of 8% AS 572, and 8% Amberlite IRC-718 with aeration

Sample	Sample aerated	*H. azteca* (% sur)	C.V. (%)
Sand control	Yes	100	0
10% ER	Yes	0	0
10% ER 8% AS 563	No	0	0
10% ER 8% AS 563	Yes	90	12.8
10% ER 8% AS 572	Yes	85	11.8
10% ER + 8% AS 572 + 8% IRC-718	Yes	90	115.5

Table 27.16 *H. azteca* toxicity results of treatments of 0.1% and 1% East River sediments with AS 572 and AS 563, Amberlite IRC-718, with (Y) and without (N) aeration

Sample	Sample aerated	*H. azteca* (% sur)	C.V. (%)
Sand Cnt	Y*	100	0
Sand Cnt 4% AS 563	Y	95	10.5
Sand Cnt 8% AS 563	Y	90	12.8
Sand Cnt 4% AS 572	Y	100	0
Sand Cnt 8% AS 572	Y	90	12.8
Sand control + 8% IRC-718	Y	95	10.5
0.1% ER	Y	100	0
0.1% ER	N*	100	0
0.1% ER + 8% AS 572	Y	95–100	10.5–0
0.1% ER + 8% AS 572	N	100	0
0.1% ER + 8% AS 563	Y	100	0
0.1% ER + 8% IRC-718	N	100	0
0.1% ER + 8% IRC-718	Y	100	0
1% ER	Y	0–45	0
1% ER	N	0	0
1% ER + 8% AS 572	Y	40–90	40.8–12.8
1% ER + 8% AS 572	N	5–35	200–71.9
1% ER + 8% AS 563	Y	40–70	70.7–28.6
1% ER + 8% AS 563	N	45	42.6
1% ER + 8% IRC-718	N	20	115.5
1% ER + 8% IRC-718	Y	75	25.5
1% ER + 8% AS 572 + 8% IRC-718	Y	45–80	42.6–20.4
1% ER + 8% AS 563 + 8% IRC-718	Y	90	12.8

Y = Afrated, * N = Non-Afrated

treated sediments: dilution/aeration/purging > dilution/aeration/purging + AS 563/AS 572 treatment and/or dilution/aeration/purging + Amberlite IRC-718 treatment > dilution/aeration/purging + AS 563/AS 572 + Amberlite IRC-718 treatment.

Table 27.17 illustrates the survival data of *H. azteca* following the aerobic biodegradation treatments of NY/NJH and East River sediments using non-amended samples and samples amended with ethanol, salicylic acid, and the surfactant Triton

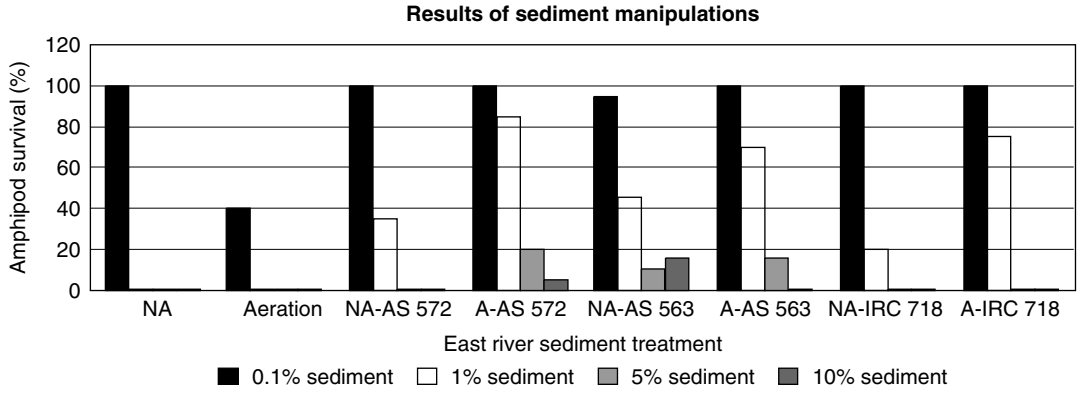

Figure 27.11 Toxicity results based on sediment manipulations.

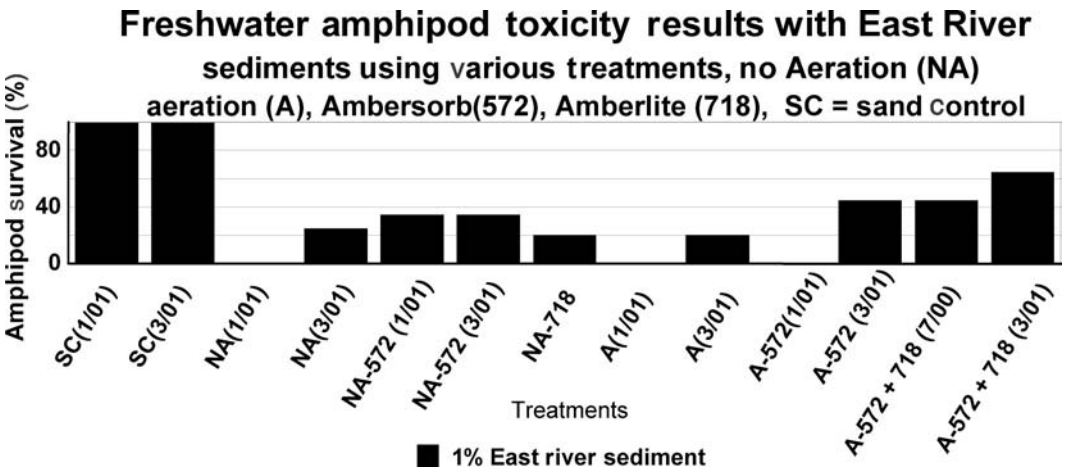

Figure 27.12 Toxicity results based on sediment manipulation without aeration.

Table 27.17 Results from *H. azteca* sediment toxicity tests conducted using East River and NY/NJH sediments treated using an aerobic biodegradation slurry system (bioslurry) and the addition of materials to stimulate the PAH biodegradation activity of the indigenous microbiota in the sediments

Sample	Treatment	*H. azteca* (% sur)	C.V. (%)
Sand control	None	90	22
NY/NJH	Bioslurry	65	15.4
NY/NJH	Bioslurry + ethanol	90	12.8
NY/NJH	Bioslurry + salicylic acid	90	12.8
NY/NJH	Bioslurry + triton	90	12.8
ER 1%	Bioslurry	80	20.4
ER 10%	Bioslurry	85	22.5
ER 100%	Bioslurry	40	40.8
ER 1%	Bioslurry + ethanol	65	29.5
ER 10%	Bioslurry + ethanol	75	33.6
ER 100%	Bioslurry + ethanol	60	47.1
ER 1%	Bioslurry + salicyclic acid	85	22.5
ER 10%	Bioslurry + salicyclic acid	60	47.1
ER 100%	Bioslurry + salicyclic acid	65	52.5

X-100. Figure 27.13 shows the same percentage of survival data as histograms for the varied aerobic biodegradation treatments. The aerobic biodegradation treatments of the NY/NJH and East River sediments have significantly increased the survival of the amphipod, *H. azteca*. The incorporation of the sediment manipulation procedures and the use of the aerobic biodegradation treatments of PAH contaminated sediments was shown to significantly eliminate the cause of toxicity due to the PAHs, metals, and hydrogen sulfide and thus increased the survival of the amphipod, *H. azteca*.

Conclusions

Bench-scale serum bottle sediment slurry studies with aged East River sediment were undertaken to determine the biodegradation of PAH contaminants, with and without the

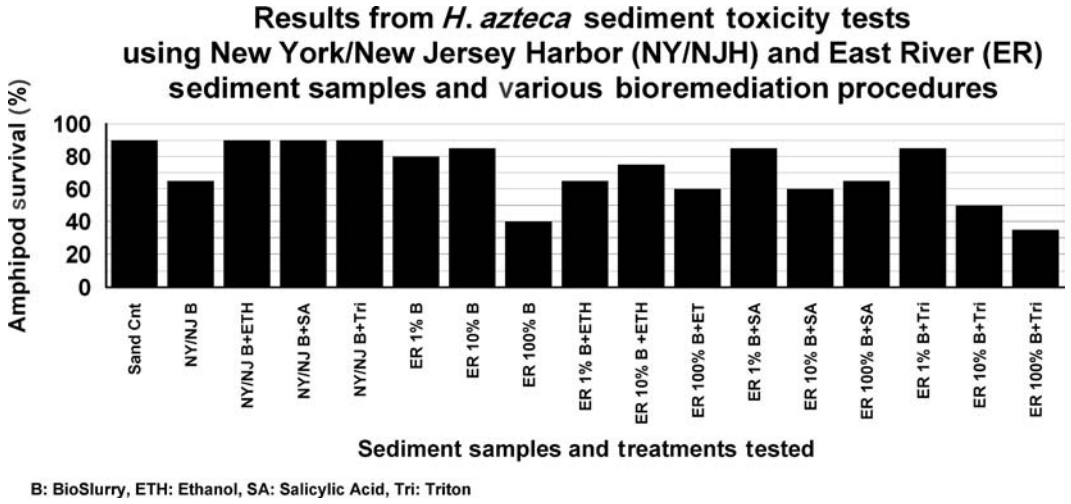

Figure 27.13 Toxicity results based on various biodegradation procedures.

addition of nutrients/co-substrates under both aerobic and sulfate reducing conditions. In the aerobic slurry systems with and without supplemental nutrients (nitrogen and phosphorus) and with pure oxygen in the headspace, appreciable biodegradation (biotransformation) of 2-, 3-, 4-, and 5-ring PAHs occurred within 17 weeks of incubation. Under sulfate reducing conditions, 2- and 3- ring PAHs were biodegraded simultaneously with sulfate reduction, after a lag period of 2 months but the rate of biodegradation was much lower than that under aerobic conditions. The addition of the co-substrates, ethanol, and acetate led to an increase of rate of biodegradation of 2- and 3-ring PAHs, particularly phenanthrene within the same incubation period.

Sediment toxicity tests were undertaken to measure baseline and residual toxicity of the NY/NJH and East River sediments to fresh water and marine amphipods, aquatic worms, FHM-EL, SHM-EL, and a duckweed vascular plant. Tests using five sediment manipulations (dilution, aeration, purging, AS 563 and AS 572, and Amberlite IRC-718 treatments) were preformed to determine the cause of toxicity in the sediments. The East River sediment was shown to be acutely toxic to all the organisms tested, as well as to plants (duckweed). The NY/NJH sediment was slightly toxic to FHM-EL and the aquatic worm *L. variegatus*, but was not toxic to one marine amphipod *A. abiota*. The East River sediment significantly reduced front production (−58.2%) and chlorophyll a levels (−35.4%) in the fresh water duckweed tests. A miniaturized toxicity test, developed by us, shows survival results similar to the old EMAP method for lethality. This miniaturized method could logistically be very desirable for determining toxicity of sediments where only small samples of sediment can be provided. Results from sediment manipulation tests showed that freshwater amphipod, *H. azetca*, survival was improved with aerobic biotreatement and with sediment manipulation procedures consisting of sediment dilution and aeration combined with 8% AS 563 or AS 572 resin treatment and/or 8% Amberlite IRC-718 treatment. The studies revealed that hydrogen sulfide, organics, including PAHs, and metals were factors contributing to East River sediment toxicity.

Studies on bioavailability, biodegradation, and toxicity of NY/NJH and East River sediments stress the importance of understanding the impact of these factors on bioremediation of PAH contaminated sediments and provide an awareness that contaminated

sediments with different characteristics may require modification in the biorestoration strategy.

It is essential that the toxicity tests be performed before and after the biotreatments of the contaminated sediments in order to determine the baseline and residual toxicity of these sediments and to determine the cause of toxicity. The toxicity assessment of the contaminated sediments before and after use of biotreatment procedures and sediment manipulation strategies is important for the development of an efficient bioremediation approach for a specific contaminated sediment site.

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chapter twenty-eight

Application of solid-phase microextraction fibers as biomimetic sampling devices in ecotoxicology

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Contents

Introduction

A number of passive sampling devices (PSDs) have been used during the past 20 years for environmental monitoring of organic chemicals. Semi-permeable membrane devices (SPMDs) are composed of low-density polyethylene (LDPE) layflat tubing filled with a

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known weight of neutral lipid (triolein). LDPE has been shown to effectively mimic the function of bipolar lipid membranes of biological systems in uptake of hydrophobic organic chemicals, and the triolein in SPMDs represents the neutral or storage lipid pool in organisms.[1] SPMDs are the original PSDs used successfully to extract hydrophobic compounds (e.g., PCBs, OCs) from water, sediment, air, and even soil.[1–5] The introduction of solid-phase microextraction (SPME) fibers in 1990[6] provided a new tool for the environmental monitoring of organic chemicals. The SPME comprises a core of fused silica fiber that is coated with a thin polymer phase for the sampling of analytes and subsequent introduction into a GC system via thermal desorption. The polarity of the coating can be varied to optimize the sampling of analytes of varying polarities. For example, a C18 coating (very hydrophobic) is best for extremely hydrophobic organic chemicals (e.g., PAHs, PCBs), while a more polar coating, such as polyacrylate, would be more suitable for detecting more polar compounds (e.g., TNT, herbicides). SPME has been used to distinguish between dissolved and bound fractions of organic pollutants in the environment.[7] A number of other solid-phase extractants (e.g., Tenax beads, C18 disks) and other formats with thin polymer coatings have also recently been reported for use in environmental monitoring.[8]

PSDs have a number of advantages over live organisms for aquatic environmental monitoring, including ease of deployment, low production, and maintenance costs, transportability, and applications to a wide variety of environmental systems (e.g., freshwater, marine, sediments, soils). These devices absorb organic contaminants from the exposure medium following the general pattern of a one-compartment first-order kinetics (1CFOK) model (Figure 28.1) that can be described by the general equation:

$$C_{\mathrm{PSD}}(t) = C_{\mathrm{medium}} \times k_1/k_2 \times (1 - \mathrm{e}^{-k_2 t}) \tag{28.1}$$

where $C_{\mathrm{PSD}}(t)$ is the concentration of the contaminant on the sampler as a function of time, t, C_{medium} is the dissolved contaminant concentration in water, and k_1 and k_2 are uptake and elimination rate constants, respectively. The uptake rate of chemicals by a PSD is a function of its sampling rate that is proportional to the number of volumes sampled per day per unit volume of the device.[9] The time taken to reach a near-equilibrium state (Figure 28.1) is a function of the area/volume (A/V) ratio, with a higher A/V providing a faster sampling rate. For this reason, equilibrium would theoretically be reached much faster in an SPME (e.g., 7 μm PDMS coating) compared to an SPME (e.g., 100 cm × 2.54 cm containing 1 g of triolein). In practice, this theory is correct, with SPMDs taking weeks to months to reach equilibrium and SPMEs reaching equilibrium in hours, given a chemical of the same log

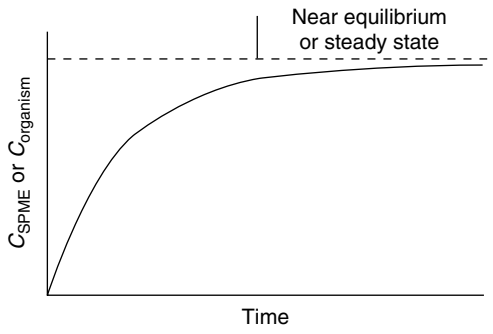

Figure 28.1 Theoretical kinetics of chemical uptake by a PSD (e.g., SPME) or an organism.

$K_{ow,}$ and similar exposure conditions (e.g., water flow). The time taken to reach equilibrium is important for environmental monitoring to help avoid the risks associated with fouling, degradation, and vandalism of the device. Of all the types of PSDs currently commercially available, SPME fibers exhibit the most rapid response time and reach equilibrium in a matter of hours to days for all except the most hydrophobic compounds. PSDs that reach equilibrium during their deployment can be referred to as equilibrium sampling devices (ESDs) and the general theory of ESD application in environmental monitoring is thoroughly described elsewhere.[9,10] It should be noted, however, that SPMDs are not designed to reach equilibrium, and relative to SPMEs, SPMDs have a high sampling matrix volume and high analyte capacity for non-polar organics. Thus SPMDs are ''integrative'' samplers where relatively large amounts of analytes are sequestered, and equilibrium is generally not approached. On the other hand, SPMEs have very low sampling matrix volumes and capacities, and approach equilibrium much more rapidly than SPMDs. Thus, SPMEs are ESDs and do not truly represent grab samples or point-in-time samples, but exposure times required to reach equilibrium can be very brief relative to SPMDs, which integrate exposure over many days to weeks. The time to equilibrium of SPMEs will vary depending upon phase thickness, exposure conditions, and hydrophobicity of the target molecule.

Another factor to consider during ESD deployment is depletion of the sampling medium. Since uptake of chemicals by ESDs is strictly by passive diffusion, it is imperative that the sampler does not deplete the sampling medium and alter diffusion gradients. This is especially important when water samples with low levels of contaminants are measured in laboratory situations. In field deployments, with a constant renewal of the aqueous medium due to bulk water movement, depletion of the sample medium is usually not as much of an issue. Under conditions of non-depletive biomimetic sampling using SPME, less than 5% depletion of mass balance of sample is suggested so that equilibrium between fiber and solid phases is not disturbed due to altered diffusion gradients.[10,11]

SPME as a biomimetic sampling device

Although the value of SPMEs as a tool for monitoring environmental levels of organic chemicals is presented earlier, their rapid chemical uptake kinetics presents an opportunity not realized by other PSDs for comparison to chemical uptake kinetics by organisms. Bioaccumulation of organic chemicals by aquatic organisms follows the same 1CFOK model as chemical uptake by the SPME. For a given concentration of an organic chemical, both the SPME and the organism will achieve an equilibrium or steady-state concentration, respectively, facilitating a correlation between the two values when different concentrations of the same chemical are compared.[5] In this way, the bioaccumulation potential of a chemical can be predicted from the equilibrium concentration of the chemical in SPME, and the SPME acts as a biomimetic sampling device (BSD). A further extension of this concept is the application of chemical uptake by SPMEs to predict the toxicity of organic chemicals. Chemical kinetics and residues in organisms related to biological responses, such as growth and survival, are termed critical body residues (CBRs)[12,13] and can be compared to kinetics and residues of chemicals in SPMEs in order to make bioavailability comparisons and predict toxicity. Recent research has established such relationships between SPME chemical uptake and CBRs for 1,2,3,4-tetrachlorobenzene and 2,4,5-trichloroaniline in the midge larvae (*Chironomus riparius*),[14] petroleum hydrocarbon mixtures and toxicity to shrimp,[15] pyrene (PYR) and earthworms (*Eisenia fetida*),[15] and 1,2,3,4-tetrachlorobenzene and enchytraeids (*Enchytraeus crypticus*).[16]

The focus of this chapter is the examination of SPME fibers as biomimetic surrogates for estimating the bioavailability of organic chemicals in sediments and soils. SPMEs provide an indirect measure of bioavailability[17] and can be correlated with chemical bioaccumulation by organisms. We discuss two different approaches for the application of SPMEs in environmental matrices. The first method describes the deployment of the SPME in an aqueous suspension of soil while it is contained in the commercially available SPME holder, while the second method describes an approach where a piece of SPME fiber is placed directly in the sediment testing medium during toxicity tests. Each of these approaches has its advantages and disadvantages that will be discussed in the remainder of the chapter.

Validating assumptions for equilibrium sampling with SPME fibers

Prior to conducting chemical determinations in a test system of interest using SPME fibers, it is necessary to determine the time taken to reach equilibrium with respect to the concentration of analyte on the SPME fiber and to validate that sampling in your test system is non-depletive. The theory behind these measurements will not be discussed here and the reader is referred to a detailed treatment of these topics by Mayer et al.[9] Our discussion in this chapter focusses on the practical aspects of conducting these measurements in a laboratory setting.

Determining time taken to reach equilibrium

In order to facilitate a meaningful comparison of chemical levels in a medium or with accumulation by an organism, the chemical concentration on the SPME fiber must be at near-equilibrium conditions with the chemical in the medium (Figure 28.1). This can be achieved by measuring the chemical concentration in the SPME until it is constant, usually involving a number of replicated parallel samples. As an example, for PYR in a soil test system, triplicate samples were taken at 4, 48, and 96 h (Figure 28.2) and showed no increase in PYR concentration over that time period, suggesting that 4 h would be sufficient to achieve equilibrium for PYR determinations in this system. It would appear that in order to determine the kinetic phase of PYR partitioning to the SPME fiber, measurements would need to be made in a geometric time series at durations shorter than 4 h.

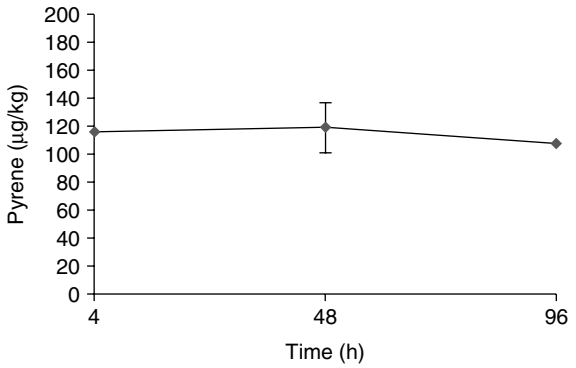

Figure 28.2 SPME exposure time for Webster soil spiked at 1000 mg/kg.

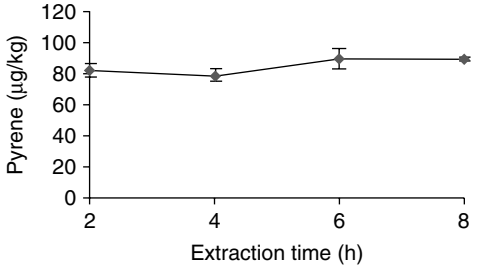

Figure 28.3 Reextraction of Perkins soil spiked at 300 mg/kg.

Validation of sample non-depletion

In order to validate that the SPME is not depleting the available chemical in the sample, resampling of the same sample is required. In the example below (Figure 28.3), PYR concentrations are determined in the same sample for duration of 2 h, followed by two 4-h exposures, followed by an 8-h exposure. The PYR concentration measured by the SPME did not change, suggesting that resampling the soil preparation did not affect the amount of PYR available for partitioning to the SPME, affirming that sampling in this system was indeed non-depletive.

Experiment 1: Determination of phenanthrene residues in soils using SPME fibers exposed in a vial-stir bar system

Procedure

The general experimental design entailed exposing earthworms (*Eisenia fetida*) to phenanthrene (PHE) in artificial soil containing organic matter at two levels (10% or 1% OM, w/w, as peat moss) to establish differences in PHE bioavailability due to differences in OM. The tests were conducted with three nominal concentrations (0.31, 2.05, and 3.10 mmol/kg dry mass soil) of PHE with an acetone solvent control (in triplicate) for each soil OM level, following ASTM guidance for conducting soil toxicity tests with earthworms as detailed in Reference 5.

Solid phase microextraction fiber analysis

The goal of SPME analysis was to determine the potentially bioavailable fraction of PHE in the soil rather than total PHE. In this example, SPMEs were deployed in commercially available SPME holders in order to take advantage of the increased analyte sensitivity due to direct injection into the GC with no solvent extraction. SPME fibers (7 µm polydimethylsiloxane (PDMS) with manual holders, Supelco, Bellefonte, PA) were used to assess uptake of PHE directly from the aqueous phase of soil at the earthworm toxicity test temperature (24 $\pm$ 1°C). For the measurement of analytes in soil, the SPME fiber must be exposed to a soil suspension in water. The soil/water ratio may vary with soil type and analyte concentration. For each SPME determination of PHE in soil in this study, 0.500 g of PHE-spiked freeze-dried artificial soil, 15 ml reagent grade water (RGW), and a Teflon®-coated magnetic stir bar (0.3 cm $\times$ 1.3 cm) were placed in a screw-top amber SPME vial (15-ml headspace with Teflon

septum, Supelco). In order to increase sample throughput, a ten-place magnetic stirrer (1200 rpm, IKA) was used with ten sample vials and ten SPME fiber assemblies to obtain steady-state data for PHE concentrations in soil suspensions. A support stand was constructed to hold ten SPME manual holders simultaneously during exposure. The needle of the SPME apparatus was inserted through the Teflon septum of the sample vial when the fiber was deployed. Each vial was aligned on the magnetic stirrer for optimum stirring velocity (~1000 rpm) and SPME fibers were exposed until steady state was achieved. Residue analysis can be accomplished on any GC fitted with an SPME adapter. In this study, this was accomplished using a Tracor 565 GC-FID, megabore fused silica capillary column (DB-5, 30 m × 0.53 mm ID × 1.5 μm, J&W Scientific), 0.75-mm ID SPME-inlet liner (Supelco), JADE septum-less injector with SPME adapter (0.56 mm ID, Alltec). Helium (High Purity, Sooner Airgas) was used as the carrier and makeup gas. The flow rate for the carrier gas was set to 35 cm/s linear velocity and makeup flow rate was set to 45 ml/min. Hydrogen (fuel for FID, High Purity, Sooner Airgas) flow rate was 35 ml/min, and breathing air (oxidant, Grade D, Sooner Airgas) flow rate was 350 ml/min. The temperature program for direct injection GC analysis was: injection port temperature 290°C, detector temperature 300°C, initial oven temperature 160°C (5-min hold) with 35°C/min ramp to 210°C (7-min hold).

It is critical that prior to initial use, each new fiber be conditioned (320°C) for 4 h to remove any adhesive or other contaminants from the fiber as per instructions from the manufacturer. Subsequent thermal desorption and conditioning of the SPME fiber was accomplished by exposing the fiber while inserted into the heated injection port (290°C) of the GC for 5 min. This resulted in adequate desorption followed by blank analyses of each fiber to ensure no PHE carryover problems existed. SPME fiber performance was determined before and after each soil determination by measuring a reference standard solution (1 mg PHE/l RGW). Integration of peaks was done by external calibration using injections from PHE standards with a certified PHE check standard (Chem Service, F81MS). Chromatogram data were collected and analyzed using PeakNet® chromatography software (Version 5.1, Dionex 1999).

One advantage of this method is that SPME fibers attached to the commercially available fiber holders could be reused until their performance degraded and was unsatisfactory. One of the major issues related to the reuse of SPME fibers to determine PHE in soil suspensions was the degradation of the bonded phase of the fibers due to erosion by soil particles during stirring. To monitor fiber performance, fiber apparatus identification and labeling was critical, since all fibers are not identical and degrade at different rates with use. Permanently labeling each SPME injector/holder and very carefully recording the history of use for each fiber accomplished identification. All SPME fibers will erode over time when exposed in an agitated medium, regardless of the exposure medium, due to general use and numbers of collisions with stirred material. This results in degraded performance with repeated use. In media, such as filtered water or air, this is not a big problem. Performance checks were conducted before and after each soil suspension exposure by measuring a reference standard solution (1 mg PHE/l RGW) to ensure that fiber sensitivity had not decreased.

PHE quantification was conducted using external calibration by conventional solvent injections with PHE standards. The quantification can be thought of as the amount of analyte on the GC column. For example, if standards were injected using 1 μl of solution at concentrations of 1, 5, 10, 50, and 100 ng/μl, the resulting mass of analyte on the analytical column would be 1, 5, 10, 50, and 100 ng, respectively. Once

the fiber was analyzed, the amount of analyte detected would be that amount on the fiber. The results may be expressed in any number of ways (i.e., amount of *X* on the fiber, amount of *X* per volume of phase on the fiber, the amount of *X* per mass of phase on the fiber, etc.).

For PHE determinations in this study, SPME fiber assemblies with a 7-μm thick PDMS bonded phase were used. SPMEs are available with bonded phases of various thickness, but analyte kinetics are much faster with thinner phases, and this was the only phase thickness that was commercially available bonded to the fiber (phase bonding results in a much more durable fiber).

We constructed a support stand to hold ten SPME manual holders simultaneously during exposure. Alternatively, one could remove the SPME fiber/needle apparatus from the holder and deploy without the need for a support stand. This will only work with SPME fibers for automated systems [SUPELCO-57303] (without the retractable spring included for the operation of manual fibers [SUPELCO-57302]). Residue analysis would require that the fiber apparatus be reinstalled to the holder. Each deployment of the SPME required that the fragile needle must be retracted on insertion and removal from the sampling vial. The SPME apparatus must be firmly supported while being inserted through the Teflon septum of the sample vial. The Teflon septum must be replaced after each use if the sample vials are reused. The fiber was placed near the bottom one-third of the vial where the agitation was the greatest. The fiber was also placed between the vial wall and the center of the vial for greatest agitation potential. It was critical to align each vial on the magnetic stirrer for optimum stirring velocity (~1000 rpm) or just below the vortex point. Creating a vortex will cause cavitation and limit the contact of the fiber with the exposure medium and should be avoided.

Each SPME fiber was exposed to a solution containing PHE until steady state was achieved (5 h). Steady state was determined by exposing SPME fibers to 1 mg PHE/l RGW over a geometric time interval (e.g., 0.5, 1, 2, 4, 8, and 16 h). No differences of PHE uptake existed between the 4, 8, and 16 h exposure ($p < 0.05$).

After the appropriate exposure period, the fiber was retracted into the needle and the device removed from the vial. The bonded phase on the fibers is very fragile and will be stripped if it comes in contact with the vial septum or GC septum or inlet device.

An additional issue related to the reuse of SPME fibers with the commercial SPME holder is water being drawn inside the sheathing needle of the fibers by capillary action during sampling. Fine particles and associated contaminants may also be drawn into the sheathing needle, and contaminants may be thermally desorbed onto the GC column during analysis, possibly compromising the analysis. The debris may also be heat-fixed to the fibers degrading its performance. To reduce this problem, the fiber is removed from the sample vial as described above, exposed, and rinsed with a stream of ultra-high purity (UHP) RGW or placed into a vial with UHP RGW under high stirring velocity for a very short period of time to dislodge any attached particles.

The addition of water into a GC is something to be avoided as when water is heated in the injection port, water expands violently and can damage the GC, injection port, analytical column, or detector. The addition of water may also cause sample analytical problems. To minimize problems caused by water injection, it is necessary to remove the water, and this was accomplished by exposing the fiber and placing an absorptive material (e.g., Kimwipe) at the interface of the needle and plunger sheathed within.

Most of the water will be drawn to the Kimwipe via capillary action. Caution should be used with this technique as the sequestration phase may be eroded easily if the Kimwipe touches the fiber itself.

Immediately upon removal of the fiber from the exposure medium in the sample vial, analytes begin to partition to the air. The rate of loss depends on the fugacity of the analyte, volatility, vapor pressure, and temperature. One way to slow down the loss is to freeze the fiber. Another way is to use a GC septum cored lengthwise to create a ''cap.'' The septum material will have some contaminants on it that will partition to the fiber using this method. The freezing method in addition to capping with a septum is a very good method for extended storage periods. The best way to guard against analyte loss is to desorb (analyze) the fiber immediately after exposure, minimizing analyte loss.

Results and discussion

Comparing SPME measures with biological responses

The application of SPME technology for evaluating dose–response relationships between PHE exposure and earthworm responses allows the examination of the effects of organic matter in artificial soil on PHE bioavailability. Even though total PHE levels were similar in the different soils, there was a dramatic difference in earthworm survival (Table 28.1) with increased organic matter increasing earthworm survival. The effect of organic matter on SPME-measurable PHE can be useful in explaining the observed results. Earthworm body residues are well correlated with SPME-measurable PHE ($r = 0.93$) (Figure 28.4), while no significant correlation between earthworm PHE residues and total PHE levels in the artificial soil was evident.

The determination of PHE in soils using this method that allows the reuse of fibers for multiple determinations provides a cost-effective method for the determination of the potentially bioavailable fraction of PHE in the test soils. The cost of the fibers is ~$60 (US) per fiber, and at least eight bioavailability determinations could be made in a soil matrix without a reduction in sensitivity due to fiber degradation. The design and intended use of SPME fibers was for the determination of total chemical measurements from various media, such as water and air (headspace analysis). However, modifying factors of toxicity, such as dissolved organic matter and suspended particulate matter, reduce the amount of chemical available for SPME measurements in a manner similar to the reduction of chemical bioavailability for organisms.

Table 28.1 Total PHE, SPME-measurable PHE, and mortality of earthworms exposed to PHE in artificial soil varying in organic matter content

Soil OM (%)	Total PHE (mmol/kg)	SPME PHE (mmol/cm^2)	Mortality (%)
1	0	5.7E – 07 (2.1E – 07)	0
1	0.31	2.1E – 06 (2.1E – 06)	97
1	2.05	2.7E – 05 (9.2E – 06)	100
1	3.10	3.1E – 05 (7.0E – 06)	100
10	0	1.2E – 07 (1.4E – 07)	0
10	0.31	6.6E – 07 (4.7E – 07)	0
10	2.05	6.2E – 06 (1.9E – 06)	6.7
10	3.10	1.2E – 05 (5.0E – 06)	68

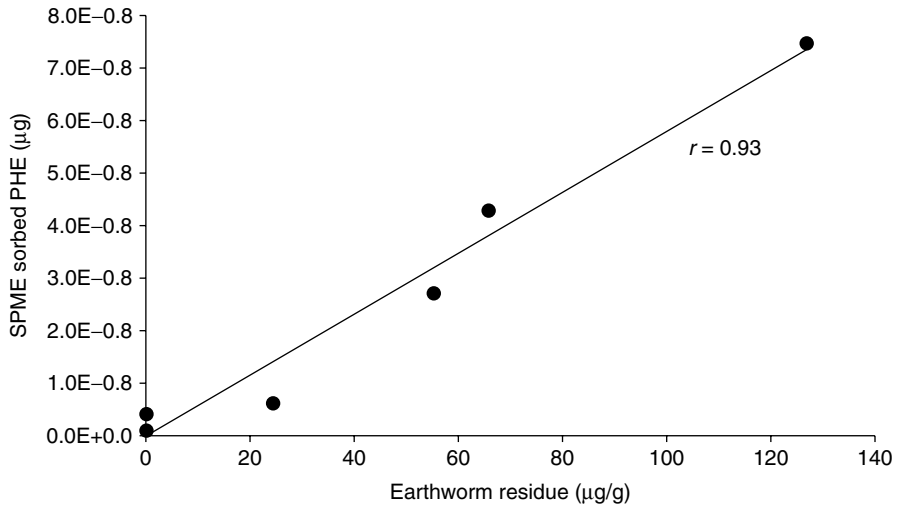

Figure 28.4 Relationship between SPME-measurable PHE and earthworm body residues. Reprinted from Ecotoxicology and Environmental Safety, Vol. 57, Lanno, R.P., Wells, J., Conder, J., Bradham, K., and Basta, N., The bioavailability of chemicals in soil for earthworms, p. 39–47, Copyright 2004, with permission from Elsevier.

Experiment 2: Determination of TNT in sediment using a disposable SPME fiber technique

Procedure

The static, direct-burial, disposable SPME approach for measuring TNT and nitroaromatics (NAs) in sediment is described in detail by Conder et al.[18] Briefly, 85-μm polyacrylate SPME fiber is purchased in bulk lengths, which are cut into 1.00-cm pieces using a double bladed, stainless steel razor blade apparatus. To facilitate handling and retrieval of the thin, transparent fiber, SPMEs are inserted through a stapled, small Teflon disc (Figure 28.5a) or placed in a small (2 cm × 2 cm) 60-μm stainless steel mesh envelope (Figure 28.5b). Chemical uptake by the fiber is independent of the type of holder. Before deployment, the SPME and holder are rinsed with 50:50 HPLC-grade acetonitrile/ultrapure water (Milli-Q Purification System), rinsed with ultrapure water, and allowed to dry at room temperature.

Measurement of NAs by SPMEs can be conducted directly in sediment during organism exposures.[18] SPMEs can be suspended in the overlying water, placed at the sediment/water interface, or buried within the sediment at the desired depth. Exposure time is 48 h, which is sufficient for TNT and its major NA transformation products to reach steady state in static exposures to NA-spiked water at room temperature (23°C). We assume that SPME extractions in sediment and sediment porewater are non-depletive, and that absorption kinetics is similar to that in pure water. Although not as rugged, the stapled Teflon disc holder is less expensive and easier to manipulate than the stainless steel mesh envelope holder, and the disc holder also has the advantage that it can be retrieved easily with a strong magnet.

After exposure, SPMEs are removed from their holders and placed into HPLC autosampler vials. The fibers can be stored (4°C) for up to a week or immediately extracted with 400 μl of 50:50 HPLC-grade acetonitrile/ultrapure water to desorb the compounds from the SPMEs. After desorption (10 min), the SPME is discarded and the solvent analyzed for NAs

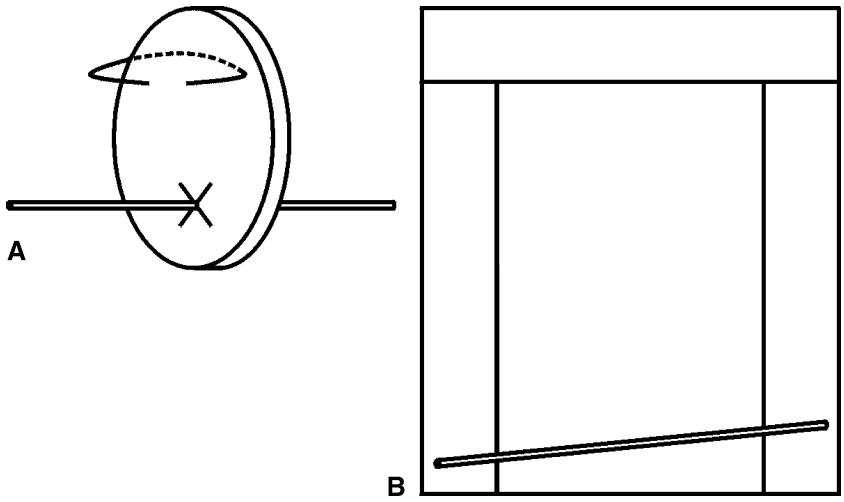

Figure 28.5 Stapled Teflon disc (a) and steel mesh envelope (b) holders for disposable SPMEs.

by HPLC. The 1-cm lengths of polyacrylate SPME fiber contain 0.521 μl polyacrylate; it is best to present SPME data as concentrations (number of molecules absorbed by the polyacrylate divided by volume of the polyacrylate) as in the previous studies.[10,14,19,20]

Routine quality assurance/quality control (QA/QC) procedures include assessment of fiber quality and extraction/handling procedures. It is advantageous to sacrifice 2–3 cm of each bulk SPME order to assess fiber performance. The 1-cm fibers can be exposed to 15-ml spiked water (e.g., 50 nmol TNT/ml) for 48 h and uptake compared to published fiber/solution partition coefficients.[18] In experiments in which disposable SPMEs are used to measure contaminants, extraction procedures should also be assessed. SPMEs can be spiked (coated directly on fiber or placed in their extraction vials) prior to receiving extraction solvent to determine percent recovery of the analytes.

Results and discussion

TNT concentrations in SPMEs were accurate predictors of TNT bioavailability in sediment. Sediments were spiked at the same TNT spiking level, but received five different levels of powdered activated carbon amendments (0–1000 μg carbon/g sediment, dw) to insure differences in bioavailability among the sediments. Each 100-g sediment replicate received 500 ml water and was aged for 14 days.[21] After aging, 10 adult *Tubifex tubifex* (sediment-dwelling oligochaetes) and one SPME were exposed for 48 h. SPMEs were exposed in overlying water at the sediment/water interface in steel mesh envelopes (Figure 28.5b). Because of differences in bioavailability (activated carbon amendments), sediment TNT concentrations determined by the acetonitrile liquid/liquid extraction were not good predictors of *Tubifex* TNT concentrations, which were subject to square root transformation to satisfy assumptions of regression analysis (Figure 28.6a). The regression model was significant; however, the slope was negative, indicating that as sediment TNT concentrations increased, *Tubifex* TNT concentrations decreased. If sediment TNT concentrations are normalized by the nominal amount of activated carbon added, the regression model improves, and the slope becomes positive (Figure 28.6b). However, if the *x*-axis is replaced by SPME-measurable TNT concentrations (Figure

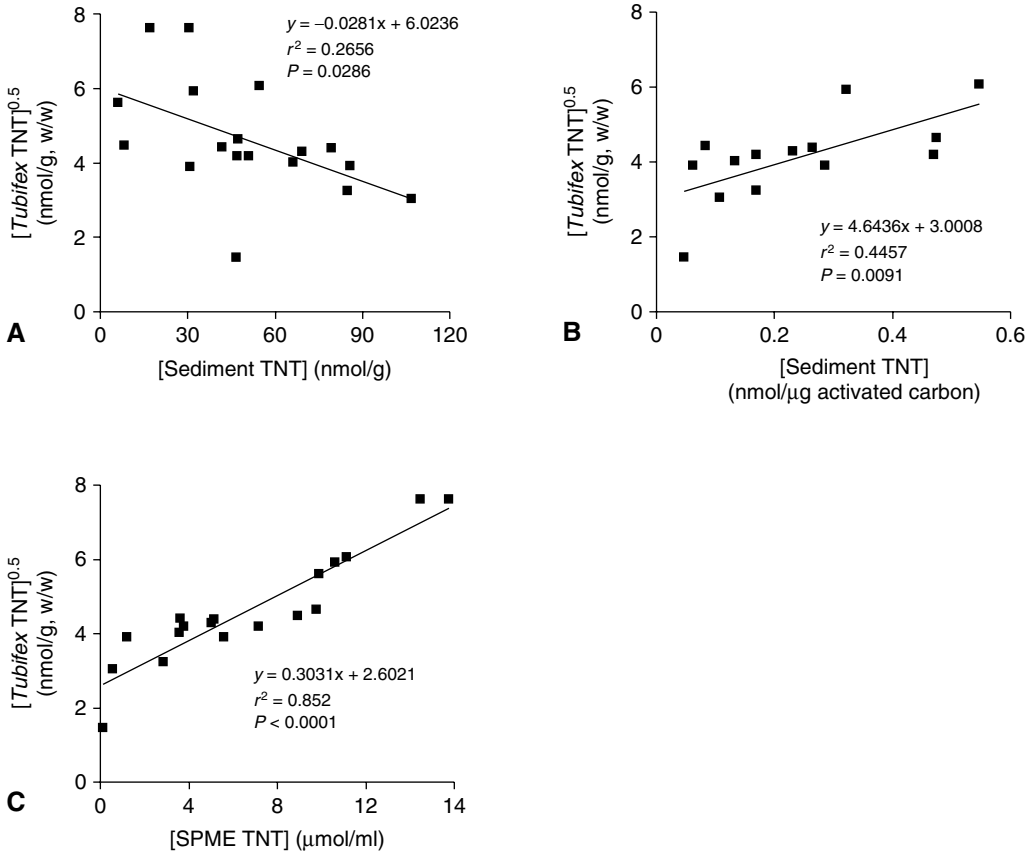

Figure 28.6 *Tubifex* TNT concentrations versus sediment TNT concentrations (a), activated carbon-normalized TNT concentrations (b), and SPME TNT concentrations (c) in TNT-spiked, activated carbon-amended sediment.

28.6c), the coefficient of determination improves from 0.4457 to 0.8520. Thus, SPMEs more accurately predict TNT bioavailability (*Tubifex* TNT concentrations) than either sediment TNT concentrations or sediment TNT concentrations normalized for carbon content.

Aside from the ability to more accurately predict TNT bioavailability in sediment than the liquid–liquid extraction to determine ''total'' NAs, the disposable SPME technique is slightly less expensive. By using the more rugged stainless steel mesh envelope holders, it may be possible to deploy SPMEs *in situ*. Also, SPMEs may be able to provide matrix-independent measures of toxicity and bioavailability. Median lethal toxicity (LC_{50}s based on SPME concentrations) for *Tubifex* exposed in TNT-spiked water and sediment were within a factor of 1.1.[22]

Even though SPMEs offer some advantages over traditional measures of exposure, they also have some limitations in estimating available compounds. SPMEs cannot mimic dietary uptake in cases where dietary contaminants are metabolized differently or partition to different compartments within an organism compared to chemicals taken up across external surfaces (epidermis or gills). SPMEs cannot simulate the complex compartmentalization of organics among different tissues, organs, and/or biomolecules, nor can they mimic biotransformation due to metabolic reactions with biomolecules, active

elimination, or detoxification systems. SPMEs cannot mimic organism movement or other behaviors that alter exposure scenarios. While the static burial method for measuring the relatively hydrophilic NAs ($\log K_{ow}$ values ≤ 2) is rapid (48 h), more hydrophobic compounds may need longer times to reach equilibrium in fibers. Time to reach steady state will vary by compound hydrophobicity, agitation method, and fiber type.[23] For example, Leslie et al.[14] found that time to reach equilibrium in static exposures using disposable 2-cm long, 15-μm PDMS fibers took from 10 h to 2 weeks with compounds ranging in $\log K_{ow}$ from 3 to 6. If minimization of sampling time is paramount, SPMEs (in holders) can be agitated in a small sediment sample, similar to the technique described previously for soil. We found that SPME exposure time for NAs could be reduced to 24 h with SPMEs exposed to 5–10 g sediment samples in 15-ml vials (topped up with water) in an end-over-end rotating mixer (60 rpm).

Summary and conclusions

Although the application of SPME technology to measure concentrations of organic chemicals in water and air is quite advanced, the application of SPME technology to sediments and soils is in its infancy. The examples of various methods for determining concentrations of PHE in soils and TNT and its metabolites in sediments presented in this chapter are promising but are only initial forays into applying SPMEs in complex environmental matrices. Both the methods discussed in this chapter, the reusable SPME in the commercially available holder and the one-time-only use and disposal of SPME fragments, have advantages and disadvantages. However crude these methods, the results are very promising in being able to detect potentially bioavailable levels of organic contaminants that correlated well with organism uptake and responses. Further research examining relationships between SPME-detectable organic chemicals, bioaccumulation, and organism responses is needed to establish SPMEs as a useful tool for monitoring organic chemicals in the environment.

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chapter twenty-nine

Passive dosimeters for measurement of ultraviolet radiation in aquatic environments

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Contents

Introduction

Ultraviolet radiation (UVR) is subdivided into UVA (320–400 nm), UVB (280–320 nm), and UVC (220–280 nm). Like visible light (430–790 nm), the longer rays of UVA pass through the atmosphere with little attenuation, while stratospheric ozone absorbs most UVB and all of the dangerous UVC. High-energy rays of UVA and UVB initiate photochemical reactions (e.g., smog generation) and can cause molecular and cellular damage to biological organisms.

Excessive exposure to UVA and UVB may induce the formation of cyclobutane pyrimidine dimers, alkaline-labile lesions, DNA crosslinks or breaks, and lesions in proteins.[1–4] In humans, excessive exposure to UVB leads to skin damage, skin cancer, cataracts, and

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immune system suppression.[5] Occurrences of skin lesions, squamous cell carcinomas, and infectious keratoconjunctivitis in domestic animals are attributed to exposure to UVB.[6–10] Once thought to be relatively innocuous, evidence now suggests that UVA exposure may be as damaging as UVB.[11] UVB exposure primarily damages the surface layers of tissue, while UVA penetrates further and causes damage much deeper in human tissues.[12,13]

Despite the partial attenuation of UVR due to reflection at the air/water interface and absorption by dissolved and particulate matter, aquatic organisms are susceptible to UVR induced damage. Calm, clear water allows for deeper penetration of UVR with detrimental biological effects detected at depths of 20 m.[14–20] Sessile organisms (e.g., corals) that lack the ability to move into more sheltered areas and organisms that live suspended in the water (e.g., larval stages of fish and invertebrates, plankton) may be at greatest risk of damage. Many of these at-risk organisms, from Cyanobacteria to teleosts, are protected from excessive UVR exposure by mechanisms, such as melanin pigmentation in amphibian eggs[21,22] and mycosporine-like amino acids in marine organisms,[19,23–26] but limits to this protection may exist. Short-term exposure to increased levels of UVR leads to reduced coral calcification and skeletal growth, decreased photosynthetic activity by zooxanthellae, and reduced survivorship among coral larvae.[27–32] UVR exposure may also contribute to coral bleaching.[29,33–36] Benthic echinoids, *Strongylocentrotus droebachiensis* (green sea urchin) and *Echinarachnius parma* (sand dollar), show decreased survivorship and delayed development in addition to DNA damage when exposed to environmentally relevant levels of UVR.[37] UVB exposure is also reported to reduce activity of bacterioplankton, which consume labile dissolved organic material in the surface layers of the ocean.[38]

Progressive thinning of the protective ozone layer has prompted the initiation of a number of studies on the effects of UVR in aquatic environments. Current technology for measuring UVR in aquatic environments is somewhat limited by cost, electrical power requirements (e.g., batteries), maintenance, deployment depths, and measurable wavelengths. Inherent restrictions of the current technology make it unsuitable for long-term monitoring or sensing in remote locations. Gleason[39] suggests that the ideal instrument for measuring UVR in marine environments would be ''small, self-contained, submersible to at least 50 m, able to scan to at least 300 nm, and be within the budget of most research labs''. Recently, passive sensors for detection and quantification of UVR and visible light have been developed.[40,41] These passive sensors require no power, can be used in air or water (salt or fresh), are small and self-contained, and have no temperature dependence between 0–50°C making them ideal for many long-term monitoring or remote sensing applications.

Materials required

Dosimeters: Colyott et al.[40] describe the design of the passive dosimeters used throughout this study (Figure 29.1). Briefly, the dosimeters are constructed with a Teflon cap containing an interference filter, an attenuator, and a diffuser. The Delrin base contains the thin-layer aluminum oxide detectors developed by Landauer, Stillwater Crystal Growth Division (formerly Stillwater Sciences), 723 1/2 Eastgate, Stillwater, Oklahoma 74074 (phone: 405–377–5161, www.landauerinc.com/affiliates).[42] A bayonet fitting attaches the base to the cap and provides an air and watertight seal. Dosimeters are 25.4 mm in diameter and 19.1 mm long with a 12.7-mm window through which the radiation passes to reach the detector. Dosimeters are read using either thermoluminescence or optically stimulated luminescence technology.[40,41,43]

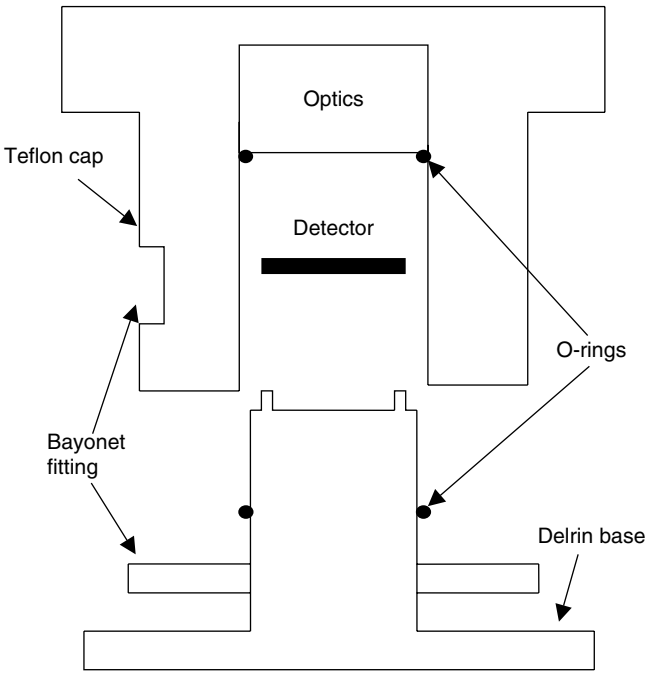

Figure 29.1 Passive dosimeter.

Procedures

Sensor deployment: For the purpose of illustration, we describe a pilot study conducted in a coral reef ecosystem at a remote location. Forty dosimeters were deployed on the edge of a patch reef within Apra Harbor, Guam 13′ 28° N, 144–48° E, on February 19, 2001. Prior to shipping, the manufacturer calibrated the dosimeters to measure UVA or UVB radiation. Dosimeters were fixed in custom-fabricated PVC mounts (Figure 29.2) and attached to the substrate by elastic straps. A bubble level was used to orient the sensors vertically with the collecting window pointed towards the water surface. The most difficult and critical (as supported by the data presented in the following) portion of this process is the stabilizing and leveling of the dosimeters. Even a slight deviation of a few degrees from the plane of the water surface will impact measurements. In shallow water, currents and wave action can have a significant impact. This is especially true for short-term data collection. To this end, it is advisable for dosimeter deployment to be done while on SCUBA if the depths are greater than 1 m. The dosimeters were placed at depths of 3, 9, and 18 m, and exposed for 30 min, 48 h , or 480 h (see Table 29.1). The 30-min exposure was done at a depth of 3 m as the sun approached zenith to achieve optimal penetration.

Climatological data for the period of deployment were obtained from the NOAA website (www.prh.noaa.gov). The availability of such information reinforces the utility of passive dosimetry in remote locations where collection of climatological data on a daily basis would be difficult or impossible. In fact, with appropriate filtering, dosimeters could be left in a remote location without any attention for many months, all the while collecting data. Filtering is pre-set at the factory prior to shipping and can be calibrated based on intended length of deployment. However, it should be pointed out that we have not investigated the potential impact of fouling. It is anticipated that future generations of

Figure 29.2 Passive dosimeter mounted underwater.

Table 29.1 Depth, exposure time, and number of passive dosimeters deployed at each site for UVA or UVB monitoring

Depth (m)	Length of exposure (h)	Number of sensors deployed
3	0.5	2
3	48	3
3	480	3
9	48	3
9	480	3
18	48	3
18	480	3

sensors will be impregnated with anti-fouling compounds and thereby enhance the feasibility of long-term studies. The average air temperature was 33.6°C (range 26.4–39.2°C). Atmospheric conditions ranged from sunny to partly cloudy with no days of complete cloud cover recorded. Average wind was 12.9 kn (range 8.5–15.7 kn). Total precipitation for the duration of sensing was 5.4 cm with a maximum of 2.4 cm falling on one day.

Data retrieval: Upon completion of exposures, the dosimeters were shipped to the manufacturer for data retrieval.

Results and discussion

Cumulative UVA and UVB exposure was determined for three depths, 3, 9, and 18 m, at three exposure times, 30 min (3 m only), 48 h, and 480 h. The results are shown in Figure 29.3 where the average measured dose of UVA or UVB (J/m^2) is plotted against the duration of exposure (h). As expected, the average measured dose decreased with the increase in depth for both UVA and UVB. Average doses of UVA were 2- to 7-fold higher

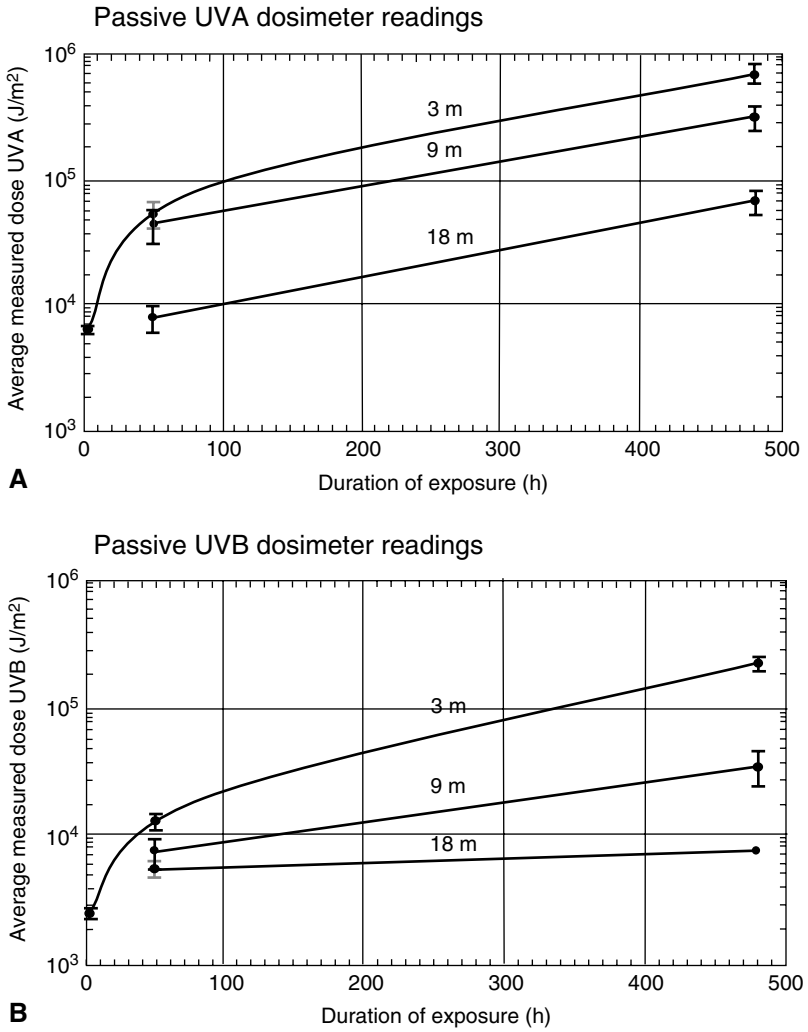

Figure 29.3 Average measured UVR dose as a function of duration of exposure and depth of deployment. Each data point is the mean of measured doses from three separate sensors at the same location with the exception of the 0.5 h time point for both UVA and UVB with a mean calculated from two sensor readings, and 18 m UVB doses calculated from one sensor at 480 h. The error bars represent 1 SD from the mean (A) UVA; (B) UVB.

than UVB levels at the same depths, which is indicative of the higher levels of UVA passing through the atmosphere and the differential attenuation of UVA and UVB as the rays penetrate the water.[44]

The average measured dose of UVA increases almost 10-fold from 48 to 480 h of exposure for all three depths of deployment (Figure 29.3A). An 8-fold difference in average UVA dosage was detected between dosimeters exposed for 30 min and those exposed for 48 h. We would expect to see a much greater difference between these data points. However, the 30-min exposure was performed during the Sun's zenith under clear skies and calm water conditions thereby maximizing the levels of UVA and UVB entering the water. In contrast, the 48 and 480 h exposures include extended periods of partial cloud cover and darkness resulting in a lower cumulative UVA dosage. Decreases in the average measured dose of UVA in relationship to the increase in depth, 1.3-fold for 3–9 m and 6-fold for 3–18 m, suggests that similar levels of UVA rays are able to penetrate to 3 and 9 m. Under clear, calm conditions these results are highly likely.[45]

Average measured UVB exposure for dosimeters placed at a depth of 3 m showed an approximate 8-fold increase from 30 min to 48 h and a 10-fold increase from 48 to 480 h (Figure 29.3B). The 8-fold increase from 30 min to 48 h is identical to the UVA data for the same exposure comparison, which suggests that similar factors might have affected UVB exposure. In contrast, a 5-fold increase and a 1.4-fold increase were measured for 48–480 h UVB exposures at 9 and 18 m, respectively. The dramatic decrease in UVB dosage with the increase of depth is most likely due to increased attenuation of the short UVB rays[44,46,47] and the inability of UVB to penetrate effectively into depths greater than 6 m.

Data from two of the three sensors exposed to 480 h of UVB at 18 m were not used to calculate average dose due to values that suggest a misalignment might have occurred during sensing. Since the UVA dosimeters located in the same areas provided exposure measurements similar to the third UVA dosimeter measurement, we eliminated the possibility that an increase in suspended particles or wave activity is responsible for the unusual UVB readings. This illustrates the importance of properly securing and aligning the dosimeters at the initiation of the sensing period.

Excessive exposure to UVR can lead to reduction in reproductive capacity, impaired larval development, and even death in aquatic organisms. The ability of researchers to perform long-term or remote studies of UVR in aquatic environments has been limited by sensors that require a continuous input of power, are prohibitively expensive, or difficult to use. Recent technological advances have led to the development of passive sensors that function without an active power source. The passive sensors offer researchers a reliable and cost-effective tool for long-term and remote monitoring of UVR and photosynthetically available radiation in aquatic environments.

Acknowledgments

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section four

Techniques for aquatic toxicologists

chapter thirty

Spectral models for assessing exposure of fish to contaminants

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Contents

Introduction

Fourier transform-infrared (FT-IR) spectroscopy is capable of identifying a wide variety of chemical structures on the basis of their unique vibrational and rotational properties.[1–3] DNA has a characteristic ''signature'' spectrum, the peaks, shoulders, and other spectral properties of which have been identified by spectroscopists as corresponding to specific structures in the DNA molecule.[1] In addition, statistical models of FT-IR spectra have the

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remarkable ability to reveal subtle changes in complex cellular structures resulting from various biological and chemical stresses.[3,4] Recent examples include the ability to discriminate, with high sensitivity and specificity, between the DNA of healthy and cancerous prostate tissues, thus providing a basis for predicting the probability of prostate cancer.[5] Furthermore, the unique ability of the FT-IR statistical models to differentiate between diverse groups of tissues was evident when it was shown that primary prostate tumors could be readily distinguished from metastasizing primary tumors. This achievement was the basis for statistical models for predicting which tumors are most likely metastasizing without having to wait for metastatic cells to be detected at distant sites in the body (e.g., the groin), at which point treatment options are limited.[5]

We recognized that the FT-IR statistical models had the potential for studying the effects of toxic chemicals on fish. In 1997, we showed that liver DNA of English sole (*Parophrys vetulus*) from the chemically contaminated Duwamish River (DR) in Seattle, WA was structurally different from that of English sole from the relatively clean, rural environment of Quartermaster Harbor (QMH) in Puget Sound, WA.[4] A subsequent study, conducted in October 2000,[6] showed that the DNA from the gills of English sole from the DR could be readily distinguished from the gill DNA of the same species from QMH. The FT-IR spectral differences between groups were consistent with a marked increase in the sediment contamination (e.g., concentrations of polychlorobiphenyls [PCBs] and aromatic hydrocarbons) and the degree of CYP1A expression in the gills. A logistic regression analysis of the spectral data sets resulted in the development of a *DNA damage index* with high sensitivity and specificity.

The present report illustrates the application of the FT-IR statistics technology to assess differences in the DNA structure of various fish tissues between reference and contaminated environments. The resulting data can be used for assessing the quality of marine environments, toxic effects on fish, and the effectiveness of remediation protocols. Overall, the FT-IR statistics technology is best used in conjunction with other markers of exposure or toxicity, such as CYP1A expression[7–9] and various histological[10] and histochemical indices.[7,11] Although initially applied to fish, this technology has the potential for application to various other aquatic organisms, in addition to a variety of human diseases.

Materials required

Tissues

Groups of fish (preferably sex-matched and not differing significantly in size and mass) are obtained from contaminated and essentially non-contaminated reference sites. Females should be restricted to those with quiescent gonads to minimize the effects of reproductive stage (e.g., suppression of CYP1A by estradiol) on the biomarker data. Each fish should be given a unique identification and carefully weighed and measured. In field studies, fish are kept alive until sacrificed via decapitation aboard the vessel. The desired tissue (e.g., gill, liver) is removed and immediately frozen in liquid nitrogen. Tissues should be maintained in a $-80^{\circ}C$ freezer until DNA extraction. Prior to freezing, a few milligrams of the tissue are preserved in neutral formalin for histological examination or histochemical determinations or both (e.g., CYP1A).[7,11,12] Otoliths may be removed for subsequent age determinations.[13]

Supplies and equipment for DNA extraction

- Scalpels, forceps, spatulas
- Mortars and pestles
- Liquid nitrogen
- Qiagen Genomic DNA Buffer Set, #19060
- Qiagen Genomic-tip 100/G, #10043
- Falcon 15-ml graduated polypropylene tubes (conical bottom), #352096
- Osmonics Cameo 30N syringe filter, nylon, 5.0 μ, 30 mm, #DDR50T3050
- Roche RNAse A (1 mg ml^{-1}), #109169
- Worthington Proteinase K (20 mg ml^{-1}), #LS004222
- Isopropanol
- Ethanol 70% (ice cold)
- Microcentrifuge tubes 2 ml, polypropylene
- Transfer pipettes 1.5 and 3 ml, disposable, polyethylene
- 50°C water bath
- Refrigerated (4°C) centrifuge
- Microcentrifuge at 4°C
- Optima grade water (Fisher), #W74LC

Supplies and equipment for FT-IR spectral analysis

- FT-IR microscope spectrometer (System 2000, Perkin-Elmer)
- BaF_2 plate, 38.5 mm × 19.5 mm × 2 mm (International Crystal Laboratories, Garfield, NJ)
- Aluminum BaF_2 plate holder (Custom-made for the Pacific Northwest Research Institute, Seattle, WA; See Figure 30.1)
- 0.1–2.5 μl pipetter with tips
- Dissecting microscope

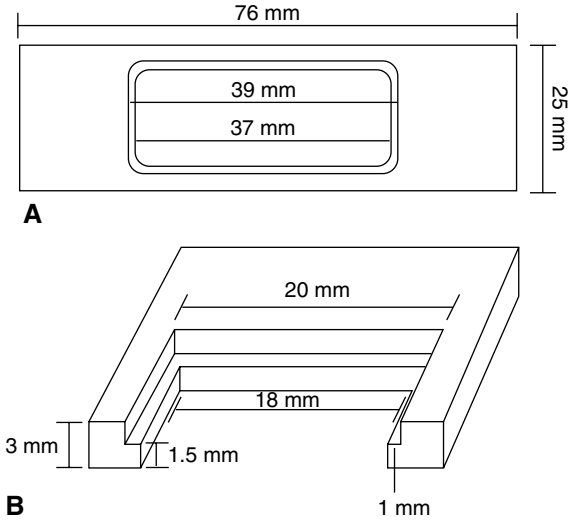

Figure 30.1 (A) Diagram of custom-made aluminum BaF_2 plate holder and (B) cross-section schematic. Dimensions are given; images are not to scale.

Procedures

DNA extraction

Frozen tissue (~100 mg; −80°C) is ground to powder with a mortar and pestle while submerged in liquid nitrogen. DNA (~50 μg) is then extracted from each sample with Qiagen 100/G Genomic-tips (Qiagen, Chatsworth, CA) using the standard Qiagen extraction protocol with the following modification: after elution, the DNA solution (eluate) is passed through a 5.0-μ Cameo 30N filter (Osmonics, Minnetonka, MN) to remove residual resin from the Qiagen Genomic-tip prior to precipitation. After filtration, the Qiagen protocol is resumed. In preparation for FT-IR spectral analysis, the DNA pellet is dissolved in 10–40 μl (depending on size) of optima grade water (Fisher Scientific). The DNA is allowed to dissolve overnight at 4°C. The Qiagen procedure is an ion-exchange system and does not constitute a source for artifactual oxidation of purines during extraction.

FT-IR spectroscopy

A 0.2-μl aliquot of the DNA solution is spotted directly on a BaF_2 plate and allowed to spread, forming an outer ring that contains the DNA. Two separate spots are created for each DNA sample. The spots are allowed to dry. Spotting is repeated until the ring is at least 100 μ wide, the width of the aperture of the System 2000 microscope spectrometer (Perkin-Elmer). The plate is then placed in a lyophilizer for 1 h to completely dry the DNA. Initially, a background energy reading (percent transmittance) is determined from a blank area of the BaF_2 plate. Energy readings are then taken at various points around the ring (Figure 30.2), and the points for spectral determinations are selected where the energy readings are 15–25% less than the background energy (optimally close to 15% less). Ten spectral determinations are made around each of the two rings per sample and the percent transmittance values are converted (Fourier-transformed) into absorbance values (Figure 30.2). The spectral data obtained are saved in a database for subsequent statistical analysis. Using MS Excel, each spectrum is baselined by taking the mean absorbance across 11 wave numbers, centered at the minimum absorbance value between 2000 and 1700 cm^{-1}, and subtracting this value from the total absorbance at each wavenumber. Each spectrum is then normalized by dividing the entire baselined absorbance values by the mean absorbance between 1750 and 1550 cm^{-1}. Baselining and spectral normalization adjust for the optical characteristics of each sample (e.g., related to film thickness). The mean absorbance value of the 20 spectral determinations for each sample is then calculated at each wavenumber between 1750 and 1275 cm^{-1}.

Other considerations

To avoid any batch effects, it is recommended that samples from reference and contaminated sites be randomized during DNA extraction and FT-IR spectroscopy. Tissues may be ground and stored at −80°C prior to DNA extraction. One technician can extract 6–12 samples, using the modified Qiagen protocol, in about 6 h, including a 2-h incubation time. FT-IR analysis should be performed on the extracted samples within a day or two. For long-term storage, we do not recommend that the DNA be kept in water, but rather in the dry state at −80°C.

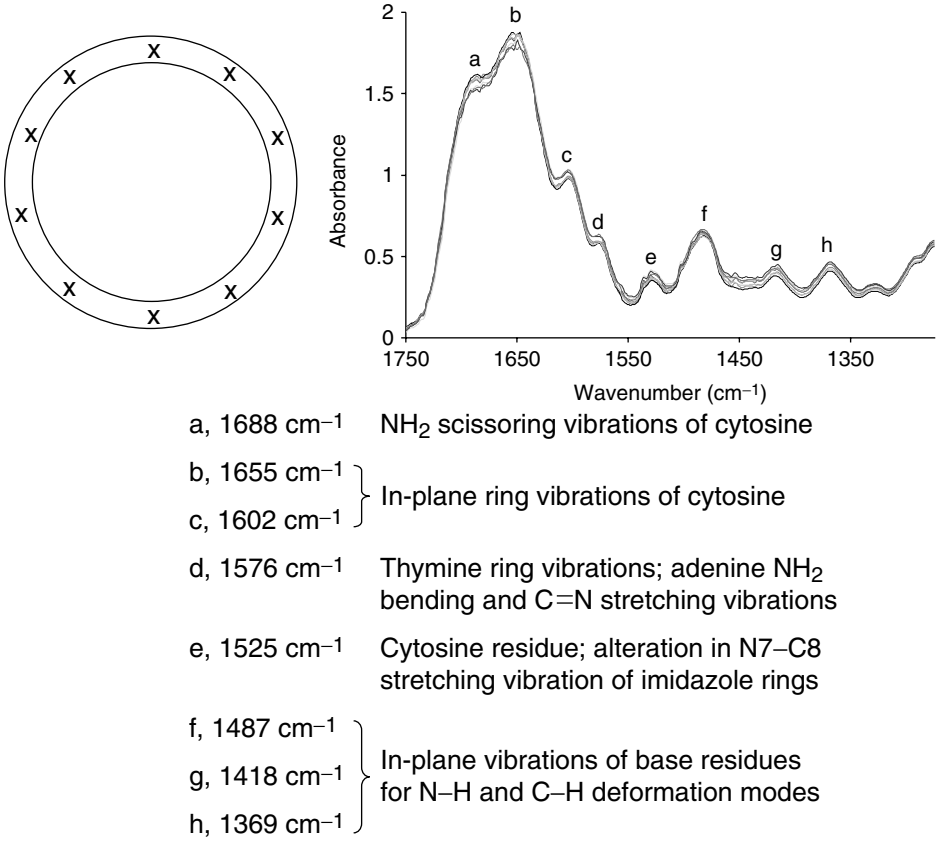

Figure 30.2 A DNA ring with 10 spectral determination points (x), the subsequent DNA spectra obtained, and the wavenumber and structural assignments for designated peaks.

Statistical analyses

FT-IR mean spectra

A mean spectrum is determined for each fish group (i.e., from contaminated and reference sites; Figure 30.3A). A *t* test is then performed at each wavenumber to establish statistical differences (*P* values) between the mean spectra (Figure 30.3B). Over the wavenumbers used, spectral regions with $P < 0.05$ are likely to represent real structural differences between groups when they comprise 5% of the spectral range.[3] These structural differences represent alterations in various aspects of the DNA molecule (e.g., as illustrated in Figure 30.2).

Principal components analysis

Statistical model development is accomplished by first conducting principal components analysis (PCA) on the mean spectrum of each individual DNA sample (S-Plus 2000 Professional Release 1, Mathsoft Engineering & Education, Cambridge, MA). PCA entails nearly 1×10^6 correlations between ~1000 independent variables relating to the absorbance, wavenumbers, and other properties of the spectrum.[14] PCA results in 10 principal

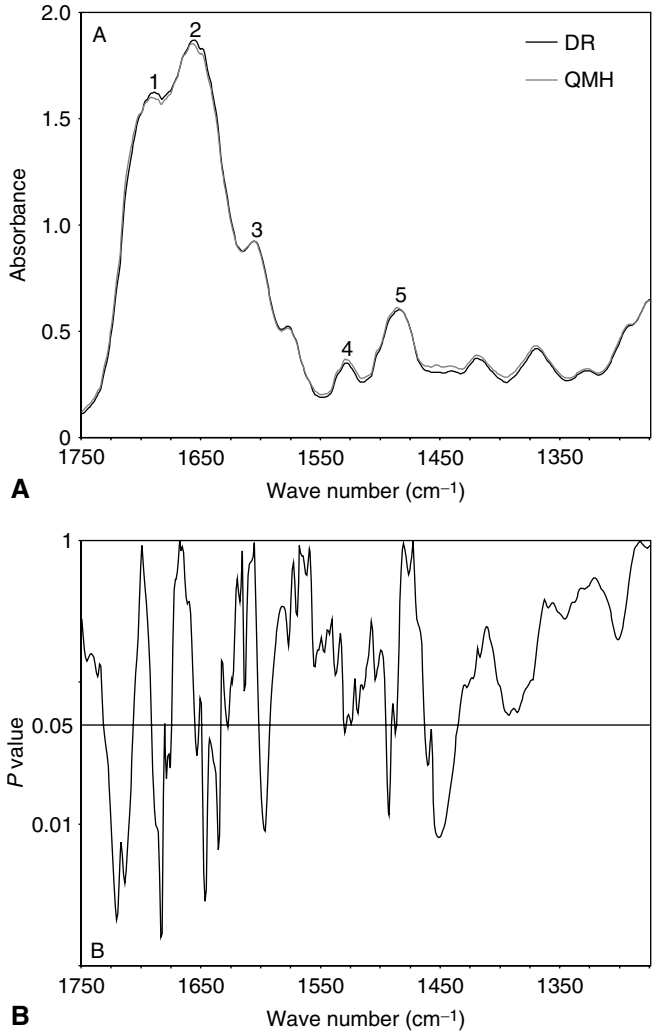

Figure 30.3 (A) Comparison of mean FT-IR spectra of gill DNA from DR and QMH fish; (B) *P* values from a *t* test comparing mean spectra at each wave number. The peak wavenumber designations are as follows: 1, 1688 cm^{-1}; 2, 1655 cm^{-1}; 3, 1602 cm^{-1}; 4, 1525 cm^{-1}; 5, 1487 cm^{-1}. (Modified and reprinted from Malins, D.C., Stegeman, J.J., Anderson, J.W., Johnson, P.M., Gold, J. and Anderson, K.M., *Environ. Health Perspect*. (Perspect., 2004, 112: 511–515.)

component (PC) scores for each sample. Significant differences ($P \leq 0.05$) in each PC score between groups are determined using *t* tests. The PC scores showing the most significant differences between the groups are used to construct two- or three-dimensional PC plots (Figure 30.4). Those PC scores representing fish with similar DNA structures will cluster together and be separated from other clusters reflecting a different DNA structure.

DNA damage index

Logistic regression analysis is performed (SPSS statistical package 10.0, SPSS, Chicago, IL) using a single, significant ($P \leq 0.05$) PC score to establish a DNA damage index. Using a

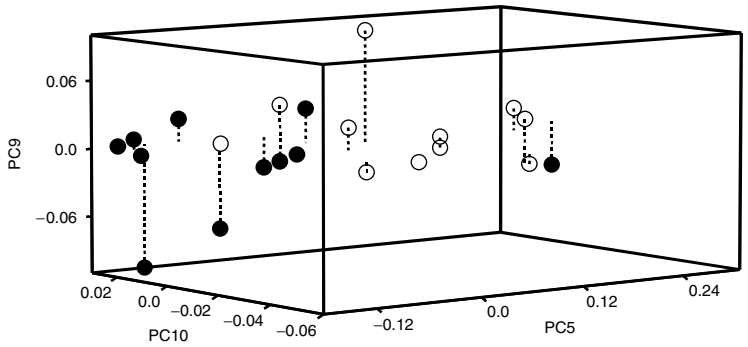

Figure 30.4 Three-dimensional separation of PC scores from the FT-IR spectra of gill DNA from the DR (● ; $n = 11$) and QMH (○ ; $n = 11$) fish. Dotted drop lines represent the distance from the PC9 baseline level of 0. (Reprinted with permission from Malins, D.C., Stegeman, J.J., Anderson, J.W., Johnson, P.M., Gold, J. and Anderson, K.M., *Environ. Health Perspect*. (Perspect., 2004, 112: 511–515.)

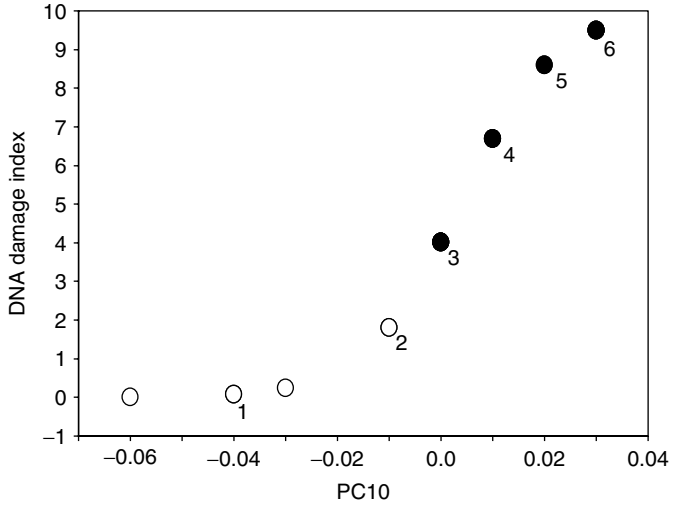

Figure 30.5 DNA damage index for gill DNA from the DR (● ; $n = 11$) and QMH (○ ; $n = 11$) fish. Overlapping points: 1, two QMH; 2, one DR and one QMH; 3, one DR and five QMH; 4, four DR; 5, one DR and one QMH; 6, four DR. (Reprinted with permission from Malins, D.C., Stegeman, J.J., Anderson, J.W., Johnson, P.M., Gold, J. and Anderson, K.M., *Environ. Health Perspect*. (Perspect., 2004, 112: 511–515.)

scale of 1–10, this index is based on the different spectral and structural properties of DNA from each fish group (Figure 30.5) and is a measure of DNA damage that provides a means to discriminate between fish from reference and contaminated sites.[6]

Results and discussion

Comparison of mean DNA spectra

As an example, comparison of the mean FT-IR spectra for the DNA from the DR ($n = 11$) and QMH ($n = 11$) fish is given in Figure 30.3A. Some of the differences in the mean

spectra may appear to be almost imperceptible; however the P values at each wavenumber shown in Figure 30.3B indicate that significant differences ($P \leq 0.05$) were found for the five peaks identified in Figure 30.3A. The peak differences occurred at the following wave numbers: 1688, 1655, 1602, 1525, and 1487 cm^{-1}. The structural assignments for these peaks are given in Figure 30.2 and represent an array of differences in the structures of the nucleotide bases between the two DNA groups. Significant differences between the mean DNA spectra for the two fish groups represented 26.5% of the spectral range (differences <5% may occur by chance).[3] Although a broader range of wavenumbers can be used (i.e., from 1750 to 700 cm^{-1}),[4] we have found that the narrower range used here (i.e., from 1750 to 1275 cm^{-1}) has given the most reliable results with studies of fish DNA. This spectral range primarily represents vibrations of the nucleotide bases, thus spectral differences between groups of DNA would include structural changes associated with the genome. Comparison of mean spectra is a procedure for identifying the nature and extent of DNA structural differences (Figure 30.2) in fish from reference and contaminated environments. These comparisons also allow for initial evaluation of whether sufficient differences exist between the group mean spectra to justify further statistical analyses. However, the technique of PCA described in the following paragraph has the ability to discriminate between groups, even when the spectral means show few, or even no differences.

Principal components analysis

PCA is a powerful means of discriminating subtle differences in DNA structures between groups of fish from different environments. Groups of DNA samples representing fish from contaminated and reference environments will cluster in different areas of the PC plots by virtue of their different spectral and structural properties. An example of this discrimination is a three-dimensional projection of PC scores (PC10, PC5, and PC9) representing the gill DNA from the DR (contaminated) and QMH (reference) fish (Figure 30.4). Despite the high degree of separation, the relatively small number of overlapping points between the groups may reflect fish migrations or other aberrations known to exist in natural fish populations.

DNA damage index

In this example with gill DNA, logistic regression analysis was conducted based on PC10, selected for its significance ($P < 0.01$). The resulting sigmoid-like curve shows a distinct separation between the DR and QMH fish groups with 9/11 DR scores falling above ~5.0 on the index and 10/11 of the QMH scores falling below this value. This indicates that the DR fish may have more damage to their gill DNA than the fish from QMH, which is further substantiated by the sediment chemistry and CYP1A data.[6] This results in an 82% probability of correctly identifying a DR sample and a 92% probability of correctly identifying a QMH sample.

The DNA damage index provides a means of quantifying the DNA damage between fish from reference and contaminated environments. This index can be determined for any two fish populations to assess environmentally induced DNA damage using a variety of tissues (e.g., gill, liver, gonads, and kidney). The example described employing fish gill[6] has the added advantage of non-lethality if tissues are obtained via punch biopsies.[15] One attractive application of the FT-IR statistics technology would be to determine the

effects of remediation on chemically contaminated aquatic environments. After remediation, DNA samples would be evaluated using the damage index developed for that specific environment, species, and tissue type to determine whether the spectral and structural characteristics had improved to closely match the index values established for the reference site. The usefulness of the DNA damage index has so far been limited to the study of English sole in Puget Sound.[6] We look forward to the application of the FT-IR statistics technology, including the DNA damage index, to other fish and aquatic species, as well as to other environments having different contaminant profiles.

Acknowledgments

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chapter thirty-one

Design and use of a highly responsive and rigidly controllable hypoxia exposure system

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Contents

Introduction

Broad changes in oxygen concentrations are characteristic of aquatic ecosystems impacted by eutrophication. Daily cycles of oxygen levels in estuaries can range from daytime supersaturation reaching as high as 300% to predawn anoxia (0%).[1–3] To test the effects of hypoxia in aquatic animal models, we have designed an exposure system capable of rapidly changing dissolved oxygen (DO) conditions in experimental tanks. The system was assembled from readily available components and allows precise, programmable control of DO concentrations in the laboratory setting.

When dealing with sublethal stressors, complicating factors, such as time to effect and spatial relevance, make field studies impractical. The mobility of free-ranging species, and the dynamic variability of aquatic systems makes clearly characterizing the exposure history of an individual challenging. Exposure to stressors may occur days prior to and miles apart from a fish kill or sampling site.[4–6] Controlled laboratory studies, however, can be

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used to document the contribution of individual environmental factors to specific health problems.

Periods of hypoxia or anoxia can have profound effects on aquatic organisms. Anoxia has been associated with the loss of benthic invertebrates in eutrophied ecosystems,[7] as well as fish kills.[8] In addition, hypoxic events have been proposed to play a role in more subtle disease conditions, such as reproductive or developmental abnormalities.[9,10] The spectrum of health effects observed in fish populations, although implied, is poorly understood mechanistically. An example is the association of hypoxic events with epizootic ulcerative syndrome (EUS), a disease which has affected countless Atlantic menhaden (*Brevoortia tyrannus*) along the east coast of the United States from Chesapeake Bay to the estuaries of the Carolinas.[11–13] Originally ascribed to the *Aphanomyces* fungus[14] and more recently to dinoflagellate *Pfiesteria* spp.,[5] the root cause(s) of EUS remain largely unknown.[15–17] Histopathological studies performed in our laboratory on hundreds of specimens frequently showed no evidence of a specific pathogen that could be associated with the disease.[18] Moreover, EUS occurrence in the estuaries of North Carolina follows a seasonal trend with temperature and hypoxic events.[19]

Fish have gained popularity as animal models in aquatic toxicology as recent advances have increased our knowledge of normal physiologic conditions and responses to various stressors.[20] Fish models are also increasingly being used in research leading to information regarding human diseases and genetic and reproductive responses.[21] In this chapter, we describe a laboratory system for examining the response of aquatic species to hypoxia. Experiments with wild caught Atlantic menhaden and laboratory-reared Nile tilapia (*Oreochromis niloticus*) were used to demonstrate the functionality and limits of the system. The tilapia served as a resistant species and the menhaden, based on the association of estuarine hypoxia with ulcerative skin lesions in these fish, served as a susceptible species.

The system described provides a means to investigate responses to hypoxia as a single variable or in combination with other stressors. The use of nitrogen gas to reduce the partial pressure of dissolved gases is not novel in aquatic research. The reduction in oxygen tension can be isolated and studied if the proper mixture of gases is used to emulate atmospheric carbon dioxide, argon, and nitrogen ratios. This method works because fish, unlike other vertebrates, sense O_2 in their environment in place of CO_2.[22–24] The system can readily be used for aquatic organisms other than fish or for complex mesocosm studies. Using O_2 in place of N_2 or component air can also rapidly create hyperoxic experimental conditions. Other advantages this system has over traditional methods include its controllability, monitoring systems, rapid oxygen partial pressure changes, and automated data logging and graphing. Herein, we describe the system and provide some example data from two experiments to demonstrate its use. DO levels for a longer-term hypoxia exposure were based on acute LC_{50} values for the two fish species. We chose a series of endpoints to test the hypothesis that hypoxia and subsequent reperfusion create oxidative cellular damage as a factor in the development of ulcerative skin lesions in fish.[25–28]

Material requirements and setup

A wide range of tank sizes can be used with this system depending on the size and flow rating of the protein skimmer, or foam fractionator, employed and species-specific requirements. Our system includes 260-l fiberglass circular tanks for the 1- and 2-h acute exposures and 855-l dark blue, circular polyethylene tanks for the long-term exposures.

Each tank has a bulkhead located on the bottom (center) and on the sidewall at the bottom. The center bulkhead was for drainage and tank cleaning purposes. The sidewall bulkhead is plumbed into an external Iwaki-Walchem 55RLXT pump (Walchem Corp., Holliston, MA). Two or more tanks should be employed, each as an independent unit, to house control and exposed animals.

The system functions by use of a Neptune Systems (San Jose, CA) Aquacontroller Pro unit connected to laboratory grade pH, DO, temperature, and conductivity probes (Figure 31.1). Data from the probes are monitored by the controller and logged both by the controller and by Aquanotes (Neptune Systems) software on a computer connected directly to the controller via a serial port. The controller can be operated via the computer (local or internet) or directly on the controller via its simple programming language. User-defined settings toggle system components on and off remotely. For example:

$$\begin{aligned} \text{DO} &> 1.0\,\text{mg/l} = \text{on} \\ \text{DO} &< 0.9\,\text{mg/l} = \text{off} \end{aligned} \tag{31.1}$$

When toggled on, the system functions by opening a solenoid and turning on the Iwaki-Walchem feed pump. The skimmer (AE Tech, ETSS Professional 800) is a passive device that forces air (or compressed gases) and water to mix at high rates via a downdraft mechanism. The skimmer must be placed at or above the level of the exposure tank's resting water position to allow for gravity feedback to the tank. This allows efficient and rapid mixing of the exposure tank water with contained gases, in this case pre-purified grade nitrogen.

Standard 300-ft^3 nitrogen tanks with nitrogen-rated regulators are used as a source of nitrogen. (*Note*: Compressed gas tanks should be properly secured to a wall or other stationary structure according to current institutional safety regulations.) The nitrogen gas passes from the tank and regulator through a stainless steel and glass flow meter

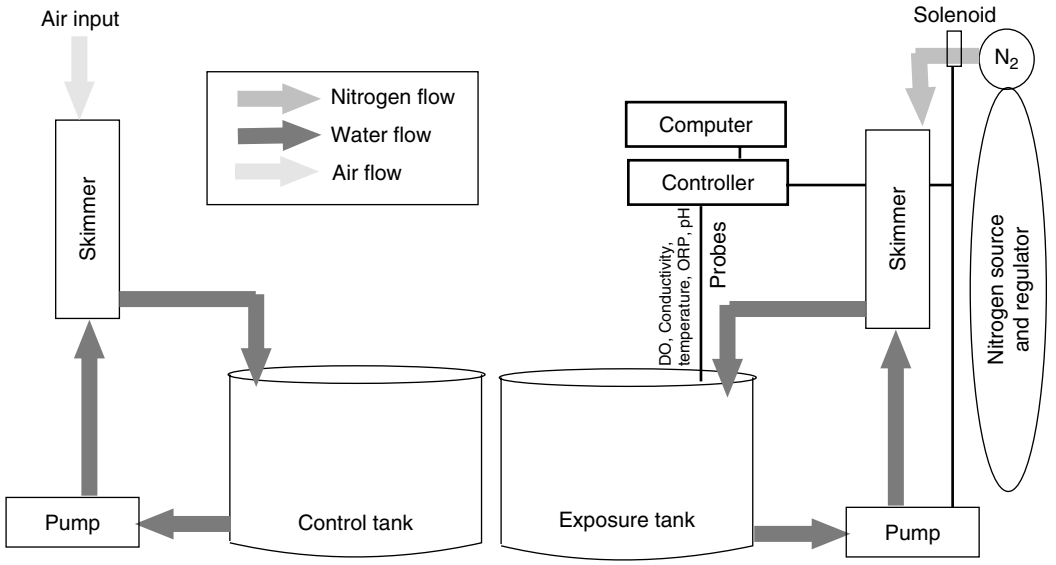

Figure 31.1 DO exposure system schematic showing sequence of controller and direction of laminar fluid and gas flow. Tanks of any size from 150 to 1500l can be used on this system depending on the capacity and volume rating of the skimmer used.

(Dwyer, model SS-DR12442). Flow meters bracket ranges of gas volume per time so the choice of flow meter model necessarily depends on the volume of nitrogen released per hour into the skimmer. The flow meter has a needle valve for controlling 5–20 standard cubic feet per minute (scfm) input into the system, so that flow rates can be finely adjusted while the regulator is static. The solenoid valve, placed between the regulator on the N_2 source and the skimmer, is turned on and off based on signals from the controller to allow gas to flow to the skimmer.

Signals are relayed from the controller via an x10 control module (www.x10.com). This module codes the signal and passes it along the electrical lines of the building allowing for remote control of x10 appliance modules. The controller specifies the channel, and each module set to that channel will respond with an ON/OFF switch. For example, when the controller reads DO at 1.1 mg/l, it then sends a signal to the unit(s) connected to the solenoid and the feed pump for the skimmer thereby initiating the scrubbing of the exposure tank water that circulates from the tank, through the skimmer, and returns to the tank via PVC plumbing with a reduced oxygen concentration. (Table 31.1)

Water for the menhaden exposures was made with synthetic sea salt (Instant Ocean, Mentor, OH) to 12.5 ppt (19 mS/cm) and allowed to mix for at least 24 h prior to use. Water was mixed between tanks before introducing the randomized fish to eliminate slight differences between water parameters. Each tank was equipped with a 2 mil clear plastic cover secured by elastic lines and clamps to prevent fish from gulping air at the surface during exposure. The clear plastic allowed easy observation of the fish during the study.

Atlantic menhaden were collected from a reference site, the White Oak River, NC. The fish were cast netted, placed into filtered, flow-through tanks and held for a minimum of 2 weeks. Menhaden had a mean fork length of 17.5 cm and mean weight of 71.8 g. Feeding commenced on the second day of holding and continued twice daily with salmon starter crumble (Zeigler Bros., Gardners, PA) ground to a fine consistency. After acclimation, fish were transported to the laboratory in a large, round, enclosed transport tank with heavy aeration and circulation to minimize stress. Temperature was maintained during transport by frozen blocks of water in sealed containers or by aquarium heaters. At the laboratory, fish were acclimated for a further 2 weeks prior to the experiment and remained apparently healthy. In parallel experiments, healthy tilapia with a mean tail length of 19 cm and mean weight of 156 g were randomized into the exposure tanks using aged and dechlorinated tap water. Tilapia were a gracious donation from NC State University Fish Barn.

Table 31.1 Required materials for exposure system setup

Item	Model	Manufacturer
Skimmer	ETSS Pro 800	AE Tech
Pump	55RLXT	Iwaki-Walchem
Solenoid	115 V a/c static off/powered on type	National Welders
Tanks	260 l circular fiberglass	Custom
	855 l circular polyethylene	PolyTank, MN
Regulator	Nitrogen-rated, M1-960-PG	National Welders
Controller	Aquacontroller Pro and lab grade probes	Neptune Systems, CA
Computer	Any Pentium model	Any
PVC pipe	1.5 in. rigid piping	Any
PVC tubing	1 in. flexible tubing	Any

Validation of DO concentrations was performed daily using a handheld YSI-85 portable meter (Yellow Springs, OH). All DO probes had new membranes at the start of each experiment and were calibrated daily. All other probes were calibrated at the start of each experiment.

Procedures

All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC), NC State University. Acute exposures were performed in 260-l round fiberglass custom tanks with either menhaden or tilapia. Each tank was prepared as mentioned earlier and contained 5 and 7 randomly selected fish, respectively. Fish were acclimated to the exposure tanks for a minimum of 2 days prior to initiating the experiment. Feeding was halted and tanks were cleaned 24 h prior to initiation. Water quality parameters [ammonia, nitrite, nitrate, and hardness (for tilapia only)] were monitored daily. A separate tank was used for each oxygen saturation level. Menhaden were exposed to 84/6.7 (control), 20/1.59, 15/1.19, 10/0.79, and 5/0.39 (% oxygen saturation/mgL^{-1}) for 1 h in independent tanks. Tilapia were exposed to 82/6.9 (control), 20/1.68,10/0.83,7/0.58, and 3/0.24 (% oxygen saturation/mgL^{-1}) in independent tanks for 2 h.

Exposures were initiated, and a log was kept of DO (% saturation and mg/l), pH, temperature, and mortality at 5-min intervals. Moribund fish, as indicated by uncontrolled swimming behavior or lack of response to physical stimuli, were removed for sampling. At the end of 1 or 2 h of exposure, remaining fish were euthanized and sampled.

Euthanasia was performed by overdose of MS222 (Argent Chemical Laboratories, Redmond, WA) in water from the tank in which the fish was exposed to maintain the oxygen saturation level. Sampling consisted of taking length and weight measurements, drawing blood for clinical pathology, and taking tissue samples for histopathology, oxidative stress, and immune function measurements. Fish were examined for gross abnormalities upon dissection. Blood and spleen samples were taken from all fish for immune function analysis (not covered herein). Samples of heart, liver, anterior kidney, intestine, gonads, gills, and spleen were fixed in 10% neutral buffered formalin for 24–48 h and then held in 70% ethanol for histopathology. Samples of muscle, liver, and blood were placed in 2-ml cryovials and snap-frozen in liquid nitrogen for later analysis of oxidative stress endpoints.

Clinical pathology

To determine blood parameters, we used a Portable Clinical Analyzer (i-Stat Corp., East Windsor, NJ) with expendable cartridges that self-calibrate upon insertion into the unit. The EG7+ cartridge employed displays results from analysis of a few drops of fresh blood for the following parameters: sodium, potassium, ionized calcium, hematocrit, pO_2, and pCO_2. Blood was drawn from the caudal vein of the fish in a syringe free of anticoagulant. Analyses for lactate dehydrogenase (LDH), creatinine kinase (CK), aspartate aminotransferase (AST), glucose, and total protein were performed by the Clinical Pathology Laboratory at the NCSU College of Veterinary Medicine. Blood samples for clinical pathology were taken in heparin-coated syringes, kept on ice, and sent directly for plasma chemistry analysis.

Histopathology

For histopathological examination, tissues were fixed in 10% neutral buffered formalin for 24–48 h, routinely processed by paraffin embedment, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Tissue sections were evaluated by a single pathologist and assigned a grade from 0 (no remarkable abnormalities) to 5 (severely lesioned).[29]

Data analysis

Results of clinical pathology and histopathology were analyzed using JMP (SAS, Cary, NC). ANOVA and Dunnett's tests were used for all comparisons using 84% and 80% oxygen saturation levels as controls for menhaden and tilapia, respectively.

Results and discussion

The controlled DO exposure system described here is very efficient and less demanding of personnel for operation than traditional systems. It responds rapidly to computer or controller commands. Monitoring can be performed remotely and data logging is automated, allowing for better control and replication of experiments. DO was reduced to 20% saturation (1.6 mg/l) in 1 h in an 855-l tank, and levels remained steady over a period of 96 h with an SD ± 0.091 mg/l during that time (Figure 31.2). Effective exposure of animals to stressors in toxicologic research hinges on controlled applications, and this system increases the control by reducing variability. Isolation of factors from extraneous

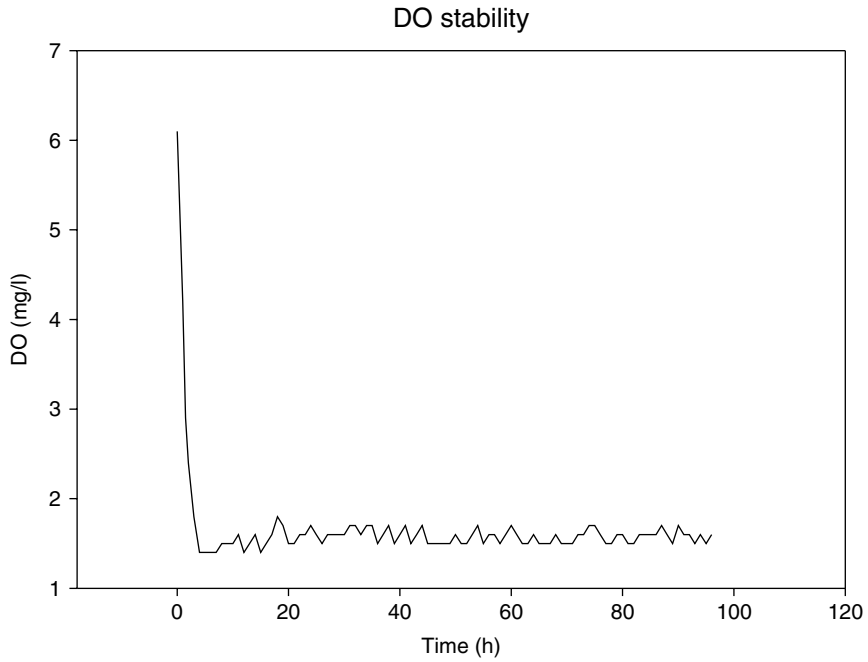

Figure 31.2 DO stability over time in laboratory trials. Trials proceeded for 96 h from initiation time. DO level of 1.6 mg/l (20% oxygen saturation) was held within 0.091 mg/l over the time course after initial oxygen reduction.

inputs is also critical for reproducibility and a realistic determination of the biological effects of each factor.

LC_{50} determination with Atlantic menhaden in these experiments mirrored the previously published data indicating a DO level of 16% saturation (1.2 mg/l) for 1 h as the approximate lethal concentration. Tilapia were highly resistant to challenge by hypoxia evidenced by a moderate response at 3% saturation (0.24 mg/l). Tilapia mortality was low, only 28% over the 2-h exposure period, such that the data generated were insufficient to determine an accurate LC_{50} (Figure 31.3).[6]

Fish showed behavioral changes as a result of the hypoxic stress. Menhaden are a filter feeding, continually active fish. The inability to rest and preserve energy stores is obvious in comparison with tilapia regarding responses to treatment. At 10% saturation, menhaden were obviously stressed and several began to search for the surface to gasp at the air–water interface. At 5% saturation, they were visibly agitated, and all tried to reach the surface of the tank. Tilapia responded to 7% saturation by disengaging territorial behaviors and resting on the bottom and occasionally an individual fish would attempt to gasp from the surface. At 3% saturation, many of the tilapia would intermittently gasp at the surface but then return to the bottom.[30] This response indicates that they have mechanisms for reducing metabolic oxygen demand and are a valid choice as a hypoxia-resistant species.

Results of the acute exposures suggest that the exposure system works very efficiently and mimics environmental hypoxia with minimal additional stress on test subjects. Blood electrolyte changes validated the adverse effect of hypoxia on the test fish, and changed

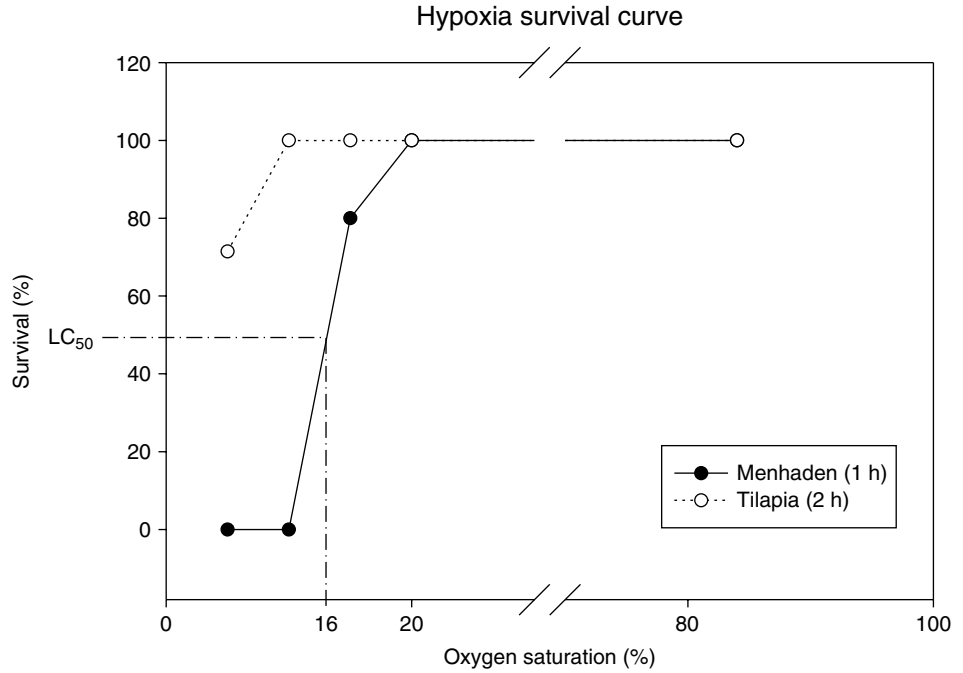

Figure 31.3 Percent survival in acute hypoxia exposures for both menhaden and tilapia (1 and 2 h, respectively). Menhaden displayed an approximate LC_{50} of 16% oxygen saturation (1.2 mg/l), while tilapia proved extremely hardy down to 3% oxygen saturation (0.24 mg/l). $N = 5$ menhaden and 7 tilapia per saturation level.

sharply as the fish passed from a mild to a severe stress state with reduced oxygen tensions. Partial pressure of CO_2 in the blood fell as oxygen saturation decreased in the exposure tank, indicating that O_2 availability is coordinately falling (Figure 31.4). Blood ion concentrations likely shift in response to a depletion of ATP stores and the lack of ability to regenerate those energy stores during a failure in oxidative phosphorylation.[31] A concomitant drop in pH suggests that anaerobic metabolism occurs as a salvage effort. Reduced ATP concentrations would also lead to a failure of the ATPases that maintain homeostasis in the blood. Failure of the sodium–potassium ATPase in many cells of the body and by the ATPases that drive chloride cell function allow for increases in sodium, calcium, and potassium in the blood. Menhaden showed a significant increase in K and Na at 10% saturation. Menhaden also responded with significant increases in ionized Ca (iCa), potassium (K), sodium (Na), and glucose at 5% saturation. Tilapia showed increases in K and iCa at 3% saturation (Figures 31.5 and 31.6).

Histopathology showed only mild parasitism in both treated and control menhaden, a reflection of being wild caught specimens. No significant difference in lesion prevalence was seen between treated and control menhaden. Likewise, no remarkable microscopic lesions were found in the tilapia specimens to suggest that hypoxia alone causes ulcerative skin lesions in fish. This is consistent with our findings from other biomarkers of oxidative stress (not discussed here) using this system. While both menhaden and tilapia showed strong physiologic responses to extremely low oxygen saturation levels, there appeared to be no overt oxidative damage to cells generated from these exposures. This may suggest an indirect or perhaps supplemental role for hypoxia in EUS and is an area in need of further study.

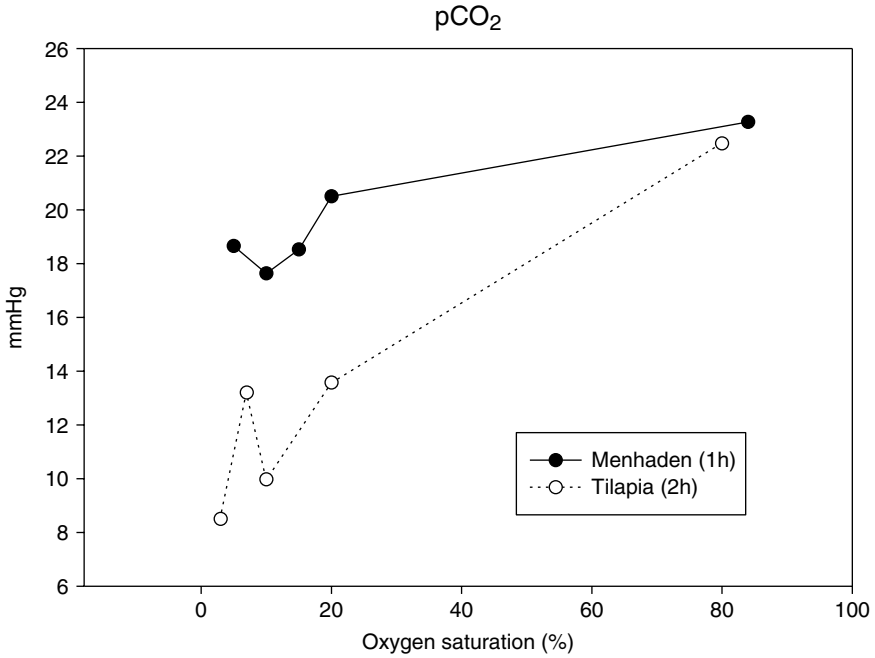

Figure 31.4 Partial pressure of carbon dioxide (pCO_2) in venous blood of exposed menhaden and tilapia. CO_2 concentrations fell as DO levels in the exposure tank decreased. A sharp drop was evident at approximately 10% saturation in both species. $N = 3$.

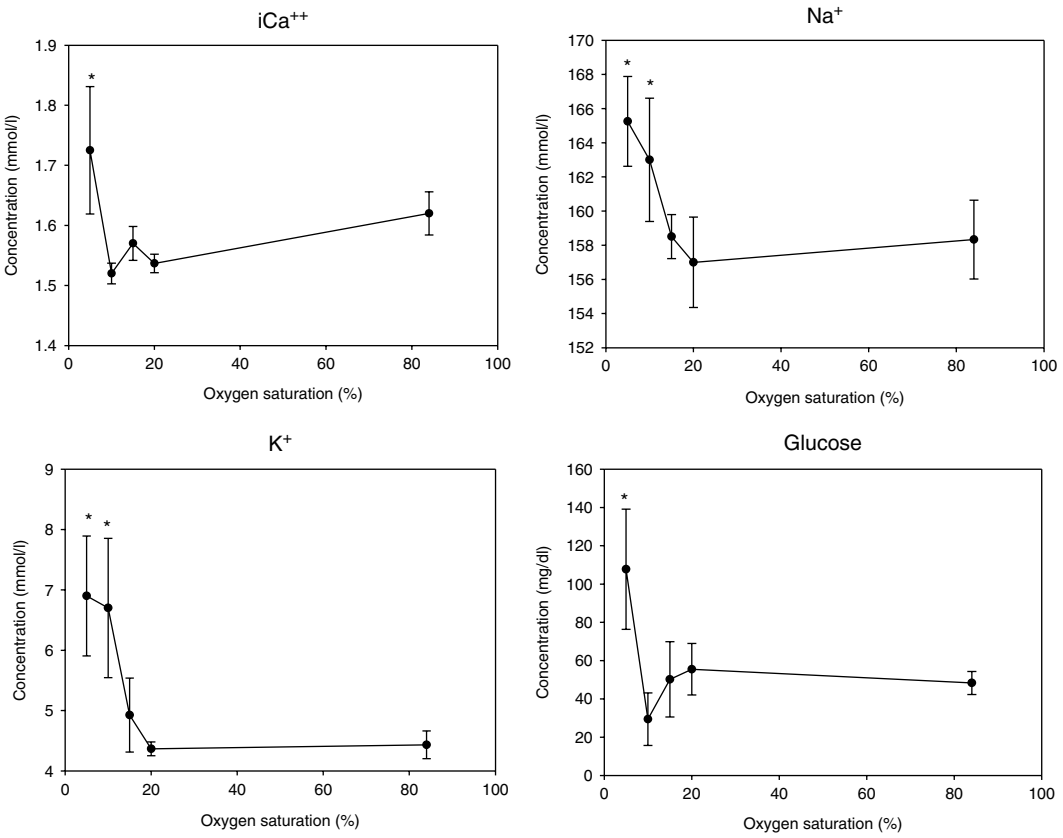

Figure 31.5 Menhaden blood chemistry parameters. Significant changes (*) were noted at critically stressful levels of hypoxia, indicating physiological failure of oxidative phosphorylation or blood ion homeostasis. Na ($p < 0.004$), K ($p < 0.0013$), iCa ($p < 0.003$), and glucose ($p < 0.0016$). Error bars are standard deviation.

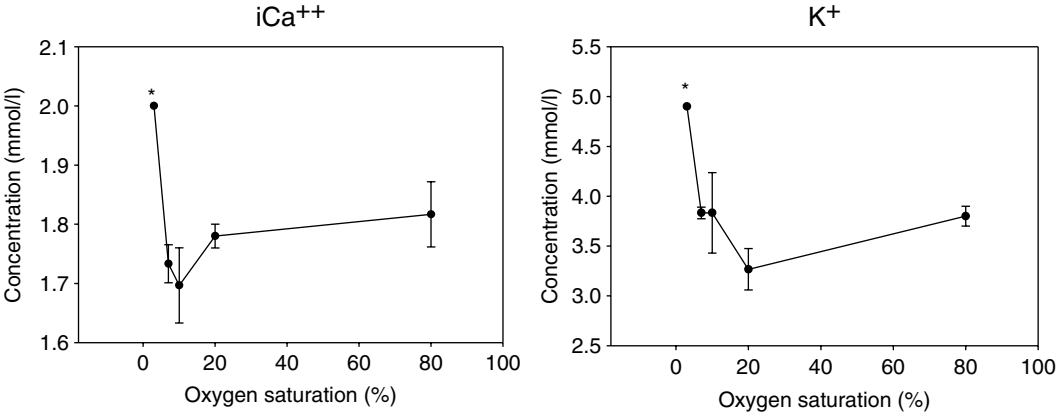

Figure 31.6 Tilapia blood chemistry parameters. Significant changes were seen in fewer ion types as compared to menhaden, indicating less loss of homeostasis and better energy management in tilapia. K ($p < 0.0041$), iCa ($p < 0.0041$). Error bars are standard deviation.

This precisely controlled hypoxia system has proven to be useful for the experimental induction of hypoxic responses in fish in our laboratory. With ever increasing influences of anthropogenic inputs into the nation's watersheds, particularly those resulting in eutrophication, this system is likely to serve broader applications that will answer questions in aquatic toxicology where hypoxia may play a role.

Acknowledgments

Development of this system was supported in part by the North Carolina Department of Environment and Natural Resources, project EW200020; North Carolina Department of Health and Human Services, project OEE 101; and NCSU College of Veterinary Medicine, state appropriated research funds. We sincerely thank the NOAA staff in Beaufort, NC, for their expertise and use of their facilities; J. Overton, L. Ausley, and M. Hale for their support in this project; the members of the Neuse River Rapid Response Team and the Tar/Pamlico Rapid Response Team for valuable technical expertise in our fish sampling efforts; M. Mattmuller for excellent histopathology support; and M. Dykstra and J. Rice for helpful discussions.

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chapter thirty-two

*Fish models in behavioral toxicology: Automated techniques, updates and perspectives**

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Contents

Introduction

Since the science of toxicology began thousands of years ago, behavioral endpoints have been used to study the effects of chemicals and drugs on humans and other mammals. In aquatic toxicology, however, the nexus of behavioral sciences with the study of toxicants

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has only become prominent within the last five decades. Behavioral endpoints have been slow to be integrated in aquatic toxicology because, until recently, there was a poor understanding of how alterations in behavior may be related to ecologically relevant issues, such as predation avoidance, prey capture, growth, stress resistance, reproduction, and longevity. Further, the ability to achieve repeatable, quantifiable data from a large number of animals or exposures has been challenging. Recent improvements in computer and video automation have made possible significant progress in the ease, utility, and affordability of obtaining, interpreting, and applying behavioral endpoints in a variety of applications from water quality monitoring to use in toxicity identification evaluation (TIE).[1–9] Consequently, behavioral endpoints in aquatic toxicology are shifting from being met with skepticism by investigators to being received with greater enthusiasm.

One of the first comprehensive reviews on aquatic behavioral toxicology was published by Rand.[10] Over the past 20 years, the field of behavioral toxicology has grown, in part, because of increased interest in the number of species used, endpoints measured, and methods to collect and interpret data. Numerous reviews have traced these advancements in the field of behavioral toxicology.[11–15] However, the recognition of behavioral toxicology as an important tool in aquatic toxicology is most clearly seen in the acceptance of behavioral endpoints in Federal regulations. In 1986, the U.S. government accepted avoidance behavior as legal evidence of injury for Natural Resource Damage Assessments under Proceedings of the Comprehensive Environmental Response, Compensation, and Liability Act of 1980.[16]

This chapter provides updated information with an added perspective on automated systems that evaluate quantitative behavioral endpoints. The chapter also provides a compilation of reference material relating to specific and nonspecific observations of behavioral alterations in fish that will be of use to both experienced and new practitioners of behavioral toxicology. The reader is reminded that much work has also been done with aquatic invertebrates,[17] including crabs,[18,19] daphnids,[20] clams,[21,22] and other animals; however, the volume of such work precludes its inclusion in this chapter.

Why study behavior?

Behavior provides a unique perspective linking the physiology and ecology of an organism and its environment.[15] Behavior is both a sequence of quantifiable actions, operating through the central and peripheral nervous systems,[23] and the cumulative manifestation of genetic, biochemical, and physiologic processes essential to life, such as feeding, reproduction, and predator avoidance. Behavior allows an organism to adjust to external and internal stimuli in order to best meet the challenge of surviving in a changing environment. Conversely, behavior is also the result of adaptations to environmental variables. Thus, behavior is a selective response that is constantly adapting through direct interaction with physical, chemical, social, and physiological aspects of the environment. Selective evolutionary processes have conserved stable behavioral patterns in concert with morphologic and physiologic adaptations. This stability provides the best opportunity for survival and reproductive success by enabling organisms to efficiently exploit resources and define suitable habitats.[15]

Since behavior is not a random process, but rather a highly structured and predictable sequence of activities designed to ensure maximal fitness and survival (i.e., success) of the individual (and species), behavioral endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors. Behavioral endpoints that integrate endogenous and exogenous factors can link biochemical and physiological processes, thus providing insights into individual- and community-level effects of environmental contamination.[24,25] Most importantly, alterations in behavior represent an integrated, whole-organism response. These altered responses, in turn, may be associated with reduced fitness and survival, resulting in adverse consequences at the population level.[26]

Rand[10] stated that behavioral responses most useful in toxicology should be: (1) well-defined endpoints that are practical to measure; (2) well understood relative to environmental factors that cause variation in the response; (3) sensitive to a range of contaminants and adaptable to different species; (4) ecologically relevant. To this list, we add endpoints that should ideally: (5) elucidate different modes of action or chemical classes; (6) be able to ''stand alone'' and be easily incorporated into a suite of assessments; (7) be simple to automate in order to maximize their utility for a broad range of applications; (8) have representation across species (e.g., reproduction, food acquisition) in order to facilitate investigations into the phylogeny and ontogeny of behavior; (9) include a suite of endpoints that focus on innate behavior of sentinel organisms that can be altered in association with stress exposure; and (10) help delineate ecosystem status, i.e., health. Although each of these considerations has merit, often the application of a specific endpoint, or suite of endpoints, is based on the ability to functionally discern exposure-related alterations, using available techniques, with the most appropriate sentinel species.

The application of behavioral endpoints in any toxicity study must also be based on the stressor(s) to be evaluated. Basic knowledge of the compound/toxicant/stressor of interest is necessary. Stress agents of interest should (a) be ''behaviorally toxic,'' (b) have a route of uptake for the aquatic species in question, and (c) structurally resemble a behavioral or neurotoxicant or one of its active metabolites. Of course, toxicants that may be a behavioral or CNS toxicant to mammals may not have similar affects on aquatic animals, and *vice versa*.[10]

The mechanism of action, route of uptake, and behavior of the compound of interest in the aquatic environment must all be understood. In adult fish, gill and gut epithelia are major routes of uptake; however, physiological differences of different life stages need to be taken into consideration. Larval fish utilize skin as a respiratory interface and may uptake more compounds than adults of the same species that utilize gills for respiration. In contrast, for compounds that directly target the gill lamellae, adult fish will have increased sensitivity compared with larvae due to the increased surface area of the gill surface.

Fish models in behavioral testing

To date, there are no standardized species or groups of species used for aquatic behavioral toxicology testing. Different species often have different behavioral and physiological responses to stress and toxicant exposure. Therefore, preliminary observations and assays are required in order to determine the feasibility of a particular

species, and if aberrant behavioral patterns can be associated with specific exposure scenarios. It is not unreasonable for preliminary testing with a novel species to take months, and in some cases, years, in order to develop biologically relevant endpoints of exposure.

Fish are ideal sentinels for behavioral assays of various stressors and toxic chemical exposure due to their: (1) constant, direct contact with the aquatic environment where chemical exposure occurs over the entire body surface; (2) ecological relevance in many natural systems[27]; (3) ease of culture; (4) ability to come into reproductive readiness[28]; (5) long history of use in behavioral toxicology. Alterations in fish behavior, particularly in nonmigratory species, can also provide important indices for ecosystem assessment.

Ideally, test organisms should have the following characteristics: (1) high ecological relevance; (2) susceptibility to the stressor(s) in question, both in the field and in the laboratory; (3) have wide geographical distributions; (4) be easy to culture and maintain under laboratory conditions; (5) have relatively high reproductive rates and, should have relatively early maturation and easy fertilization in order to produce sufficient numbers of organisms of the proper age and size for testing; (6) have environmental relevance to the potential exposure (have been exposed to the test contaminant in the wild); (7) have the ability to yield reproducible data under controlled laboratory conditions.

Once the model test species is determined, exposure-related behavioral alterations can be distinguished as fixed action patterns (FAPs) or through specific behaviors discerned from a species' ethogram. FAPs are specific, innate behavioral sequences initiated by specific stimuli that are not a result of gene–environment interactions.[29] These ''hard-wired'' behaviors are typically under genetic control, may be species specific, go to completion upon initiation, are not regulated through feedback loops, and are not reflexes but complex, coordinated behaviors.[14] As such, alteration(s) of FAPs are good endpoints to include in a suite of behavioral tests.

Quantifiable behavioral changes in chemically exposed fish provide novel information that cannot be gained from traditional toxicological methods, including short-term and sublethal exposure effects, mechanism of effect, interaction with environmental variables, and the potential for mortality.[12,28,30–33] Ecologically relevant behaviors affected by sublethal concentrations include: altered vigilance, startle response, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviors.[12,34] Changes in behavior may also alter juvenile recruitment, thereby disrupting population demography and community dynamics over time.[26]

Researchers wishing to develop a new fish model for behavioral toxicology must consider the life history and ecology of the species. For example, herring form tight schools in nature, yet if kept solitary in the laboratory, will die after a few days.[35] Other clupeids, such as menhaden (*Brevoortia tyrannus*), can be laboratory-maintained over long periods of time (months to years) in large holding tanks, but when transferred to behavior/exposure arenas, become highly stressed and succumb within 72 h due to sepsis prior to any toxicant/stressor exposure. These examples demonstrate the importance of having insight into the sentinel species' niche, i.e., habitat, diet, foraging strategies, reproductive strategies, home range, and social structures (school versus shoal versus solitary swimmers). These factors will help discern (1) which types of behavior are important for study, (2) appropriate experimental design and exposure parameters, and (3) the ecological importance of the behavior on the life history of the fish. Channel catfish (*Ictalurus punctatus*), brown bullhead

(*Ictalurus nebulosis*), and striped bass (*Morone saxatilus*), for example, may at times share similar geographical habitats but differ greatly in life history characteristics (diet, position in the water column, migration, and social structures). These differences directly translate into differential exposure to chemicals in the environment. Of course, there may also be vast physiological and biochemical differences between species in the metabolism, tissue distribution, and elimination profiles, all of which can alter exposure concentrations at target tissues.

Different species of fish will have different suites of behaviors and adaptive behavior patterns, i.e., responses to stimuli. These patterns may also vary widely under different holding and exposure conditions within individuals of the same species. Therefore, it is critical to carefully document observations of normal baseline behavior under controlled conditions prior to behavioral testing with a chemical or other stress agent. Further, it is important to recognize changes in behavior that are not only associated with controlled, laboratory stress exposure, but also with sub-optimal health. Table 32.1 provides a consolidation of qualitative comments from the literature that indicate behavioral changes associated with a broad variety of different scenarios. This table illustrates the often-nonspecific nature of many different behavioral alterations associated with disease agents, biologicals, sub-optimal water quality, and

Table 32.1 Behavioral alterations observed in fish associated with different stress agents

Host species	Stressor	Behavior/movement comments	References
	Biologicals		
Salmonids	*Aeromonas salmonicida*	Lethargy, inappetence, loss of orientation, abnormal swimming behavior	(37, 38)
Fish	*Bacillus* spp.	Weakness, lethargy	(38)
Cold freshwater fish	Bacterial gill disease	Lethargy, flared opercula, coughing, dyspnea	(39)
Fish	Bacterial gill disease	Lethargy, anorexia, increased respiration	(40, 41)
Fish	Blood flukes	Lethargy, flashing	(39)
Bluegill	40 ppb brevetoxin	Altered ventilatory responses; reversible after 1 h	(42)
Channel catfish	Channel catfish virus disease	Hanging head up in the water, disorientation, corkscrew swimming	(39)
Fish	*Chryseobacterium scopthalmum*	Lethargy	(43, 44)
Coho salmon and farmed trout	*Clostridium botulinum*	Sluggishness, erratic swimming, listlessness, may alternately float and sink before showing temporary rejuvenation	(45)
Warm and cold freshwater fish, cold water marine fish	Columnaris	Dyspnea	(39)
Striped bass	*Corynebacterium aquaticum*	Inappetence, swimming more slowly	(38)

Table 32.1 Continued

Host species	Stressor	Behavior/movement comments	References
Deep angelfish	Deep angelfish disease (herpes-like)	Loss of equilibrium, headstanding	(46)
Catfish	*Edwardsiella ictaluri*	Fish hang listlessly at surface in a head-up-tail down posture, sometimes swimming rapidly in circles; corkscrew spiral swimming, depression	(39, 47)
Lake trout, lake trout × brook trout	Epizootic epitheliotropic disease (herpes virus-like)	Sporadic flashing, corkscrew swimming	(48)
Striped mullet	*Eubacterium tarantellus*	Erratic swimming, loss of equilibrium, spiral swimming; floating at surface and sinking to the bottom repeatedly	(49)
Salmonids	*Exophiala salmonis*	Erratic swimming	(50, 51)
Farmed barramundi	*Flavobacterium johnsoniae*	Listlessness, anorexia	(52)
Coho salmon	*Flexibacter psychrophilus*	Nervous spinning	(53)
Fish	*Gill Cryptobia* infestation	Anorexia	(39)
Cherry salmon	*Hafnia alvei*	Slow swimming	(54)
Salmonids	Infectious hematopoietic necrosis virus	Lethargy, sporadic hyperactivity	(39)
Salmonids	Infectious pancreatic necrosis virus	Corkscrew spiral swimming, whirling	(39)
Fish	*Lactococcus gravieae*	Moribund fish swim erratically just below the surface of the water	(38)
Fish	Motile aeromonads	May swim normally or hang in the water, on their sides	(55)
Fish	Mycobacteria	Listlessness, anorexia, dyspnea, inappetence	(38, 56–59)
Mainly salmonids	*Myxobolus cerebralis*	Whirling or frenzied, tail-chasing behavior, impaired balance	(60)
Fish	*Nocardia*	Inactivity, anorexia	(61)
Bluegill	*Pfiesteria piscicida* (laboratory exposure to non-axenic cultures)	Decreased aggression and social interactions, followed by solitary time spent on or near the bottom. Subsequent sporadic bursts of activity, including tailstanding, bobbing, corkscrewing in place, breaking at the surface, followed by inactivity at a 45° angle in water column, or resting on the bottom prior to morbidity. Strong elevations in cough rate and % movement without notable changes in respiratory rate	(36, 42)
Fish	*Pfiesteria piscicida* cultures	Pigmentation changes, lethargy, episodic hyperactivity and decreased respiration	(62–65)

Table 32.1 Continued

Host species	Stressor	Behavior/movement comments	References
Fish	*Plesiomonas shigelloides*	Inappetence	(66)
Fish	*Pricirickettsia salmonis*	Gathering at the surface of cages, sluggishness, inappetence	(38)
Fish	Protozoan ectoparasites	Dyspnea	(39)
Rio Grande cichlid; zilli cichlid	Rio Grande cichlid rhabdovirus disease	Lethargy	(67)
Salmonids	Salmonid rickettsial septicaemia	Lethargy, swimming near the surface or at the side of the net	(68)
Rabbitfish	*Shewanella putrefaciens*	Lethargy	(69)
Carps; sheatfish; guppy; Northern pike	Spring viremia of carp (*Rhabdovirus carpio*)	Decreased swimming ability	(70)
Fish	*Staphylococcus aureus*	Lethargy	(38)
Fish	*Streptococcus*	Erratic swimming	(71)
Tilapia	*Streptococcus difficilis*	Lethargy, erratic swimming, showing signs of dorsal rigidity	(38)
Farmed Atlantic salmon	Unidentified Gram-negative rod	Lethargy, swimming close to surface, loss of balance	(38)
Warm marine fish	Uronemosis	Dyspnea, hyperactivity, then lethargy	(39)
Rainbow trout	*Vagococcus salmoninarum*	Listless behavior, impaired swimming	(72)
Fish	*Vibrio alginolyticus*	Sluggishness	(73, 74)
Fish	*Vibrio anguillarum*	Anorexia, inactivity	(38, 75)
Sharks	*Vibrio harveyi*	Lethargy, stopped swimming, appearing disorientated	(38)
Fish	*Vibrio salmonicida*	Inappetence, disorganized swimming	(75)
Japanese horse mackerel	*Vibrio trachuri*	Erratic swimming	(38)
Cold freshwater fish (mainly salmonids)	Viral hemorrhagic septicemia	Lethargy, congregating away from the current on the edges of the pond or raceway, looping swimming behavior, darting through the water and spiraling at the bottom of the pond	(39)
Atlantic salmon	*Yersinia intermedia*	Lazy movements, congregating at the surface of the water	(76)
Rainbow trout	*Yersinia ruckeri*	Sluggishness	(77)
	Contaminants		
Rainbow trout	Al	Avoidance behavior(s)	(78)
Rainbow trout	Al	2 minute clips, position holding, slow and burst type swimming	(79)
Atlantic salmon, rainbow trout	Cu and Zn (salmon); zinc sulfate (trout)	Avoidance behavior(s); 53 ppb (salmon); 5.6 ppb (trout)	(80)
Three-spined sticklebacks	BBP (butyl benzyl phthalate)	Shoal choice	(81)
Rainbow trout	Carbaryl	Velocity, school size NNA at 1 fps for 1 min (60 frames)	(82)
Rainbow trout	Carbaryl	Swimming capacity, feeding activity, strikes	(12)
Fathead minnow	Cd	Decreased predator avoidance, 25–375 ppb	(83)

Table 32.1 Continued

Host species	Stressor	Behavior/movement comments	References
Rainbow trout	Cd	Altered dominance, feeding and aggression	(84)
Zebra fish	Cd	Avoidance behavior(s)	(85)
Bluegill	Cd, Cr, Zn	Hyperactivity	(86)
Green sunfish	Chlordane	Avoidance behavior(s) 20, 10, 5 mg l^{-1}	(87)
Rainbow trout	Co	Dominance hierarchy, growth, food intake and coloration	(88)
Rainbow trout	Copper sulfate, dalapon, acrolein, dimethylamine salt of 2,4 D, xylene	Avoidance behavior(s)	(89)
Pink salmon	Crude oil	Avoidance behavior(s) 1.6 mg l^{-1}	(90)
Estuarine fish	Cu	8 ppb olfactory disruption	(91)
Goldfish	Cu	Velocity, TDT, turning angles	(92)
Rainbow trout	Cu	Attraction 460–470 ppb, avoidance 70 ppb	(93)
Salmon	Cu	Altered chemoreception and home stream recognition	(94)
Rainbow trout	Cu and Ni	Attraction 390 ppb (Cu), 6 ppb (Ni), avoidance 4.4 ppb (Cu), 24 ppb (Ni)	(95)
Rainbow trout	Cu, Co	Avoidance behavior(s)	(96)
Atlantic salmon	DDT	Alteration in temperature preference, 5–50 ppb	(97)
Bluegill	DDT	Hyperactivity	(98)
Brook trout	DDT	Biphasic concentration–response relationship for temperature preference and DDT	(99)
Croaker	DDT	Effects on the F_1 generation vibratory/visual stimuli, burst speed	(100)
Goldfish	DDT	Increases in velocity, turns and area occupied	(82)
Brook trout	DDT and methoxychlor analogs	Alteration in temperature preference for methoxychlor analogs	(101)
Mosquito fish	Endrin, toxaphene, parathion	Avoidance behavior(s)	(102)
Mummichog	Environmental MetHg	Prey capture (strikes and captures), predator avoidance, lab and field validation	(103)
Rainbow trout	Heavy metal mix	Avoidance behavior(s)	(104)
Chinook salmon	Kraft mill extract	Avoidance behavior(s) 2.5–10%	(105)
Smelt	Kraft mill extract	Avoidance behavior(s) 0.5%	(106)
Three-spine stickleback	Lead nitrate	Attraction at high concentration, avoidance at low concentration	(107)
Shiners	Malathion	Concentration-dependent decrease in temperature selection	(108)
Medaka	OPs	Vertical path analysis in 1-min clips, velocity, meandering, TDT, smooth versus erratic swimming, 4 fps	(109)

Table 32.1 Continued

Host species	Stressor	Behavior/movement comments	References
Mosquito fish	OPs	Avoidance behavior(s)	(102)
Goldfish	Parathion	Hypoactivity and alteration in angular change	(110)
Mummichog	Pb	Feeding activity and performance, predator avoidance	(111)
Rainbow trout fingerlings	Phenol	Decrease in predator avoidance to adults, 0.5–18 mg l^{-1}	(112)
Minnow	Phenol and *p*-chlorophenol	Avoidance behavior(s)	(113)
Herring	Pulp mill extract	Avoidance behavior(s)	(114)
Rainbow trout	Rotenone	Avoidance behavior(s)	(115)
Roach	2,4,6 trinitrophenol	Attraction	(116)
Three-spined stickleback	Zinc and copper sulfate	Avoidance, ''stupefied and motionless''	(107)
	Water quality		
Brook trout	Acidification	Avoidance behavior(s)	(117)
Trout	Acidification	Female reproductive behavior, nest digging	(118)
Carp	Ammonia	Center of gravity of a group of fish/vertical location	(119)
Largemouth bass and mosquito fish	Ammonia	Decreased prey consumption of bass, less effect on mosquito fish	(120)
Fish	Ammonia poisoning	Hyperexcitability, fish often stop feeding	(121)
Fish	Chlorine poisoning	Dyspnea	(39)
Fish	Environmental hypoxia	Fish piping for air, gathering at water inflow, depression	(122, 123
Cold freshwater fish	Hypercarbia	Dyspnea	(39)
Bluegill	Hypoxia	Altered ventilatory and cough responses	(42)
Mullet, menhaden, spot, croaker, pinfish, and mummichog	Hypoxia	Avoidance behavior(s)	(124)
3-spine sticklebacks, minnows, and brown trout	Hypoxia and temperature change	Avoidance behavior(s)	(125)
Fish	Nitrite poisoning	Lethargy, congregating near the water surface	(39)
Fish	Low pH	Hyperactivity, dyspnea, tremors	(126)
Fish	Total suspended solids	Coughing to clear gills	(39)
Fish	Low temperature stress	Inactivity, depression	(39)

contaminants. Further, these collective references suggest the need to provide quantitative data when reporting behavioral alterations to facilitate comparison with other studies or observations.

The requirement to carefully document baseline ''normal'' behavior should be viewed as strength of behavioral testing. Traditional (LC_{50}) tests do not require stringent documentation of baseline behavior, other than visual observations of whether the test subjects were ''healthy,'' and verification that a minimal amount of mortality (i.e., $\leq$10%)

occurs in the control and treatment group(s). Behavioral toxicology testing, however, allows the control group to subsequently be exposed, if careful documentation on baseline behavior is made. The statistical power of behavioral tests can be greatly improved by using repeated measures analyses, using each animal as its own control. This type of analysis greatly reduces the inherent variability between individuals.

Descriptive behavioral alterations

Behavioral assays provide biologically relevant endpoints to evaluate sublethal exposure effects and may compliment traditional toxicity testing. In order to evaluate behavioral endpoints, specific descriptive observations regarding behavioral alterations in response to low-level stress (deviation from baseline) need to be demonstrated. The degree of alteration that can be experimentally meaningful is typically based on the ability to statistically discriminate differences between treatment and control groups. However, it should be noted that a common misuse of statistics is to find differences between treatment groups solely due to low *p*-values without empirically observable, exposure-related changes. Factors that can influence *p*-values include, in addition to sound experimental design, use of proper controls (negative, solvent, and positive), time frame of observation(s), sample size, response precision within treatment groups, and reproducibility between experiments (refer also to ''Preliminary studies'' section, page 578).

Ultimately, the question addressed in behavioral toxicology is: How do alterations in a species' behavior, resulting from sublethal stress exposure, alter individual fitness and have a biologically relevant effect? Even when biologically and statistically significant data are derived from an exposure study, it is typically difficult to extrapolate alterations observed under controlled laboratory conditions to ecologically relevant field scenarios. The answer to bridging this gap lies in the appropriate selection of behavioral endpoints and adding a similarly controlled comparison with the same species under more naturally complex or field exposure conditions (e.g., using predator–prey interactions and avoidance–attractance responses).[26,127,128]

Individual movement and swimming patterns

Avoidance and attractance

When a contaminant triggers a stimulus response, the resulting behavioral reaction (avoidance or attraction) significantly regulates exposure duration of the organism. If the contaminant is perceived by the fish as noxious, the fish responds by avoiding the area containing the chemical. In contrast, if the contaminant triggers an attractance response, the fish will stay in the area, thus increasing exposure duration. Avoidance–attractance responses depend on: (1) the substance activating the receptor; (2) sufficient exposure history of the species to evolve adaptive responses or sufficient experience by the organism to acquire a response to the stimulation; (3) sufficient directional information from the chemical concentration gradient to orient in the proper direction from the chemical plume.[15]

Avoidance and attractance behavior in fish has proven to be an easy and realistic behavioral endpoint of exposure because many contaminants induce avoidance or attractance behavior. The utility of avoidance behavior as an indicator of sublethal toxic exposure has been demonstrated over the past 50 years, and chemically induced

avoidance or attractance may significantly alter the distribution and migration patterns of individuals and groups of fish.[129]

Gray[11] demonstrated the avoidance of oil-contaminated water and gas-supersaturated water by free-ranging fish in the field. Avoidance of heavy metals (e.g., cadmium, copper, cobalt, and aluminum) by a variety of freshwater fish has been documented at low environmentally relevant concentrations.[78,85,96,104,130] In addition, fish can actively avoid fluctuations in water quality conditions, such as hypoxia, temperature, acidification, and ammonia.[117,119,124,125] Fish also maintain the ability to avoid anthropogenic compounds released into the environment, including certain pesticides and rotenone.[102,115]

Through the use of elaborate experimental designs, coupled with qualitative and quantitative measures of behavior, avoidance behavior can provide an endpoint that directly correlates to the field. Similar avoidance responses were observed in laboratory and field tests with fathead minnows (*Pimephales promelas*) when exposed to metals characteristic of the Coeur d'Alene River (Idaho, ID) downstream from a large mining extraction operation. Telemetry studies conducted at the confluence of the river with an uncontaminated tributary of the river revealed a similar avoidance of the contaminated water within the concentration range that induced avoidance responses in laboratory studies.[128] Hartwell et al.[127] conducted integrated laboratory and field studies of avoidance and demonstrated that fathead minnows avoided a blend of heavy metals (copper, chromium, arsenic, and selenium) that are typical of effluent from fly ash settling basins of coal-burning electrical plants. Fish avoided a 73.5 $\mu g\ l^{-1}$ mixture of these metals in a natural stream and 34.3 $\mu g\ l^{-1}$ in an artificial stream.

It may appear at first glance that most studies demonstrate avoidance behavior when fish are exposed to contaminants. However, it is difficult to generalize about the avoidance of aquatic contaminants by fish because of the variety of species and experimental designs used to test behavioral responses, as well as variations in the modes and sites of action of the chemicals studied.[95] Beitinger[131] reviewed the published literature on avoidance for over 75 different chemicals. Roughly one-third of the chemicals were avoided, whereas the others either failed to elicit a response or induced inconsistent responses. Many contaminants may cause avoidance reactions but some may attract aquatic organisms: these include detergents,[132] some metals,[30,93,133] and petroleum hydrocarbons.[134,135] Also, different species may have different avoidance responses. Largemouth bass (*Micropterus salmoides*) have been shown to be insensitive to 50 $\mu g\ l^{-1}$ copper sulfate, whereas goldfish (*Carassius auratus*) and channel catfish were attracted to this concentration.[133] Part of the explanation for these apparent conflicting results may be the contaminant-induced alterations of chemosensory systems. Hansen et al.[96,130] found simultaneous alterations in chemosensory-mediated behavior, in the physiologic responsiveness of the olfactory system of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*O. mykiss*) to L-serine, and evidence of damage to the olfactory tissue responsible for mucosa production and olfactory receptor cells. McNicol and Scherer[136] determined that whitefish (*Coregonus clupeaformis*) avoided cadmium concentrations of 1 $\mu g\ l^{-1}$ and less, and also avoided cadmium at 8 $\mu g\ l^{-1}$ and greater, but showed little response to concentrations between this range.

In recent physiological and behavioral studies (McNichol and Hara, personal communication), electro-olfactorygram (EOG) responses to the lower concentrations were shown to be mediated by the olfactory system. The olfactory system apparently became injured at cadmium concentrations greater than 1 $\mu g\ l^{-1}$, when avoidance responses ceased to occur and EOG responsiveness to L-serine were abolished. The renewed avoidance response to 8 $\mu g\ l^{-1}$ was likely induced by generalized irritation.

Likewise, Hansen et al.[96,130] found that even brief exposure of chinook salmon and rainbow trout to copper (25 $\mu g\ l^{-1}$) was associated with a significant reduction in EOG responses that recovered over several days; however, exposure to higher concentrations (44 $\mu g\ l^{-1}$ chinook salmon; 180 $\mu g\ l^{-1}$ rainbow trout) abolished behavioral responses. Furthermore, physiologic recording revealed these higher concentrations diminished both the EOG responses recorded on the mucosa and electro-encephalogram (EEG) responses to L-serine recorded from the olfactory tract. Necrosis and reduced density of olfactory receptors were evident injuries to the olfactory epithelium. These studies highlight the versatility and importance of integrating behavioral endpoints into a suite of toxicological studies that include relevant physiological and pathological endpoints helped to elucidate mechanism of action. We refer the reader to the many thorough reviews of the well-studied endpoint of avoidance–attractance behavior.[15,131,137,138]

Swimming patterns

Avoidance behavior is an amalgam of many behaviors that may culminate in a single endpoint, whereas movement analysis is a finer scale technique investigating the components of movement. Neurotoxicity is frequently observed in changes in form, frequency, or posture of swimming movements, with changes often occurring much earlier than mortality.[12,15] Sublethal metal and pesticide exposures have demonstrated alterations in swimming behaviors and serve as models for additional stressors.[79,92,109,139] When bluegill received pulsed doses of the pyrethroid insecticide ES-fenvalerate (0.025 $\mu g\ l^{-1}$), the first indication of toxicity was caudal fin tremors as fish initiated movement.[140] Exposure of rainbow trout to sublethal concentrations of 40 $\mu g\ l^{-1}$ malathion resulted in convulsive movements.[139] A review by Little and Finger[12] revealed that the lowest behaviorally effective toxicant concentration that induced changes in swimming behavior of fish ranged from 0.1% to 5.0% of the LC_{50}. When observations were made over time, behavioral changes commonly occurred 75% earlier than the onset of mortality. Development of locomotory responses, frequency of swimming movements, and duration of activity were significantly inhibited before effects on survival or growth were observed in brook trout (*Salvelinus fontinalis*) alevins exposed to aluminum concentrations (300 $mg\ l^{-1}$) under acidic conditions ($pH \leq 6.1$).[141]

Movement analysis of individuals and groups of fish continues to be refined as computer technology advances. Swimming responses have been used in automated biomonitoring systems because of their consistent sensitivity to numerous contaminants.[142,143] Studies of fish movement typically involve videography and quantification of movement parameters. Movement endpoints are designed to discern alterations in general swimming patterns in response to stressor exposure. Behavioral endpoints quantified through movement analysis typically include total distance traveled, velocity, acceleration, turning angles and frequency, time spent swimming, as well as horizontal and vertical distributions of individuals.

The measurements of swimming behavior are usually limited to the laboratory. Assessment of fish at contaminated field sites is currently not possible as species-typical responses have not been defined to permit the evaluation of behavioral function except for the most extreme aberrations.[15] In the laboratory, subtle changes that arise from exposure can be confirmed through comparisons with controls or with responses observed during a preexposure period.

Intra- and interspecific interactions

For hazard assessment and environmental regulation, it is important to show a causal linkage with the population in order to provide a predictive index of population-level effects. Recently, behavioral toxicology has focused more on complex behaviors, such as prey capture, predator avoidance, and courtship, and mating. These behaviors maintain high environmental relevance and direct fitness consequences to the individual. Hypoactivity and hyperactivity, as well as deviations in adaptive diurnal rhythmicity, may disrupt feeding and increase vulnerability to predation.[144,145] However, studies on these more complex behaviors are multifaceted and difficult to conduct depending on the amount of ecological realism the researcher wishes to achieve, but the experimental design can also be readily adapted to standard toxicity testing procedures.[146] The advantages, however, can be great and ecosystem effects of toxicant and stressor exposure may be more readily implicated. For example, fish exposed to heavy metals (cobalt, lead, and cadmium) displayed alterations in dominance, feeding behavior, growth, and predator avoidance.[84,111] Faulk et al.[100] demonstrated that the F_1 generation of fish exposed to DDT had deficits in their response to vibratory and visual stimuli, as well as altered swimming behavior. Feeding and prey vulnerability have been used to examine sublethal contamination because predator and prey may be differentially affected by toxicants.[147] Exposure to environmental mercury resulted in alterations in foraging (prey strikes and captures), as well as predator avoidance.[103] Female reproductive behavior and nest digging were found to be disturbed upon exposure to increasing levels of acidification.[118] Alterations in these behaviors can have serious effects to the individual and population of fish exposed and may induce changes in gene flow and demography.[148,149] To date, there are no well-characterized examples of automated systems to detect and evaluate predator–prey interactions. Certainly, this is not to say that the technology is not currently attainable.

Respiratory patterns

Respiration is a rhythmic neuromuscular sequence regulated by an endogenous biofeedback loop, as well as by external environmental stimuli. Acute contaminant exposure can induce reflexive cough and gill purge responses to clear the opercular chamber of the irritant, and can also increase rate and amplitude of the respiratory cycle as the fish adjusts the volume of water in the respiratory stream. As exposure continues, the respiration cycle can become irregular, largely through decreased input, as well as alterations in the endogenous pacemaker. Diamond et al.[3] found that the frequency and amplitude of bluegill opercular rhythms and cough responses were altered following exposure to different contaminants. For example, dieldrin, an organochlorine insecticide, increased ventilatory frequency at concentrations above 24 $\mu g\ l^{-1}$ and caused cough responses and erratic movements. In contrast, zinc at 300 $\mu g\ l^{-1}$ reduced the amplitude of the respiratory response.

A variety of biomonitoring systems have been developed to assess changes in respiratory rate relative to stress exposure. These systems have the great advantage of sensitivity since many waterborne stressors, even at low environmental concentrations, affect gill tissue and respiratory function. Respiratory frequency, depth (volume), and cough frequency can be measured, noninvasively, using physiological signals from restrained sentinel fish. One such system to accomplish this with good repeatability has been described by Shedd et al.[150] Briefly, small flow-though exposure vessels house

individual small fish in their respective chambers. Electrical signals generated by the respiratory and body movements of individual fish are detected by electrodes suspended above and below each fish. The signals are amplified, filtered, and analyzed using various algorithms on a personal computer. The muscular electrical output (0.05–1 mV) from each fish is independently amplified by a high-gain, true differential-input, instrumentation amplifier by a factor of 1000. Signal interference by frequencies above 10 Hz is attenuated by low-pass filters. The ventilatory parameters monitored by the computer include ventilatory rate, ventilatory depth (mean signal height), and gill purge (cough) rate.

Since fish are poikilotherms, temperature may also play an important role in determining exposure effects on a given fish species. Efforts at the UM Aquatic Pathobiology Laboratory to validate a respiratory response system, as described above, have recently demonstrated temperature-dependent differences in bluegill (*Lepomis macrochirus*) exposed to brevetoxin at 19°C versus 25°C. Interestingly, respiratory responses (increased ventilatory, cough and ''other movement'') were altered at 25°C but not at 19°C (Figure 32.1).

As with other behavioral systems, it is essential to properly integrate responses over time in order to achieve a good signal-to-noise ratio. The accuracy of any computer ventilatory parameter can be established by comparing the computer-generated values with concurrent strip chart recorder tracings.[150] Biomonitoring systems that measure fish ventilatory patterns have further application as early warning signals of water quality changes and toxicity.[9]

Social behavior and group dynamics

Toxicology studies typically focus on the exposure of single fish in the laboratory, when in reality, many fishes tend to congregate in groups and interact with many components of their environment. Group living is a basic life history characteristic of many fishes, with 25% of all species forming schools or shoals during their life, and 50% during larval and juvenile stages.[35,151] Pavlov and Kasumyan[151] define a fish school as having all individuals oriented in the same direction, situated at a certain distance from each other, and unitary in all movements (polarized). Shoaling, in contrast, is a simple, spatial aggregation of fish attracted by a stimulus occurring independently of each other with no mutual attraction between individuals (nonpolarized). Schooling and shoaling behaviors are complex social behaviors utilized by a wide diversity of fish species to increase individual fitness and propagate their genes in the population[152] by providing defense from predation, while increasing reproductive, foraging, and migration efficiencies.

These behaviors have predictable structures, shapes, and responses to threats and environmental fluctuations. In addition, these behaviors are intimately tied to, and regulated by, the visual and lateral line systems, and are developed as soon as fish are able to swim and feed.[151] Alterations in school structure and density can be caused by individual differences in motivation, physiology, and abiotic and biotic factors of the environment. It has been demonstrated, through the use of shoal choice experiments and frame capture, that pesticide exposures can alter shoaling and schooling behaviors. Atlantic silversides (*Menidia medidia*) exposed to an acetyl cholinesterase inhibiting insecticide, carbaryl, displayed alterations in parallel orientation and increased distances between fish when compared to controls.[82] In addition, swimming orientation in schools

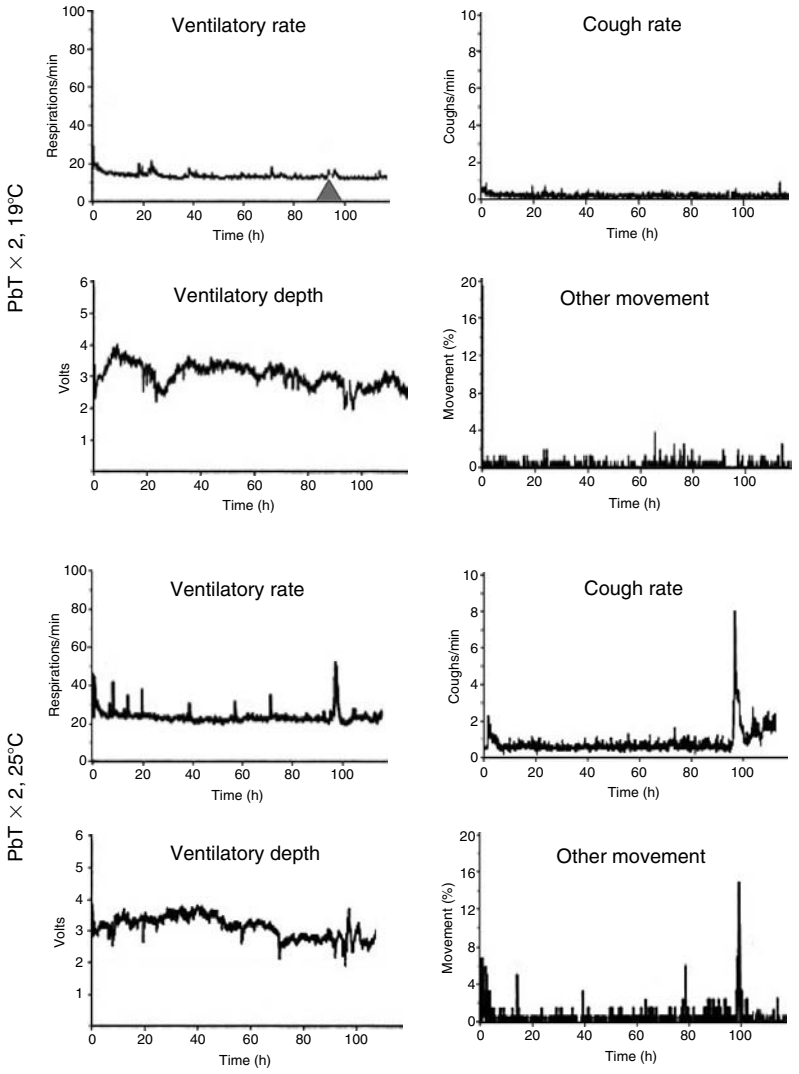

Figure 32.1 Ventilatory responses of bluegill to brevetoxin (PbT × 2, 40 μg l^{-1}) at 19°C and 25°C using a flow-through exposure system as described by Shedd et al.[150] These data illustrate the importance of water quality variables (temperature in this case) in determining the behavioral effects of certain toxicants. The upper panel of four graphs shows respiratory responses of bluegill at 19°C; no significant responses are noted in these data (gray triangle in upper-right graph indicates start of exposure after 96 h of baseline acclimation). At 25°C, bluegill respond to exposure with significantly increased ventilatory rate (but not ventilatory depth), cough rate, and ''other movement.'' This latter category represents data that do not fall into one of the three previous categories, based on the analytical algorithms; it often reflects whole-body movement in the exposure chambers.

of three-spined sticklebacks (*Gasterosteus aculeatus*) was disturbed following exposure to the organotin bis(tributyltin)oxide.[81] Schooling declined following exposure of yearling common carp to 0.05 mg l^{-1} DDT and of fathead minnows to 7.43 mg l^{-1} of the herbicide 2,4-dinitrophenol at a pH of 7.57.[153]

Behavioral analysis systems

Rand[10] extensively reviewed different exposure and tank designs that have historically been used to evaluate fish responses to stress exposures. These systems include both static and flow-through designs, as well as tube, Y-shape, rectangular, square, round, and maze configurations.[80,89,92,105,107,129,154–167] The different exposure designs permit gathering data relevant to avoidance and attraction, ability to detect gradients, orientation to changes in light, sound and temperature, altered performance/stamina and learning. Changes associated with toxicant or stress exposure may be gathered visually, or with electrodes,[168–170] photocells or photoresistors,[171] or videography. Data have been recorded using event recorders, strip chart recorders, polygraphs, and video processors and computers that can integrate signals and generate x, y coordinate data.

Recent hardware and software updates have been used to develop integrated exposure systems to test suites of behavioral endpoints.[172] These exposure systems are used to investigate the effects of sublethal stressors on fish movement and responses to stimuli. For example, simultaneous video capture from multiple exposure arenas can be used to digitally track movement. Video data can then be used to address questions regarding differences between treatment groups or differences between pre- and postexposure behavior using repeated measures analyses.

Figure 32.2 schematically depicts such a system using video cameras with multiple, dedicated analog video decks. In this system, twelve 10-l exposure arenas were constructed from 14-in. (35.6 cm) diameter polyvinyl chloride (PVC) pipes and end caps. Each arena had two 0.25-in. (0.64 cm) threaded nipples that served as an input and drain. The input was bifurcated to accept both toxicant and dilution water flow lines. Toxicant and dilution water flow were electronically controlled with digital, multichannel peristaltic pumps (Masterflex L/S, Cole-Parmer, Vernon Hills, IL) that were supplied by multiple, aerated 600-l carboys. The exposure arenas were illuminated with 14 h:10 h (light/dark) shadow less fluorescent lighting combined with a computer-controlled dusk and dawn cycle provided by incandescent lights. Lighting was controlled using X-10 computer hardware with an X-Tension (Sand Hill Engineering, Geneva, FL) software interface on a Macintosh operating system. Dusk–dawn lighting systems are very useful in reducing stress in aquatic holding and testing facilities, and can be developed, relatively inexpensively, using other techniques and hardware systems.[173] Twelve color charged-coupled device (CCD) cameras with manual iris and focus control were mounted above the respective arenas and were connected to dedicated VCR decks with time-lapse and dry contact closure capability for recording and computer control. All 12 VCRs were connected to a multiplexer that supported real-time observation (Figure 32.2). VCR recording and stop functions were synchronously activated remotely using X-10 technology.

Analog video data are then digitized in real-time at three frames per second on a Macintosh platform (various hardware and software systems, both Macintosh- and Windows-based are available for this task). Movement data are subsequently imported into a commercial tracking program (Videoscript Professional, version. 2.0©) and converted into x, y coordinate data. This program uses a custom algorithm designed for fish movement, which identifies and tracks the head of each fish target. The x, y coordinate data are then analyzed, using proprietary software designed at the Aquatic Pathobiology Laboratory, to obtain the desired behavioral endpoints. There are a variety of ''off the shelf,'' commercially available motion tracking and analysis systems (e.g., *Ethovision*, Noldus Information

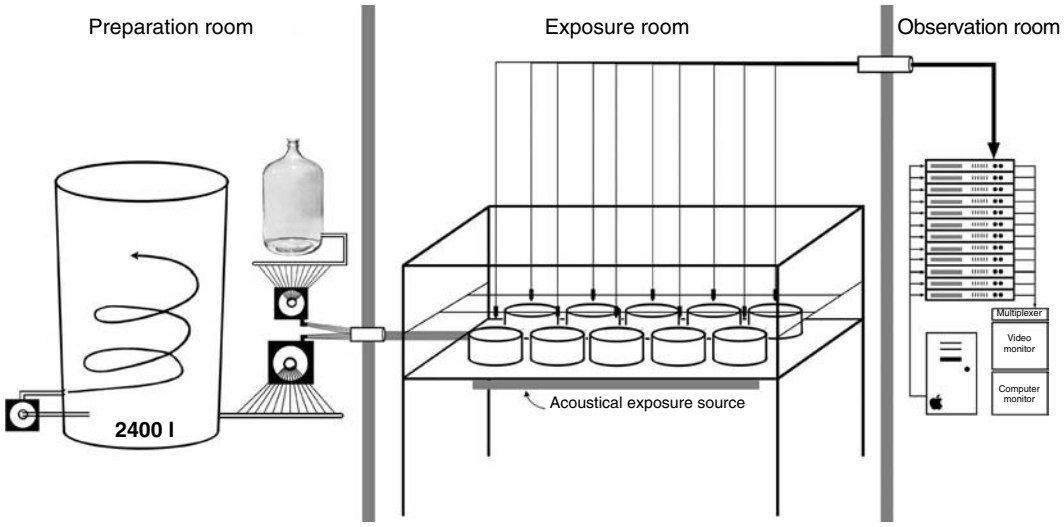

Figure 32.2 Schematic showing flow-through exposure arenas with dedicated, overhead video cameras (shadow less lighting and drainage not shown) in the behavioral toxicology exposure suite at the Aquatic Pathobiology Laboratory, University of Maryland. Water is prepared, temperature adjusted, and delivered from an adjacent ''preparation room,'' while video data are amassed by computer-controlled video decks. Toxicants or aqueous stress agents, and flow-through dilution water, are pumped from the preparation room by computer-controlled peristaltic pumps. An instantaneous acoustic/vibratory stimulus can be provided to discern differences in startle response behavior. In this diagram, an acoustical exposure source is indicated under the platform supporting the exposure arenas. The source in one experiment consisted of three carefully positioned, spring-loaded mousetraps that could be tripped remotely with conjoined pull strings. Video response data are transformed into x, y coordinate data, and relevant endpoints are discerned using our tracking and analytical software. Each of 12 arenas can house individual or multiple fish (up to 120 fish can be monitored simultaneously), or individual arenas can be replaced with multichamber arenas to aid in identifying individual animals that respond (or fail to respond) to different stimuli (see Figure 32.4 describing chambers used to discern startle response with small fish).

Technology; and *Expert Vision*, Motion Analysis Corporation) that can then be customized for particular research requirements.

A major benefit of this behavioral hardware and analysis system (Figure 32.2) is that investigators can take conventional behavioral analyses, which have previously been limited to ranks and counts, and quantify it using computer technology. The behavior and hardware analysis system has potential for greater flexibility in behavioral measurements than commercial behavioral quantification systems, but requires that a programmer be on staff. Current system capabilities and development areas include quantification of a wide range of behaviors, including daily swimming patterns, startle-type responses, avoidance behaviors, and social interactions (Table 32.2). In addition, the system can remotely dose and record up to 12 individual fish or 12 groups of up to 10 individuals simultaneously, for up to 1 h, without the need to mark or tag the animals, thus reducing variance in behavior due to observer or handling disturbances. Finally, the system can be adapted for static and flow-through exposures of environmentally relevant contaminants, and has the ability to be mobile for real-time field assessment.

Table 32.2 Individual and group endpoints for movement analysis

	Definition
Individual endpoints	
Percent movement	The number of seconds the fish satisfies movement criteria divided by the total number of seconds spent swimming, multiplied by 100
Velocity	Average velocity (cm s^{-1}) while the fish is moving during the experimental period
Angular change	The difference (0–180°) between the angular components of two consecutive 1 s movement vectors (degrees per second) divided by the total number of consecutive 1 s movement events. Angular change was only calculated when two consecutive movement vectors met the movement criteria
Path tortuosity (fractal dimension)	Fractal dimension (D) is calculated using the hybrid divider method (Hayward et al., 1989) and is an indicator of path complexity. A series of path generalizations are created at various step sizes, and the data are mathematically drawn on a Richardson plot (log path length versus log step length). D is then calculated as 1 minus the slope of the Richardson plot. $D = 1$ if the path is a straight line and $D = 2$ if it completely fills the 2-dimensional plane
Space allocation	The number of frames fish spend in predefined regions of the exposure arena divided by the total number of frames
Distance from center	Sum of individuals distance from the center of the exposure arena (cm) divided by the total number of frames. This is a measure of how close the fish swims to the walls of the arena
Relative burst frequency	The number of frames that velocity is >3 SD above mean velocity
Startle response	Duration of movement, latency to response, percent response, and burst swimming (response to vibratory/auditory stimulus)
Anti-predator response	Percent fish halting movement, latency to response, percent exhibiting startle-type response, direction of movement (toward or away visual stimuli), and group endpoints (response to overhead ''fly-by'' of bird silhouette)
Group endpoints	
Interactions	The number of times two fish swim within 0.1 body lengths of each other
Percent shoaling	Number of frames satisfying shoaling criteria divided by the total number of frames
Shoal NNA	Angle of trajectory between two fish in a shoal, must be greater than 45°
Shoal NND	Distance between nearest and second nearest neighbor for each fish in a shoal (minimum three fish)
Percent schooling	Number of frames satisfying schooling criteria divided by the total number of frames
School NNA	Angle of trajectory between two fish in a shoal, must be less than or equal to 45°
School NND	Distance between nearest and second nearest neighbor for each fish in a shoal (minimum three fish)
Percent solitary	Number of frames not satisfying shoaling or schooling criteria divided by the total number of frames
Solitary NND	Distance between nearest and second nearest neighbor for each individual fish not in a shoal or school
Velocity	Speed of fish calculated in centimeters per second

Recent efforts have refined a set of behavioral endpoints in order to investigate the effects of sublethal stressors on fish movement and responses to stimuli. A suite of behavioral endpoints has been developed to evaluate the effect(s) of specific compounds at low levels on fish (Table 32.2). In addition, individual and group models can be utilized with the ability to test different species of small fish. To illustrate the importance of using a suite of exposure endpoints, data from killifish (*Fundulus heteroclitus*) exposed at 25°C to an environmentally relevant concentration (40 $\mu g\ l^{-1}$) of dissolved brevetoxin failed to produce exposure-associated alterations in any nondirected movement parameter. (Brevetoxin is an important biotoxin produced by harmful algae (dinoflagellates) associated with ''red tides.'') However, this acute, sublethal, low-level exposure significantly altered startle responses in killifish. Conversely, a low anesthetic dose (60 mg l^{-1}) of the common anesthetic and model toxicant, methane tricanesulfonate,[174] was associated with significantly altered movement patterns (Figure 32.3), as well as startle responses.

In addition to exposure-related alterations in movement patterns, changes in startle response parameters can lend valuable insight into changes in the CNS that may have significant environmental consequences. Startle response parameters include, but are not limited to: response frequency, response latency, and average velocity. Startle responses can be elicited using a vibratory stimulus and quantified from small to medium-sized fish using the exposure system described previously in this chapter (Figure 32.2). Alternatively, more precise acoustical tone pips, generated through underwater speakers, can be used as a stimulus with small fish (Figure 32.4). Startle response testing can also yield data sets that can be used for screening large groups of animals.

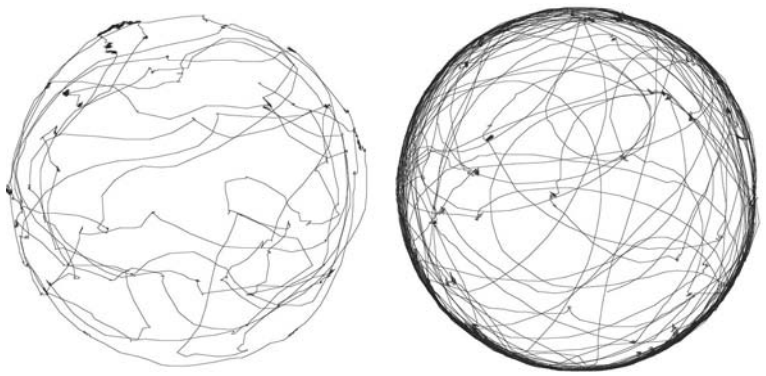

Figure 32.3 Paths of an individual killifish (*F. heteroclitus*) before (left) and after (right) exposure to a sub-anesthetic dose (60 mg l^{-1}) of MS-222. Analysis of these 30-min paths indicate that exposure to low doses of this anesthetic agent causes an increase in percent time in motion (from 12% to 49%) and movement velocity (9.1–11.9 cm s^{-1}), a decrease in path complexity (fractal dimension of 1.082–1.027), and a tendency to swim close to the arena periphery (change in distance from center). All of these endpoints describe quantifiably significant alterations in movement associated with exposure. Functionally, exposed animals tended to increase their speed and stay in motion to compensate for slight loss of equilibrium. The ''intoxicated state'' was depicted by ''hugging'' the vessel walls during movement and failing to maintain vigilance (loss of path complexity). MS-222 exposure also significantly altered the startle response of exposed fish such that there was a decrease in the number of responses, a decline in the response duration, and an increased response latency ($p < 0.02$). Startle responses to MS-222 and other chemical stressors were elicited using an instantaneous vibratory stimulus.

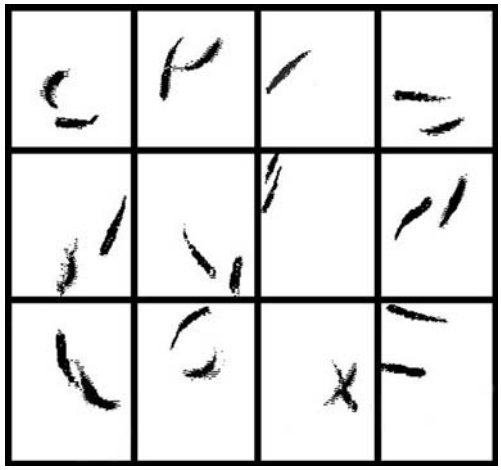

Figure 32.4 An alternative, more precise mechanism to elicit startle response. Video frame captures showing startle response from zebra fish (*Danio rerio*) exposed to instantaneously pulsed, tone pip (400 Hz, 150 db). The stimulus was generated using a tone generator, an amplifier, and a calibrated underwater speaker. Fish were acclimated individually in each of 12, small chambers. Video frames were captured 33 ms prior to the stimulus and 33 ms after the stimulus to visualize the position of the fish prior to and after eliciting startle response. A fish doublet is seen in all chambers where the animal startled in response to the stimulus. All animals startled in this image except for one (top row, 3rd column). In this trial, typical of others at this sound pressure level, 12 out of 12 fish in 8 out of 10 replicate trials startled; 11 out of 12 fish in the other two replicates startled. Note that the acoustic stimulus described here is different from the ''acoustical sound delivery board'' as illustrated and described in Figure 32.2.

Preliminary studies

When developing or applying automated systems, preliminary studies are essential in order to optimize and validate behavioral hardware and maximize the sensitivity of individual endpoints. For example, it is important to know (1) how a given species will acclimate to the exposure arenas, (2) effects of flow or toxicant infusion relative to distribution within the exposure arena, as well as the sentinel's response to flow (attractance/avoidance to dilution flow), and (3) the optimal duration of observation time intervals. Discerning appropriate observation time intervals (both timing and duration) is critical. Shorter observation time tends to introduce more noise (and a tendency for false positives; type I errors), while longer observation time tends to average out potentially important exposure-related differences (tendency for negatives; type II errors). Figures 32.5 and 32.6 provide sample data indicating changes in movement patterns during an acclimation study, and toxicant distribution visualized in a dye experiment, respectively.

The importance of understanding flow and mixing dynamics in test chambers should not be underestimated.[30] Most studies use steep gradient test systems, in which the chemical concentration increases sharply over a short distance; in most instances, the gradient is unknown to the experimenter but not to the fish. Fish must choose between clean water and contaminated water. When the gradient is steep, it may not mimic what fish actually experience in the field, except under unusual conditions, for example, immediately downstream from an effluent pipe or mine tailing. Kleerekoper et al.[30]

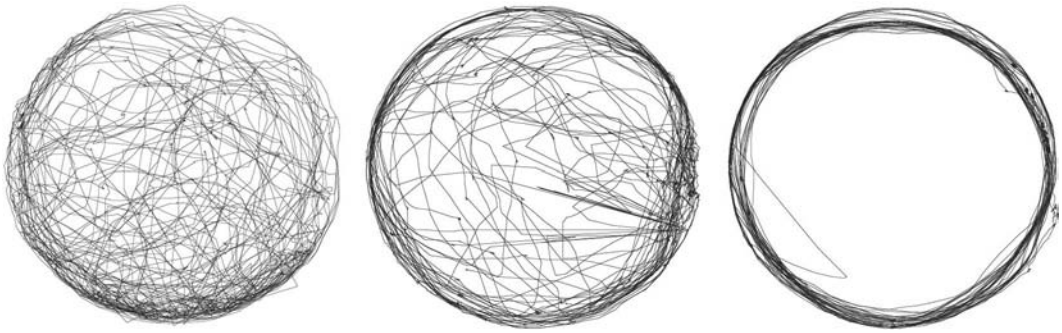

Figure 32.5 Acclimation experiment with killifish showing plotted x, y coordinate data for 30 min of movement from a single animal 1 h after entry into control exposure area (left), after 24 h (center), and after 48 h (right). These plots indicated that, over time, there was a significant, repeatable decrease in path complexity (fractal dimension), a reduction in angular change and time spent in the center of the vessel (swimming close to vessel wall). These data were useful in determining appropriate timing to expose and observe these fish. We found that after 24 h of arena acclimation, animals maintained sufficient movement complexity and repertoire to discern changes in nondirected swimming patterns in response to certain stress exposure scenarios.

showed that when goldfish (*C. auratus*) were attracted to copper concentrations between 11 and 17 $\mu g\, l^{-1}$ under shallow gradient conditions, but when the gradient was steep, fish avoided concentrations as low as 5 $\mu g\, l^{-1}$.[175]

Conclusions

Behavioral toxicology is a useful indicator of sublethal contamination because behavioral endpoints frequently occur below concentrations that are chronically lethal and at lower concentrations than those that affect growth.[140,141] As such, careful selection and design of behavioral tests could produce definable, interpretive endpoints for use in regulatory applications of product registration, damage assessments, formulation of water quality criteria and standards, and understanding changes in fish population dynamics based on anthropogenic effects. Behavioral endpoints could also, through the increased use and decreasing costs, be included in regulation of effluent standards. Although behavioral endpoints may often provide excellent sensitivity, utility, or biologic significance, behavioral responses have historically not been routinely included in standard aquatic toxicity assessment programs.

In developing behavioral test methods for contaminant assessment, behavioral toxicological endpoints must address one or more of three assessment questions: (1) How well does the response measure effect or injury arising from exposure? (2) Does the response aid in the identification of the toxic agent? (3) Does the use of the behavioral response increase the capability of contaminant assessments to predict the ecologic consequences of exposure? About 11 years ago, Little et al.[27] explicitly defined the challenges for behavioral toxicology: ''(1) more and better behavioral testing procedures ... must be developed and refined into effective tools which can be readily and clearly applied in contaminant assessments; (2) establishment of the links between behavioral effects observed in the laboratory and ecological effects observed in the field to aid in the development of contaminant assessment that are adequately predictive of population and community response; and (3) behavioral toxicologists must continue to work to dispel the

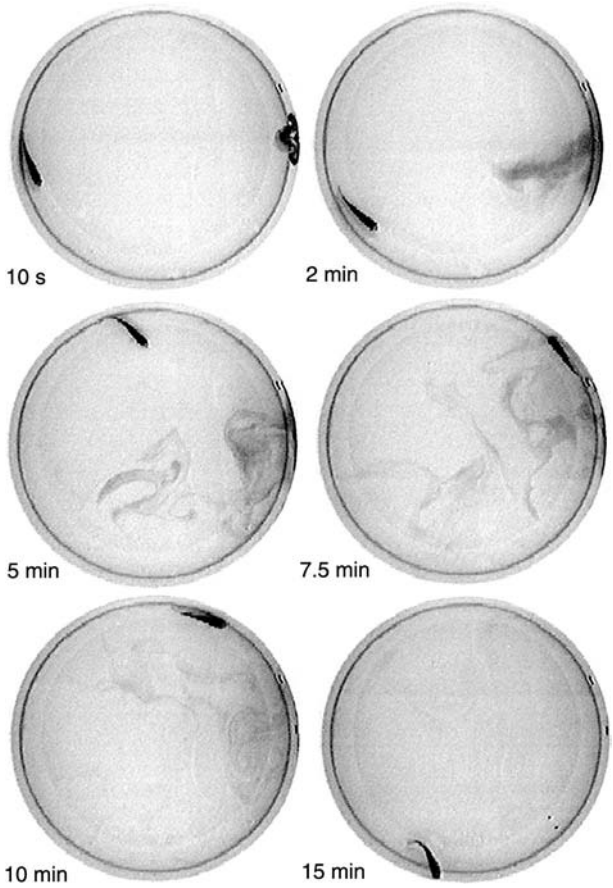

Figure 32.6 Preliminary experiment using nontoxic food-grade dye to discern patterns and timing of ''toxicant'' dispersion in exposure arenas. A 5-ml bolus of diluted dye was infused into multiple vessels and video frames were captured at 10 s, 2 min, 5 min, 7.5 min, 10 min, and 15 min. These sequential images from a single arena indicate that avoidance/attractance behavior can be observed within the initial 2–5 min of exposure, while the ''toxicant'' is not yet dispersed throughout the arena and there is still a concentration gradient in the ''sector'' of the arena where the stressor is pumped. After 15 min the dye is well mixed with the dilution water throughout the vessel. A number of factors will influence the rate of dispersion throughout the vessel, including the flow rate of the infused stress agent, the density and temperature of the mixtures, the number and species of test fish in the arena, and the shape and volume of the arena.

erroneous paradigm prevalent in aquatic toxicology that suggests that behavioral responses are not suitable for inclusion in contaminant evaluation and assessment because they are either unquantifiable, too complex, too variable, extraordinarily difficult to measure, not biologically significant, or lacking in ecologic relevance.''

Great strides have been made towards elucidating and developing better testing procedures, in large associated with improvements in videography and the power of personal computers, and with the availability of video equipment to fit almost any budget and work requirement. However, additional work is required to validate the use of many individual and group behaviors as endpoints of sublethal toxicant and stressor exposure.

Today, the relationship between many exposure-related behavioral alterations observed in the laboratory and their relevance to ecological effects observed in the field remains poorly understood. A notable exception is avoidance responses that have been intensively studied in the laboratory and verified in the field through the use of telemetry. As a result, avoidance is the only behavioral response that has legal standing in the United States as evidence of environmental injury under proceedings of the Comprehensive Environmental Response and Compensation Liability Act. Consequently, there is opportunity for researchers to actively contribute to the development of contaminant assessment paradigms that are predictive of population and community response. To accomplish this goal, sound experimental designs, combined with proper statistical analyses, are essential for developing approaches using fish movement for the bioindication of stressors or assessing mechanism of action.

Continued interest and new developments in automated behavioral monitoring and analysis techniques, especially those that can be verified in field trials, are being used to make functional inroads to dispel the erroneous paradigm that ''behavioral responses are not suitable for inclusion in contaminant evaluation and assessment.''[27] Behavioral responses should be more routinely included and integrated in contaminant evaluation as researchers continue to learn about and develop new automated techniques that allow for relatively inexpensive and rapid collection, processing, and verification of behavioral disruptions associated with contaminant exposure.

The behavioral exposure systems and endpoints described in this chapter can be applied to quantify differences in behavior associated with exposure to metals, organics, pesticides, algal bloom toxins, alterations in water quality, and agricultural waste. Behavioral endpoints also provide a valuable tool for the quantification of differences in reproductive behaviors, predator–prey interactions, behavioral changes across environmental gradients, and differences in swimming behavior between species. Analysis of behavioral responses to a variety of stimuli can provide quantitative measures of neural and mechanical disruption, reflecting biochemical and physiological alterations.[25] These additional tools support toxicological investigation with endpoints other than traditional LC_{50}'s, and may aid in determining new no observed effect levels (NOELs) and lowest observed effect levels (LOELs), as well as investigating the environmental relevance of various low-level exposures. Behavioral toxicology may ultimately provide sensitive, noninvasive, and broadly applicable endpoints for the description of integrated, whole animal responses associated with exposure to a wide variety of stressors.

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chapter thirty-three

Measuring metals and metalloids in water, sediment, and biological tissues

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Contents

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Introduction

Outbreaks of cadmium (Itai–Itai disease) and mercury (Minamata disease) poisoning during the 1950s made us acutely aware of the adverse consequences of high metal concentrations in our environment. Measurement of metals and metalloids became an integral component of our efforts to monitor and correct effects of anthropogenic emissions. The widespread introduction of commercial atomic absorption spectrophotometers (AAS) in the early 1960s[1,2] contributed enormously to the rapid increase in essential data. Today flame and flameless capabilities are incorporated together in most AAS units, allowing convenient measurement of elements present in ''milligram per gram'' to ''microgram per kilogram'' concentrations. Well-established preconcentration procedures are used to remove analytes from interfering matrices, as well as to concentrate them in small volumes. Flame and furnace chemistries are now sufficiently well understood to allow effective matrix modification for most elements.

Inductively coupled plasma-mass spectrometry (ICP-MS), a more recent technology, can also be used for rapid ultratrace multielement analysis. It consists of an ICP ion source, a quadrupole or magnetic sector mass filter, and an ion detection system.[3] The detection sensitivity of ICP-MS is generally better than the graphite furnace AAS. One important feature is that it can detect and quantify small variations on isotopic compositions in geological and environmental samples. (Relative isotope abundances of the metals are shown in Appendix I.) Only the most fundamental techniques for measuring metals dissolved in waters or present in solid samples are described herein. More up-to-date information about measurement of metals with ICP-MS can be found in the literature, e.g., References 4–6.

Materials required

General

Chemical safety

The analyst should not prepare or use any of the following reagents without fully understanding steps necessary for their safe use. Eye protection, protective clothing, and plastic gloves should be worn when handling strong acids. Always handle strong acids in a fume hood. The methods described here avoid the use of perchloric acid as it can react violently with organic materials. Nitric acid is a safer and spectroscopically cleaner; however, digestion of some samples with nitric acid may be unacceptable.

High purity acids

High purity nitric and hydrochloric acids may be purchased or produced. Commercially available acids include Ultrex® (Baker, Phillipsburg, NJ), Suprapur® (Merck, Darmstadt, Germany), and Aristar® (BDH Chemicals, Poole, England). Sub-boiling distillation with a Teflon® still, such as that sold by Berghof/America, Inc. (Raymond, NH), may also be used to generate these acids. Store distilled acids in acid-cleaned Teflon containers.

Acid cleaning of laboratory ware

No one procedure ensures both optimal allocation of effort and contamination-free analyses for all situations. Initially, the analyst should use procedural blanks to gain an understanding of the effort required for efficient, contamination-free analyses. Contamination control is one of the most crucial aspects in analysis of many trace metals.

Although expensive, it is preferable to use Teflon containers and laboratory ware. Linear polyethylene, polycarbonate plasticware and, perhaps, Pyrex are less costly alternatives. For example, some analysts may find a Pyrex filtration apparatus more convenient than the suggested polycarbonate unit in the dissolved metals procedure below. Pyrex volumetric flasks are a necessary compromise for careful volumetric measurement, although liquids may be weighed to avoid potential contamination from volumetric glassware. Rubber stoppers or seals, soft glass, Bakelite® caps, and caps made with contaminating seals, such as paper or aluminum, should not be used.

Clean all plasticware and glassware with a noncationic detergent (e.g., Acationox®, American Scientific Products, Boston, MA, or Citranox®, Alconox, White Plains, NY, 10603) before acid cleaning. Acid soak all materials that will touch the sample at least 24 h in 50% (v/v) concentrated nitric acid in deionized water. Rinse items 7–10 times with deionized water and place them in a contamination-free area to dry (e.g., under a Class 100 laminar flow clean hood). Adjust the duration of soaking, rinsing, and level of contamination control during drying in balance with the concentration of analyte expected. Items for the analysis of lead in water may require extreme attention to restricting contact with dust particles during drying, yet those used for the analysis of calcium in sediment will require much less attention. Use of procedural blanks aids in adjusting procedures to the appropriate level of rigor. The results from procedural blanks should dictate the specific details of washing and preparation, not rote adherence to any prescribed cleaning steps.

Many problems are eliminated if laboratory wares used for trace element measurement are used only for those analyses. Such dedicated laboratory wares should be stored in sealed plastic bags, e.g., Ziploc bags. Acid-cleaned pipette tips can be conveniently stored in an acid-cleaned, Teflon or linear polyethylene bottle with a polyethylene cap. Use these items as soon as reasonable after acid cleaning to minimize contamination during storage.

Soak membrane filters for dissolved metals analysis in dilute, high purity hydrochloric acid at least for 4 h. A 0.5 *N* HCl soaking solution can be made for this purpose by carefully adding 4 ml of concentrated, high purity hydrochloric acid to 92 ml of deionized water. Soak the filters for longer duration if blanks indicate contamination. Soaking can be done in a covered, acid-cleaned Teflon beaker (100 ml). Rinse filters thoroughly with metal-free deionized water prior to use. Acid-washed plastic or Teflon-coated tweezers should be used carefully to handle the filters.

If dissection is required in preparation of biological samples, metal-cutting tools can easily introduce metals, such as iron, zinc, and chromium. The authors use plastic, disposable utensils, such as those sold in grocery stores for picnics, as dissecting utensils. Plastic knives may be sharpened with nonmetallic abrasives, but they should be carefully cleaned and checked for contamination before use. These plastic utensils may be destroyed by prolonged soaking in strong acid. A dilute (10%, v/v) concentrated nitric acid soak for 12 h is probably adequate. However, the researcher should test one utensil in any cleaning procedure prior to general implementation, as some are destroyed by more concentrated acid solutions.

Standard materials

Standard reference materials are the most effective means of troubleshooting analytical problems during method development and of documenting accuracy during sample analysis. Sample spikes are useful in the absence of a standard material or to augment method troubleshooting. The U.S. National Institute of Standards and Technology (NIST) has standard materials for water, sediment, and animal tissue. The author also uses

materials supplied by the National Resource Council of Canada. Veillon[7] and Van Loon[8] list other sources of standard materials for biological tissues. At present, convenient suppliers include:

Standard reference materials

- National Institute of Standards and Technology, 100 Bureau Drive, Stop 2322, Gaithersburg, MD 20899-2322, USA
- U.S. Geological Survey, Crustal Imaging & Characterization Team, Box 25046, MS 973, Denver, CO 80225, USA
- Brammer Standard Co., Inc., 14603 Benfer Rd, Houston, TX 77069-2895, USA
- National Research Council of Canada, Institute for National Measurement Standards, 1200 Montreal Road, Bldg. M-36, Ottawa, Ontario, Canada K1A 0R6
- BSI, Customer Services, 389 Chiswick High Road, London, W4 4AL

Standard metal solutions

Stock solutions of 1000 μg/ml for preparing AAS standards can be purchased from several sources, including Fisher Scientific (Pittsburgh, PA), Sigma (St. Louis, MO), and Perkin-Elmer (Norwalk, CT). Standards have a finite shelf life and should not be used beyond their expiration dates.

Matrix modifiers

Atomic absorption spectrophotometry measures the absorption of light at a specific wavelength (λ) by the ground state atom (M^0) during excitation: $M^0 + E_\lambda \rightarrow M^*$. The absorption is proportional to the number of ground state atoms in the light path. In turn, the number of ground state atoms is a function of the concentration of the element in the sample and physicochemical reactions occurring in the flame or graphite furnace (Figure 33.1). The reactions occurring in the flame or furnace can be manipulated with matrix modifiers to optimize production of ground state atoms and, thus, enhance absorption (sensitivity).

For flame AAS, the sample is aspirated to form small droplets in a premix chamber. These droplets are swept into the flame and form dry ''clotlets'' of salts and other solids. The clotlets melt and vaporize in the flame. Simple metal compounds, such as the metal monoxides illustrated here, undergo a sequence of transitions that, at equilibrium, determine the measured population of ground state atoms (M^0):

$$MO + E_{\text{heat}} \leftrightarrow M^0 + O^0 \tag{33.1}$$

$$M^0 + E_\lambda \leftrightarrow M^* \tag{33.2}$$

$$M^0 + E_{\text{heat}} \leftrightarrow M^+ + e^- \tag{33.3}$$

Selection of a hot flame (e.g., nitrous oxide–acetylene, 2900°C) instead of a cool flame (e.g., air–acetylene, 2300°C) for elements with metal monoxide dissociation constants greater than approximately 5 eV assures that reaction (33.1) will produce ample amounts of M^0.[2] (Pertinent dissociation constants are listed in Appendix I.) A reagent (e.g., La) may also be added, which reacts with components in a droplet that would otherwise combine with the analyte to make it resistant to dissociation. If the flame is too hot, ionization in reaction (33.3) shifts the equilibrium to decrease the amount of M^0 present (Figure 33.2).

Elements with low ionization energies (see Appendix I), such as the alkali and alkaline-earth metals, are most prone to ionization.[9] Rare earth elements can also have ionization problems with a hot (nitrous oxide–acetylene) flame.[9] Adding excess amounts

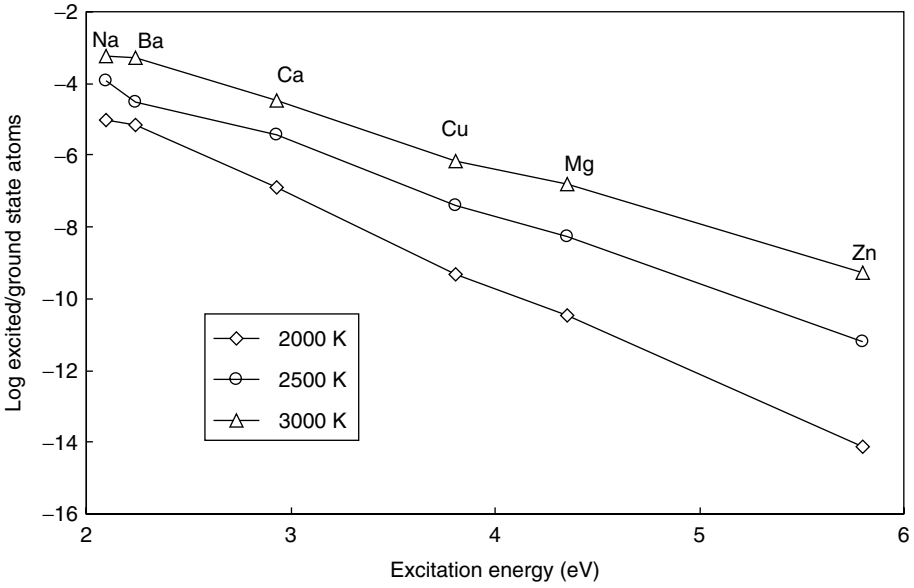

Figure 33.1 The relative amount of atoms excited and at ground state for flames with three different temperatures (Na, Ba, Ca, Cu, Mg, and Zn). Both temperature of the flame and the excitation energy determine the proportion of atoms that are excited. Regardless of excitation energy, the log (number of excited atoms/number of ground state atoms) increases with flame temperature. For any given temperature, the proportion of atoms excited decreases with increasing excitation energies. (Data from Table VIII-5 of Reference 60.)

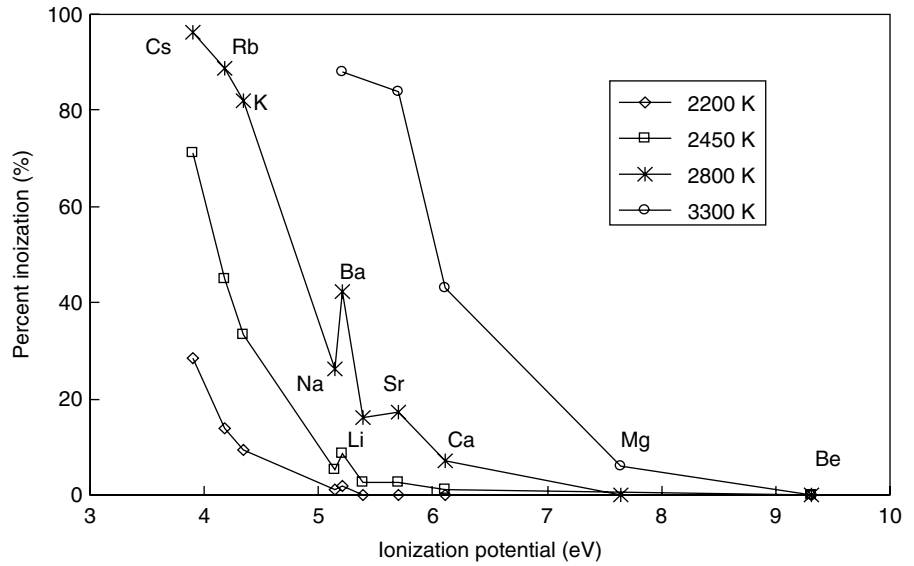

Figure 33.2 Percent of the total number of atoms that are ionized for 10 elements aspirated into different temperature flames. Note that some (e.g., Cs) have high proportions of atoms ionized even in a lower temperature (2450 K, oxygen–hydrogen) flame. Others (e.g., Mg) have minimal ionization even in the hottest (3300 K, nitrous oxide–acetylene) flame. (Data from Table 12-3 of Reference 61.)

of any element that ionizes readily (i.e., an ionization buffer) can shift the equilibrium back to favor M^0 (Figure 33.3). As is evident from their ionization potentials (E_i), cesium ($E_i = 3.89$), potassium ($E_i = 4.34$), sodium ($E_i = 5.14$) or, even, lanthanum ($E_i = 5.58$) can be used as ionization buffers.

For flameless AAS, a matrix modifier might be added for several reasons. In the furnace, a sample is dried onto a graphite surface and then heated to drive off any compounds that would interfere during subsequent measurement of analyte. After such pyrolysis, the sample is finally heated to a temperature sufficient to dissociate compounds containing the analyte. The amount of ground state atoms is measured at this atomization stage. Obviously, any material remaining after the pyrolysis stage can interfere with measurement during atomization. The presence of several salts of the analyte with their different dissociation constants may also change the shape (broaden or skew) of the absorbance curve during atomization and compromise quantification. Matrix modifiers (e.g., the Pd–Mg modifier described in the following or thiourea addition for cadmium analysis[10]) can be added to the sample that increase the temperature at which the analyte dissociates. In this way, more potentially interfering compounds can be driven off with higher temperatures during the pyrolysis stage without significant loss of analyte. This is especially helpful for elements with low boiling temperatures (see Appendix I) or those that form volatile compounds.

A matrix modifier can also be added to make components of the sample more volatile. Ediger[11] recommended additions of ammonium nitrate to samples containing large amounts of sodium chloride because the resulting sodium nitrate and ammonium chloride salts could be driven off at low pyrolysis temperatures.

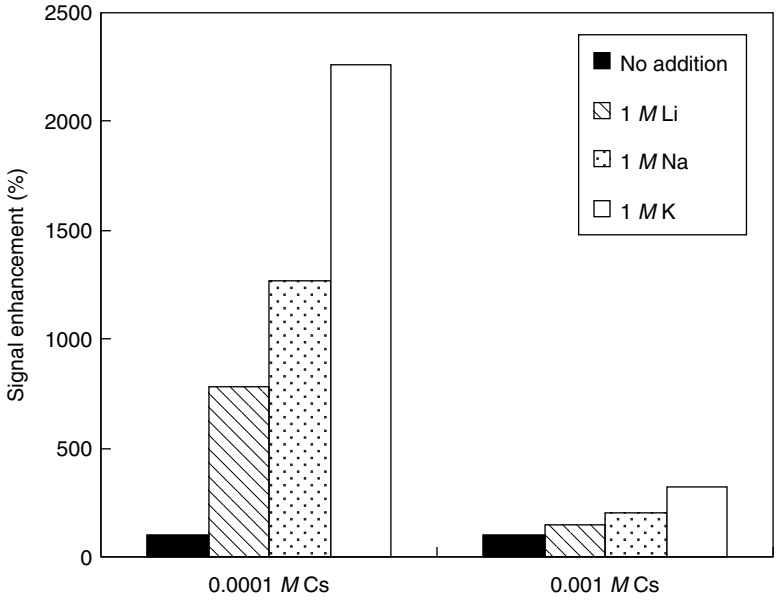

Figure 33.3 Signal enhancement (percentage increase relative to the signal for the solution with no addition of Li, Na, or K) for two different concentrations of Cs. Cesium, an easily ionized element (Figure 33.2), has a greatly enhanced signal if an excess of another easily ionized element is added. Elements with the lowest ionization potentials (see Figure 33.2) produce the best enhancement. The enhancement increases as more ionization buffer is added relative to the amount of Cs, e.g., 1 *M* ion buffer to 0.0001 *M* Cs versus 1 *M* ionic buffer to 0.001 *M* Cs. (Air–acetylene flame data from Table 12-4 of Reference 61.)

The excess of matrix modifier can also result in the preponderance of only one salt to dissociate during atomization. For example, the addition of excess H_3PO_4 to lead samples greatly improves the shape of the atomization curve by favoring formation of a single lead phosphate salt prior to atomization.[12] Similarly, Ediger[11] suggested that cadmium can be stabilized during furnace AAS by adding ammonium phosphate.

In general, the reagents recommended by Perkin-Elmer[13,14] and Schlemmer and Welz[15] are used here. Additional discussion of matrix modifiers can be obtained from References 1 and 12. The reagents described in Appendix I for analysis of each element are produced and used as described in the following. Preparation of $Pd(NO_3)_2$ and $Mg(NO_3)_2$ solutions are taken from Schlemmer and Welz.[15] The $Pd(NO_3)_2$ and $Mg(NO_3)_2$ reagents may also be purchased from Perkin-Elmer.

$Pd(NO_3)_2$ *stock solution* (1% w/v Pd): A minimum volume of Ultrex® or equivalent concentrated nitric acid is used to dissolve 1.0 g of palladium metal powder (Aldrich Chemical Co., Milwaukee, WI), and the dissolved metal is brought to 100 ml with deionized water. A small amount of metal may be dissolved first with mild heating and additional small amounts added until all is dissolved. $Pd(NO_3)_2$ can also be purchased as a salt (Sigma or Aldrich) to make this solution.

$Mg(NO_3)_2$ *stock solution* (1% w/v $Mg(NO_3)_2$): Dissolve 1.729 g of $Mg(NO_3)_2 \cdot 6H_2O$ in 100 ml of deionized water.

$NH_4H_2PO_4$ *stock solution* (1% w/v $NH_4H_2PO_4$): Dissolve 1.000 g of ammonium phosphate dibasic in 100 ml of deionized water.

5 μg Pd + 3 μg $Mg(NO_3)_2$ *reagent*: Use Eppendorf or similar calibrated pipettes with acid-cleaned tips to add the following volumes of stock solutions to a 25-ml, acid-cleaned volumetric flask: 1.250 ml of 1% Pd stock solution and 0.750 ml of the 1% $Mg(NO_3)_2$ stock solution. Bring to volume with deionized water. Add 10 μl per furnace injection for matrix modification as indicated in Appendix I.

5 μg $Mg(NO_3)_2$ *reagent*: Use Eppendorf or similar calibrated pipettes with acid-cleaned tips to add 1.250 ml of the 1% $Mg(NO_3)_2$ stock solution to a 25-ml, acid-cleaned volumetric flask. Bring to volume with deionized water. Add 10 μl per furnace injection for matrix modification as indicated in Appendix I.

15 μg $Mg(NO_3)_2$ *reagent*: Use Eppendorf or similar calibrated pipettes with acid-cleaned tips to add 3.750 ml of the 1% $Mg(NO_3)_2$ stock solution to a 25-ml, acid-cleaned volumetric flask. Bring to volume with deionized water. Add 10 μl per furnace injection for matrix modification as indicated in Appendix I.

50 μg $NH_4H_2PO_4$ + 3 μg $Mg(NO_3)_2$ *reagent*: Use Eppendorf or similar calibrated pipettes with acid-cleaned tips and acid-cleaned volumetric pipettes to add the following volumes of solutions to a 25-ml, acid-cleaned volumetric flask: 12,500 ml of 1% $NH_4H_2PO_4$ stock solution and 0.750 ml of 1% $Mg(NO_3)_2$ stock solution. Bring to volume with deionized water. Add 10 μl per furnace injection for matrix modification as indicated in Appendix I.

$LaCl_3$ *reagent* (50 g/l La): (Caution: the following solution reacts vigorously during preparation.) Dissolve very small amounts of lanthanum oxide (La_2O_3) at a time and, after complete dissolution of each small amount, add a small amount more. Cautiously continue this process until all is dissolved. Carefully and slowly dissolve a total of 29.3 g of La_2O_3 in 250 ml of concentrated hydrochloric acid. Then cautiously dilute to 500 ml with deionized water. Add 1 ml of this $LaCl_3$ solution to 10 ml of each sample, blank and standard prior to analysis. Alternatively, 133.6750 g of lanthanum chloride heptahydrate ($LaCl_3 \cdot 7H_2O$) can be added to 1 l of deionized water to make this 50 g/l La solution.

KCl *reagent* (50 g/l K): Dissolve 95 g of KCI in 1 l of deionized water. Add 200 μl of this reagent to 10 ml of each sample, blank and standard prior to analysis.

Dissolved metals in water

- Acid-cleaned 500-ml Teflon or linear polyethylene sample bottles
- Acid-cleaned 60-ml, wide mouth, Teflon or linear polyethylene bottles for filtered samples
- Acid-cleaned 47-mm, polycarbonate filtration funnel and receiving flask
- Acid-soaked 0.45-μm membrane filter (e.g., Millipore HAWP 047 or AAWP 047 filters)
- Acid-cleaned plastic or Teflon-coated tweezers for handling the filters
- Acid-cleaned 500-ml squirt bottle containing deionized water
- Source of ultrapure deionized water with consistently low concentrations of the elements of interest (e.g., ASTM Type I reagent grade water)
- Vacuum pump with a liquid trap
- Class 100 laminar flow hoods if required
- Plastic gloves (without talc powder)
- Calibrated fixed-volume pipettes (e.g., Eppendorf pipettes) of appropriate volumes
- Acid-cleaned pipette tips of appropriate sizes
- An appropriate standard material
- Insulated chest containing ice for field chilling of samples
- Plastic Ziploc® bags

Metals in biological tissue or sediments

- Acid-cleaned Teflon beakers with a spout to allow effective pouring of the digest (25, 50, or 100 ml volume depending on the weight of sample to be digested, one per sample, including blanks and standard materials)
- Acid-cleaned Teflon watch glasses adequate to completely cover the Teflon beakers or loose-fitting beaker lids, such as those sold by Berghof/American, Inc. (Raymond, NH)
- Acid-cleaned Teflon funnels for digest transfer from beakers to volumetric flasks
- Acid-cleaned 60-ml, Teflon or linear polyethylene bottles for sample digests
- Acid-cleaned, plastic dissecting utensils if required
- Acid-cleaned, Class A Pyrex 25-ml volumetric flasks with stoppers
- Acid-cleaned, Class A Pyrex 5-ml volumetric pipette
- Acid-resistant pipette bulb
- Calibrated fixed-volume pipettes (e.g., Eppendorf pipettes) of appropriate volumes
- Acid-cleaned pipette tips of appropriate sizes
- Acid-cleaned 500-ml squirt bottle of deionized water
- Source of ultrapure deionized water with consistently low concentrations of the elements of interest (e.g., ASTM Type I reagent grade water)
- Plastic gloves (without talc powder)
- 0° to l00°C thermometer (preferably traceable to an NIST thermometer)
- Hot plate capable of holding a constant temperature of 80°C
- Pyrex pan to serve as a water bath into which the Teflon beakers may be placed
- Appropriate standard materials

Procedures

Dissolved metals in water

Dissolved metals are procedurally defined as those passing through a 0.45-μm filter.

Sampling

Sufficient numbers of acid-cleaned sample bottles should be carried to the field inside sealed plastic bags. Bring enough to take replicate samples at all or a subset of sample sites. Travel blanks may be produced by pouring a deionized water ''sample'' into acid-cleaned containers in the field. Travel blanks should be handled like all other samples. Field spikes or field processing of solutions of known metal concentrations are also helpful in tracking accuracy and precision of the entire sampling and measurement process.

Rinse each sample bottle (including the cap) with sample at least four times prior to filling with the final sample. Do not allow the sample to touch your hand or any other contaminating materials prior to entering the sample bottle. Do not agitate sediments or dislodge materials from vegetation during sampling. Do not take a sample near the water surface as some metals are concentrated in the surface microlayer. Seal the sample bottle in a Ziploc® plastic bag and place it into an ice-filled cooler chest. Process the sample as soon as reasonable, usually within 24 h of sampling. Ideally, the sample should be filtered immediately. Adjust the above sampling instructions based on results from spiked samples and travel blanks.

Filtration

Ideally, one acid-cleaned filtration apparatus should be used for each sample. If this is unreasonable, samples could be filtered in sequence through one or a few filtration apparatus. If information is available about the relative concentrations expected in the samples, samples suspected to have low concentration should be filtered first so as to minimize potential contamination among samples. If there are extreme differences in metal concentrations among samples, samples can be grouped and filtered through separate apparatus. Regardless, results from filtration blanks should be used in any final judgment of the method adequacy.

With deionized water, thoroughly rinse all surfaces of the filtration apparatus that will contact the sample. Rinse the filter with deionized water and place it into the apparatus. Filter 50–100 ml of deionized water through the unit and discard the filtrate. Repeat this process at least three times. Use caution when disconnecting the vacuum tubing from the filtration flask because contaminating particulates can be sucked into your sample during the abrupt equalization of pressure. Initially use blanks and known metal solutions to assess the adequacy of these procedures for your specific needs and adjust as required.

Place 50 ml of water sample into this thoroughly rinsed apparatus. Allow at least 25 ml to filter into the receiving flask. Rinse the receiving flask with this filtered sample and discard the rinse. Repeat this sample rinsing process. Place more samples into the filtration funnel and collect approximately 60 ml of filtered sample. Transfer approximately 10 ml of this filtered sample into an acid-cleaned, Teflon or polyethylene bottle. Use this sample aliquot to completely rinse the bottle and then discard the rinse. Fill the bottle with the remaining 50 ml of sample. Add 100 μl of concentrated, ultraclean nitric acid to the sample using a pipette with an acid-cleaned tip. This 0.2% (v/v) nitric acid

preserved sample should have a pH less than 2. (Do not place the pH probe into the sample to check the pH as this could result in gross contamination. Instead use another aliquot of filtered sample for assessing the adequacy of acidification.) Add more acid if required for adequate adjustment of your sample type.

Other methods exist for sampling dissolved metals. Windom et al.[16] pumped samples through cartridge filters in the field. The filtrate flowed directly into a polypropylene sample bottle and was acidified under a portable clean bench. This procedure requires considerable field effort and equipment but, if feasible, eliminates many ambiguities regarding changes in unfiltered water during transport and potential contamination upon contact with various laboratory wares.

Often, dissolved element concentrations are below the detection limit or, in the case of marine waters, present in a high salt matrix. The metals must then be removed from their original matrix and concentrated before analysis using one of several methods. They may be removed with solid resins (e.g., References 17 and 18), chelated and extracted with a solvent (e.g., References 19–25), or chelated and co-precipitated (e.g., Reference 26). If the chelation-extraction procedure (e.g., Reference 27) is used, it is important to keep in mind that pH of the sample is critical to effective extraction[25] that humic acids can interfere,[24] and that the potential for contamination increases with preconcentration.

Storage and analysis

Minimal storage time of dissolved metal samples is recommended. Although regulatory guidelines for maximum storage times range from 28 days to 6 months, spiked samples and sample blanks should be used to assess the best storage time for your particular needs. Store samples at 4°C, away from any potential source of contamination. They may be stored in the dark to avoid precipitation if silver concentrations are to be measured. Evaporation during storage can increase sample concentrations and should be avoided. A mark indicating the sample volume on the outside of each container can help in monitoring any potential volume changes.

Allow the sample to come to room temperature before analysis. To avoid contamination, never sample directly from the sample bottle. Instead, pour an aliquot from the bottle into an acid-cleaned container and discard the aliquot after use. Analyze the sample according to Appendix I and the AAS manufacturer's instructions.

Calculation of free metal concentration

The method described here applies to the measurement of dissolved metal, which includes free metal ions, metal–ligand complexes, metal monoxides, and other species. Notionally, the most bioavailable and toxic form is the free metal ion.[28–30] The free ion concentrations can be estimated with chemical equilibrium calculations, either manually or with computer software (e.g., Visual MINTEQ[31]). Figure 33.4 illustrates the fraction of free copper or silver to the dissolved copper or silver at different pH values, in both seawater and river water.

Metals in biological tissue or sediments

Sampling, dissection, and storage

It is especially important with solid samples, such as sediments or tissues, that a large enough sample be taken so as to be representative. Too often treated as homogeneous

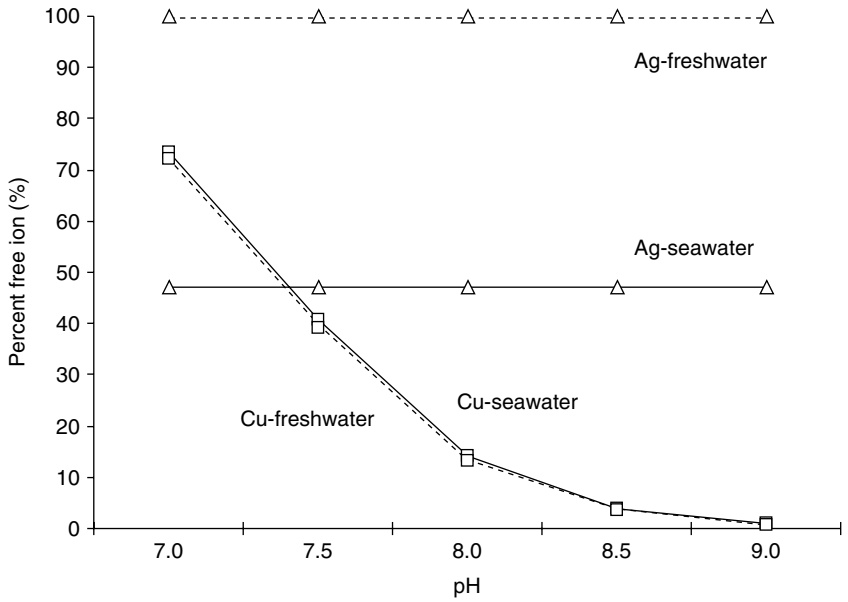

Figure 33.4 Percent of dissolved copper that is free copper ion and percent of dissolved silver that is free silver ion in normal seawater and river water at different pH conditions (calculated with Visual MINTEQ). The percent of free Cu^{2+} to dissolved Cu varies with pH of the water. In contrast, that of dissolved Ag is much less variable with the same water component.

materials, these materials range from poorly mixed to well-mixed, heterogeneous samples. Methods for calculating representative weights are provided by Ingamells and Switzer,[32] Ingamells,[33] Wallace and Kratochvi1,[34] and Newman.[35]

Sediments should be taken from a specified depth as metal concentrations and species can change rapidly with depth. If possible, nonmetallic or samplers with nonmetallic coatings (e.g., a Teflon-coated dredge) should be used to minimize contamination. The sediment may be mixed, put into an acid-cleaned, Teflon or plastic bottle, and stored on ice until return to the laboratory. It should be stored at 4°C, away from any potential source of contamination until processed.

Biological samples could be tissues, organs, or entire organisms depending on the goals of the study. If the entire animal or the gut alone is to be analyzed, the organism must be allowed ample time to clear its gut of contaminating food. Tissues and organs may be carefully dissected from the animal using acid-cleaned, plastic utensils. Whole animals or dissected tissues should be placed into acid-cleaned, wide mouth containers (Teflon or plastic) and stored frozen until freeze-drying can be performed. The 20-ml polypropylene scintillation vials with linerless caps are ideal for small samples. Placing sample vials in a Ziploc® bag along with several ice cubes can reduce sublimation from samples during prolonged storage. Appropriate standard reference materials should be processed in tandem with samples from this step onward. They will reflect the quality of the process from storage through measurement.

Freeze-drying

Loosening or removing caps from storage vials allows freeze-drying of samples in their storage containers. Freeze-dry until no more weight (water) is lost from the sample. Tighten caps onto vials prior to storage under desiccation. (Do not use desiccant that

sheds small particulates that could contaminate the sample.) Digest the samples, including the standard reference materials, as soon as reasonable after freeze-drying. Note that volatile metals, including those forming volatile organic compounds, such as mercury, can be lost during freeze-drying.[36] Wet tissue can be analyzed for mercury and expressed later as dry weight using a wet-to-dry weight ratio. Relative to volatile elements, it is important to realize that most standard reference materials are freeze-dried materials that may have lost volatile compounds of analytes before they were analyzed for certified concentrations. Consequently, the lack of any apparent loss during freeze-drying of a standard material in tandem with samples may not accurately reflect loss from sample. Splits of materials analyzed before and after freeze-drying would better reflect such loss.

Digestion

Wear proper eye protection, protective clothing, and plastic gloves while working with strong acids. Digestion should be done in a hood capable of efficiently removing the generated acid fumes.

Weigh 0.1000–0.5000 g of freeze-dried tissue or sediment and record the exact weight for later calculations. (The optimum weight needed to be representative can be estimated as described by Newman.[35]) It is best to use the same weight for all samples. Place the sample into an acid-cleaned Teflon beaker and cover immediately with an acid-cleaned Teflon watch glass or lid. Approximately 5–10% of all samples to be digested should be reference materials and an additional 5–10% should be procedural blanks (beakers without sediment or tissue added). Whether 1 in 5 or 10 samples is a blank or standard material will depend in the element of interest and its concentration in the materials being analyzed. Low concentrations of easily contaminated analytes require numerous blanks and standard materials.

After all samples have been placed into beakers, slowly add 5 ml of concentrated, ultraclean nitric acid to each. More acid may be required to completely wet, and cover some types of samples, e.g., light powders. Make certain that all of the material contacts acid and no internal pockets or edges remain dry. Allow the covered beakers containing sample and acid to sit for 1 h at room temperature. This predigestion reduces the risk of vigorous foaming and loss of sample upon heating. Next, carefully place the covered beakers onto the hot plate and allow them to reflux at 80°C for 4 h. (A beaker filled with water and placed among the beakers containing samples can be used to monitor digestion temperature.) If there are wide deviations in temperature on the surface of the hot plate, a Pyrex pan partially filled with water can be placed onto the hot plate to provide more uniform heating of samples. Rotation of samples on the hot plate may also ensure that heating is less variable among samples. Periodically check to ensure that the entire bottom of each beaker remains wet and that the partially digested samples remain covered with acid. Add more acid if required to keep the entire sample wet with hot acid.

After 4 h, carefully remove the watch glass or lid from each beaker. (Some samples may require additional time to digest adequately. Use the digests of standard materials to assess the completeness of digestion or loss of analyte by excessive digestion.) Gently and carefully rinse the droplets of acid from the watch glass or lid back into the beaker using deionized water in a squirt bottle. Place the samples back onto the hot plate and allow the digest volume to evaporate to approximately 2.5 ml. After cooling, quantitatively transfer the digest to a 25-ml, acid-cleaned Class A volumetric flask using successive rinses with deionized water. Allow each digest to cool in the volumetric flask and bring the volume to nearly 25 ml with deionized water. Gently mix the digest in the flask and allow it to cool

again. Carefully bring the digest to 25 ml volume. This digest has a 10% (v/v) nitric acid matrix. If concentrations allow further dilution, a 50-ml volumetric flask can be used instead to produce a 5% (v/v) nitric acid matrix. Carefully transfer the digest to an acid-cleaned Teflon, polypropylene, or polyethylene storage container.

This procedure has been used successfully by Newman and Mitz[37] and Newman et al.[38] for Pb and Zn analysis in biological tissues. Standard materials were used to indicate the adequacy of the digestion for Pb[37] (National Research Council Canada TORT-l standard) and Zn[38] (U.S. EPA Quality Control Samples, Metals in Fish). However, it may not be adequate for samples with high concentrations of lipids. Incompletely digested lipids produce a turbid digest with a surface film. An inadequate digestion will be indicated by spurious values upon repeated analysis of digest aliquots or unacceptably low concentrations for standard reference materials. More rigorous digestion is required in this case. Clegg et al.[39] and Sinex et al.[40] compare alternative digestion procedures for biological tissue and sediments, respectively. Siemer and Brinkley[41] and Chung and Tsai[42] describe simple refluxing systems for acid digests, and Uhrberg[43] describes an acid digestion bomb. Very effective methods are described that use conventional microwave ovens[44,45] or microwave ovens specifically designed for sample digestion (see the following).

Microwave ovens specifically designed for sample digestion (e.g., CEM MARS 5 Microwave Digester, Matthews, NC) are widely used at this time.[46–48] This method can provide more complete digestion than the method introduced above. The associated procedures for the CEM MARS 5 Microwave Digester are briefly introduced here. After freeze-drying, sample aliquots are weighed on an analytical balance and the exact weight recorded for use in later calculations. Transfer the entire sample to an acid-cleaned, microwave digestion vessel. Add 5 ml of concentrated, ultraclean nitric acid to the vessel and rinse the vessel sides with the acid to sweep the entire sample to the bottom. Place 5 ml of concentrated, ultraclean nitric acid as a blank in another vessel and cover it with a special top with glass tubing for the temperature probe. Make sure the rupture membrane is inserted into each blue Teflon vent cover. Insert the vessel into the support module and fasten it with a torque wrench. Thread the temperature probe into the blank vessel lid and attach the pressure sensor to the top of the vented lid. Insert the vessel modules into the turntable, make sure the weight is evenly balanced, and place the turntable into the microwave oven. Close the door firmly after attaching the temperature probe and pressure sensor to the microwave oven. Select the appropriate method and begin the digestion. During the first few minutes of the digestion, observe the vessels to ensure that no steam is venting. Venting indicates missing or damaged rupture membranes and the digestion should be stopped. Caution should be taken when removing things from the microwave oven because the vessels may be hot and pressured. After the programmed digestion is finished, wait until the temperature has dropped to room temperature. Disconnect the pressure and temperature probes. Place the turntable under an acid fume hood. When removing the module, point the vent away from yourself and slowly open the vent.

Carefully decant the sample from the vessel into a 25-ml volumetric flask. Rinse the vessel sides thrice with small volumes of ultrapure deionized water and transfer that water into the 25-ml volumetric flask. Do not exceed the 25 ml volume. Use the ultrapure deionized water to bring the sample volume to 25 ml, mix well, and store in a clean sample container. These samples are ready to be analyzed.

The nitric acid digest of sediments might not accurately reflect the concentration of bioavailable metal in many cases. Digestive enzyme, gut amino acids, and other factors can affect the bioavailability. Chemical procedures have also been developed for determining metal concentrations in various sediment fractions. Concentrations in the various fractions

may be related to solid phase species and bioavailability. Tessier et al.[49,50] describe one such technique in current use. If applied, it is important to acknowledge that the resulting fractions are procedurally defined ones and artifacts might be present.[51,52] Pai et al.[53] provide specific recommendations for graphite furnace AAS analysis of the complex sediment extract matrices generated with this procedure. Digestive solubilization, which is a major route of exposure for many metals in sediments (e.g., Reference 54), can be qualified with biomimetic techniques. The digestive fluids extracted from the gut lumens of deposit feeders are added to sediments. The mixture is incubated for a certain period of time before the dissolved metals in the digestive fluids are measured by AAS or ICP-MS.[55]

Storage and analysis

Digests should be analyzed as soon as reasonable. Storage under refrigeration will reduce change in volume due to evaporation. Store the digests away from any contaminating materials.

Allow the sample to come to room temperature before analysis. To avoid contamination, never sample directly from the sample bottle. Instead, pour an aliquot from the bottle into an acid-cleaned container. Discard the aliquot after use; do not return it to the sample bottle. Analyze the sample according to Appendix I and the AAS or ICP-MS manufacturer's instructions. Any dilutions of sample digests should be performed in acid-cleaned containers with special attention given to changes in the matrix, for example, a significant change in viscosity associated with dilution. Matrix matching is often required for many elements and sample dilutions. The method of standard addition can be used to assess the amount of matrix matching required.

Results and discussion

Concentrations are estimated using aqueous or solid standards matching the sample. The method of standard addition can be used if there are matrix effects. Particularly with graphite furnace AAS and samples requiring various dilutions, it is prudent to assume that a matrix effect is present unless shown to be otherwise.

Although method detection limit is more commonly reported, measurement relative uncertainty also provides the end user with sufficient information to assess data quality.[35] If the data include ''below detection limit'' observations, they must be treated as censored data in subsequent statistical analyses.[56,57] It is also essential that results of standard reference materials or, minimally, spiked samples recoveries, be reported. Expected reference material values and acceptable limits should also be reported. Comment must also be made regarding results from procedural blank analysis. Without this information, the end user has no means of determining the quality of measurement.

Appendix: Analytical details for specific elements

Extracted information from the works of Dean,[58] Lide,[59] Van Loon,[8] Varma,[1] Perkin-Elmer,[13,14] and Price.[2] Sensitivities are expressed in milligram per liter to produce a 0.0044 Abs unit (1% absorption) for flame AAS. For furnace AAS, characteristic masses are picogram to produce a 0.0044 Abs unit for a Perkin-Elmer transverse heated graphite atomizer.[14] Because values for sensitivity and characteristic mass vary among instruments, those given reflect only general ranges and relative values among wavelengths: AW, atomic weight; D_0, dissociation energy of the metal monoxide in eV; BP, boiling

point in °C; and E_i, ionization energy in eV. For those using ICP-MS to determine isotopic compositions, published relative abundances of each elements' isotopes are listed as percentages of total element abundance, e.g., Ag-107 51.84% below indicates that 51.84% of Ag in a sample will be the Ag-107 isotope.

Ag (silver, AW 107.868, D_0 2.2, E_i 7.57, BP 2163, Ag-107 51.84%, Ag-109 48.16%)

General: Silver solutions should be stored in an amber or opaque container as light could cause silver to precipitate.[13] Hydrochloric acid should not be used because silver will precipitate with chloride.

Flame technique: Use a lean air–acetylene flame to obtain sensitivities of 0.1 mg/l (λ = 328.1 nm) and 0.2 mg/l (λ = 338.3 nm).[2] Large amounts of aluminum can cause signal suppression.[2] Bromide, chromate, iodate, iodide, permanganate, tungstate, and chloride may precipitate silver from solution.[13]

Flameless technique: Perkin-Elmer[14] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 800°C and 1500°C, respectively. The characteristic mass is approximately 4.5 pg at 328.1 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is less than 1 ng/l.

Al (aluminum, AW 26.98154, D_0 5.3, E_i 5.99, BP 2520, Al-27 100%)

General: During preparation of working standards, aluminum can quickly adsorb to glass volumetric flasks. Always add aliquots of the stock solution to acidified (nitric acid) deionized water at the bottom of each volumetric flask to avoid adsorption.

Flame technique: Respectively, the 308.2, 309.3, and 396.2 nm wavelengths have sensitivities of approximately 1.5, 1.1, and 1.1 mg/l[13] with the use of a rich nitrous oxide–acetylene flame and the presence of excess La or K to suppress ionization. ($LaCl_3$ and KCl solutions described above.) Price[2] reported slight signal suppression by calcium, silicon, and perchloric and hydrochloric acids. Acetic acid, titanium, and iron may enhance signal.[13]

Flameless technique: Perkin-Elmer[14] recommends addition of 15 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1200°C and 2300°C, respectively. The characteristic mass is approximately 31 pg at 309.3 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

As (arsenic, AW 74.9216, D_0 5.0, E_i 9.81, BP 615 (hydride AsH_3-55, As-75 100%)

Flame technique: Generally, a hydride generation method is used. However, a rich air–acetylene flame will produce sensitivities of 0.78, 1.0, and 2.0 mg/l at the 189.0, 193.7, and 197.2 nm wavelengths. Background absorption is high at these wavelengths.

Flameless technique: Perkin-Elmer[10] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization

temperatures should be in the ranges of 1200°C and 2000°C, respectively. The characteristic mass is approximately 40.0 pg at 193.7 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Ba (barium, AW 137.33, D_0 5.8, E_i 5.21, BP 1805, Ba-130 0.11%, Ba-132 0.10%, Ba-134 2.42%, Ba-135 6.59%, Ba-136 7.85, Ba-137 11.23%, Ba-138 71.7%)

Flame technique: Price[2] and Varma[1] suggest a rich nitrous oxide–acetylene flame with an ionization buffer (KCl solution described above) at 553.6 nm to obtain a sensitivity of approximately 0.4 mg/l. Price[2] recommends analysis without an ionization buffer if the less sensitive 455.4-nm wavelength is used (sensitivity = 2.0 mg/l).

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 1200°C and 2300°C, respectively. The characteristic mass is approximately 15.0 pg at 553.6 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is less than 1 ng/l.

Be (beryllium, AW 9.01218, D_0 4.6, E_i 9.32, BP 2472, Be-9 100%)

General: This toxic element should be handled with appropriate caution.

Flame technique: A rich nitrous oxide–acetylene flame is recommended. Price[2] reports a sensitivity of 0.05 mg/l at 234.9 nm. Absorption may be enhanced by nitric and sulfuric acids.[2] High concentrations of aluminum, sodium, silicon, and magnesium can depress sensitivity.[1,2,13]

Flameless technique: Perkin-Elmer[14] recommends addition of 15 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1500°C and 2300°C, respectively. The characteristic mass is approximately 2.5 pg at 234.9 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Bi (bismuth, AW 208.9804, D_0 3.6, E_i 7.29, BP 1564, Bi-209 100%)

Flame technique: Price[2] reports the following approximate sensitivities: 0.8 (223.1 nm), 1.5 (222.8 nm), 10 (227.7 nm), and 2.2 (306.8 nm) mg/l with a lean air–acetylene flame.

Flameless technique: Perkin-Elmer[14] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1100°C and 1700°C, respectively. The characteristic mass is approximately 60.0 pg at 223.1 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is less than 1 ng/l.

Ca (calcium, AW 40.08, D_0 4.8, E_i 6.11, BP 1494, Ca-40 96.95%, Ca-42 0.65%, Ca-43 0.14%, Ca-44 2.86%, Ca-46 0.004%, Ca-48 0.19%)

Flame technique: With a lean nitrous oxide–acetylene flame and La (or alkali salt) additions, sensitivity is approximately 0.03 mg/l but increases twofold with use of a stoichiometric

or lean air–acetylene flame at 422.7 nm.[2] Sensitivity is 20 mg/l at 239.9 nm (air–acetylene flame). [KCl and $LaCl_3$ solutions described above can be used to reduce ionization (KCl) or chemical interferences ($LaCl_3$).] With the lean air–acetylene flame, high concentrations of aluminum, beryllium, phosphorus, silicon, titanium, vanadium, or zirconium can interfere with analysis.[1,2,13]

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 1100°C and 2500°C, respectively. The characteristic mass is approximately 1.0 pg at 422.7 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 10–100 ng/l.

Cd (cadmium, AW 112.41, D_0 1.5, E_i 8.99, BP 767, Cd-106 1.25%, Cd-108 0.89%, Cd-110 12.49%, Cd-111 12.80%, Cd-112 24.13%, Cd-113 12.22%, Cd-114 28.73%, Cd-116 7.49%)

General: With graphite furnace AAS, contamination can be a significant problem. Use special care in acid cleaning and sample preparation. This element is relatively toxic and should be handled appropriately.

Flame technique: With a lean air–acetylene flame, sensitivity is 0.015 mg/l at 228.8 nm and 20 mg/l at 326 nm.[2] High concentrations of silicate interfere with analyses.[13]

Flameless technique: Perkin-Elmer[10] recommends addition of 50 μg $NH_4H_2PO_4$ and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 700°C and 1400°C, respectively. The characteristic mass is approximately 1.3 pg at 228.8 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Co (cobalt, AW 58.9332, D_0 3.8, E_i 7.88, BP 2928, Co-59 100%)

Flame technique: Sensitivities using a rich air–acetylene flame for the 240.7, 242.5, 252.1, and 341.3 nm wavelengths are 0.08, 0.2, 0.5, and 4.0 mg/l, respectively.[2] Perkin-Elmer[13] and Varma[1] note that large amounts of transition and heavy metals depress signal.

Flameless technique: Perkin-Elmer[14] recommends addition of 15 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1400°C and 2400°C, respectively. The characteristic mass is approximately 17.0 pg at 242.5 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Cr (chromium, AW 51.996, D_0 4.4, E_i 6.77, BP 2672, Cr-50 4.35%, Cr-52 83.79%, Cr-53 9.50%, Cr-54 2.36%)

Flame technique: Sensitivities using a rich air–acetylene flame for the 357.9, 359.3, 360.5, and 425.4 nm wavelengths are approximately 0.05, 0.15, 0.2, and 0.5 mg/l, respectively.[2] Phosphate, nickel, and iron may interfere with an air–acetylene flame.[1,2,13] Perkin-Elmer[13] and Varma[1] indicate that the addition of calcium can eliminate the effect of phosphates and the addition of 2% (w/v) ammonium chloride can reduce the effect of iron.

Flameless technique: Perkin-Elmer[14] recommends addition of 15 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1500°C and 2300°C, respectively. The characteristic mass is approximately 7.0 pg at 357.9 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Cu (copper, AW 63.546, D_0 3.6, E_i 7.73, BP 2563, Cu-63 69.17%, Cu-65 30.83%)

Flame technique: Using a lean air–acetylene flame, Price[2] reports the following sensitivities for 217.9, 222.6, 244.2, 249.2, 324.8, and 327.4 nm wavelengths are approximately 0.6, 2.0, 40, 10, 0.04, and 0.1 mg/l, respectively.

Flameless technique: Perkin-Elmer[14] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1200°C and 1900°C, respectively. The characteristic mass is approximately 17.0 pg at 324.8 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Fe (iron, AW 55.847, D_0 4.2, E_i 7.90, BP 2862, Fe-54 9.59%, Fe-56 91.72%, Fe-57 2.20%, Fe-58 0.28%)

Flame technique: Price[2] lists the following sensitivities for the 248.3, 252.3, 271.9, 296.7, 302.1, 344.1, 372.0, 382.4, and 386.0 nm wavelengths: 0.08, 0.3, 0.5, 1.0, 0.7, 5.0, 1.0, 30, and 2.0 mg/l, respectively, using a lean air–acetylene flame. Cobalt, copper, nickel, or nitric acid can depress sensitivity, but their effects are much reduced by adjusting the flame to a very lean flame.[1,13] Organic acid, such as citric acid, may depress signal, but this effect may be minimized by using phosphoric acid.[1] Silicon effects can be reduced by the addition of 0.2% (w/v) calcium chloride.[1,13]

Flameless technique: Perkin-Elmer[14] recommends addition of 15 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1400°C and 2100°C, respectively. The characteristic mass is approximately 12.0 pg at 248.3 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

K (potassium, AW 39.0983, D_0 2.5, E_i 4.34, BP 759, K-39 93.2%, K-40 0.012%, K-41 6.73%)

Flame technique: Price[2] lists the following sensitivities for 404.4, 766.5, and 769.9 nm wavelengths with a lean air–acetylene flame: 10.0, 0.2, and 0.1 mg/l, respectively. Ionization is overcome by the addition of La, Na, or Cs.[1,2,13] The $LaCl_3$ solution described above may be used for this purpose.

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 900°C and 1500°C, respectively. The characteristic mass is approximately 2.0 pg at 766.5 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 10–100 ng/l.

Mg (magnesium, AW 24.305, D_0 4.1, E_i 7.65, BP 1090, Mg-24 78.9%, Mg-25 10.00%, Mg-26 11.10%)

Flame technique: Use a lean nitrous oxide–acetylene flame to get approximate sensitivities of 25, 0.2, and 0.005 mg/l for the 202.5, 279.6, and 285.2 nm wavelengths.[2] La ($LaCl_3$ solution described above) or K (KCl solution described above) additions may be required, as well as the hot nitrous oxide–acetylene flame. Perkin-Elmer[13] recommends a lean air–acetylene flame. The $LaCl_3$ solution can reduce chemical interference from aluminum, silicon, titanium, zirconium, and phosphorus.[1] The KCl solution reduces ionization.

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 800°C and 1900°C, respectively. The characteristic mass is approximately 0.4 pg at 285.2 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Mn (manganese, AW 54.9380, D_0 4.2, E_i 7.43, BP 2062, Mn-55 100%)

Flame technique: Price[2] reports sensitivities of 1, 0.025, 100, and 0.5 mg/l using a stoichiometric to lean air–acetylene flame for the 222.2, 279.5, 321.7, and 403.1 nm wavelengths. Perkin-Elmer[13] suggests that interference by silicon may be overcome by the addition of 0.2% (w/v) calcium chloride. Varma[1] notes interferences from phosphate, perchlorate, iron, nickel, and cobalt that are reduced or eliminated using a lean air–acetylene or nitrous oxide–acetylene flame. Varma[1] suggests addition of K (KCl solution described above) to eliminate any ionization.

Flameless technique: Perkin-Elmer[14] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1300°C and 1900°C, respectively. The characteristic mass is approximately 6.3 pg at 279.5 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Mo (molybdenum, AW 95.94, D_0 6.3, E_i 7.09, BP 4639, Mo-92 14.84%, Mo-94 9.25%, Mo-95 15.92%, Mo-96 16.68%, Mo-97 9.55%, Mo-98 24.13%, Mo-100 9.63)

Flame technique: Price[2] reports sensitivities of 0.5, 1.5, 10.0, and 2.0 mg/l for the 313.3, 317.0, 320.8, and 379.8 nm wavelengths using a rich air–acetylene flame. A rich nitrous oxide–acetylene flame has a sensitivity of 0.2 mg/l for the 313.3 nm wavelength. Interference from calcium, chromium, manganese, nickel, strontium, sulfate, and iron can be reduced by using a nitrous oxide–acetylene flame.[1,2,13] Varma[1] suggests the addition of either 0.5% (w/v) aluminum, 2% (w/v) ammonium chloride, or 0.1% (w/v) sodium sulfate to reduce interferences.

Flameless technique: Perkin-Elmer[14] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization

temperatures should be in the ranges of 1500°C and 2400°C, respectively. The characteristic mass is approximately 12.0 pg at 313.3 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is less than 1 ng/l.

Na (sodium, AW 22.98977, D_0 2.7, E_i 5.14, BP 883, Na-23 100%)

Flame technique: Sensitivities of 5.0 and 0.02 mg/l are reported by Price[2] for 330.2 and 589.0 nm wavelengths using a lean air–acetylene flame. The addition of an alkali salt (KCl solution described above) is recommended.[1] Noise produced by calcium can interfere at the 589.0 nm wavelength.[1]

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 900°C and 1500°C, respectively. The characteristic mass is approximately 1.2 pg at 589.0 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Ni (nickel, AW 58.69, D_0 4.0, E_i 7.64, BP 2914, Ni-58 68.27%, Ni-60 26.1%, Ni-61 1.13%, Ni-62 3.59%, Ni-64 0.91%)

Flame technique: For the wavelengths 232.0, 234.6, 247.7, 339.1, 341.5, 346.2, and 352.5 nm, Price[2] reports sensitivities of 0.1, 0.5, 50, 5, 0.2, 1.0, and 2.5 mg/l, respectively with a lean air–acetylene flame. Perkin-Elmer[13] and Varma[1] reports that high concentrations of iron, cobalt, and chromium can depress the signal. A nitrous oxide–acetylene flame can be used to reduce this interference.

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 1100°C and 2300°C, respectively. The characteristic mass is approximately 20.0 pg at 232.0 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Pb (lead, AW 207.2, D_0 3.9, E_i 7.42, BP 1750, Pb-204 1.40%, Pb-206 24.10%, Pb-207 22.10%, Pb-208 52.40%)

General: This element is toxic and should be handled appropriately. Low concentration work requires extreme care to avoid contamination.

Flame technique: Price[2] reports sensitivities of 0.12, 6.0, and 0.2 mg/l for the 217.0, 261.4, and 283.3 nm wavelengths with a lean air–acetylene flame. Note that, although the 217.0 nm wavelength is more sensitive than the 283.3 nm wavelength, the associated noise is higher than that of the 283.3 nm wavelength. The lower noise and only marginally lower sensitivity of the 283.3 nm wavelength make it slightly more useful.

Flameless technique: Perkin-Elmer[14] recommends addition of 50 μg $NH_4H_2PO_4$ and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 850°C and 1500°C, respectively. The characteristic mass is approximately 30.0 pg at 283.3 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is less than 1 ng/l.

Pt (platinum, AW 195.08, D_0 3.6, E_i 9.0, BP 3827, Pt-190 0.01%, Pt-192 0.79%, Pt-194 32.90%, Pt-195 33.80%, Pt-196 25.30%, Pt-198 7.20%)

Flame technique: Using a lean air–acetylene flame, Price[2] reports respective sensitivities for the 217.5, 265.9, 299.8, and 306.5 nm wavelengths of 10, 2.5,15, and 5 mg/l. Interferences from high concentrations of many elements, ammonium ion, sulfuric acid, perchloric acid, and phosphoric acid can be reduced by adding 0.2% (w/v) La in 1% (v/v) hydrochloric acid.[1,13] Perkin-Elmer[13] also note that a nitrous oxide–acetylene flame will reduce these interferences.

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 1300°C and 2200°C, respectively. The characteristic mass is approximately 220.0 pg at 265.9 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is less than 1 ng/l.

Sb (antimony, AW 121.75, D_0 3.9, E_i 8.64, BP 1587 (hydride SbH_3-17), Sb-121 57.3%, Sb-123 42.7%)

Flame technique: For the 206.8, 217.6, and 231.2 nm wavelengths and a lean to stoichiometric air–acetylene flame, Price[2] reports sensitivities of 0.5, 1.1, and 1.9 mg/l, respectively. Although the 206.8 nm wavelength is more sensitive, it is noisier than the 217.6 nm wavelength.

Flameless technique: Perkin-Elmer[14] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1300°C and 1900°C, respectively. The characteristic mass is approximately 55.0 pg at 217.6 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is less than 1 ng/l.

Se (selenium, AW 78.96, D_0 4.4, E_i 9.75, BP 685, Se-74 0.90%, Se-76 9.00%, Se-77 7.60%, Se-78 23.50%, Se-80 49.60%, Se-82 9.40%)

Flame technique: Price[2] reports a sensitivity of 0.8 mg/l for a ''just luminous'' lean air–acetylene flame with the 196.1 nm wavelength. At this wavelength, there is much light scatter from the flame and background correction is essential.[13]

Flameless technique: Perkin-Elmer[14] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1300°C and 1900°C, respectively. The characteristic mass is approximately 45.0 pg at 196.1 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 10–100 ng/l.

Sn (tin, AW 118.69, D_0 5.7, E_i 7.34, BP 2603 (hydride SnH_4-52), Sn-112 0.97%, Sn-114 0.65%, Sn-115 0.36%, Sn-116 14.70%, Sn-117 7.70%, Sn-118 24.30%, Sn-119 8.60%, Sn-120, 32.40%, Sn-122 4.60%, Sn-124 5.60%)

Flame technique: Sensitivities at 224.6 and 286.3 nm using a rich nitrous oxide–acetylene flame are 0.8 and 2.5 mg/l, respectively.[2] Price[2] reports sensitivities for the 270.6, 286.3,

and 303.4 nm wavelengths to be 25, 10, and 50 mg/l with a rich air–acetylene flame, respectively. A rich nitrous oxide–acetylene flame and 328.1 nm wavelength seem the best combination because of the slightly lower noise at this wavelength relative to the slightly more sensitive 224.6 nm wavelength.

Flameless technique: Perkin-Elmer[13,14] recommends addition of 5 μg Pd and 3 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 1400°C and 2200°C, respectively. The characteristic mass is approximately 90.0 pg at 286.3 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Ti (titanium, AW 47.88, D_0 6.9, E_i 6.83, BP 3289, Ti-46 8.00%, Ti-47 7.30%, Ti-48 73.80%, Ti-49 5.50%, Ti-50 5.40%)

Flame technique: Price[2] recommends a rich nitrous oxide–acetylene flame for all three wavelengths (364.3, 365.4, and 337.2 nm). At 364.3, he gives a sensitivity of 1.5 mg/l. Addition of an excess of alkali salt (KCl solution described above) is recommended to reduce ionization.[1,2] The presence of hydrofluoric acid, iron, and many other elements will enhance the signal for this element.[1,2] Sulfuric acid will greatly reduce sensitivity.[1]

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 1500°C and 2500°C, respectively. The characteristic mass is approximately 70.0 pg at 364.3 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

V (vanadium, AW 50.9415, D_0 6.7, E_i 6.75, BP 3409, V-50 0.25%, V-51 99.75%)

Flame technique: Price[2] gives a sensitivity of 1.0 mg/l for the doublet (318.39 and 318.34) using a stoichiometric nitrous oxide–acetylene flame. High concentrations of aluminum, titanium, iron, and phosphoric acid can enhance the signal.[1,2] An ionization buffer (KCl solution described above) is required.

Flameless technique: The pyrolysis and atomization temperatures should be in the ranges of 1200°C and 2400°C, respectively. The characteristic mass is approximately 42.0 pg at 318.4 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

Zn (zinc, AW 65.38, D_0 2.9, E_i 9.39, BP 907, Zn-64 48.60%, Zn-66 27.90, Zn-67 4.10%, Zn-68 18.80%, Zn-70 0.60%)

General: Zinc contamination can be a problem when working with low concentrations.

Flame technique: Using a stoichiometric to lean air–acetylene flame, Price[2] reports sensitivities of 0.012 and 150 mg/l for the 213.9 and 307.6 nm wavelengths, respectively.

Flameless technique: Perkin-Elmer[13,14] recommends addition of 5 μg of $Mg(NO_3)_2$ to the sample. (Reagent described above.) The pyrolysis and atomization temperatures should be in the ranges of 700°C and 1800°C, respectively. The characteristic mass is approximately 1.0 pg at 213.9 nm.

ICP-MS technique: With a Perkin-Elmer SCIEX ELAN 6000 ICP-MS, the detection limit is 1–10 ng/l.

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chapter thirty-four

Estimation of inorganic species aquatic toxicity

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Contents

Introduction

Background

Researchers, manufacturers, and regulating agencies must evaluate properties for chemicals that are either present in or could be released into the environment. The routine use of over 70,000 synthetic chemicals stresses the need for this information. However, minimal physical and toxicity data are available for only about 20% of these compounds. The cost of testing all of these chemicals is prohibitive, so researchers and managers increasingly rely on predictive models [i.e., quantitative structure–activity relationships (QSARs)] for chemical property estimation, hazard evaluation, and information to direct research and set priorities. While property estimation is routine for organic species, QSARs usable for inorganic materials are few.

The linear solvation energy relationship (LSER) developed by Kamlet and coworkers (Reference 1 contains a compilation of the Kamlet et al. LSER development references) for neutral organic species is suitable for estimation of environmental properties. The form of the LSER equation used here relates many chemical solution properties to the solute's LSER variables (Table 34.3):

$$\log(\text{property}) = mV_i/100 + s\pi^* + b\beta_m + a\alpha_m \tag{34.1}$$

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where V_i is the intrinsic (van der Waals) molecular volume, π^* is the solute ability to stabilize a neighboring charge or dipole by nonspecific dielectric interactions, and β_m and α_m are the solute ability to accept or donate a hydrogen in a hydrogen bond. The coefficients m, s, b, and a are constants for a particular set of conditions, determined by multiple linear regression of the LSER variable values for a series of chemicals with the measured value for a particular chemical property.

Inorganic LSER variable value development

Since LSER has proven to be a useful QSAR for organics,[2] LSER variable values were devised for the remaining periodic table elements and ions.[3] For the steric parameter $V_i/100$, atomic parachors[4] were transformed through characteristic molecular volumes V_x to intrinsic molecular volumes, V_i.[5] The use of a calculated volume was preferable, and any coordination sphere of water was considered to be included in the LSER theory (see References 1, 2, 6, and 7, also references therein). For the other variables (dipolar π^* and hydrogen bonding β and α), reasonably complete sets of appropriate elemental property values (*vide infra*) were not available to serve as a proportional reference for value calculation for the remaining periodic table elements. A heuristic development process was used, in which descriptive solution chemistry[8,9] served as a frame of reference to correlate trends from measured physical property data with existing LSER parameter values in order to suggest parameter values for unassigned elements. The property data used were: (1) for π^*, dipole moments, polarizabilities, and electronegativity; (2) for β and α, ionization potentials (basicity), electron affinities (acidity), and pK values.

Inorganic structures were taken as likely solution species.[8,9] The LSER values for the neutral inorganic molecule or salt were calculated as the sums of the neutral molecule or salt component contributions (see Table 34.1 for component values, Table 34.2 for whole salt/molecule values, and the section ''Method'' for a calculation example). The volumes of a salt's component ions were summed with no compensation for bonds between

Table 34.1 Inorganic component species LSER values

Ion	$V_i/100$[a]	π^*	β	α
Li^{+1}	0.158	0.05	0.00	0.10
Na^{+1}	0.229	0.00	0.05	0.00
K^{+1}	0.360	0.10	0.06	0.00
Rb^{+1}	0.417	0.17	0.06	0.03
Cs^{+1}	0.533	0.20	0.10	0.03
Be^{+2}	0.144	0.00	0.00	0.00
Mg^{+2}	0.216	0.00	0.00	0.10
Ca^{+2}	0.349	0.00	0.00	0.10
Sr^{+2}	0.406	0.00	0.00	0.10
Ba^{+2}	0.529	0.00	0.00	0.10
B^{+3}	0.131	0.03	0.00	0.40
Al^{+3}	0.202	0.06	0.00	0.20
V^{+5}	0.316	0.05	0.10	0.10
$Cr^{+3/+6}$	0.305	0.05	0.05	0.20
Mo	0.362	0.10	0.05	0.10
Mn^{+2}	0.294	0.10	0.10	0.00
$Fe^{+2/+3}$	0.283	0.05	0.10	0.05

Co^{+2}	0.272	0.10	0.05	0.35
Ni^{+2}	0.261	0.10	0.05	0.30
Cu^{+2}	0.251	0.10	0.05	0.35
Ag^{+1}	0.307	0.15	0.05	0.25
Zn^{+2}	0.240	0.15	0.00	0.25
Cd^{+2}	0.296	0.20	0.00	0.25
Hg^{+2}	0.319	0.19	0.00	0.55
Tl^{+1}	0.311	0.03	0.00	0.15
Pt^{+2}	0.337	0.15	0.05	0.25
As^{+3}	0.207	0.23	0.13	0.05
Sb^{+3}	0.263	0.35	0.09	0.07
Bi^{+3}	0.294	0.18	0.10	0.08
Sn^{+2}	0.274	0.05	0.03	0.00
Pb^{+2}	0.302	0.00	0.00	0.00
Ce^{+3}	0.507	0.00	0.00	0.20
Yb^{+3}	0.405	0.04	0.03	0.00
Th^{+4}	0.496	0.02	0.02	0.10
U^{+6}	0.479	0.03	0.03	0.10
F^{-1} covalent	0.077	0.08	0.19	0.06
F^{-1} ionic	0.077	0.18	0.29	0.06
Cl^{-} covalent	0.149	0.35	0.15	0.06
Cl^{-1} ionic	0.149	0.60	0.40	0.06
Br^{-} covalent	0.185	0.43	0.17	0.05
Br^{-1} ionic	0.185	0.68	0.32	0.05
I^{-} covalent	0.242	0.45	0.18	0.04
I^{-1} ionic	0.242	0.70	0.33	0.04
ClO_3^{-1}	0.269	0.50	0.40	0.30
ClO_4^{-1}	0.309	0.00	0.40	0.42
BrO_3^{-1}	0.458	0.60	0.30	0.41
BrO_4^{-1}	0.549	0.20	0.57	0.53
IO_3^{-1}	0.402	0.50	0.45	0.40
IO_4^{-1}	0.515	0.25	0.50	0.57
−OH ionic	0.105	0.45	0.50	0.00
−OH covalent	0.105	0.40	0.47	0.33
−SH ionic	0.176	0.25	0.20	0.00
−SH covalent	0.176	0.35	0.16	0.03
=O	0.091	0.34	0.10	0.12
=S	0.162	0.24	0.05	0.05
−O−	0.091	0.27	0.45	0.00
O^{-2} ionic	0.091	0.10	0.15	0.00
S^{-2} covalent	0.162	0.50	0.23	0.00
S^{-2} ionic	0.162	0.10	0.00	0.00
Se^{-2}	0.196	0.20	0.00	0.00
SO_3^{-2}	0.282	0.65	0.82	0.36
SO_4^{-2}	0.322	0.65	0.82	0.00
$S_2O_3^{-2}$	0.597	1.62	1.28	0.17
−OS(=O)2OH	0.336	1.00	0.80	0.75
PO_4^{-3}	0.336	0.45	0.87	0.00
HPO_4^{-2}	0.350	0.95	0.80	0.75
$H_2PO_4^{-1}$	0.364	0.95	0.75	0.75
$_2$(−O)(H)P(=O)	0.310	0.75	0.75	0.00

Continued

Table 34.1 Inorganic component species LSER values

Ion	$V_i/100$[a]	π^*.	β	α
(−O)(HO)(H)P(=O)	0.325	0.68	0.47	0.33
$P_2O_7^{-4}$	0.581	0.90	1.74	0.00
VO_3^{-1}	0.436	1.07	0.40	0.46
CrO_4^{-2}	0.465	1.41	0.45	0.68
$HCrO_4^{-1}$	0.505	1.52	0.85	0.56
$Cr_2O_7^{-2}$	0.839	2.41	1.15	1.12
MnO_4^{-1}	0.454	1.46	0.50	0.48
WO_4^{-2}	0.531	0.67	1.00	0.36
SeO_4^{-2}	0.356	1.14	0.84	0.24
SeO_3	0.316	0.80	0.74	0.12
AsO_3	0.327	1.25	0.43	0.41
AsO_4	0.367	1.59	0.53	0.53
$HAsO_4$	0.375	1.65	0.90	0.74
$B_4O_7^{-2}$	0.498	1.04	1.54	1.60
NO_2^{-1}	0.184	0.53	0.49	0.00
NO_3^{-1}	0.224	0.50	0.49	0.00
$[S{=}C{=}N]^{-1}$	0.282	0.63	0.22	0.00
$[S{=}N{=}C]^{-1}$	0.282	0.85	0.42	0.00
−OC(=O)H	0.212	0.62	0.37	0.00
$-OC(=O)CH_3$	0.308	0.65	0.80	0.06
CO_3^{-2}	0.230	0.44	0.55	0.00
−OC(=O)OH	0.252	0.55	0.48	0.55
$[-O_2CCO_2-]^{-2}$	0.345	1.10	0.45	0.24
−C#N covalent	0.171	0.45	0.11	0.22
$C\#N^{-1}$ ionic	0.171	0.70	0.30	0.22
H_2O	0.119	0.45	0.45	0.45
Coordinated water	0.068	0.25	0.00	0.55
NH_3	0.146	0.15	0.65	0.00
NH_4^{+1}	0.160	0.00	0.00	0.05

[a] Volumes computed from V_x as explained in the section ''Inorganic LSER variable value development'' and used in Table 34.2.

cationic and anionic species. These suggested values were processed through a solubility estimation equation[7] [Equation (34.2), Table 34.3] and compared with the large table of solubility values (aqueous solubility at 20°C) from the 44th Edition of the *Handbook of Chemistry and Physics*.[10] Overall agreement within approximately an order of magnitude between observed and estimated solubilities for the range of compounds containing the element(s) in question was the criterion for acceptance of the parameter value. This criterion was adopted since no error limits were reported for the solubility listing, which was compiled from numerous sources. Use of this large series of test compounds ensured numerous cation/anion permutations. Variable values for elements with the simplest solution chemistry (e.g., alkali and alkaline earth metals as halides), as well as for those elements with solution chemistry analogous to the 11 traditional LSER elements, were developed first. These parameter values were then used to develop values for more complex elements, in more complex salts or molecules. The process was repeated until all elements and common atom aggregates (e.g., anions, such as sulfates, metal oxides, etc.) were assigned LSER values.

Table 34.2 Inorganic compound LSER values and organism acute toxicities

Salt	LSER values				Golden Orfe, log LC_{50} (mM/l)				*Daphnia magna*, log EC_{50} (mM/l)				Microtox, log EC_{50} (μM/l)			
	$V_i/100$	π	β	α	%[a]	Pred.[b]	P − O[c]	Obs.[d]	%	Pred.[e]	P − O	Obs.[f]	%	Pred.[g]	P − O	Obs.[h]
$Na_2B_4O_7$	0.956	1.04	1.64	1.60	0.92	1.47	0.87	0.60	1.00	−1.14	−0.37	−0.77				
$Na_2Cr_2O_7$	1.297	1.49	1.25	0.64	0.91	−1.13	−1.00	−0.12	1.00	−3.19	−0.86	−2.33				
$K_2Cr_2O_7$	1.559	1.69	1.27	0.64					1.00	−4.73	−2.97	−1.76				
Na_2WO_4	0.989	0.67	1.10	0.36					1.00	−0.32	−0.01	−0.30				
$KClO_3$	0.629	0.60	0.46	0.30	0.75	0.29	−1.17	1.46	1.00	0.60	−0.25	0.86				
$KClO_4$	0.669	0.10	0.46	0.42	0.75	0.35	−0.96	1.31	1.00	1.14	0.31	0.83				
NaC#N	0.400	0.70	0.35	0.22	0.92	1.12	3.97	−2.85								
KC#N	0.531	0.80	0.36	0.22					0.50	0.33	2.42	−2.09				
$NaNO_2$	0.413	0.53	0.54	0.00	0.92	2.22	1.30	0.91	1.00	2.14	1.74	0.40				
$LiNO_3$	0.382	0.55	0.49	0.10									0.97	5.39	0.08	5.30
$NaNO_3$	0.453	0.50	0.54	0.00									0.92	5.01	−0.14	5.15
KNO_3	0.584	0.60	0.55	0.00									0.75	3.29	−1.06	4.35
$CsNO_3$	0.757	0.70	0.59	0.03									0.75	2.30	−1.91	4.20
$Be(NO_3)_2$	0.592	1.00	0.98	0.00	0.95	2.73	2.80	−0.07	0.80	0.93	1.94	−1.02				
$Mg(NO_3)_2$	0.664	1.00	0.98	0.10									0.62	2.89	−0.17	3.06
$Ca(NO_3)_2$	0.797	1.00	0.98	0.10									0.60	2.21	−0.78	2.99
$Sr(NO_3)_2$	0.854	1.00	0.98	0.10									0.39	1.27	−0.82	2.10
$Ba(NO_3)_2$	0.977	1.00	0.98	0.10									0.40	0.94	−1.05	1.99
$Cr(NO_3)_3$	1.247	1.55	1.52	0.20									0.91	1.30	−0.76	2.06
$Mn(NO_3)_2$	1.012	1.10	1.08	0.00									0.94	2.34	−0.66	3.00
$Fe(NO_3)_3$	1.225	1.55	1.57	0.05									0.22	0.45	0.00	0.44
$Cu(NO_3)_2$	0.969	1.10	1.03	0.35									0.76	1.55	−0.13	1.68
$Co(NO_3)_2$	0.990	1.10	1.03	0.35									0.87	1.64	−0.92	2.56
$Ni(NO_3)_2$	0.979	1.10	1.03	0.30									0.77	1.58	−0.54	2.12
$AgNO_3$	0.801	0.65	0.54	0.25	0.98	−0.23	2.40	−2.63	1.00	−0.13	4.57	−4.70	0.98	2.21	2.24	−0.03
$Zn(NO_3)_2$	0.958	1.15	0.98	0.25									0.80	1.63	0.39	1.24
$Cd(NO_3)_2$	1.014	1.20	0.98	0.25	0.40	−0.02	0.63	−0.64	1.00	−1.40	1.31	−2.71	0.71	1.10	0.09	1.02
$Hg(NO_3)_2$	1.037	1.19	0.98	0.55												
$La(NO_3)_3$	0.981	1.53	1.52	0.00									0.19	0.71	0.10	2.99

Continues

Table 34.2 Continued

Salt	LSER values				Golden Orfe, log LC_{50} (mM/l)				*Daphnia magna*, log EC_{50} (mM/l)				Microtox, log EC_{50} (μM/l)			
	$V_i/100$	π	β	α	%[a]	Pred.[b]	P − O[c]	Obs.[d]	%	Pred.[e]	P − O	Obs.[f]	%	Pred.[g]	P − O	Obs.[h]
$TlNO_3$	0.535	0.53	0.49	0.15	0.40	0.52	0.69	−0.17	1.00	1.35	3.57	−2.22				
$Pb(NO_3)_2$	0.750	1.00	0.98	0.00									0.40	1.68	1.65	0.02
$BeSO_4$	0.466	0.65	0.82	0.00					1.00	2.10	2.61	−0.51				
$MgSO_4$	0.538	0.65	0.82	0.10					1.00	1.67	0.87	0.80				
$BaSO_4$	0.851	0.65	0.82	0.10					1.00	0.19	1.05	−0.86				
$MnSO_4$	0.886	0.75	0.92	0.00					1.00	0.10	1.36	−1.26				
$FeSO_4$	0.88	0.70	0.92	0.05					0.40	0.07	0.96	−0.89				
$CuSO_4$	0.843	0.75	0.87	0.35	0.76	0.57	2.87	−2.30	0.80	−0.08	3.22	−3.30				
$ZnSO_4$	0.832	0.80	0.82	0.25					0.80	−0.09	1.98	−2.07				
$CdSO_4$	0.888	0.85	0.82	0.25					1.00	−0.46	1.32	−1.78				
NaF	0.306	0.18	0.34	0.06	0.92	2.20	1.01	1.20	0.80	2.30	1.40	0.91				
NaCl	0.378	0.60	0.45	0.06					1.00	2.00	0.76	1.24				
KCl	0.509	0.70	0.46	0.06					1.00	1.23	0.67	0.56				
$MgCl_2$	0.514	1.20	0.80	0.22									1.00	4.63	−0.69	5.32
$CaCl_2$	0.647	1.20	0.80	0.22					1.00	0.10	−0.89	0.98	1.00	3.65	−1.71	5.36
$SrCl_2$	0.704	1.20	0.80	0.22					0.40	−0.07	−0.10	0.03				
$BaCl_2$	0.827	1.20	0.80	0.22	0.40	0.12	−0.50	0.62								
$MnCl_2$	0.772	0.80	0.40	0.12									0.73	1.43	−0.85	2.28
$CoCl_2$	0.750	0.80	0.35	0.47					1.00	−0.62	0.97	−1.60				
$NiCl_2$	0.739	0.80	0.35	0.42	0.83	−0.88	−1.53	0.64	0.83	−0.43	0.64	−1.07	0.83	1.27	−0.83	2.10
$CuCl_2$	0.729	0.80	0.35	0.47									0.87	1.31	0.93	0.39
$ZnCl_2$	0.718	0.85	0.30	0.37	0.86	−0.98	−0.17	−0.81	0.86	−0.46	0.53	−0.99	0.86	1.29	0.21	1.08
$CdCl_2$	0.774	0.90	0.30	0.37									0.41	0.42	−1.48	1.90
$HgCl_2$	0.797	0.89	0.30	0.67	0.10	−0.20	2.53	−2.73	0.11	−0.13	4.19	−4.32	0.11	0.04	0.08	−0.04
$SnCl_2$	0.572	0.75	0.33	0.12					0.40	0.24	0.98	−0.74				
$PbCl_2$	0.600	0.70	0.30	0.12									0.74	2.21	2.26	−0.05
NaBr	0.414	0.68	0.37	0.05	0.92	1.34	−0.64	1.99	1.00	1.59	−0.25	1.83				
NaI	0.471	0.70	0.38	0.04	0.92	1.08	−0.75	1.82	1.00	1.31	2.37	−1.06				
Na_2S	0.620	0.50	0.33	0.00	0.92	0.31	0.81	−0.49	1.00	0.90	1.94	−1.04				
Na_2SeO_3	0.774	0.80	0.84	0.12	0.92	1.15	1.37	−0.22	1.00	0.31	1.35	−1.03				

NaH_2PO_4	0.579	0.95	0.85	0.75	0.92	1.08	−0.06	1.14				
Na_2HAsO_4	0.833	1.44	1.01	0.05	0.92	−0.87	0.12	−0.26	1.00	−0.79	0.47	−1.26
Na_3AsO_3	1.014	1.25	0.58	0.41					1.00	−2.23	−1.15	−1.08
$Pb(OAc)_2$	0.918	1.30	1.60	0.12	0.40	1.31	1.09	0.22	0.40	−0.03	2.15	−2.18
NaOAc	0.537	0.65	0.85	0.06					0.50	0.88	−1.06	1.94

[a] Percentage, as (free + MCl_x), the bioavailable metal fraction, from References 11–13. For metal nitrates, $[MCL_x] = 0$.

[b] Estimated using Equation (34.5) (Table 34.3), from Reference 6.

[c] P – O = (predicted – observed), the prediction accuracy.

[d] Observed values from Reference 19.

[e] Estimated using Equation (34.4) (Table 34.3), from Reference 2.

[f] Observed values from References 15–18.

[g] Estimated using Equation (34.3), from Reference 2.

[h] Observed values from References 11, 12, and 14.

Acute aquatic toxicity estimation development

With solubility a fundamental factor in aquatic acute toxicity, the utility of these inorganic species LSER values were proven using published toxicity equations. Lastly, bioavailable metal fraction values for the various inorganic solution species adapted from the works of McCloskey et al.,[11] Newman and McCloskey,[12] and Tatara et al.,[13] were applied to the organism toxicity estimations. These refined toxicities were then compared with literature endpoint data to determine the prediction accuracy.

Equations (34.3)–(34.5) in Table 34.3 were used to demonstrate the estimation of aquatic baseline toxicities (narcoses) for three organism types spanning eukaryotic, invertebrate, and vertebrate species: the phosphorescent algae Microtox (*Vibrio fischeri*, Beijerinck 1889),[2] the water flea *Daphnia magna*,[2] and the decorative goldfish Golden Orfe (*Leuciscus idus melanotus*).[6] For the Microtox test, data from References 11 and 12 were supplemented with data from Reference 14. *D. magna* data reported by Bringmann and Kuhn[15,16] were supplemented with data from the works of Khangarot and Ray[17] and LeBlanc.[18] Golden Orfe data were obtained from the work of Juhnke and Ludemann.[19]

In this chapter, these new LSER variable values are made available to estimate acute aquatic toxicities for a range of inorganic salts and molecules for aquatic species ranging from algae through fish using published LSER equations. These inorganic LSER variable values will enhance the application and utility of LSER (e.g., for estimation of their environmental behavior and accurate inorganic compound hazard screening).

Materials required

A PC or Mac, EXCEL or other version of a spreadsheet, and any storage media the user is familiar with. No laboratory work is involved, as this is purely an estimation method. For the calculation(s), the user will need the table of LSER values presented in this chapter (Table 34.1), the two-step example with comments provided under the section ''Method,'' and any published (or unpublished) LSER equation(s) for aquatic toxicity the user may wish to use. The user is also referred to earlier works[1,2] for calculating properties for organic compounds using LSER.

Table 34.3 LSER equations

1. General LSER equation (see References 1, 2, 6, and 7, also references therein):
   $\log(\text{property}) = mV_i / 100 + s\,\pi^* + b\,\beta_m + a\,\alpha_m$ (34.1)
2. Solubility estimation[7]: (M/L, 20°C)
   $\log(S_w) = 0.05 - 5.85V_i/100 + 1.09\pi^* + 5.23\beta$; $r^2 = 0.9889$, $n = 115$, SD = 0.153 (34.2)
3. Microtox (*Vibrio fischeri* Beijerinck 1889) (u*M*/l, 15 min, 20°C)[2]:
   $\log(EC_{50}) = 7.49 - 7.39V_i/100 - 1.38\pi^* + 3.70\beta - 1.66\alpha$; $r^2 = 0.97$, $n = 40$, SD = 0.319 (34.3)
4. *Daphnia magna* (m*M*/l, 48 h, 20°C)[2]:
   $\log(EC_{50}) = 4.18 - 4.73V_i/100 - 1.67\pi^* + 1.48\beta - 0.93\alpha$; $r^2 = 0.95$, $n = 53$, SD = 0.221 (34.4)
5. Golden Orfe (*Leuciscus idus melanotus*) (M/L, 48 h, 20°C)[6]:
   $\log(LC_{50}) = 2.90 - 5.71V_i/100 - 0.92\pi^* + 4.36\beta - 1.27\,\alpha$; $r^2 = 0.94$, $n = 32$, SD = 0.246 (34.5)

Method

Inorganic LSER variable value determination

The calculation of the LSER values for any inorganic species, using the values for various components in Table 34.1 is a simple sum of the parts. In some cases, principally the halogens and chalcogens, the best professional judgment must be used to determine whether some components are covalently bonded in solution, or exist as anions or cations in solution. But the process is straightforward as shown by the following example.

Example: Calculation of LSER values for $BaCl_2$, using Table 34.1.

Ion	$V_i/100$	π^*	β	α
Ba^{+2}	0.529	0.00	0.00	0.10
Cl^{-1} (ionic)	0.149	0.60	0.40	0.06
Cl^{-1} (ionic)	0.149	0.60	0.40	0.06
$\Sigma(BaCl_2)$	0.827	1.20	0.80	0.22

To estimate the acute toxicity of an inorganic species to an organism, process the LSER values determined above through predetermined QSAR equations from the literature that correlate the LSER structural and electronic parameters to overall toxicity (such as those presented in Table 34.3), and then apply a metal species bioavailability factor to the result. The following example demonstrates the relative simplicity of the calculation.

Example: Calculation of the acute toxicity of $BaCl_2$ to the Golden Orfe (European goldfish) as shown in Table 34.2.

To estimate the acute toxicity of $BaCl_2$ to the Golden Orfe (mM/l, 48 h, 20°C),[6] use the LSER parameter values derived above, and Equation (34.5)[6] in Table 34.3:

$$\begin{aligned}\log(LC_{50}) &= 2.90 - 5.71V_i/100 - 0.92\pi^* + 4.36\beta - 1.27\alpha \\ &= 2.90 - 5.71(0.827) - 0.92(1.20) + 4.36(0.80) - 1.27(0.22) \\ &= 2.90 - 4.722 - 1.10 + 3.49 - 0.28 \\ &= 0.288, \text{ if all of the salt were bioavailable}\end{aligned}$$

As presented in Table 34.2, the barium bioavailability, as percentage or (free metal + MCl_x), is 0.40. So,

$$\log(LC_{50}) = 0.228 \times 0.40, \text{ or } 0.12$$

The observed toxicity as $\log(LC_{50})$ from Table 34.2 is reported to be 0.62. Prediction accuracy ($P - O$) is 0.50, within half of an order of magnitude. For systems with several prominent solution components (e.g., phosphate salts), the above procedure is followed with the LSER values for each component determined, and the bioavailability factor applied. The toxicity for each component is then multiplied by its relative proportion in solution, and the results are summed for an overall toxicity. The major components, their relative proportions, and bioavailability factors for solution equilibria can be determined for the solution conditions with available software, such as MINEQL+.[11–13]

Results and discussion

To demonstrate the utility of the process described in this chapter, toxicities, as baseline narcosis [log(EC_{50}) and log(LC_{50})] estimated by Equations (34.3)–(34.5) in Table 34.3 to three organisms for numerous inorganic species, are tabulated in Table 34.2 as bioavailable fractions (%), predicted values (Pred.), prediction accuracy (P – O), and observed values (Obs.). Prediction accuracy is presented graphically in Figures 34.1–34.3, plotted

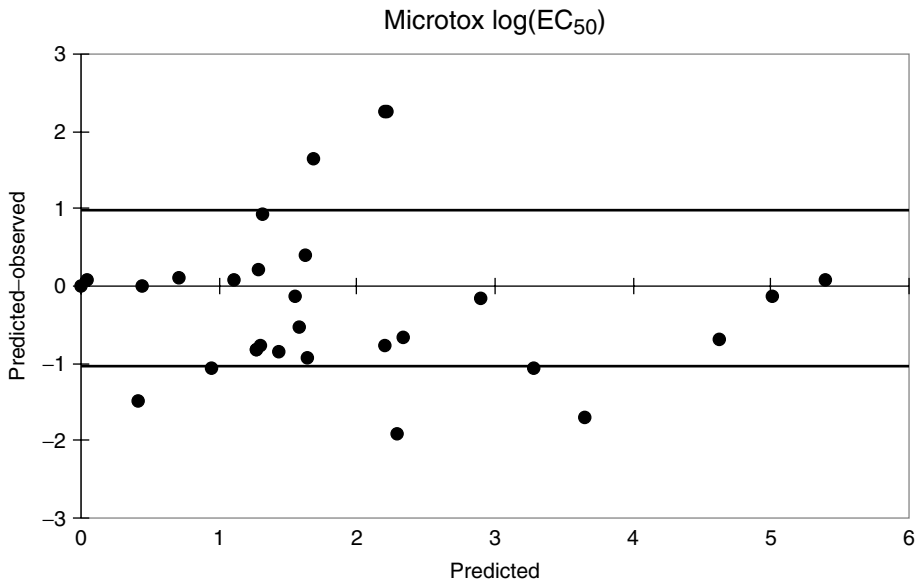

Figure 34.1 Prediction accuracy for inorganic toxicities to Microtox.

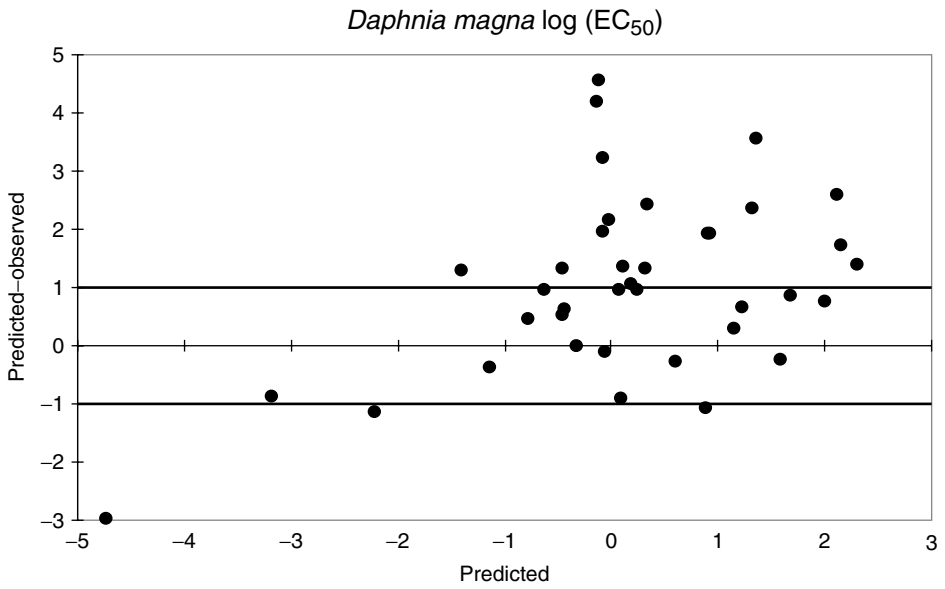

Figure 34.2 Prediction accuracy for inorganic toxicities to *Daphnia magna*.

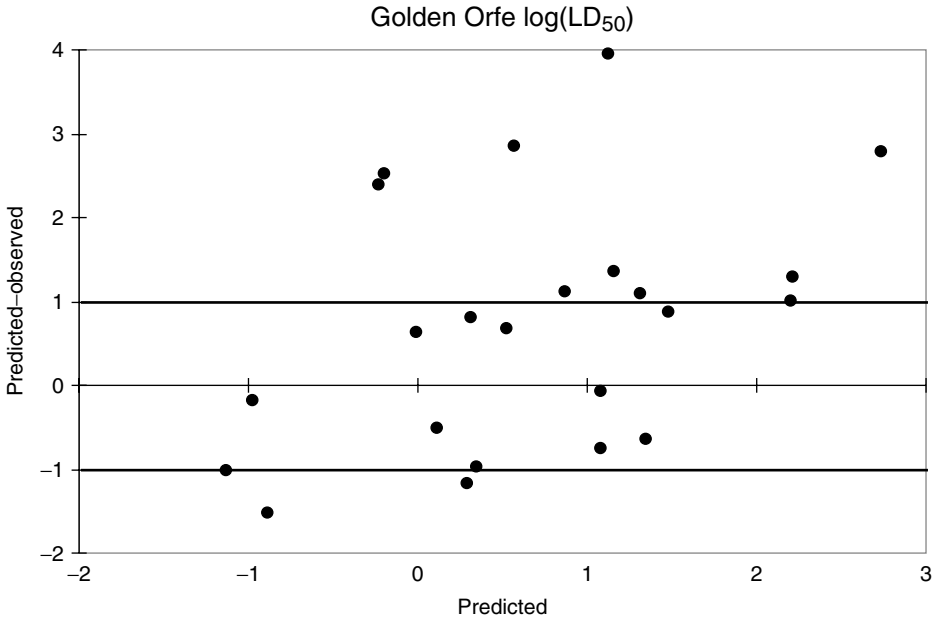

Figure 34.3 Prediction accuracy for inorganic toxicities to Golden Orfe.

against the predicted value. The tighter the data point cluster around the 0-line or *x*-axis, the closer the agreement between prediction and observation, and the better the LSER values serve to estimate the observed property with the particular equation used.

Estimated toxicities for many of the compounds were at or within an order of magnitude of the measured value. For the Golden Orfe and *D. magna*, two-thirds (16 in 23 and 26 in 39, respectively) of the estimated acute toxicities were within 1.3 log-units of the measured value; for the Microtox test, 80% were within 1.3 log-units, or 33 in 39 tested. Outliers in common for all organisms that were more toxic than the narcosis toxicity estimated for their tested species included cyanides, Pb^{+2} and Be^{+2} salts, $AgNO_3$, $CuSO_4$, and $HgCl_2$. Each organism also had a small number of other outliers more or less toxic than estimated.

LSER can be used as a much-needed, simple although unorthodox tool for estimating aquatic baseline toxicities (narcosis) for inorganic and organometal compounds when used with LSER equations that represent a wide range of organism types. Since the toxicity equations were developed for nonionic organic compounds, the whole salt as a neutral material was used to address this criterion and to indirectly address the question of elemental valence. As the reference data here were gathered from a number of sources for each organism, with each source having an anticipated variation in data measurement accuracy, an arbitrary outer difference limit of $\pm$ 1.3 log-units was used to define a reasonable fit. Use of a factor to account for the bioavailable metal fraction[11–13] improved the narcosis prediction accuracy, often markedly. Where multiple solution species are possible, the dominant species is not necessarily the toxic species. The toxic species will likely resemble the species successfully transported across the cell membrane, the bioavailable fraction.[20] A better definition of both the toxic solution species structure(s) and the bioavailable fraction could yield a more accurate toxicity estimation.

Compounds with a positive prediction accuracy (P – O) greater than 1.3 log-units likely have a dominant specific mode of action other than narcosis. A considerable deviation from the 0-line is considered to demonstrate the action of an enhanced toxicity over narcosis. As LSER equations are developed for the more specialized modes of action, the inorganic compounds that act accordingly should be modeled better by those equations. The different responses to a given inorganic compound (Table 34.2) between such a wide range of organism types are expected[18,21] and are reflected in the coefficients for toxicity equations (Table 34.3). For the outliers that were less toxic than estimated, the reasons for each were not understood. The deviations may reflect the error limits unavailable from the data sources, the error limits of the equations, improper species modeling, including bioavailability, or the predominance of another mode of action.

Conclusions

The acute aquatic toxicities (narcoses) for a range of organism types may be estimated with LSER for a large number of inorganic and organometal species, many with an accuracy at or within an order of magnitude. Optimum estimations make use of a bioavailable metal fraction and a more accurate structure(s) for the toxic solution species. The estimated toxicities for a number of salts were quite different from the observed values, likely due to a dominant specific toxicity mechanism other than baseline narcosis. The tool still requires fine-tuning.

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chapter thirty-five

Determining aromatic hydrocarbons and chlorinated hydrocarbons in sediments and tissues using accelerated solvent extraction and gas chromatography/mass spectrometry

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Contents

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Introduction

Numerous and varied environmental studies have involved analyses of marine sediments and tissues (e.g., marine mammal blubber, shellfish, fish muscle or liver, etc.) for toxic contaminants, such as chlorinated pesticides, polychlorinated biphenyls, and aromatic hydrocarbons (AHs). High quality data with documented quality assurance (QA) are needed from such studies in order for valid conclusions to be drawn and appropriate decisions reached, for instance, in damage assessment situations, for policy making, and to facilitate comparisons of the results with those of other studies. New techniques are continually being sought and evaluated to further optimize analytical accuracy, precision, sensitivity, robustness, efficiency, and safety, as well as to minimize contamination from laboratory materials and from sample-to-sample carry-over.

Recent improvements assessed and adopted by this laboratory for determining selected chlorinated hydrocarbons (CHs, Table 35.1) and AHs (Table 35.2) at ''nanogram per gram'' concentrations are presented. They are among the latest revisions[1] to the methods of Sloan et al.,[2] and Krahn et al.,[3] which updated the method of MacLeod et al.[4] Here, we provide detailed descriptions of the improved procedures for sample extraction, cleanup by gravity-flow silica/alumina columns and by size-exclusion high-performance liquid chromatography (HPLC), and quantitation of CHs and AHs by on-column injection gas chromatography/mass spectrometry (GC/MS). One of the recent procedural modifications is the extraction of samples using accelerated solvent extraction (ASE), which reduced time, labor, solvent use, hazardous waste, and potential exposure of analysts to extraction solvent (in this case, dichloromethane). This ASE method provides an exhaustive extraction of organic compounds from various matrices, while excluding water. Thus, both AHs and CHs are quantitatively recovered in a single extract, and the percent of nonvolatile extractable material, as well as lipid classes, can be determined from a portion of the ASE extract. Another major modification is the use of GC/MS selected-ion monitoring (SIM)[5] in place of GC/electron-capture detection for CH analyses. Thus, the concentrated cleaned-up extracts are analyzed for both AHs and CHs (separately) using the same GC/MS system and configuration, as well as similar operating conditions. As MS instrumentation has improved, the sensitivity of GC/MS SIM for CH detection has approached that of electron-capture detection while being more selective, thus reducing interferences. Alterations made to the GC/MS system that allow greater instrument stability and accuracy include replacing the electron ionization filaments with chemical ionization filaments in order to use a higher source temperature, using a cool on-column injection system in the GC in place of a splitless injection system, and adding a guard column before the analytical column. Quantitation has also been optimized by using point-to-point calibration (also known as pointwise linear calibration), which provides a better data fit over the entire range of GC/MS calibration standards than would be given by a single equation, such as linear, weighted linear, or quadratic regression.

The method described here incorporates QA measures (including a method blank, a sample of a reference material that has certified concentrations for many analytes, and, as

Table 35.1 Chlorinated hydrocarbon analytes including polychlorinated biphenyl (PCB) congeners (by IUPAC number)

PCB 17	2,4′-DDD
PCB 18	4,4′-DDD
PCB 28	2,4′-DDE
PCB 31	4,4′-DDE
PCB 33	2,4′-DDT
PCB 44	4,4′-DDT
PCB 49	Hexachlorobenzene
PCB 52	α-Hexachlorocyclohexane
PCB 66	β-Hexachlorocyclohexane
PCB 70	γ-Hexachlorocyclohexane
PCB 74	Aldrin
PCB 82	Dieldrin
PCB 87	Endosulfan I
PCB 95	Endosulfan II
PCB 99	*trans*-Nonachlor
PCB 101 + PCB 90[a]	*cis*-Nonachlor
PCB 105	Heptachlor
PCB 110	Heptachlor epoxide
PCB 118	Oxychlordane
PCB 128	*trans*-Chlordane
PCB 138 + PCB 163 + PCB 164[a]	*cis*-Chlordane
PCB 149	Mirex
PCB 151	
PCB 153 + PCB 132[a]	
PCB 156	
PCB 158	
PCB 170 + PCB 190[a]	
PCB 171	
PCB 177	
PCB 180	
PCB 183	
PCB 187 + PCB 159 + PCB 182[a]	
PCB 191	
PCB 19	
PCB 195	
PCB 199	
PCB 205	
PCB 206	
PCB 208	
PCB 209	

[a] These analytes are quantitated and reported as the sum of their concentrations because they co-elute on GC by this method.

needed, replicate samples) into each batch of samples. Internal standards (also known as surrogate standards) are added to the samples before extraction to monitor and account for any losses during sample preparation. Additional internal standards are added

Table 35.2 Aromatic hydrocarbon analytes

Naphthalene
1-Methylnaphthalene
2-Methylnaphthalene
Biphenyl
2,6-Dimethylnaphthalene
Acenaphthylene
Acenaphthene
2,3,5-Trimethylnaphthalene
Fluorene
Dibenzothiophene
Phenanthrene
Anthracene
1-Methylphenanthrene
Fluoranthene
Pyrene
Benz[*a*]anthracene
Chrysene + triphenylene[a]
Benzo[*b*]fluoranthene
Benzo[*j*]fluoranthene + benzo[*k*]fluoranthene[a]
Benzo[*e*]pyrene
Benzo[*a*]pyrene
Perylene
Indeno[1,2,3-*cd*]pyrene
Dibenz[*a*,*h*]anthracene + dibenz[*a*,*c*]anthracene[a]
Benzo[*ghi*]perylene

[a] These analytes are quantitated and reported as the sum of their concentrations because they co-elute on GC by this method.

before sample cleanup to account for the amount of total extract fractionated by HPLC, and before GC/MS to measure the recovery of the extraction and HPLC internal standards. Analytes are measured based on multiple concentration levels of GC/MS calibration standards. These procedures can be applied to a variety of sediment and tissue matrices across a wide range of contaminant concentrations and with any moisture or lipid content. Low limits of quantitation (on the order of a nanogram per gram or lower) can be achieved even when only small sample amounts (2 g or less) are available, such as when analyzing individual, small fish tissues or marine mammal biopsy samples.

Materials required

Reference to a company or product does not imply endorsement by the U.S. Department of Commerce to the exclusion of others that may be suitable.

Equipment

Samples are extracted using an Accelerated Solvent Extractor® (ASE 200, Dionex, Salt Lake City, UT) with 33-cc extraction cells (048764, Dionex). Nitrogen (Grade 4.8, PraxAir, Danbury, CT) is used for ASE pneumatics and cell purging. The HPLC system used for

sample cleanup consists of several components: a Waters 515 HPLC pump (Waters, Milford, MA), a Waters 717 Plus autosampler (Waters), an in-line filter (2-μm particle size, 7302, Rheodyne, Inc., Cotati, CA) after the autosampler, a six-port valve (7030, Rheodyne, Inc.), an Envirosep ABC® size-exclusion guard column (60 mm × 21.2 mm, Phenomenex, Inc., Torrance, CA), an Envirosep ABC size-exclusion preparatory column (350 mm × 21.2 mm, Phenomenex, Inc.), a Spectra 100 UV/VIS detector (Spectra-Physics, San Jose, CA), a Gilson FC 204 fraction collector (Gilson Co., Middleton, WI), and a computer system with Rainin Dynamax® DA software (Version 1.2B4, Rainin Instrument Co., Inc., Woburn, MA). It also includes a ventilated solvent reservoir, in which the solvent is degassed by helium (Grade 5.0, 99.999%, PraxAir). The helium is purified by an in-line hydrocarbon trap (Big Supelco Carb HC®, 24564, Supelco, Bellefonte, PA) and an in-line oxygen trap (Supelco Pure O, 2-2449, Supelco, Bellefonte, PA). The GC/MS system consists of an Agilent 7683 autosampler with an on-column injection syringe and needle guide (Agilent Technologies, Wilmington, DE), an Agilent 6890N gas chromatograph with an cool on-column injection port (Agilent Technologies), a deactivated fused-silica guard column (10-m × 0.53-mm, 460-2535-10, Agilent Technologies), a DB-5® (J & W Scientific, Folsom, CA) GC column (60-m × 0.25-mm, 0.25-μm film thickness, 122-5062, Agilent Technologies), an Agilent 5973N Mass Selective Detector® (Agilent Technologies), and a computer system with ChemStation® software (Version DA, Agilent Technologies). The helium carrier gas (grade 5, ultra-high purity, 99.999%, PraxAir) is filtered through an indicating moisture trap (GMT-4-HP, Agilent Technologies), a disposable oxygen trap (803088, Supelco, Inc.) and an indicating oxygen trap (4004, Alltech Associates, Inc., Deerfield, IL). The guard column is connected to the GC column by a glass, universal column union (705-0825, Agilent Technologies) using polyimide-sealing resin (500-1200, Agilent Technologies). Other necessary equipment includes a gas leak detector (Leak Detective II, 20413, Restek Corporation, Bellefonte, PA), an analytical balance, a muffle furnace, drying ovens at 120°C and 170°C, a steam table, tube heaters (720000-0000, Kontes, Vineland, NJ, with glass-cylinder shroud, wire tube holder, and aluminum inserts bored out to accept 50-ml conical tubes), and a vial heater (we use a HPLC column heater, SP 8792, Spectra-Physics) modified with a rack to hold 2-ml GC vials, which allows the solvent level in the vials to be visually monitored).

Reagents

Only high purity solvents, i.e., dichloromethane (High Purity, 300-4, Burdick & Jackson, Muskegon, MI), isooctane (Optima, 0301-4, Fischer Scientific, Fair Lawn, NJ), and methanol (Optima, A454-4, Fischer Scientific) are used in sample analysis. The following reagents are heated in a muffle furnace at 700°C for 18 h then stored at 120°C: sodium sulfate (reagent grade, anhydrous, granular, S1461, Spectrum, Gardena, CA), magnesium sulfate (anhydrous, M65-3, Fischer Scientific), and Davisil® (WR Grace & Co.) silica (100–200 mesh, grade 634, S734-1, Fischer Scientific). Immediately prior to use, alumina (80–200 mesh, O537-01, JT Baker, Phillipsburg, NJ) is heated at 120°C for 2 h. Sand (Ottawa, kiln-dried, 30–40 mesh, SX0075-3, EM Science, Gibbstown, NJ) is soaked overnight in a 1:3 v/v mixture of concentrated nitric acid (reagent grade, A200-500, Fischer Scientific), concentrated hydrochloric acid (reagent grade, A144-212, Fischer Scientific), then it is washed three times each with water, methanol, and dichloromethane, in that order, then dried, and stored at 120°C. All heated reagents are allowed to cool to room temperature in a desiccator immediately prior to use. Glass wool (Pyrex®, Corning, Inc., Corning, NY) is

heated in a muffle furnace at 400°C for 18 h then cooled and stored at room temperature. Copper (reagent grade, granular, 20–30 mesh, 1720-05, JT Baker) is activated before use by submersing it in concentrated hydrochloric acid, stirring it with a glass rod, and allowing it to stand for 5 min. The copper is then washed three times each with water, methanol, and dichloromethane, in that order, and covered with dichloromethane until used to avoid contact with air.

Disposable labware

The following items are low-cost, one-use only. ASE collection vials (60-ml with cap and septa, 048784, Dionex) are purchased pre-cleaned. Boston round bottles (250-ml, 28-mm i.d. mouth) are rinsed with acetone. The following items are heated in a muffle furnace at 400°C for 18 h then stored at room temperature before use: ASE glass fiber filters (047-017, Dionex), conical centrifuge tubes (50-ml, 18-mm i.d. mouth), chromatography columns (plain, custom, 22-mm i.d., 25-cm length, DJ's Glass Factory, San Jose, CA), jars (10-oz, 2.5-in. i.d.), GC vials (2-ml, C4000-1W, National Scientific Co., Duluth, GA), HPLC vials (4-ml, 99300-A, Sun International, Wilmington, NC), low-volume vial inserts for the GC vials (C4000-54R, National Scientific Co.), and low-volume vial inserts for the HPLC vials (200756, Sun International). The bottles, jars, and tubes are fitted with caps lined with Teflon® (E.I. Du Pont de Nemours & Company, Inc., Wilmington, DE), and the vials are assembled with caps and Teflon-lined septa. Teflon boiling chips (AW0919120, All-World Scientific, Lynnwood, WA) are rinsed three times with dichloromethane, allowed to dry, and stored at room temperature.

Standard solutions

All solutions are prepared in-house using isooctane as the solvent, except for the HPLC retention-time standard, which is prepared using dichloromethane. Concentrations given here are approximate; actual concentrations vary slightly from batch to batch of the solutions. The GC calibration standards and the internal standard solutions, including GC and HPLC internal standards, are prepared such that the concentrations of the internal standards in final sample extracts analyzed by GC/MS are approximately equal to the concentrations of the internal standards in the GC calibration standards. The internal standard for quantitating CH analytes (CH I-Std) contains PCB 103 and 4,4′-dibromooctafluorobiphenyl (DOB) at 1 ng/μl each compound. The HPLC internal standard for CHs (CH HPLC I-Std) contains tetrachloro-*m*-xylene (TCMX) at 1 ng/μl. The GC internal standard for CHs (CH GC I-Std) contains tetrachloro-*o*-xylene (TCOX) at 1 ng/μl. The internal standard for quantitating AH analytes (AH I-Std) contains naphthalene-d_8, acenaphthene-d_{10}, and benzo[*a*]pyrene-d_{12} at 1.7 ng/μl each. The HPLC internal standard for AHs (AH HPLC I-Std) contains phenanthrene-d_{10} at 1.6 ng/μl. The GC internal standard for AHs (AH GC I-Std) contains hexamethylbenzene (HMB) at 10 ng/μl. The HPLC retention-time standard contains DOB and perylene at 1.5 ng/μl each compound. The CH GC calibration standards contain the CHs as in Table 35.1. They are prepared at the following concentrations: Level 1 at 0.001 ng/μl each compound, Level 2 at 0.003 ng/μl each compound, Level 3 at 0.01 ng/μl each compound, Level 4 at 0.03 ng/μl each compound, Level 5 at 0.1 ng/μl each compound, Level 6 at 0.3 ng/μl each compound, Level 7 at 1.0 ng/μl each compound, Level 8 at 4.0 ng/μl each compound, Level 9 at 10 ng/μl each compound, Level 10 at 20 ng/μl each compound, Level 11 at

100 ng/μl each compound; plus all levels contain PCB 103, DOB, TCMX, and TCOX at 0.3 ng/μl each compound. The AH GC calibration standards contain the AHs in Table 35.2 at the following concentrations: Level 1 at 0.0011 ng/μl each compound, Level 2 at 0.0044 ng/μl each compound, Level 3 at 0.015 ng/μl each compound, Level 4 at 0.044 ng/μl each compound, Level 5 at 0.11 ng/μl each compound, Level 6 at 0.33 ng/μl each compound, Level 7 at 1.13 ng/μl each compound, Level 8 at 3.3 ng/μl each compound, and Level 9 at 10.5 ng/μl each compound; plus all levels contain naphthalene-d_8, acenaphthene-d_{10}, benzo[*a*]pyrene-d_{12}, and phenanthrene-d_{10} at 0.5 ng/μl each compound in all levels, and HMB at 3.2 ng/μl.

Reference materials

Standard Reference Materials® (SRM) are purchased from the National Institute of Standards and Technology (NIST), Gaithersburg, MD. SRM 1941b ''Organics in Marine Sediment'' is analyzed with sediment samples, SRM 1945 ''Organics in Whale Blubber'' is analyzed with marine mammal blubber samples, and SRM 1974b ''Organics in Mussel Tissue (*Mytilus edulis*)'' is analyzed with other tissue samples (e.g., shellfish, fish muscle, or liver, etc.).

Procedures

Sample extraction

Sediments or tissues are customarily analyzed in batches of 10–14 samples. Each batch includes a method blank, a sample of an appropriate SRM and, as needed, one or more replicate sediment or tissue samples. If only one of the two classes of analytes (CHs or AHs) is to be quantitated in the samples, all the internal standards for the other class of analytes are omitted. Prior to removing a portion of sample for analysis, tissues are homogenized to the extent possible. Standing water is decanted from sediment containers, and all pebbles, biota, and other extraneous material are discarded, and then the sediment is stirred. A specified amount of sediment or tissue sample is transferred to a 10-oz jar and weighed to the nearest 0.001 g. Typically, the amount of sediment or tissue analyzed is approximately 2 g of wet sample, except the amount of blubber analyzed is approximately 0.5 g. To absorb the water from the sample, allowing greater extraction efficiency of the organic compounds, 15 cc of sodium sulfate is added to the sample in the jar and mixed thoroughly with the sample. To avoid clumping and hardening of the sodium sulfate, the sample is mixed immediately after adding the sodium sulfate to the jar. The sample continues to be mixed until it appears dry, then 15 cc of magnesium sulfate is added to the sample in the jar and mixed thoroughly for increased absorption of water from the sample. Two glass-fiber filters are placed at the bottom of an ASE cell, then the sample/drying agent mixture is transferred to the ASE cell. The bottom of the cell is tapped firmly but carefully on the countertop during the transfer to completely settle the cell contents. The remaining cell volume is filled with sodium sulfate. Again, the bottom of the cell is tapped firmly but carefully on the counter top to completely settle the cell contents, leaving approximately 3 mm void depth at the top of the cell. One glass-fiber filter is placed on top of the sodium sulfate in the cell without overlapping the rim of the cell. AH I-Std solution (75 μl) and CH I-Std solution (75 μl) are added onto the top filter in the cell, then approximately 1 ml of dichloromethane is added onto the filter to rinse the

internal standards into the cell. The cell threads are cleared of sample/drying agent as necessary, and the cell is capped firmly but not forcefully. The ASE is prepared for extracting the batch of samples by loading the sample cells and collection vials into their respective carousels, and filling the ASE solvent reservoir with dichloromethane. The ASE system rinse function is activated three times to flush the solvent lines prior to extracting a batch of samples. The samples are extracted using an ASE schedule with the rinse parameter set to ''ON,'' and a method with a 0-min preheat, a 5-min heat, two 5-min static cycles at 2000 psi and 100°C, a 115%-volume flush and a 180-s purge. After the extraction is complete, AH HPLC I-Std solution (75 μl) and CH HPLC I-Std solution (75 μl) are added to the extract in the ASE collection vial, and the extract is thoroughly mixed.

Sample cleanup

Silica/alumina column chromatography

A gravity-flow silica/alumina chromatography column is used for removing extraneous polar compounds from the sample extract. The column is prepared by positioning an approximately 1-cm high plug of glass wool at the bottom of a chromatography column, then adding 10 cc of alumina, followed by 20 cc of silica, and 5 cc of sand. To slightly deactivate the column packing, which improves the recovery of high molecular weight AHs, 35 ml of 10% methanol in dichloromethane is slowly added to the column and allowed to drain into a waste container. Then, to flush the methanol from the column, 35 ml of dichloromethane is slowly added to the column and allowed to drain into the waste container. All subsequent eluant is collected in a 250-ml bottle. The sample extract in the ASE collection vial is transferred into the column without disturbing the packing. To elute the analytes, 35 ml of dichloromethane is added to the column. After the eluant is collected, several boiling chips are added to the bottle, and the sample volume is reduced to approximately 20 ml using a steam table. The sample is transferred to a 50-ml centrifuge tube, a boiling chip is added to the tube, and the sample volume is reduced to approximately 1 ml using a tube heater. For all samples in a batch of sediments, activated copper is added to the tube a few grains at a time until no further discoloring of the copper occurs, then the tube is capped and stored overnight. The sample is transferred to a HPLC vial with a low-volume insert (leaving the copper in the tube in the case of sediment samples), and the sample volume is brought to 1 ml as necessary.

Size-exclusion high-performance liquid chromatography

Size-exclusion HPLC is used for removing extraneous high molecular weight compounds (e.g., biogenic material) from the sample extracts by collecting a fraction of the eluant containing the analytes, which elute after the high molecular weight compounds. The system is operated using a mobile phase of 100% dichloromethane at 5 ml/min and ambient temperature, while the UV detector monitors the wavelength 254 nm. The HPLC system is calibrated when any component of the system (e.g., column or length of tubing) is changed, to determine the relationship between the fraction collection times and the retention times of the components in the HPLC retention-time standard.[1] In this standard, DOB is the first peak to elute, at approximately 14 min, and perylene is the last peak, at approximately 20 min. The beginning of the elution of DOB, which is determined during the system calibration, denotes the beginning of the fraction containing AHs and CHs; the ending of the elution of perylene, also determined during the system calibration,

denotes the ending of the fraction. The HPLC retention-time standard is chromatographed immediately prior to chromatographing a batch of samples in order to adjust the fraction collection times as necessary using the relationship determined from the system calibration. This standard is also chromatographed in the middle and at the end of the batch of samples to monitor the retention-time stability and adjust the fraction collection times if necessary. Samples are chromatographed by injecting 500 μl, and the ''AH/CH'' fraction of each sample is collected in a 50-ml tube. The remaining extract in the HPLC vial is reserved in a freezer in case of need. The size-exclusion guard column is backflushed for 2–3 min after the sequence of samples and standards has finished. A boiling chip is added to the 50-ml tube containing the AH/CH fraction, and the fraction volume is reduced to 1 ml using a tube heater. AH GC I-Std solution (30 μl), CH GC I-Std solution (30 μl), and isooctane (70 μl) are added to the tube, and the contents are mixed thoroughly. The ''AH/CH'' fraction is transferred from the tube to a GC vial, a small boiling chip is added to the vial, and the fraction volume is reduced to 100 μl using a vial heater. The concentrated fraction is transferred to a GC vial with a low-volume insert.

Gas chromatography/mass spectrometry

GC/MS is used to measure the concentrations of the analytes and the internal standards in the samples. Operating conditions for the GC/MS are listed in Table 35.3. The GC/MS is checked for air leaks, both spectrally and with a handheld electronic leak detector, and the MS tune is performed or checked for stability prior to analyzing a sequence of samples.

GC/MS for quantitating AHs

For AHs, the GC oven temperature is programmed for an initial temperature of 80°C held for 1 min, a first ramp to 200°C at 10°C/min, a second ramp to 300°C at 4°C/min, and a final temperature of 300°C held for 15 min. Sediment samples are analyzed using scan mode, scanning all masses from 60 to 300 amu. Tissue samples are analyzed using SIM mode, scanning only the quantitation ions during the specified time window as shown in Table 35.4. Scan mode provides greater ability to confirm analyte identification in complex sediment samples, whereas SIM mode provides greater sensitivity for tissue samples, which generally contain lower concentrations of contaminants. The quantitation ions for AHs in tissues shown in Table 35.4 are also the ions used for quantitating AHs in sediments. The time windows for SIM mode are adjusted as necessary when the GC column is changed or the guard column is trimmed. Prior to analyzing samples, the sensitivity of the GC/MS is checked by analyzing the lowest level of AH GC calibration standard that will be used (Level 1 for tissues; Level 3 for sediments). One or more batches of sediment samples are analyzed in a sequence that includes one each of AH GC calibration standard Levels 3–6, 8, and 9, alternating with the samples, plus one Level 7 before the first sample and replicate Level 7 analyses at the middle and end of each batch. One or more batches of tissue samples are analyzed in a sequence that includes one each of AH GC calibration standard Levels 1–3 and Levels, 5–7, alternating with the samples in the sequence, plus one Level 4 before the first sample and replicate Level 4 analyses at the middle and end of each batch. Included at the beginning of each sequence is an injection of a sample in the batch, which is not quantitated but serves as a GC/MS system conditioner. Alternating the standards and samples in the sequence improves the GC/MS stability, which is monitored using the replicate Level 7 or Level 4 analyses.

Table 35.3 Gas chromatograph/mass spectrometer operating conditions

Injection volume	2 μl
Injection technique	Cool on-column
Inlet temperature program	Oven temperature plus 3°C
Transfer-line temperature	300°C
Carrier gas	Helium
Carrier gas linear velocity	30 cm/s
Carrier gas flow	1.3 ml/min constant flow
Column	
Material	Fused-silica capillary tubing
Length	60 m
Internal diameter	0.25 mm
Stationary phase	DB-5® (J & W Scientific)
Phase composition	5% phenyl, 95% methylpolysiloxane
Film thickness	0.25 μm
Guard column	
Material	Deactivated fused-silica capillary tubing
Length	10 m
Internal diameter	0.53 mm
Quadrupole temperature	150°C
Ion-source pressure	$\leq 5 \times 10^{-5}$ Torr
Ion-source temperature	300°C
Ionization mode	Electron ionization (70 eV)
Filament	Chemical ionization filament
Tune mode	AHs: autotune CHs: autotune, then the multiplier voltage is increased for the desired sensitivity
Emission current maximum	35 μA
Scan rate	Approximately 10–20 scans per peak

GC/MS for quantitating CHs

For CHs, the GC oven temperature is programmed for an initial temperature of 80°C held for 1 min, a first ramp to 150°C at 10°C/min, a second ramp to 195°C at 0.5°C/min, a third ramp to 315°C at 3°C/min, and a final temperature of 315°C held for 15 min. Sediment and tissue samples are analyzed using SIM mode for optimal sensitivity, scanning only the quantitation ions during the specified time window as shown in Table 35.5. The time windows for SIM mode are adjusted as necessary when the GC column is changed or the guard column is trimmed. Prior to analyzing samples, the sensitivity of the GC/MS is checked by analyzing the lowest level of CH GC calibration standard that will be used (Level 2 for blubber; Level 1 for other tissues and sediments). One or more batches of blubber samples are analyzed in a sequence that includes one of each CH GC calibration standard Levels 2–4 and Levels 6–11 alternating with the samples, plus one Level 5 before the first sample and replicate Level 5 analyses at the middle and end of each batch. One or more batches of sediment samples or tissue samples, other than blubber, are analyzed in a sequence that includes one of each CH GC calibration standard Levels 1–4 and 6 alternating with the samples, plus one Level 5 before the first sample and replicate Level 5 analyses at the middle and end of each batch. The replicate Level 5 analyses are monitored for GC/MS stability. Included at the beginning of each sequence is an injection of a sample in the batch, which is not quantitated but serves as a GC/MS system conditioner.

Table 35.4 Selected-ion monitoring for quantitating aromatic hydrocarbons in tissue samples

Approximate time window (min)	Compounds	Quantitation ion (atomic mass units)
7–12	Naphthalene-d_8	136
	Naphthalene	128
	2-Methylnaphthalene	142
	1-Methylnaphthalene	142
12–13	Biphenyl	154
	2,6-Dimethylnaphthalene	156
13–14	Hexamethylbenzene	147
	Acenaphthylene	152
	Acenaphthene-d_{10}	164
	Acenaphthene	154
14–17	2,3,5-Trimethylnaphthalene	170
	Fluorene	166
17–20	Dibenzothiophene	184
	Phenanthrene-d_{10}	188
	Phenanthrene	178
	Anthracene	178
20–29	1-Methylphenanthrene	192
	Fluoranthene	202
	Pyrene	202
29–42	Benz[*a*]anthracene	228
	Chrysene + triphenylene	228
	Benzo[*j*]fluoranthene + benzo[*k*]fluoranthene	252
	Benzo[*e*]pyrene	252
	Benzo[*a*]pyrene-d_{12}	264
	Benzo[*a*]pyrene	252
	Perylene	252
42–53	Indeno[1,2,3-*cd*]pyrene	276
	Dibenz[*a,h*]anthracene + dibenz[*a,c*]anthracene	278
	Benzo[*ghi*]perylene	276

Calculations of results

The acquired GC/MS data for the samples and calibration standards are processed using the Agilent ChemStation software to determine the areas of the MS response for the analytes and internal standards. These areas are then used by a BASIC program written in-house to compute the analyte amounts in the samples (nanogram analyte per sample) based on point-to-point calibration. The point-to-point calibration method plots the relative response factors of the calibration standards versus their analyte areas, then the linear equations generated between each pair of consecutive points are used for the calibration,[1] i.e., for each analyte in each sample, a relative response factor is computed by using the analyte area and interpolating between the relative response factors of the analyte in the two consecutive calibration standards whose areas for that analyte bracket the area of the analyte in the sample. When a particular analyte in a given sample has an MS area that is larger than its area in the highest-level calibration standard, the analyte amount is calculated using the relative response factor of that analyte in the highest level of calibration standard used. The resulting concentration is footnoted as exceeding the

Table 35.5 Selected-ion monitoring for quantitating chlorinated hydrocarbons in tissue and sediment samples

Approximate time window (min)	Compounds	Quantitation ion (atomic mass units)
15–29	Tetrachloro-*o*-xylene	207
	Tetrachloro-*m*-xylene	207
29–35	α-Hexachlorocyclohexane	181
	Hexachlorobenzene	284
	4,4′-Dibromooctafluorobiphenyl	456
35–39	β-Hexachlorocyclohexane	219
	γ-Hexachlorocyclohexane	181
39–45	PCB 18	256
	PCB 17	256
45–55	PCB 31	256
	PCB 28	256
	PCB 33	256
	Heptachlor	272
55–65	PCB 52	292
	PCB 49	292
	Aldrin	263
	PCB 44	292
65–69	PCB 103	326
69–76	Heptachlor epoxide	353
	Oxychlordane	115
	PCB 74	292
	PCB 70	292
	PCB 66	292
	PCB 95	326
76–79	*trans*-Chlordane	373
79–86	2,4′-DDE	246
	Endosulfan I	241
	PCB 101 + PCB 90	326
	cis-Chlordane	373
	PCB 99	326
	trans-Nonachlor	409
86–93	Dieldrin	79
	PCB 87	326
	4,4′-DDE	318
	PCB 110	326
	2,4′-DDD	235
93–98	PCB 82	326
	PCB 151	360
	Endosulfan II	241
98–103	PCB 149	360
	PCB 118	326
	cis-Nonachlor	409
	4,4′-DDD	165
	2,4′-DDT	235
103–106	PCB 153 + PCB 132	360
	PCB 105	326
106–110	4,4′-DDT	165
	PCB 138 + PCB 163 +PCB 164	360
	PCB 158	360

Table 35.5 Continued

Approximate time window (min)	Compounds	Quantitation ion (atomic mass units)
110–113	PCB 187 + PCB 159 + PCB 182	394
	PCB 183	394
	PCB 128	360
113–115	PCB 177	394
	PCB 171	394
	PCB 156	360
115–121	PCB 180	394
	PCB 191	394
	Mirex	272
	PCB 170 + PCB 190	394
	PCB 198	430
	PCB 199	430
121–153	PCB 208	464
	PCB 195	430
	PCB 194	430
	PCB 205	430
	PCB 206	464
	PCB 209	498

calibration range and is therefore an estimate. When an analyte is not detected in a sample or has an area that is smaller than that analyte's area in the lowest level of GC calibration standard used, the concentration of the analyte in that sample is reported to be less than the value of its lower limit of quantitation. The lower limit of quantitation for a particular analyte in a given sample is the concentration that would be calculated if the analyte had an MS area equivalent to its area in the lowest-level calibration standard used in the calibration. Percent recoveries of internal standards are calculated using single-point calibration. The CH internal standard is PCB 103 for all analytes in Table 35.1 except when samples contain extremely high concentrations of PCBs, including significant amounts of PCB 103, in which case DOB is used as the CH internal standard. The AH internal standards are naphthalene-d_8 for the analytes naphthalene through 2,6-dimethylnaphthalene, acenaphthene-d_{10} for analytes acenaphthene through pyrene, and benzo[*a*]pyrene-d_{12} for analytes benz[*a*]anthracene through benzo[*ghi*]perylene in Table 35.2. For CH analyses, the percent recovery of each sample's CH internal standard is computed based on the CH GC internal standard and then adjusted for the CH HPLC internal standard recovery (to account for the amount injected on HPLC), which is also computed based on the CH GC internal standard. For these calculations, the Level 5 GC calibration standard is used for the relative response factors of the internal standards. Similarly, for AH analyses, the percent recovery of each sample's AH internal standards are computed based on the AH GC internal standard and then adjusted for the AH HPLC internal standard recovery, which is also computed based on the AH GC internal standard. For these calculations, the Level 4 GC calibration standard in the case of SIM analyses, or the Level 7 GC calibration standard in the case of scan analyses, is used for the relative response factors of the internal standards.

Quality assurance

Quality assurance measures are incorporated into the analyses of each batch of samples. Specific QA criteria for batches and individual samples, as well as the percent of the total

results that must meet the criteria, depend on the type of project for which the samples are analyzed and the purpose of the data. Typical criteria are presented here. When QA criteria are not met, the data may be footnoted as such or the sample(s) reanalyzed. The stability of the GC/MS is evaluated using the repetitions of the mid-level GC calibration standard analyzed periodically in the sequence of samples and other levels of GC calibration standards. The GC/MS is considered stable if the area of an analyte relative to the area of its internal standard in a given repetition is within $\pm$ 15% of the respective average for the repetitions. The percent recoveries of internal standards in samples are considered acceptable if they are 60–130%. When three or more replicate samples are analyzed, the precision of the results can be evaluated based on the relative standard deviation (RSD) of the concentrations of each analyte. The precision is considered acceptable if the RSD is <15% for at least 90% of the analytes. For duplicate samples, this translates to a relative percent difference of <30% for at least 90% of the analytes. At least one sample of an appropriate SRM from the National Institute of Standards and Technology (NIST) is analyzed in each batch of samples to indicate the accuracy of the data for the entire batch. NIST provides certified concentrations and uncertainty values (for approximately 95% confidence interval) for many of the analytes in Tables 35.1 and 35.2. For the analytes having NIST-certified concentrations, upper and lower control limits are set as is done by NIST in the Intercomparison Exercise Program for Organics in the Marine Environment, such that the control limits are 30% beyond each end of the 95% confidence interval. The accuracy of an SRM result is considered acceptable if the determined concentration falls within the upper and lower control limits. This criterion does not apply to analytes with concentrations below their limit of quantitation. The analysis of the SRM is acceptable if at least 70% of the analytes having NIST-certified concentrations are within their control limits. Analytes which do not have NIST-certified concentrations, as a result, do not have QA criteria for accuracy, but can be monitored and compared to NIST reference concentrations where available or to previous in-house results.

Results and discussion

The analytical procedures described here resulted in better data quality, improved safety, and increased cost-effectiveness as new techniques were adopted. Specifically, the ASE has provided quantitative extraction of the analytes, while decreasing (1) solvent use, (2) hazardous waste, (3) potential exposure of analysts to solvent vapors, and (4) labor for sample extraction and cleaning equipment. This ASE method uses optimal amounts of drying agents and sample sizes to robustly and reproducibly extract samples with better quantitative results than previous methods (i.e., using a homogenizing probe for tissues and a ball-mill tumbler for sediments[2]). GC/MS has been another focal point for improving analyses. GC/MS SIM eliminated many interferences with measuring CHs that were present in analyses by a comparable method using GC/electron-capture detection. Cool on-column GC injection avoids the molecular weight loading discrimination, thermal degradation, and adsorption of analytes to active sites that occurred during splitless injection. In conjunction with the cool on-column injector, a 0.53-mm i.d. guard column attached to the analytical column has maintained good GC resolution for a longer period of time by reducing build-up of nonvolatile compounds on the analytical column. The

guard column provides the added benefit of focusing the analytes, which improves peak shape and resolution for better quantitation. Setting the GC carrier gas to constant flow instead of constant pressure also improved peak shape and resolution. Increasing the MS ion-source temperature (from 230°C to 300°C) greatly reduced inconsistencies in the MS response caused by varying amounts of extraneous compounds that are present in the samples but not in the GC calibration standards. The higher source temperature was made possible by replacing the standard electron-ionization filaments with chemical-ionization filaments, which have ceramic insulators that can withstand higher temperatures. Alternating the standards and samples in the sequence, rather than analyzing all the standards before or after all the samples, further contributed to improved GC/MS stability. The mode of processing data has also had a significant impact on data quality. Quantitation using point-to-point calibration was found to be more accurate than using a single regression equation for the expansive range of analyte concentrations that can be determined by this method. This range is defined by the lowest-level calibration standard used, which is near the instrument detection limit, and the highest-level calibration standard used, which for AHs in sediment samples or CHs in blubber samples is near the level of overloading the GC column. Relatively large errors in calculating concentrations have occurred when a single quadratic regression equation, with or without weighting, was used, particularly near the extremes of the calibration range.

Before method modifications were employed, they were validated in this laboratory by analyzing and applying QA criteria to replicate samples of a variety of NIST SRMs, such as SRM 1974b ''Organics in Mussel Tissue (*M. edulis*),'' NIST SRM 1941b ''Organics in Marine Sediment,'' and NIST SRM 1945 ''Organics in Whale Blubber.'' During the application of this method in analyses of environmental sediment or tissue samples, QA measures, including the analysis of an appropriate (by matrix type) SRM, are continuously monitored and documented with the analysis of every batch of samples. The proficiency of the method has also been demonstrated and compared with other laboratories' results through participation in the annual NIST Intercomparison Exercise Program for Organics in the Marine Environment, which involves replicate analyses of both an SRM and an unknown sample of a similar matrix.

SRM 1974b is used as the reference material in batches of tissue samples other than blubber. This material is analytically challenging because of its high moisture content (89.87%) and low CH and AH concentrations. SRM 1974b has certified concentrations for 35 of our CH analytes and 17 of our AH analytes (co-eluting compounds are counted as one analyte, as noted in Tables 35.1 and 35.2, and their certified concentrations are summed for comparison). These certified concentrations range from 0.269 to 14.73 ng/g wet weight for the CHs and from 0.494 to 18.04 ng/g wet weight for the AHs. Tables 35.6 and 35.7 show representative examples of our results for these CH and AH analytes, respectively, quantitated in replicate samples of SRM 1974b as part of a recent NIST intercomparison exercise for the analysis of mussel tissue. The NIST-certified concentrations and the control limits are also shown for comparison. In these analyses, the measured concentrations of 31 CHs and 15 AHs were within the control limits, and 2 CHs were below their limit of quantitation (2,4′-DDE at <0.397 ng/g and PCB 170 at <0.385 ng/g). The measured concentrations of PCB 31, 4,4′-DDD and benz[*a*]anthracene were greater than their upper control limit, whereas that for naphthalene was less than its lower control limit. The RSDs of all measured analyte concentrations ranged from 0.4% to

Table 35.6 Measured and certified concentrations of chlorinated hydrocarbons in National Institute of Standards and Technology (NIST) Standard Reference Material® 1974b ''Organics in Mussel Tissue (*Mytilus edulis*)'' analyzed as part of the NIST Intercomparison Exercise Program for Organics in the Marine Environment, 2003

Analyte	Measured average (ng/g wet weight) ($n = 3$)	RSD[a] (%)	Certified concentration[b] (ng/g wet weight)	LCL[c]	UCL[d]
trans-Chlordane	0.98	1.7	1.14 ± 0.17	0.68	1.70
cis-Chlordane	1.12	1.6	1.36 ± 0.10	0.88	1.90
trans-Nonachlor	1.07	2.3	1.30 ± 0.14	0.81	1.87
2,4′-DDE	<0.397	–	0.336 ± 0.044	0.204	0.494
4,4′-DDE	4.27	1.0	4.15 ± 0.38	2.64	5.89
2,4′-DDD	0.87	7.2	1.09 ± 0.16	0.65	1.63
4,4′-DDD	5.07	2.4	3.34 ± 0.22	2.18	4.63
PCB 18	1.07	2.8	0.84 ± 0.13	0.50	1.26
PCB 28	2.55	5.3	3.43 ± 0.25	2.23	4.78
PCB 31	5.04	1.4	2.88 ± 0.23	1.86	4.04
PCB 44	3.75	0.5	3.85 ± 0.20	2.56	5.27
PCB 49	4.98	0.5	5.66 ± 0.23	3.80	7.66
PCB 52	6.51	0.4	6.26 ± 0.37	4.12	8.62
PCB 66	7.15	2.1	6.37 ± 0.37	4.20	8.76
PCB 70	7.45	2.0	6.01 ± 0.22	4.05	8.10
PCB 74	4.28	0.6	3.55 ± 0.23	2.32	4.91
PCB 82	1.17	1.2	1.16 ± 0.14	0.71	1.69
PCB 87	4.61	1.0	4.33 ± 0.36	2.78	6.10
PCB 95	5.85	0.5	6.04 ± 0.36	3.98	8.32
PCB 99	6.15	1.2	5.92 ± 0.27	3.96	8.05
PCB 101	11.1	0.7	10.7 ± 1.1	6.7	15.3
PCB 105	4.73	1.2	4.00 ± 0.18	2.67	5.43
PCB 110	10.3	0.7	10.0 ± 0.7	6.5	13.9
PCB 118	10.6	1.0	10.3 ± 0.4	6.9	13.9
PCB 128	1.90	1.8	1.79 ± 0.12	1.17	2.48
PCB 138 + PCB 163	13.4	1.8	11.2 ± 1.5[e]	6.8	16.5
PCB 149	7.08	1.5	7.01 ± 0.28	4.71	9.48
PCB 151	1.75	0.7	1.86 ± 0.16	1.19	2.63
PCB 153 + PCB 132	15.6	1.2	14.7 ± 1.1[f]	9.5	20.5
PCB 156	0.792	3.4	0.718 ± 0.080	0.447	1.037
PCB 158	1.112	2.4	0.999 ± 0.096	0.632	1.424
PCB 170	<0.385	–	0.269 ± 0.034	0.165	0.394
PCB 180	1.15	1.0	1.17 ± 0.10	0.75	1.65
PCB 183	1.23	1.2	1.25 ± 0.03	0.85	1.66
PCB 187	2.76	0.6	2.94 ± 0.15	1.95	4.02

[a] Relative standard deviation of the measured concentrations.

[b] NIST-certified concentration with uncertainty value.

[c] Lower control limit (ng/g wet weight) = 0.7 × (certified concentration − uncertainty value).

[d] Upper control limit (ng/g wet weight) = 1.3 × (certified concentration + uncertainty value).

[e] Certified concentration and uncertainty value of PCB 138 are summed with NIST reference concentration and uncertainty value of PCB 163, respectively.

[f] Certified concentrations and uncertainty values of PCB 153 and PCB 132 are summed, respectively.

Table 35.7 Measured and certified concentrations of aromatic hydrocarbons in National Institute of Standards and Technology (NIST) Standard Reference Material® 1974b ''Organics in Mussel Tissue (Mytilus edulis)'' analyzed as part of the NIST Intercomparison Exercise Program for Organics in the Marine Environment, 2003

Analyte	Measured average (ng/g wet weight) ($n = 3$)	RSD[a] (%)	Certified concentration[b] (ng/g wet weight)	LCL[c]	UCL[d]
Naphthalene	1.43	11.3	2.43 ± 0.12	1.62	3.32
Fluorene	0.466	2.1	0.494 ± 0.036	0.321	0.689
Phenanthrene	2.65	3.2	2.58 ± 0.11	1.73	3.50
Anthracene	0.551	1.0	0.527 ± 0.071	0.319	0.777
1-Methylphenanthrene	0.82	1.9	0.98 ± 0.13	0.60	1.44
Fluoranthene	19.6	1.3	17.1 ± 0.7	11.5	23.1
Pyrene	20.15	1.1	18.04 ± 0.6	12.21	24.23
Benz[*a*]anthracene	7.09	3.7	4.74 ± 0.53	2.95	6.85
Chrysene + triphenylene	13.9	2.3	10.6 ± 1.7[e]	6.2	16.0
Benzo[*b*]fluoranthene	7.19	3.1	6.46 ± 0.59	4.11	9.17
Benzo[*j* + *k*]fluoranthenes[f]	5.79	2.7	6.15 ± 0.47[e]	3.98	8.61
Benzo[*e*]pyrene	10.9	2.7	10.3 ± 1.1	6.4	14.8
Benzo[*a*]pyrene	2.81	1.0	2.80 ± 0.38	1.69	4.13
Perylene	1.15	1.4	0.99 ± 0.14	0.60	1.47
Indeno[1,2,3-*cd*]pyrene	2.68	1.0	2.14 ± 0.11	1.42	2.93
Dibenz[*a,h* + *a,c*]anthracenes[g]	0.656	0.8	0.539 ± 0.044[h]	0.347	0.758
Benzo[*ghi*]perylene	3.80	1.7	3.12 ± 0.33	1.95	4.49

[a] Relative standard deviation of the measured concentrations.

[b] NIST-certified concentration with uncertainty value.

[c] Lower control limit (ng/g wet weight) = 0.7 × (certified concentration − uncertainty value).

[d] Upper control limit (ng/g wet weight) = 1.3 × (certified concentration + uncertainty value).

[e] Certified concentrations and uncertainty values of coeluting compounds are summed, respectively.

[f] Benzo[*j*]fluoranthene + benzo[*k*]fluoranthene.

[g] Dibenz[*a,h*]anthracene + dibenz[*a,c*]anthracene.

[h] Certified concentration and uncertainty value of dibenz[*a,h*]anthracene are summed with NIST reference concentration and uncertainty value of dibenz[*a,c*]anthracene, respectively.

7.2% for CHs and from 0.8% to 11.3% for AHs. The percent recoveries of internal standards ranged from 102% to 107% for CHs and from 84% to 95% for AHs.

The reference material used in batches of sediments is SRM 1941b, which has certified concentrations for 34 of our CH analytes and 17 of our AH analytes. These certified concentrations range from 0.378 to 6.75 ng/g dry weight for the CHs and from 73.2 to 848 ng/g dry weight for the AHs. Measuring CHs in this material is particularly challenging because of the low concentrations of CHs in the presence of high concentrations of AHs and other contaminants. As part of a recent NIST intercomparison exercise for the analysis of marine sediment, our analyses of replicate samples of SRM 1941b (Tables 35.8 and 35.9) resulted in 25 CHs and 17 AHs being within the control limits, and 3 CHs being below their limit of quantitation (*t*-nonachlor at <0.413 ng/g, *c*-nonachlor at <0.421 ng/g and PCB 195 at <0.417 ng/g). The measured concentrations of PCB 31, PCB 105, PCB 110, 4,4′-DDD, and hexachlorobenzene were greater than their upper control limit, whereas that for *cis*-chlordane was less than its lower control limit. The RSDs of all measured analyte concentrations ranged from 0.3% to 6.8% for CHs and from 0.8% to 2.9% for AHs.

Table 35.8 Measured and certified concentrations of chlorinated hydrocarbons in National Institute of Standards and Technology (NIST) Standard Reference Material® 1941b ''Organics in Marine Sediment'' analyzed as part of the NIST Intercomparison Exercise Program for Organics in the Marine Environment, 2003

Analyte	Measured average (ng/g dry weight) ($n = 3$)	RSD[a] (%)	Certified concentration[b] (ng/g dry weight)	LCL[c]	UCL[d]
Hexachloro-benzene	8.53	0.6	5.83 ± 0.38	3.82	8.07
trans-Chlordane	0.611	2.5	0.566 ± 0.093	0.331	0.857
cis-Chlordane	0.51	4.1	0.85 ± 0.11	0.52	1.25
trans-Nonachlor	<0.413	–	0.438 ± 0.073	0.256	0.664
cis-Nonachlor	<0.421	–	0.378 ± 0.053	0.228	0.560
4,4′-DDE	4.25	4.0	3.22 ± 0.28	2.06	4.55
4,4′-DDD	8.88	2.5	4.66 ± 0.46	2.94	6.66
PCB 18	2.99	1.2	2.39 ± 0.29	1.47	3.48
PCB 28	5.31	2.9	4.52 ± 0.57	2.77	6.62
PCB 31	5.05	1.9	3.18 ± 0.41	1.94	4.67
PCB 44	4.23	1.2	3.85 ± 0.20	2.56	5.27
PCB 49	4.14	0.8	4.34 ± 0.28	2.84	6.01
PCB 52	5.93	1.4	5.24 ± 0.28	3.47	7.18
PCB 66	6.24	0.9	4.96 ± 0.53	3.10	7.14
PCB 87	1.48	6.8	1.14 ± 0.16	0.69	1.69
PCB 95	4.29	3.1	3.93 ± 0.62	2.32	5.92
PCB 99	3.24	0.6	2.90 ± 0.36	1.78	4.24
PCB 101	6.25	2.5	5.11 ± 0.34	3.34	7.09
PCB 105	2.11	2.4	1.43 ± 0.10	0.93	1.99
PCB 110	6.68	1.8	4.62 ± 0.36	2.98	6.47
PCB 118	5.21	2.6	4.23 ± 0.19	2.83	5.75
PCB 128	0.854	2.0	0.696 ± 0.044	0.456	0.962
PCB 138 + PCB 163	6.35	2.0	4.88 ± 0.34[e]	3.18	6.79
PCB 149	5.03	1.5	4.35 ± 0.26	2.86	5.99
PCB 153 + PCB 132	7.78	1.3	6.75 ± 0.59[f]	4.31	9.54
PCB 156	0.600	1.8	0.507 ± 0.090	0.292	0.776
PCB 170	1.47	4.2	1.35 ± 0.090	0.88	1.87
PCB 180	3.40	0.7	3.24 ± 0.51	1.91	4.88
PCB 183	0.860	0.5	0.979 ± 0.087	0.624	1.386
PCB 187	2.22	0.7	2.17 ± 0.22	1.37	3.11
PCB 194	1.30	5.6	1.04 ± 0.06	0.69	1.43
PCB 195	<0.417	–	0.645 ± 0.060	0.410	0.917
PCB 206	2.69	5.9	2.42 ± 0.19	1.56	3.39
PCB 209	4.71	0.3	4.86 ± 0.45	3.09	6.90

[a] Relative standard deviation of the measured concentrations.

[b] NIST-certified concentration with uncertainty value.

[c] Lower control limit (ng/g dry weight) = 0.7 × (certified concentration - uncertainty value).

[d] Upper control limit (ng/g dry weight) = 1.3 × (certified concentration + uncertainty value).

[e] Certified concentration and uncertainty value of PCB 138 are summed with NIST reference concentration and uncertainty value of PCB 163, respectively.

[f] Certified concentration and uncertainty value of PCB 153 are summed with NIST reference concentration and uncertainty value of PCB 132, respectively.

Table 35.9 Measured and certified concentrations of aromatic hydrocarbons in National Institute of Standards and Technology (NIST) Standard Reference Material® 1941b ''Organics in Marine Sediment'' analyzed as part of the NIST Intercomparison Exercise Program for Organics in the Marine Environment, 2003

Analyte	Measured average (ng/g dry weight) ($n = 3$)	RSD[a] (%)	Certified concentration[b] (ng/g dry weight)	LCL[c]	UCL[d]
Naphthalene	896	1.9	848 ± 95	527	1230
Fluorene	76	1.1	85 ± 15	49	130
Phenanthrene	453	1.5	406 ± 44	253	585
Anthracene	203	0.8	184 ± 18	116	263
1-Methyl-phenanthrene	78.5	1.3	73.2 ± 5.9	47.1	102.8
Fluoranthene	712	1.4	651 ± 50	421	911
Pyrene	604	1.4	581 ± 39	379	806
Benz[*a*]-anthracene	390	2.1	335 ± 25	217	468
Chrysene + triphenylene	490	2.9	399 ± 36[e]	254	566
Benzo[*b*]fluoranthene	520	2.3	453 ± 21	302	616
Benzo[*j* + *k*]fluoranthenes[f]	494	1.5	442 ± 23[g]	293	605
Benzo[*e*]pyrene	398	1.5	325 ± 25	210	455
Benzo[*a*]pyrene	360	2.5	358 ± 17	239	488
Perylene	492	1.0	397 ± 45	246	575
Indeno[1,2,3-*cd*]pyrene	380	1.1	341 ± 57	199	517
Dibenz[*a,h* + *a,c*] anthracenes[h]	83	1.8	90 ± 15[e]	52	137
Benzo[*ghi*]perylene	304	2.3	307 ± 45	183	458

[a] Relative standard deviation of the measured concentrations.

[b] NIST-certified concentration with uncertainty value.

[c] Lower control limit (ng/g dry weight) = 0.7 × (certified concentration − uncertainty value).

[d] Upper control limit (ng/g dry weight) = 1.3 × (certified concentration + uncertainty value).

[e] Certified concentrations and uncertainty values of coeluting compounds are summed, respectively.

[f] Benzo[*j*]fluoranthene + benzo[*k*]fluoranthene.

[g] Certified concentration and uncertainty value of benzo[*j*]fluoranthene are summed with reference concentration and uncertainty value of benzo[*k*]fluoranthene, respectively.

[h] Dibenz[*a,h*]anthracene + dibenz[*a,c*]anthracene.

The percent recoveries of internal standards ranged from 103% to 107% for CHs and from 81% to 90% for AHs.

SRM 1945 is used as the reference material in batches of blubber samples. This SRM has certified concentrations for 41 of our CH analytes, ranging from 3.30 to 445 ng/g wet weight. Measuring CHs across this range of concentrations in this material is also challenging because of the high percent of nonvolatile extractable material (74.29%) and high concentrations of other contaminants in this SRM. In another recent NIST intercomparison exercise, which was for the analysis of marine mammal blubber, our analyses of replicate samples of SRM 1945 (Table 35.10) resulted in 36 CHs being within the control limits and none being below its limit of quantitation. The measured concentrations of PCB 87, PCB 138 + PCB 163 + PCB 164, PCB 194, PCB 206, and PCB 209 were greater than their upper control limit. The RSDs of all measured analyte concentrations ranged from 0.3% to 4.5%, and the percent recoveries of the internal standard ranged from 107% to 109%.

The results for the three different SRMs summarized above met the targeted QA criteria for overall accuracy, precision, and internal standard recoveries, and demonstrate

Table 35.10 Measured and certified concentrations of chlorinated hydrocarbons in National Institute of Standards and Technology (NIST) Standard Reference Material® 1945 ''Organics in Whale Blubber'' analyzed as part of the NIST Intercomparison Exercise Program for Organics in the Marine Environment, 2003

Analyte	Measured average (ng/g wet weight) ($n = 3$)	RSD[a] (%)	Certified concentration[b] (ng/g wet weight)	LCL[c]	UCL[d]
Hexachloro-benzene	27.2	0.8	32.9 ± 1.7	21.8	45.0
α-Hexachloro-cyclohexane	16.2	1.4	16.2 ± 3.4	8.96	25.5
γ-Hexachloro-cyclohexane	3.12	4.5	3.30 ± 0.81	1.74	5.34
Mirex	38.3	0.4	28.9 ± 2.8	18.3	41.2
Heptachlor epoxide	12.4	0.4	10.8 ± 1.3	6.7	15.7
Oxychlordane	23.0	3.3	19.8 ± 1.9	12.5	28.2
cis-Chlordane	56.9	0.8	46.9 ± 2.8	30.9	64.6
trans-Nonachlor	182	0.3	231 ± 11	154	315
cis-Nonachlor	58.4	0.4	48.7 ± 7.6	28.8	73.2
2,4′-DDE	14.66	1.6	12.28 ± 0.87	7.99	17.10
4,4′-DDE	488	0.5	445 ± 37	286	627
2,4′-DDD	21.7	1.0	18.1 ± 2.8	10.7	27.2
4,4′-DDD	133	0.8	133 ± 10	86	186
2,4′-DDT	89	0.3	106 ± 14	64	156
4,4′-DDT	257	0.4	245 ± 15	161	338
PCB 18	3.08	1.3	4.48 ± 0.88	2.52	6.97
PCB 44	11.7	0.5	12.2 ± 1.4	7.6	17.7
PCB 49	16.9	0.6	20.8 ± 2.8	12.6	30.7
PCB 52	38.1	0.3	43.6 ± 2.5	28.8	59.9
PCB 66	22.3	1.8	23.6 ± 1.6	15.4	32.8
PCB 87	25.3	0.8	16.7 ± 1.4	10.7	23.5
PCB 95	39.9	0.3	33.8 ± 1.7	22.5	46.2
PCB 99	54.8	0.5	45.4 ± 5.4	28.0	66.0
PCB 101 + PCB 90	77.5	0.6	65.2 ± 5.6[e]	41.7	92.0
PCB 105	27.1	0.4	30.1 ± 2.3	19.5	42.1
PCB 110	34.8	0.4	23.3 ± 4.0	13.5	35.5
PCB 118	83.8	0.4	74.6 ± 5.1	48.7	104
PCB 128	24.5	0.3	23.7 ± 1.7	15.4	33.0
PCB 138 + PCB 163 + PCB 164	183.5	0.3	131.5 ± 7.4[e]	86.9	180.6
PCB 149	85.2	0.6	106.6 ± 8.4	68.7	149.5
PCB 151	27.5	0.8	28.7 ± 5.2	16.5	44.1
PCB 153 + PCB 132	243	0.4	213 ± 19[f]	136	302
PCB 156	12.6	0.6	10.3 ± 1.1	6.4	14.8
PCB 170 + PCB 190	50.3	0.6	40.6 ± 2.6[e]	26.6	56.2
PCB 180	143.7	0.4	106.7 ± 5.3	71.0	145.6
PCB 183	39.6	0.5	36.6 ± 4.1	22.8	52.9
PCB 187	114.2	0.5	105.1 ± 9.1	67.2	148.5
PCB 194	60.8	0.4	39.6 ± 2.5	26.0	54.7
PCB 195	11.6	0.4	17.7 ± 4.3	9.4	28.6
PCB 206	50.6	0.4	31.1 ± 2.7	19.9	43.9
PCB 209	18.4	0.6	10.6 ± 1.1	6.7	15.2

[a] Relative standard deviation of the measured concentrations.

[b] NIST-certified concentration with uncertainty value.

[c] Lower control limit (ng/g wet weight) = 0.7 × (certified concentration - uncertainty value).

[d] Upper control limit (ng/g wet weight) = 1.3 × (certified concentration + uncertainty value).

[e] Certified concentration and uncertainty value are for the co-eluting compounds combined as stated by NIST.

[f] Certified concentration and uncertainty value are for PCB 153 only; NIST does not provide a certified or reference value for PCB 132.

the applicability of this method. In each exercise, over 70% of the analytes having certified concentrations above their limit of quantitation were within the control limits (94% of CHs and 88% of AHs in SRM 1974b, 81% of CHs and 100% of AHs in SRM 1941b, and 88% of CHs in SRM 1945). Also, the RSDs for all analytes were less than the criteria of 15% maximum, and the percent recoveries of the internal standards in all samples were well within the criteria of 60% to 130%. This level of data quality has been achieved or surpassed routinely, using the techniques incorporated in the method given here. The QA measures also show where difficulties in the analyses occur, e.g., high or low biases in some analyte results. Because it is desirable to meet the SRM QA criteria for 100% of the certified analytes as an indication of attaining that level of accuracy for all sample results, further improvements in all parts of the method continue to be investigated for their impact on data quality.

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chapter thirty-six

Histological preparation of invertebrates for evaluating contaminant effects

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Contents

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Introduction

Although many studies in toxicologic pathology evaluate the effects of toxicants on fishes because of their similarities with other vertebrates, invertebrates can also provide insights into toxicant impacts on ecosystems. Invertebrates not only serve as food resources (e.g., worms, clams, shrimp, insects), but also as agents of physical habitat change (e.g., reef-building corals, bioeroding sponges, bioturbating worms), among other roles. Adverse effects on individuals and populations of any species due to toxicant exposures from water, sediment, or food can ultimately alter community relationships and have direct and indirect impacts on members of the community.

Many invertebrates are sedentary or have a limited range of mobility compared to fishes. Because of this, their health may reflect local environmental conditions better than fishes. Therefore, invertebrates can be used to study chronic or acute exposures to single toxicants or mixtures in the field and laboratory[1,2] (see also Chapters 6 and 7 in this volume). However, many such studies of invertebrate species limit their observations to gross descriptions, percent of organisms killed by the toxicant, or reductions in growth and reproduction. Knowing that an animal died or did not reproduce, however, may not be as important as knowing why. Toxicant effects can be measured using many techniques and tools, but the only techniques that can provide visual images of the internal condition of the cells and tissues of an organism are observations made using light and electron microscopy. This field is known as histology. Structure and composition of cells and tissues reflect their function and overall functioning of the organism. Histotechniques provide data that can be used in conjunction with procedures from other fields (e.g., physiology, biochemistry, molecular biology, ecology) to improve our understanding of invertebrate susceptibilities to contaminants, mechanisms of toxicant damage to target cells and organs, and impact of the exposure on the individual, population, and community.[3–8]

This chapter will discuss the preparation of invertebrate tissues for light microscopical examination using paraffin-embedding, a common procedure in many hospitals and veterinary medical diagnostic operations. The resulting product is a section of tissue typically 2–10 μm thick mounted on a glass microscope slide (histoslide). Dyes or other compounds can be used to enhance particular features of tissues for study with bright-field illumination or epifluorescence. Although basic steps for tissue fixation, dehydration, clearing, embedding, sectioning, and staining have remained similar since the late 1800s, recent developments in equipment, techniques, and the ability to identify specific

molecules in tissues could require different approaches to improve the microscopic appearance of tissues or to address specific research questions.

Three kinds of data can be generated from histoslides to test hypotheses: descriptive, qualitative, and quantitative data.[8] Study objectives must be established prior to collecting and processing organisms to identify the most appropriate methodology for producing the required information (i.e., the correct type, quantity, and quality of histological data). The hypothesis(es) being tested and the endpoints (data) that will be collected using microscopic examinations of the stained tissue sections should be framed. Qualitative (ranked) data can be compared using nonparametric statistics, and quantitative data can be compared using parametric statistics. Adequate numbers of control (unexposed) animals need to be included in every study for comparison with exposed animals. The number of replicates needed from exposed and ''pristine'' sites, or from laboratory exposures and controls, should be statistically determined to ensure valid data. In the case of endangered or threatened species, such as stony corals, a minimum of three samples from different colonies from each exposure concentration should be examined to provide an indication of individual variability and normal tissue conditions within that field site or experiment. You should plan to collect the invertebrates from the cleanest water first, then sample from the least to most contaminated, rinsing equipment between samples. Also consider limitations on resources versus data needs, i.e., how many histoslides of which portions of tissue will you need to examine to test the hypothesis(es), versus what will you be able to examine based on time, expertise, or funding constraints.

Since the ability to obtain the data is dependent on the quality of the histoslide produced, this procedure focuses on two steps: *fixation* to preserve the tissues without postmortem autolysis, and *staining* to enable the observer to see the structure and composition of the cells and tissues so that comparisons can be made with established criteria for cell damage, or with control specimens. Interpreting structure and composition seen in the tissue section under the microscope to derive these data requires special training, which cannot be covered in this chapter. Please contact any of the authors for assistance in locating comparative histopathologists and courses or workshops for such training. One resource for information, histoslides, and other materials on invertebrates exposed to contaminants or affected by other diseases is the Registry of Tumors in Lower Animals in Sterling, Virginia, VA, USA, a project funded by the National Cancer Institute (see www.pathology-registry.org, for more information).

Materials required

The equipment, supplies, and reagents needed to prepare invertebrates for histologic examination are presented in three separate lists, below. These materials are similar to those used in hospital and research histology laboratories for processing human or other vertebrate tissues. This procedure is based on the same histotechniques used in these facilities and more detailed information is presented in many books and journals (e.g., References 9–13, *Journal of Histotechnology*). You can use an internet search engine to locate resources for new and used equipment. Processing, embedding, sectioning, staining, and coverslipping can be performed manually or with the use of automated equipment. If possible, using an automated tissue processor and embedding center, at a minimum, will reduce time and artifacts, and improve the consistency of the histoslides. Be sure to keep equipment clean and in working order by following manufacturers' instructions.

Equipment

- Fume hood, providing ventilation and sufficient air exchange to protect worker health
- Tissue processor for paraffin-embedding (e.g., the Technicon Autotechnicon Mono Tissue Processor is a basic model, more features in Leica TP1020, Shandon Hypercenter, or many other models on the market)
- Embedding center (e.g., Tissue Tek III Embedding Console System)
- Microtome (e.g., American Optical, Leitz 1512, Leica RM2125T Manual Rotary Microtome, Microm HM 310 Manual Rotary Microtome)
- Blade holder for microtome disposable blades
- Temperature-controlled water bath (e.g., Fisher Model 134 Tissue Prep Flotation Bath)
- Slide warmer or oven (many models available)
- Balance (for weighing out chemicals and stains, to $\pm$ 0.01 g)
- Magnetic stirrer/heater and coated stir bars (for mixing solutions)

Supplies

- Personal protective equipment (disposable latex or nitrile gloves, goggles, lab coat, closed-toed shoes)
- Dissecting tools (e.g., Clauss poultry shears; stainless steel scissors; scalpels with disposable surgical blades, razor blades or knives; needle probes; 12 and 6-in., and fine curved and pointed dissecting forceps)
- Weighing papers or pans
- Paper towels
- Cork or plastic trimming board or wax-based dissecting pan
- Plastic ruler (or rule marked on trimming board) or calipers (for measuring animal size)
- Pencils
- Waterproof paper for labels
- Plastic or glass jars or containers with tight-fitting lids
- Sturdy resealable plastic bags
- Stainless steel or plastic cassettes (type may depend on tissue processor used)
- Stainless steel or disposable molds for embedding
- Mold-release spray (for stainless steel molds)
- Narrow- and wide-blade plastic paint scrapers (for removing paraffin from equipment, counters, floor)
- Thermometer(s) (0–100°C in 1°C gradations, for checking temperature of molten paraffin, water bath, oven)
- Embedding rings (optional, depends on type of cassette used)
- Disposable knife blades for the microtome (be sure to get proper size for holder).

Note: Traditional one-piece steel blades, which require skillful sharpening, may be used; however, due to potential sand and grit in tissues, the more expensive disposable blades save time

- Ice tray or pan filled with water and frozen
- Needle probes or small brushes

- Flat-bladed forceps
- Slide racks to fit in staining dishes
- Staining dishes (glass or plastic, 300–500 ml size, disposable plastic food storage dishes with lids that fit slide racks can be used for water rinses)
- Well-cleaned microscope slides, frosted end (use Plus (+)™ charged slides for immunohistochemistry (IHC) or stains which tend to loosen tissues from slide)
- Glass coverslips (24 × 40, 50, or 60 mm to cover the tissue section on the unfrosted area of the slide, thickness No. 1 or 1.5)
- Mounting medium, xylene-compatible (Permount® or synthetic)
- Absorbent tissues (e.g., Scotties® or Kleenex®)
- Lint-free tissues (e.g., Kimwipes®)
- Cardboard or metal flat slide holders or trays
- Microscope slide boxes (to hold 25 or 100 histoslides)

Reagents

Note: Many of these reagents are hazardous chemicals and should be handled with appropriate care and caution; review all MSDS before using.[14]

- Formaldehyde (37–40%)
- Other chemicals as noted in the selected fixative formulation(s)
- Undenatured ethanol solutions (70%, 80%, 95%, and 100%)
- Xylenes (or xylene substitute, clearing agent)
- Paraffin
- Certified stains and/or staining solutions (some can be purchased premade)
- Ammonium hydroxide (reagent-grade, in solution)
- Sodium hydroxide (reagent-grade, pellets)
- Hydrochloric acid (reagent-grade, concentrated, 12 *N*)
- Ethylenediaminetetraacetic acid (EDTA), disodium salt dihydrate, reagent-grade ($Na_2ClOH_{14}O_8N_2 \cdot 2H_2O$, CAS No. 6381-92-6)
- Deionized or distilled water

Procedure

An overview of the paraffin-embedding procedure is presented here with the steps in chronological order, as it applies to a variety of invertebrates. Each laboratory should develop written standard operating procedures (SOPs). SOPs are used to guide processing for a particular invertebrate to be studied, inform technician(s) on how the laboratory operates, how equipment should be used, tips to obtain the best results with available equipment, and describe safe handling of chemicals and emergency procedures.

1. Select and prepare fixative solution

Fixation is the most important step in the overall procedure. Chemicals are used to precipitate and denature proteins, cross-link molecules, and preserve membranes in tissue samples. The fixative solution stops all metabolic reactions, prevents autolysis by intracellular enzymes, maintains the spatial relationships of cellular components and extracellular substances, and brings out differences in refractive indexes among tissue

elements for better visibility, as well as enhancing the staining reactions.[11] Each of the chemicals used in the fixation process has advantages and drawbacks and will produce different results, both in terms of tissue preservation and in the ability to further demonstrate metabolic processes, presence of microorganisms, or immunoreactivity through the application of special stains. However, all fixatives are not compatible with all staining procedures.

(a) Use Table 36.1 to select a fixative for a particular invertebrate.
- Consider hypothesis(es) being tested and endpoints (data) that will be collected from stained tissue sections; which stains will be used and why they are necessary.
- Note special fixation and handling requirements for the chemicals.
- Determine the most appropriate fixation times for the animals and whether they can remain in fixative or require immediate rinsing and storage in another solution.
- Note the origin of the animal. Marine organisms, especially those that are osmoconformers, cannot be fixed in a low-osmolality (or osmolarity) fixative, but require the osmolality (= salt content) of the fixative to be approximately the same or slightly less than the salinity or osmolality of the water from which they were collected to prevent swelling and bursting of cells. Fixing organisms from fresh or brackish estuarine waters in a high-osmolality fixative solution can result in shrinkage of cells and tissues.
- Calculate volume of fixative needed, approximately 10–20 times the tissue volume.

(b) Prepare the fixative.
- Wear personal protective equipment.
- Weigh chemicals carefully and check calculations of proportions.
- Follow mixing instructions.
- Store the fixative in a plastic or glass jug or carboy.

2. *Inventory samples*

Planning collection efforts well in advance of a study can maximize tissue preservation and minimize unnecessary artifacts and cellular changes, which can result from improper handling. Each animal must be uniquely identified with a label, in pencil or waterproof ink. A written record of the field collection data, such as date, source or site, and other pertinent information, must accompany the animals back to the laboratory. Because postmortem autolysis increases with time after death, animals should be fresh (alive, apparently healthy or morbid), at ambient or chilled temperature. Freezing causes extensive cellular disruption and should be avoided. The species and sample collection information must be recorded in an accessioning log prior to processing. Additional notes should be kept in laboratory notebooks.

(a) Assign each specimen a unique number or code to facilitate identification and sample tracking during processing.
(b) The accessioning log should include, for example:
- Identifying number or code
- Collection location and exposure conditions
- Experimental exposure data

Table 36.1 Recommended fixatives for selected invertebrates for histologic examination using light microscopy

Fixative and source	Invertebrate group	Anticipated staining uses			Comments
		Routine	Special	Immunohisto-chemistry	
Bouin's solution (Reference 11, p. 20; can be ordered premade)	Marine, freshwater, or terrestrial invertebrates	Yes	Yes, good for stains requiring mordant	No	Excellent preservation. Do not leave in this fixative longer than 6–12 h. Wash thoroughly in 70% undenatured ethanol, changing frequently to remove the picric acid. Store in 70% ethanol
Davidson's or Dietrich's solutions (see text)	Marine or freshwater mollusks, crustaceans	Yes	Yes, but will need mordant solution for some stains	No	Good preservation in some organisms, in others more vacuolation of cells observed; intracellular granules not as well preserved. Fix at least 48 h. May be stored in this fixative up to a few months, then transfer to 70% undenatured ethanol
Helly's solution (see text)	Marine sponges, cnidarians, annelids, mollusks, echinoderms	Yes	Yes, good for stains requiring mordant	No	Excellent preservation. Fix only for 8–16 h. Tissues must be washed in water for 24 h and then stored in 70% undenatured ethanol
10% Neutral buffered formalin (Reference 10, p. 28; Reference 11, p. 13; can be ordered premade)	Freshwater or terrestrial invertebrates	Yes	Yes, but will need mordant solution for some stains	Yes, extensive time in formalin might require antigen-retrieval procedures	Fix for at least 24 h (small animals) or for IHC to 72 h. Can remain in this solution after fixation
10% Seawater-formalin solution (see text)	All marine invertebrates	Yes	Yes, but will need mordant solution for some stains	Yes, extensive time in formalin might require antigen-retrieval procedures	Preservation of tissues not optimal, use only if another fixative is not available. Fix for at least 48 h. Can remain in this solution after fixation
Z-Fix (order concentrate from Anatech, Ltd.)	All invertebrates	Yes	Yes	Yes	Dilute concentrate with freshwater for freshwater or terrestrial invertebrates and with seawater for marine invertebrates. Fix for at least 24 h. Store in this solution after fixation

Figure 36.1 Trimming tissues to fit into cassettes. (Photo by Jeffrey Greenberg, U.S. EPA, Atlantic Ecology Division.)

- Sacrifice date
- Species
- Project title or other information
- Researcher's name

(c) As each animal is inventoried, examine it carefully and record gross observations (e.g., changes in tissue color or morphology, ulcerations or abrasions, masses or cysts, degeneration or sloughing of tissue, internal or external parasites). It is better to have more information and field or experimental data, than less.

3. *Trim and fix tissue*

After recording gross observations (including size and weight), each animal must be trimmed into 2- to 3-mm thick portions so the fixative will penetrate evenly into tissues (Figure 36.1). Animals can be anesthetized[15] prior to necropsy. They can either be trimmed and fixed immediately, or partially fixed until firm enough to trim, then returned to fixative. Small specimens can be placed directly into processing cassettes for fixation. For large specimens, organs can be dissected out and sliced to permit fixative penetration. Alternatively, the animal's body cavities can be injected with fixative (use blunt end of syringe or wide-bore needle syringe) in addition to immersion. Begin working with specimens from the cleanest location first, to most contaminated, rinsing dissecting board and implements between animals to avoid cross-contamination of tissues.

(a) Wear gloves and handle animals with care.
- Two or more layers of gloves, heavy rubber, or chain-mail gloves might be needed when working with sponges, spiny crustaceans, or sea urchins.
- Long-spined urchins can be held with tongs while trimming their spines to about 1.5 cm length with scissors for easier handling.
- Gloves not only protect you from physical injury but also from exposure to potentially harmful chemicals and pathogenic microorganisms.

(b) Place animal on a dissecting board, keep moist (cover with a damp paper towel if needed).
(c) Trim, using scalpel or razor blade, according to Table 36.2 and the study plan.
(d) Mark particular locations or lesions with colored surgical marking ink (applied to blotted-almost-dry tissue) to facilitate later identification (this step can also be done after fixation).
(e) Select container large enough to hold organism(s) and add fixative (10–20 times the tissue volume).
(f) Add trimmed organism(s) to fixative solution:
- If animal sections are small enough to fit into processing cassettes without further trimming, then place sections in cassette and add a waterproof label marked with a solvent-resistant histological marking pen (not just waterproof) or a pencil recording the inventory or accessioning number or code before closing cassette.
- Multiple specimens can be fixed in a single large container of fixative (when individual identification is not needed), by placing separate specimen samples in different cassettes, or by placing larger individuals in mesh or plastic bags with holes cut in the sides to allow penetration of solutions (the latter options permit keeping a label with each specimen).
- Smaller animals can be fixed in containers with tight-fitting lids, either individually or in groups. Extremely small organisms (e.g., micromollusks, copepods, or worms) or pieces of tissue can be placed in fine mesh minicassettes or cassettes with biopsy sponges to prevent loss through the cassette holes during processing.
- Fixed material can also be enrobed in agar or HistoGel™ (see next step), and the resulting block inserted in cassette for fixation.

(g) Unused tissues can be stored in the fixative depending on the solution used (see Table 36.1), in 70% ethanol, or discarded following the protocol for your facility.
(h) To avoid contamination of tissues, rinse or wipe off dissecting board before trimming the next organism.

4. *Remove excess fixative, decalcify tissue (optional), and place in cassettes*

After fixation, tissues are ready to be rinsed and undergo a final trimming, if necessary, to fit into cassettes and embedding molds. Calcium carbonate endoskeletons or exoskeletons of invertebrates (e.g., stony corals, sclerites of gorgonians, tests and Aristotle's lantern of echinoderms, chitinous shells and stomachs of arthropods, small mollusks fixed in their shells) must be decalcified before final trimming to permit sectioning. Not all of these materials will be readily removed during decalcification, but it should help.

Acidic decalcification solutions can affect end results. Routine or special histochemical stains are not affected by this treatment, provided they are washed thoroughly to remove the acid. For delicate tissues, acid-based solutions can be diluted 1:1 with deionized water. Acid-based decalicifiers work quickly; however, preserving antigens for study using IHC requires slow decalcification of small pieces of tissue, using EDTA at neutral pH and room temperature. The formula for this solution is provided in Appendix. If you are uncertain of which decalcification method to use and have enough material, divide the specimen so more than one technique can be used.

Table 36.2 Trimming and fixation of invertebrate tissues for histological examination

Invertebrate group	Small (<5-mm thick)	Large (>5-mm thick)
Sponges *Note*: Sclero-sponges are extremely hard and cannot be sectioned with razor knives. Embedding in plastic and grinding to prepare thin sections (as is done for bone) might be useful with this group	Immerse whole in fixative	Trim into slices with disposable razor blade to 5-mm thickness, then immerse in fixative. (*Note*: Siliceous spicules in the sponges will dull the blade quickly, change frequently.) Boring sponges in shells or calcareous substratum can be fixed in entirety, but try to keep portions relatively thin to ensure penetration of the fixative
Cnidarians or coelenterates	Relax using an anesthetic, if desired.[15] (*Example*: Add a 2% solution of magnesium sulfate to the collection water of the animals. Gradually increase the concentration of the magnesium sulfate in the collection water until it reaches about 30%. Carefully drain off overlying water, then add fixative to this container. After about 30 min, change solution to fresh fixative.) If not relaxing, immerse whole animal in fixative Minimize time in Bouin's and Helly's solutions	Relax using an anesthetic, if desired[15] Stony corals: Immerse whole or portion in fixative. Tissue is thin and will easily be penetrated by the fixative. The carbonate skeleton is removed after fixation Gorgonians: Trim into 5-mm thick portions. Trim branch length to fit the size of the fixation container Sea anemones: Immerse in fixative for approximately 10 min to firm tissue, then slice open sagittally to permit fixation in the gastric cavity. Or inject fixative into the mouth, slice as needed, and immerse in fixative Jellyfish or comb jellies: Immerse whole animal in fixative. Slice larger animals at intervals to permit fixative penetration or dissect out portions needed for study and immerse in fixative All of the above: Minimize time in Bouin's and Helly's solutions
Annelids	Relax worms using an anesthetic, if desired[15] Immerse whole worms less than 3 cm long in the fixative Final trim: Cross-sections, sagittal, and longitudinal sections should be made	Relax worms using an anesthetic, if desired[15] Immerse larger worms in fixative for 15 min, then cut longitudinally to 5-mm thick sections Very large worms will require dissection of desired parts Final trim: Cross-sections, sagittal, and longitudinal sections should be made
Mollusks	Bivalves: Carefully separate shell halves slightly with a scalpel to allow fixative to penetrate. Immerse whole animal and shell in fixative Gastropods: Crack the shells of smaller snails using pliers or a hammer (gently!) to allow preservative infiltration. Immerse whole animal with shell in fixative	Bivalves: Insert a shellfish knife between the valves being careful not to damage the tissues. Separate the valves by turning the knife sideways. Gently slide the knife along the dorsal valve to detach the mantle and adductor muscle. Remove the shell. Detach the adductor muscle from the ventral valve using the same technique (see Reference 37) Univalves (e.g., limpets, slipper shells): Immerse whole animal and shell in the fixative

		Gastropods: Gastropods can be quite difficult to remove from their shells. The tip of the spire can be removed, then the shell is cracked and slowly chipped away from the opening and up along the spiral using bone shears, and carefully the animal is removed from the remaining shell fragments. Immerse whole animal in fixative Cephalopods: Immerse whole animal in fixative until firm All of the above: After the whole animal has been in fixative for approximately 30 min, the body can be sagittally or parasagittally sectioned at 5-mm intervals, leaving the sections attached at one end. Use a firm pressure and slow sawing motion with razor blade or scalpel. Return the tissue to the fixative for the specified amount of time
Crustaceans	Copepods, amphipods, crabs, hermit crabs, shrimp: Immerse whole animals in fixative[38] Zooplankton samples (can contain an assortment of crustaceans and other phyla): Add Dietrich's fixative to the collecting medium. Decant solution off after the animals die, and refill the container with fresh fixative. The animals can be stored indefinitely in Dietrich's	Immerse in fixative for 30 min or inject fixative into the animal's hepatopancreas, stomach, and midgut Barnacles: Crack shells with poultry shears and place in fixative Crabs: Cut abdomen away from carapace, pull body apart, and return to fixative Hermit crabs: Pull from their shells. Cut into several sections and immerse in fixative Shrimp: Bisect or trisect longitudinally but leave the tail intact. Return to fixative Lobsters, large shrimp: Loosen the carapace from the thorax and cut it off behind eyes. Remove the intestine, reproductive tract, heart, and hepatopancreas. Cut the body through midline from posterior to anterior. Ventral nerve fibers and ganglions lie between the muscle bundles and are easily teased out after fixation
Echinoderms	Immerse in fixative	Inject fixative through the test (exoskeleton) in several places If necessary, insert scalpel and cut around peristome to permit fixative to infiltrate the organs Test can also be split open to permit fixative penetration. Immerse in fixative

Figure 36.2 Self-siphoning water bath. Continuous filling and draining with tap water thoroughly rinses decalcified tissues (here in cassettes) for 24 h. (Photo by Jeffrey Greenberg, U.S. EPA, Atlantic Ecology Division.)

(a) Rinse fixed tissues briefly in freshwater before final trimming, unless they have been stored in 70% undenatured ethanol.
- As noted in Table 36.1, Bouin's and Helly's solutions require extensive washing to remove excess fixative. As appropriate to the fixative, either water or 70% ethanol can be used for this procedure, until the wash solution is no longer colored.
- For Helly's solution, a self-siphoning water bath is preferred (Figure 36.2); alternatively, use constant flow from a tap into a water dish containing the tissue or several changes of static water.

(b) Using fresh scalpel or razor blades, trim fixed tissue into 2- to 3-mm thick sections that fit easily into labeled cassettes.
- Use firm pressure on blade and a slow sawing motion.
- Switch to a new blade as the old one dulls to ensure a smooth cut with minimum disruption or tearing of tissue.
- Do not force sections to fit into cassettes, as this will yield incomplete penetration of reagents during subsequent processing.
- If tissue cannot be cut easily, it should be decalcified first [see Steps 4(e)–4(i) below].

(c) Place the side of the tissue that will be viewed microscopically facing down in cassette for appropriate orientation in embedding molds following processing.
- More than one piece of small tissue can be placed in a single cassette.
- Trimming must be determined based on study objectives. One study might need only a cross-section of a clam; another might need multiple cassettes to hold portions of gills, gonad, heart, intestines, hepatopancreas, and muscles from a lobster.
- Ganglia and nerve fibers from large organisms must be processed for shorter times during subsequent tissue processing. They should be placed in separate cassettes and marked to indicate this during necropsy and fixation.

(d) After final trimming of undecalcified or decalcified (and washed) invertebrate samples, place cassettes in a large container and cover them with 70% ethanol, cap tightly. They can be kept in this solution indefinitely.

(e) If the tissue will not cut easily, decalcification should be performed. Check your invertebrate carefully for possible calcareous material. For example, echinoderms that feed by scraping algae off calcareous surfaces, such as the long-spined sea urchin, may form rounded balls of calcareous material in their intestines, and the balls should be carefully removed or decalcified prior to processing.
- Copepods and amphipods often require no further decalcification after several days of fixation in Dietrich's solution or Davidson's solution as the weakly acidic nature of these fixatives aids in decalcification.
- Delicate tissues of some specimens could benefit from enrobing the specimen in low melting point (35°C) agar (dissolve 1.5 g in 100 ml at 90°C with stirring) or commercially available HistoGel (which comes with instructions) prior to embedding. This enrobing will help to maintain the architectural relationships of tissue elements after decalcification has removed the calcium carbonate. Blot the specimen dry with a paper towel first so the cooled agar (about 60°C) will adhere, place the specimen in a disposable mold and cover with the agar. Place the mold in a vacuum oven set to 50°C and apply two cycles of vacuum, while the tissue is in the molten agar to help the agar coat the specimen and remove air bubbles. Turn off the oven to let the agar cool and gel. Scrape the agar or HistoGel gently away from an area of calcified tissue to permit the decalcifying solution to penetrate.

(f) Place tissue in a glass or plastic jar filled with appropriate room temperature decalcifying solution.
- The amount of reagent needed will vary with amount of calcified tissue.
- For acid-based solution, add a tissue or paper towel to top of the solution and place container under a fume hood.
- For neutral pH EDTA solution, suspend tissues off bottom and keep them above the layer of chelated calcium that will form on the bottom as decalcification progresses.
- For either solution, loosely cap the container. Change decalcifying solution in container once or twice daily until calcium carbonate is no longer present. For some organisms, this is easily seen when an acid decalcifier stops bubbling, or when an attempt to trim the tissue results in easy cutting. Other procedures for checking the decalcification endpoint can be used, such as chemical tests or X-radiography.

(g) When decalcification is confirmed, trim tissue and place in numbered processing cassettes [see Step 4(c)].

(h) Place the cassettes of final trimmed, decalcified tissues in running tap water for 24 h to remove all acid (Figure 36.2). Rinse EDTA-decalcified tissues in tap water for about 30 min. (If not rinsed, adverse staining reactions will occur.)

(i) Store the tissues in 70% undenatured ethanol as described in Step 4(d).

4. *Process and embed tissue*

Although the steps of dehydration, clearing, and infiltration with paraffin can be performed manually, using glass jars and an oven (for melting the paraffin), automation provides more controlled timing, heating, and cooling options and greatly improves the speed and consistency of tissue processing (Figure 36.3). We have used both ''simple''

Figure 36.3 A basic tissue processor can speed up the dehydration, clearing, and infiltration steps and provide consistent results. (Photo by E.C. Peters.)

(e.g., graduated series of ethanol and xylene) and special formulations (e.g., S-29 and UC-670; 2-2-DMP) for dehydration and clearing of invertebrate tissues and did not find appreciable differences for the many species processed.

The length of time in dehydrating and clearing solutions does, however, depend on the thickness and density of tissues being processed and can greatly affect results. For example, bivalves require up to 1 h in each solution, including 3 changes of molten paraffin, whereas delicate coral tissue should be restricted to approximately 15–30 min in each change of dehydrating and clearing agent, with no more than 15 min in each change of molten paraffin. A reduced-time processing run, such as those used for biopsies in clinical settings, should also be used for marine cell-blocks and fine micromollusk samples, with no more than 10–15 min per solution. A low melting point (56–58°C) paraffin-based embedding medium that will produce a moderately firm block is preferred for most tissues. If available, gentle vacuum should be applied throughout processing, or minimally for the final change of paraffin. Table 36.3 summarizes suggested processing times for the solutions typically used. Do not allow the cassettes to dry out at any point of the process.

(a) *Automated processing*: Set up the processor and follow operating instructions to program the times for each solution. Fill processor chamber or basket with cassettes directly from storage solution and immediately immerse in the first solution. Gentle agitation or stirring helps the solutions infiltrate the tissues.
(b) *Manual hand processing*: Carefully transfer tissues or cassettes into each solution. Gently swirl or agitate containers every 10 min to aid infiltration of solutions into the tissues.
    - Due to their small size, copepods or other small invertebrates can be easily transferred from one solution to the next by using a Pasteur pipette under a dissecting microscope.
    - A dye (e.g., phloxine or eosin) added to the last 95% ethanol station will stain the tissue and aid in providing color contrast in paraffin during embedding and sectioning. The dye will wash out as the microscope slide goes through the staining process.

Table 36.3 Suggested times in solutions for processing invertebrates. Times are guidelines only and should be altered if needed. RT, room temperature, approximately 21°C

Station	Solution	Tissue <2 mm all dimensions[a]		Tissue <2 mm thick, delicate[b]		Tissue 2–5 mm thick, dense	
		Time (min)	Temperature	Time (min)	Temperature	Time (h)	Temperature
1	70% ethanol	10	RT	30	RT	1	RT
2	80% ethanol	10	RT	30	RT	1	RT
3	95% ethanol	10	RT	15	RT	1	RT
4	100% ethanol	10	RT	15	RT	1	RT
5	100% ethanol	10	RT	15	RT	1	RT
6	100% ethanol	10	RT	15	RT	1	RT
7	Xylene	10	RT	15	RT	1	RT
8	Xylene	10	RT	15	RT	1	RT
9	Xylene	10	RT	15	RT	1	RT
10	Paraplast	10	58°C	30	58°C	1	58°C
11	Paraplast	10	58°C	15	58°C	1	58°C
12	Paraplast with vacuum, if available	Not needed		15 min, if available	58°C	1, if available	58°C

[a] Such as tissue biopsies, zooplankton, eggs, nerve fibers, and ganglions.

[b] Such as corals.

(c) While tissues are processing, set up the embedding center (Figure 36.4). Be sure the paraffin has melted before starting to process tissues, as this can take several hours or overnight.

(d) When tissues have completed their last paraffin, transfer them to clean molten paraffin in the embedding center or another container in an oven.

(e) Using heated forceps, remove one cassette from container, open it, and remove paper label and set it aside. If using embedding rings with base molds, write inventory number from label on ring using pencil.

- *Embedding center*: Keep forceps in heated wells when not in use.

Figure 36.4 Basic embedding center consists of molten paraffin dispenser (top center), paraffin bath for tissues (back right), mold warming tray (front right), forceps warmers (front center right), hot plate (front center), and cold plate for hardening the blocks (left). (Photo by Jeffrey Greenberg, U.S. EPA, Atlantic Ecology Division.)

- *Manual embedding*: A small alcohol lamp may be used to warm forceps. Warning — overheated forceps can burn tissues.

(f) Half-fill a pre-warmed clean base mold with molten paraffin and quickly transfer the tissue piece(s). Keep orientation of tissue the same as was trimmed into the cassette, so the area of interest is placed on bottom of the mold.
- Keep forceps warm between handling of specimens. Paraffin congeals on cold forceps causing specimen to stick.
- Should this occur, put forceps and tissue back into molten paraffin until congealed paraffin remelts and specimen is free.

(g) Cool base mold on cold plate or an ice tray, gently holding tissue against bottom with forceps until trapped in hardening paraffin. Do not let surface paraffin become hardened.

(h) Quickly place numbered cassette or embedding ring on top of base mold and fill with molten paraffin.

(i) Place paper label on top and keep filled mold on cold plate or ice tray until completely hardened.

(j) When completely hardened, remove block from mold. Do not force out of mold. Blocks can be stored at room temperature indefinitely.

5. *Section and mount tissue*

Set the thickness setting on microtome (Figure 36.5) at 4–6 μm. Disposable microtome blades are preferred when sectioning most invertebrates, especially for tissues that might contain spicules, debris, sand particles, or calcified areas.

A pan of ice, ice tray, or cold pack should be placed on one side of the microtome and a water bath is placed on the other side (Figure 36.5). A water bath filled with deionized water and heated to approximately 45°C should be warm enough to soften thin paraffin sections, but not hot enough to cause rapid melting and distortion of tissues (about 10–15°C below the melting point of the paraffin). Remove dust particles and debris from surface of water bath at regular intervals to keep debris from getting on or under tissue sections. This is accomplished by dragging an unfolded paper towel or Kimwipe on

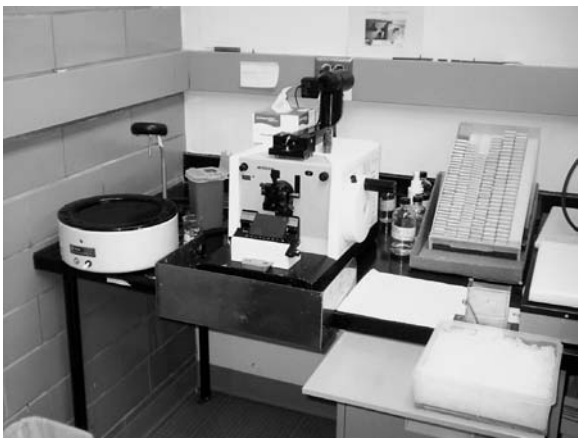

Figure 36.5 Basic microtome set up. A water bath is placed to the left of the microtome. (Photo by Jeffrey Greenberg, U.S. EPA, Atlantic Ecology Division.)

surface to pick up debris. Place blocks to be chilled tissue side down on ice. Microscope slides can be labeled with the inventory number before or after sections are mounted. The number of slides made for each block will depend on the number of stains to be applied to the sections.

(a) Place cooled block in microtome chuck and be sure all adjustments are finger-tight on block, blade, and holder.
(b) Trim block face into tissue by taking about 10 thick sections, then form a ribbon of about 10 sections using the automatic feed.[10,11]
(c) Use fingers and a brush or dissecting needle to lift ribbon and float it on the warm water bath.
(d) Select suitable section(s) and gently separate them. Hold a clean microscope slide at a 45° angle and insert into water bath. Use the needle to bring the section(s) to the slide. Holding the section(s) over the slide, slowly draw the slide out of the water with the section(s) attached.
    - In general, no adhesive is necessary for attaching tissues to slides; however, for long or harsh staining procedures use adhesives, such as gelatin, albumin, and poly-L-lysine, or Plus (+) charged slides.
    - Mounting two or more sections on one slide ensures that differences seen in one section can be confirmed and traced in adjacent sections.
    - Change blade as frequently as necessary to achieve good sections (with long blades, they can often be moved two or three times in the holder to use all portions of the blade surface).
    - Nicks in the blade will tear sections. With difficult sections, enough tissue can often be recovered by careful and slow placement of partial ribbons on the slide to obtain the necessary tissue sections for diagnosis and observation.
    - Soaking the block with a water-dampened wipe between sections can often compress tears and yield a suitable ribbon.
    - If the tissue has not been completely decalcified, the block can be melted down, and tissues run through a reverse process so further decalcification can be accomplished, or the cut block can be placed in decal solution for several hours to several days to decalcify it enough to permit sectioning.
    - Another aid to manipulating difficult tissues is the use of an intermediate room temperature 5% ethanol solution before the warm water bath. Float sections on the surface of the room temperature ethanol solution and tease small folds and wrinkles out prior to transferring with a clean glass slide onto the warm water bath.
    - Dry serial sectioning is another useful technique. The paraffin ribbon containing the tissue sections is not floated on a water bath but carefully placed in a shallow cardboard box until all required sections are cut. (Air currents can be treacherous for this method.) Place a microscope slide on a slide warmer and flood with water. Cut the paraffin ribbon into appropriately sized lengths and float them on the slide. Multiple ribbons can be placed on the same slide in this manner and, if done skillfully, can even provide a means of measuring structures within the organism. The slide must remain flat on warming table until water has completely dried and sections have adhered to slide.
    - Arthropods present challenges in sectioning, because chitin, a dense nitrogen-containing polysaccharide related to cellulose, forms the exoskeleton of these organisms, and it can easily destroy microtome blades. Face the block,

then apply either a water-dampened tissue or a thin film of a depilatory cream for a few minutes, wipe off, then try sectioning with a new blade. Alternatively, try using 4% phenol, as recommended for insects.[10] These tissues often require more care and attention when sectioning, but good sections can be obtained with patience.

(e) Drain each slide vertically for a few minutes, then place it on the slide warmer at 45–50°C for up to 4 h, or place it in a slide rack and put it in an oven at 60°C for up to 4 h.

(f) After they are thoroughly dried, store the mounted tissue sections in slide racks or slide boxes at room temperature until ready to be stained.

(g) Once an adequate number of slides have been made, blocks can be sealed by dipping in molten paraffin. Should additional sections become necessary at a later date, carefully realign the face of the block with the microtome blade.

6. *Stain tissue*

Excellent texts are available that explain the chemistry involved in applying dyes to highlight specific features of the cells and tissues. Staining enables changes in structure and composition, whether natural or induced by toxicants or pathogenic microorganisms, to be visualized with microscopy.[9–13] This section discusses the basic steps and provides details on routine and special stains that have proven helpful in aquatic toxicology studies of invertebrates, as well as one of the fastest growing areas of specialization, immunohistochemical protocols, which identify the location of specific molecules within cells and tissues. The term ''biomarkers'' includes measurable molecular, biochemical, and cellular changes caused by chemical contaminants or other stressors that might help identify causal mechanisms underlying observed effects at the population or community levels. Some biochemical biomarkers under investigation are molecules that indicate the structural integrity or functional integrity of enzyme pathways or that are induced during exposure to detoxify and remove contaminants. These biochemical biomarkers hold promise in identifying the most subtle irreversible changes occurring in cells and tissues before they are evident during histopathological examinations with light microscopy.

All staining processes share the same basic steps, whether performed manually (Figure 36.6a) or by using an automated stainer (Figure 36.6b):

(a) Place slides in racks.

(b) Move the racks through various solutions contained in glass or plastic slide dishes.
- Paraffin is removed with several changes of xylene and the sections are rehydrated by passing them through graded ethanol solutions (100%, 95%, and 80%) to deionized or distilled water.
- The time spent in each staining solution might need to be adjusted for some invertebrates, depending on the fixative used and whether the tissue was decalcified, as well as how recently the staining solution was prepared.
- If the protocol indicates the staining reaction should be checked under a microscope, do this and adjust intensity of the staining reaction by increasing

A

B

Figure 36.6 Slide staining options: (a) Performing the routine H&E procedure in glass staining dishes. Dishes containing xylene are under the fume hood to prevent human exposure. (Photo by E.C. Peters.) (b) Automated slide stainer. (Photo by Jeffrey Greenberg, U.S. EPA, Atlantic Ecology Division.)

time in the stain or in the differentiation solution (which removes excess stain).

(c) Following staining, dehydrate the sections again through graded ethanols and 3 changes of xylene.

(d) Apply a coverslip over the stained section(s) using an appropriate mounting medium, such as Permount or one of the newer acrylic compounds.

- Most people develop their own coverslipping techniques.
- The finished slide should not have any air bubbles under the coverslip, excess mounting medium on the sides, or streaks of medium on the surface.
- Clean the surface of the coverslipped slide with a folded absorbent tissue dipped in xylene or xylene substitute.

Routine staining

General tissue staining procedures use one to three dyes to differentiate the nucleus from the cytoplasm and to distinguish types of tissue. Most staining procedures call for one stain to demonstrate an important cell component's structure, such as the nucleus, and one or more counterstains to act as a contrast color on the rest of the tissue. Hematoxylin and eosin (H&E) is known as a ''routine'' staining protocol, and should always be performed on one section from each block to interpret the condition of the nuclei and cytoplasm.

- Hematoxylin is a basic dye that binds to nucleic acids and other basophilic substances, such as mucopolysaccharides, tinting them various shades of blue or purple.
- A slide is properly stained with hematoxylin when cytoplasm and connective tissue are clear or light tan, and nuclei are distinctly purplish, but chromatin inside the nuclei is visible as a darker purple dust or streaks.
- Note that basophilic secretions, such as those of mucous secretory cells, may or may not stain purplish and should not be used as an indicator.
- The tissue is typically counterstained with eosin, an acidic dye, which stains acidophilic components, such as cytoplasm and connective tissue, with shades of red to pink (i.e., it should not be a single bright pink color).
- A slide is properly stained with eosin when cytoplasm and connective tissue are a clear and strong pink, but not heavy, i.e., cytoplasmic inclusions and pigments should be distinguishable; and different shades of pink are seen in connective tissue and muscle bundles.

Various H&E protocols can be found in reference books,[9–11] and some premade solutions are now available commercially. Two types of hematoxylin staining procedures have been developed: *regressive*, in which the tissue is first overstained with hematoxylin, then differentiated by removing excess stain with acid alcohol; and *progressive*, which relies on ionic bonding to only stain the acidic nuclear material. One popular regressive formulation is Harris's hematoxylin (Figure 36.6a) and results in crisp nuclear staining with distinctive chromatin. It works well with tissues that have been decalcified. Progressive hematoxylins, such as Mayer's or Gill's, should only stain the basophilic components and do not require the differentiation step. A number of formulations for eosin also exist, some of which are also commercially available, and it is often combined with phloxine to increase the intensity.

Special histochemical staining

Additional staining protocols can be performed on replicate tissue sections from each block to highlight certain structural features, confirm composition of secretions, or aid in identifying parasites and pathogens.[9–13] We provide two protocols here that have been helpful in toxicologic pathology studies. Modified Cason's is a quick trichrome stain primarily used to distinguish connective tissue from muscle fibers. It can also assist in examining the condition of symbiotic algae in corals, gonad development, and fine ducts. Some toxicants are irritants, provoking mucus secretions as a protective mechanism in invertebrates. Modified Movat's pentachrome protocol distinguishes changes in the

mucopolysaccharides of mucous secretory cells. Changes in molecular structure, composition, and pH are reflected in differential binding of the alcian blue and saffron solutions, resulting in blue, yellow, or green.

Modified Cason's trichrome for connective tissue stain

Adapted from Reference 16. Prepare Cason's trichrome solution (see Appendix). Use a control tissue slide (a section known to have connective tissue present).

(a) Deparaffinize and hydrate to water (3 min each in 3 changes of xylenes and 3 changes of 100% undenatured ethanol, 1 change each 95% and 80% ethanol, and distilled or deionized water).
(b) If tissue sections were not fixed in Bouin's solution, Helly's solution, Z-Fix, or Zenker's-type, put the slides into a staining dish overnight in a solution of Helly's without the formaldehyde. The picric acid or metal ions in the Helly's will bind to the connective tissue and enhance the binding of the aniline blue.
(c) Stain 5 min in Cason's trichrome solution.
(d) Wash 3–5 s in running tap water.
(e) Dehydrate rapidly through 95% ethanol (1 min) then 3 changes of 100% ethanol (2 min each). Water removes the Cason's trichrome solution from the tissues, so be careful that it does not wash out too much.
(f) Clear in 2 changes of xylene (3 min each).
(g) Mount in Permount or other synthetic resin.

Results:

- Collagen — blue
- Nuclei, muscle fibers, yolk proteins — red

Modified Movat's pentachrome stain

This modification of Movat's procedure, which was developed by P.P. Yevich and C.A. Barszcz at the EPA's Narragansett, Rhode Island, laboratory, does not include the stains and steps for elastin found in mammalian tissues and is more appropriate for marine invertebrates. The formulas for the necessary solutions are in Appendix.

(a) Deparaffinize and move through 3 changes of 100% ethanol.
(b) Place slides in alcian blue solution for 30 min.
(c) Wash slides in running tap water for 3 min.
(d) Place slides in alkaline alcohol for 120 min.
(e) Transfer slides to rinse in distilled water for 2 min.
(f) Place slides in Weigert's iron hematoxylin for 1 min.
(g) Wash slides in running tap water, 15 min.
(h) Rinse slides in deionized water, 2 min.
(i) Stain slides with woodstain scarlet solution, 1 min.
(j) Rinse in 0.5% aqueous glacial acetic acid, 2 min.
(k) Differentiate in 5% aqueous phosphotungstic acid solution, 7–10 min. Check slides under the microscope.

(l) Rinse in 0.5% aqueous glacial acetic acid, 2 min.
(m) Place slides in 3 changes of 100% undenatured ethanol, 2 min each.
(n) Transfer slides to alcoholic saffron solution, for 7–10 min. Cover the staining dish and seal edges with tape after inserting slides to prevent rehydration with moisture in the air. After staining, return solution quickly to bottle and cap tightly.
(o) Rinse slides in 100% ethanol, 3 changes, 2 min each,
(p) 3 changes of xylene, 2 min each. Coverslip.

Results (different fixatives and decalcification solutions may result in color variations in your sections):

- Nuclei — black
- Connective tissue — pale blue-green to yellow
- Mucous secretory cells — green, blue, yellow
- Muscle fibers — red

Price's modified Twort's procedure for Gram-positive and Gram-negative bacteria

Tissue damage can be caused by pathogenic microorganisms. A toxicant can damage the animal's immune system or cause tissue necrosis, which then attracts microorganisms that feed on or use released tissue compounds as substrates. This stain will help identify Gram-positive and Gram-negative bacteria. Formulas for the solutions in this staining procedure are in Appendix.

(a) Deparaffinize and rehydrate tissue sections to water.
(b) Stain with filtered crystal violet solution, 2 min.
(c) Rinse in tap water, then drain.
(d) Place in iodine solution, 2 min.
(e) Rinse in tap water, then drain.
(f) Do a half-second dip in acetone, then without pausing to drain, plunge immediately into running tap water for 2–3 min.
(g) Counterstain in Twort's stain for 3–5 min.
(h) Wipe off the back of the slide, then carefully blot front of slide by placing tissue side down on bibulous paper or a lint-free Kimwipe. (Use a rolling motion to avoid damaging tissue section.)
(i) After blotting, place the slides in a clean, dry rack to avoid transferring any excess Twort's stain into the alcohol.
(j) Dehydrate rapidly: about 15 gentle dips in 100% alcohol, then 2 changes of fresh 100% alcohol for 2 min each.
(k) Clear in 2 or 3 changes of xylene, 3 min each, and mount coverslip with permanent media.

Results:

- Gram-positive organisms — blue
- Gram-negative organisms — pink to red
- Nuclei — red
- Most cytoplasmic structures — green

Immunohistochemical staining

Histological dyes cannot uniquely stain particular cells, cell structures, or tissue components. IHC developed from the discovery of antigens that induce a detectable immune response in a vertebrate by producing a specific antibody capable of binding to the epitope of the antigen (proteins, polysaccharides, nucleic acids, and polymers). An antigen present in a tissue section on a microscope slide can be demonstrated by applying a solution containing the appropriate antibody, which then binds to the epitope(s) of the antigen. The antibody is visualized by attaching either a fluorochrome or an enzymatic chromogen to the antibody. A fluorochrome is a substance that absorbs light not visible to the human eye (short wavelengths, such as UV) and emits light that is visible (longer wavelengths). An example of a fluorochrome is fluoroscein isothiocyanate (FITC), and epifluorescence microscopy must be used to see where the antibody is binding. Enzyme IHC is used for light microscopic detection. An enzyme is used that, in the presence of substrate and chromogen, produces visible color. For example, horseradish peroxidase is used with hydrogen peroxide and 3-amino-9-ethylcarbazole (AEC) to form a colored compound. Mayer's hematoxylin is used as a counterstain in this procedure because it does not contain alcohol that would decolorize the section.

While IHC has become an extremely valuable tool in the diagnostic process of human disease, it is unclear how far down the evolutionary chain this antigen–antibody complex is conserved. IHC is typically species-specific and most antibodies are made against human or other mammalian (and a few fish) antigens, although many have been successfully used in lower animals. Some investigators are now producing invertebrate-specific antibodies. Antibodies should always be fresh (note expiration dates) and stored as directed by the manufacturer.

It is beyond the scope of this chapter to cover more than the basics of IHC, and there are many excellent texts[17] journal articles [e.g., *Journal of Histotechnology*, Special Issue on Diagnostic Immunohistochemistry, Vol. 25 (4), 2002] available. When performing a preliminary investigation, the primary decision will be whether the direct method or indirect method of staining is most appropriate. In either case, fixation is important to anchor the water-soluble antigens in place but can mask the epitopes. A variety of fixatives have been developed that limit masking reactions and procedures have also evolved to unmask antigens by using heat from a microwave oven or by proteolytic digestion, but these are not always successful.

Several different approaches may need to be undertaken to determine the most effective protocol. The methods used, how the antibody is prepared (monoclonal — more specific but less sensitive; polyclonal — more sensitive but less specific), the need for unmasking and how it should be done are variables that need to be addressed. From a technical standpoint, the procedure is fairly straightforward but interpretation of staining results requires skill and experience.

When evaluating the usefulness of an antibody, the use of several different control slides is essential as numerous factors can impact results. Control slides serve to verify both positive and negative staining.

- *Positive control*: a slide containing tissue that has the antigen of interest. This slide verifies the viability of the antibody. If the positive control fails to stain, then the antibody solution may be suspect. The tissue used as a control should have undergone the same fixation and processing protocol as the tissue under investigation.

- *Procedural control*: a slide of the tissue being investigated treated with a ubiquitous antibody, such as vimentin or ubiquitin. This will verify that the tissue being tested is immunoreactive, and the procedure being followed is conducive to achieving a positive result.
- *Negative control*: two slides — one of the verifiable positive control tissue, and one from the investigative tissue. Both slides follow through all the same procedural steps as the test slides, but plain buffer is substituted for the primary antibody. This will ensure that any positive reaction obtained is due to the application of the primary antibody.

Dilution factors for the antibody must also be determined. Optimal staining is achieved when background staining is at a minimum and specific staining is of medium to high intensity. For direct immunostaining, only the primary antiserum needs to be tested at different dilutions. Use of the indirect method will require a checkerboard titration to determine the optimal concentrations of the antibody solutions.[17] Seventeen test slides will need to be used: sixteen for the dilutions plus one that receives neither the primary nor the secondary antiserum. If the optimal set of dilutions seems to fall between tested examples, set up a second checkerboard titration within the indicated range. Include control slides in the dilution tests. Once staining has started, do not allow the tissue sections to dry out.

Secondary antiserum dilution	Primary antiserum dilution			
	1:50	1:100	1:500	1:1000
1:50	1	2	3	4
1:100	5	6	7	8
1:500	9	10	11	12
1:1000	13	14	15	16

(a) Cut sections at 3–4 μm and use poly-L-lysine-coated or positively charged slides to help tissue sections adhere to the slides. Dry at 58°C for 2 h.
(b) Deparaffinize (3 changes xylene, 5 min each) and rehydrate slides (2 changes 100% ethanol, 5 min each; 2 changes 95% ethanol 5 min each) to distilled water.
(c) Circle the tissue sections with a wax pencil or immuno-marker to act as a dam, to prevent the antibody solution from running off the tissue. This will also reduce the amount of antibody solution needed for the procedure.
(d) Place two glass rods in a shallow dish with lid or special IHC tray that contains a paper towel moistened with IHC buffer. Lay the slides horizontally on top of the two parallel glass rods.
(e) Inhibit endogenous peroxidase activity by applying 3% hydrogen peroxide solution for 5–10 min.
(f) Wash slides in distilled water for 1 min.
(g) Wash slides in buffer for 5 min.
(h) Incubate with primary antibody for 20 min.
(i) Rinse with 3 changes of buffer, 5 min each. Discard buffer after each rinse.
(j) Apply and incubate with the link antibody (if used) for 20 min.

(k) Rinse with 3 changes of buffer, 5 min each. Discard buffer after each rinse.
(l) Apply and incubate chromogen substrate or fluorochrome solution for 10 min.
(m) Rinse with 3 changes of buffer, 5 min each. Discard buffer after each rinse.
(n) Counterstain, if desired, and coverslip with water-based media.
(o) Examine the tissue sections with brightfield or epifluorescence microscopy, as appropriate, and record the intensity of the immunostaining to the background on a scoring sheet for quality control purposes. If the staining is too intense or shows too much background staining, increase the dilution of the antibody.
(p) Seal edges of histoslides with glue, cement, or fingernail polish for longer storage when using aqueous mounting methods to prevent drying out.

Results and discussion

Knowledge of invertebrate histology is critical to understanding their physiology, reproduction, biochemistry, systematics, molecular biology/genetics, immunology, embryology, and ecology. Examination of tissues by light microscopy can be used to evaluate the health of an animal following exposures to contaminated materials or infectious agents, or to determine the presence or absence of pathological changes. These changes can be identified by morphological alterations, changes in the rate of occurrence of normal features (e.g., mitotic figures), reactions to special histochemical or immunohistochemical stains, or through variations in normal staining characteristics, which might be indicative of changes in biochemical composition or the existence of degenerative processes.[3,4,7] The study of such changes in cells and tissues — histopathology — is important in the invertebrates, as well as in the vertebrates. The normal range of appearance of cells and tissues must be understood first, because cellular appearance can change due to metabolic activity, diet and feeding times, daily light–dark cycles, or the reproductive cycle.

Study of vertebrate[3] and invertebrate pathology resources[18,19] is required, as well as consulting experts on different organisms. Although there are few atlases on the anatomy and histology of invertebrates, they need to be consulted.[20–25] Two papers have reviewed toxicant effects on invertebrates.[4,7,26] The mechanisms by which changing environmental conditions, toxicants, biotoxins, or pathogens cause disease appear varied, and will also differ with the species and individual (genetic polymorphisms), their defense mechanisms, and immune systems. Interpretation also involves converting the 2-dimensional images seen in light and transmission electron microscopy to their original 3-dimensional structures.

A study of a small burrowing clam, *Macoma balthica*, collected in the Gulf of Riga, provides one instructive example of the application of invertebrate histopathology in aquatic toxicology and illustrates some of the decisions that must be made regarding processing tissues, data collection efforts, and interpretation of multiple parameters.[27,28] This study, one of six subprojects to look at pollution in this Gulf, was undertaken in collaboration with Dr. Kari Lehtonen of the Finnish Institute of Marine Research, as part of the multinational Nordic Environmental Research Programme financed mainly by the Nordic Council of Ministers.[29] The Gulf of Riga is a semi-enclosed estuary under a strong influence of the Daugava River, with water exchange with the Baltic Sea. The eastern and southern (city of Riga) watersheds are heavily industrialized, contributing nutrients, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and metals to the water and sediments of the Gulf. The clam larvae settled at a particular location and were chronically exposed to potentially toxic organic and inorganic chemicals. Contact

with and ingestion of water and sediment, and ingestion of food particles as deposit/detritus feeders[30] resulted in a bioaccumulation of these chemicals in the tissues. The plan to collect and examine clams from sites throughout the Gulf was not coordinated with sediment evaluation or clam tissue collection for analyses of chemical contaminants, which was unfortunate, based on the preliminary histopathology results.

The fixative selected was Helly's, which nicely preserves the intracytoplasmic vesicles and provides crisp staining. However, these clams live in very low salinity water (average of 5.7 practical salinity units) and the high-osmolarity of the fixative caused vacuolation and distortion of oocytes and other changes that were consistently observed in the tissues. Otherwise, cells and tissues were well fixed. The small clams (*ca*. 15 mm average maximum diameter) were carefully removed from their shells using a scalpel and fixed for 12–14 h, rinsed in water for 24 h, and stored in 70% ethanol. Clams were trimmed by slicing sagittally through the mid-line and both halves embedded (one cut side up, the other cut side down) in Paraplast. Because of their small size, two to four clams were included in each cassette and paraffin block. On sectioning the clams, it was apparent that particulate material in the stomach caused microtome blade damage, indicating that depuration of gut contents did not occur as expected when the animals were held in buckets of clean ambient water for 24 h before fixation. Sections were stained with hematoxylin and eosin, and on some, Cason's, periodic acid-Schiff (with and without diastase digestion to demonstrate glycogen), and modified Movat's pentachrome procedures were used on additional sections from the blocks. Despite some difficulties with the fixation and sectioning, sections of sufficient quality were obtained for histopathologic examination.

Collection of data involved examining each of the 558 clams and recording lesions and other observations using a lesion coding system[31] modified for bivalves. The first challenge of the study occurred during processing and embedding when the clam halves became mixed up. This resulted in confusion about which lesion belonged to which of the two to four clams in the block, so to solve this, each histoslide was placed on a light table and the locations of the tissue sections traced onto the data sheet. Microscopic examination then permitted the halves to be assigned to one clam, based on the minor differences in tissue structure and composition that can be used to distinguish among individuals. Each clam was then assigned a subnumber for the block; and all observations from each clam could then be recorded on the data sheets (Figure 36.7). The lesion coding system also permitted recording which tissues and organs were present on the sections for each clam. Not all organs were present for each clam on the histoslide; thus, deeper sections needed to be cut from those blocks before statistical comparisons of the intensity/severity, prevalence, and distribution of some of the organ-specific lesions could be performed (e.g., kidney concretions, because the kidney was not sectioned for all clams).

Although the lesion coding system provided a concise way to record all histopathological information for each clam, the amount of data generated for 558 clams was daunting. Both qualitative (presence/absence, descriptions) and semiquantitative data (extent: focal, multifocal, diffuse; severity: minimal $= 1$ to severe $= 5$) were recorded. Image analysis, to provide count and measurement data, was not undertaken, although possible proliferation of blood vessels with hypertrophy of endothelial cells and variable size of lysosomes in digestive tubule cells could be examined this way.

As noted previously, the amount of time available for data collection and analysis must be factored into the study. In this case, time ran out and a complete multivariate analysis and comparison with data from other studies in the Gulf has yet to be performed. The limited analyses conducted revealed that neoplasms (of gill and gonad), blood vessel proliferation, thickened connective tissue, and extracellular kidney concretions with

PAGE 1

HISTOPATHOLOGY RECORDED BY ECP DATE 12/25/95

Species Macoma balthica Location Gulf of Riga Date Collected 4/6/94

Lesion Codes	Pathology Numbers									
Examined = X, Incomplete = I, No Section = N, Autolyzed = A; Number code for severity is entered in box by the organ name for each lesion seen. SYS.ORG.SIT.SUB-CHG.TYP.SUT.SST.SSS- EXT	95-2-C-1	95-2-C-2	95-2-C-3	95-2-D-1	95-2-D-2	95-2-D-3	95-2-E-1	95-2-E-2	95-2-E-3	
NS.GA (GANGLIA)	I	N	N	N	N	N	N	N	N	
.PE SN-IC, PH	1									
.VI, SN. - IC, PH	2									
.CE										
.VI, LN-DC, LO?	2									
.PE. LN-DC, LO?	2									
RS.SI (SIPHON)	I	I	N	X	X	I	N	I	I	
RS.GI (GILLS)	X	N	N	N	X	X	X	X	X	
.AR										
.OD										
.ID										
.FI										
CS. BS-RS. GI - IC, PH brown masses	3									

Location of animal(s) on slide(s):

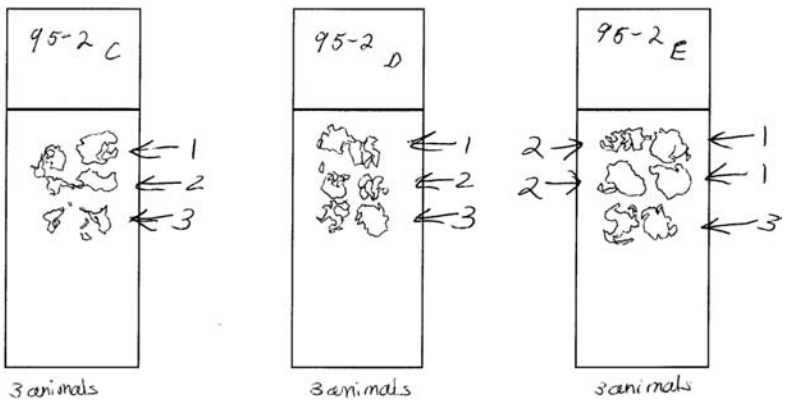

Figure 36.7 First page of three-page data sheet for recording lesion codes for each clam, identified by the pathology numbers at the top of the columns and on the sketches of each histoslide at the bottom of the page. Numbers indicate the severity of the lesion; an ''X'' means the tissue was present on the histoslide, an ''N'' means it was not present, and a ''I'' means that the amount of tissue was insufficient to score. (Data sheet designed by E.C. Peters.)

atrophy and necrosis of the renal epithelium were found at stations in the industrialized area of the Gulf (Figure 36.8a–d). The relationship of bivalve neoplasms and other lesions to chemical contaminants is unclear but appears to be likely, especially for PCBs and metals at the mouth of the Daugava River.[29] However, sediment analyses indicated that the Gulf overall was not significantly polluted compared to the Baltic Sea.

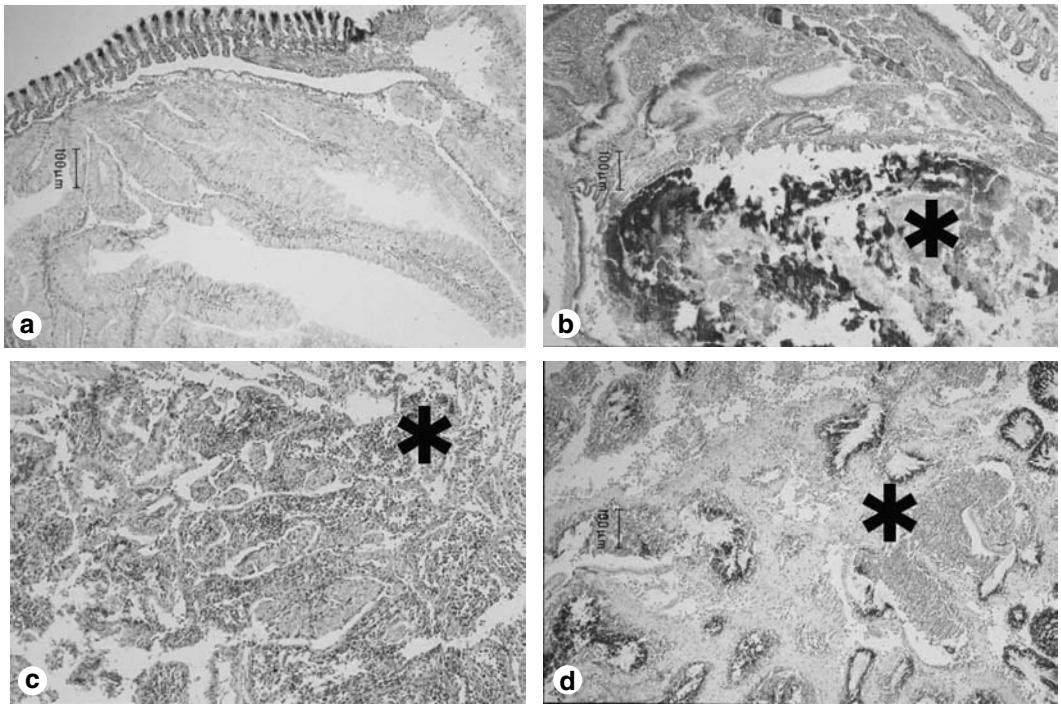

Figure 36.8 **(see color insert following page 464)** Example lesions affecting the health of individual clams and population status in the Gulf of Riga: (a) normal kidney, gill at top; (b) kidney concretion at ''*''; (c) germinoma, undifferentiated cells filling gonadal follicles to left of ''*''; (d) abnormal thickening of connective tissue among gonadal follicles in male clam to left of ''*'', infiltration of hemocytes in follicles to right of ''*''. (Photomicrographs by E.C. Peters.)

Although preliminary research on induction of stress proteins began using tissues from other samples of these clams, the use of Helly's fixative did not permit examination of the induction of stress biomarker proteins using immunohistochemical procedures. A study of *M. balthica* exposed to contaminated sediments from Sydney Harbour, Cape Breton, Nova Scotia, Canada, for 28 days in the laboratory used sediment chemistry analyses, histopathological examinations, and immunohistochemical staining for the enzymes acid phosphatase, adenosine triphosphatase (ATP), salmon CYP1A (P450), and γ-glutamyl transpeptidase (GGT) in the digestive gland.[32] The enzymes generally showed increased activity in sediment-exposed animals compared to controls. Some of the differences were dependent on the condition of the tissues, as revealed by the histopathological examinations. The responses of these clams tested using the U.S. EPA bioaccumulation test method[33] showed early indication of stress due to chronic biologic exposure to organic and inorganic contaminants.

Despite the issues raised in this discussion, it should be noted that the large number of animals in the Gulf of Riga study from diverse stations provided insights into individual variability and also revealed new information on the life histories of these clams. For example, no juveniles were found at the easternmost station where neoplasms were present in the clams, but juveniles dominated at the westernmost station nearest the Baltic Sea. Gonadal follicles might have been damaged by gymnophallid trematode sporocysts or metacercaria at most stations except the most industrialized southeastern

location in the Gulf, and all clams, no matter when collected during the year, had mature gonads (*M. balthica* in the Baltic Sea has a pronounced seasonal reproductive cycle). The ''Swiss cheese'' appearance of digestive diverticular tubule cells might have been related to the unusually high lipid content of the clams in some areas of the Gulf [disturbed lipid metabolism, neutral lipid/lipofuscin accumulation(?)]. However, a special fixative would have to be used when clams were collected to verify that the clear circular vacuoles had been filled with lipid, since lipid is removed during tissue processing. Different nutritional conditions in different parts of the Gulf were suggested in the biochemistry studies and aid in the interpretation of the histopathology and analytical chemistry data.[27,32,34,35]

Microscopic observations provide additional clues to verify whether gross observations of invertebrate mortality or morbidity, reduced growth and reproduction, or other effects were actually due to the toxicant, or were the result of parasites or pathogenic microorganism infestations or infections, starvation, genetics, or other factors. Sometimes toxicant-exposed animals might survive better than controls, because the toxicant preferentially kills parasites or pathogens that damage the host. Impacts on invertebrates can produce delayed, chronic, and indirect effects on the long-term survival and reproduction of their predators, influencing recovery from contamination events.[36] Research correlating body burdens of contaminants with histopathologic biomarkers of cellular alteration (contaminant-specific or nonspecific lesions) is needed to link exposures with mechanisms of action (e.g., necrosis, neoplasia, metaplasia, oocyte atresia, skeletal deformities). Laboratory exposure and field verification of the lesions that develop in invertebrates, as well as measurements of contaminant levels, will provide the necessary information for ecological risk assessments.

Appendix

The following fixatives are either not readily available in vertebrate-oriented histotechnique manuals or are modifications of similar fixative solutions and are presented here. Each formula makes 1 l of fixative. Each volume (in cm^3) of tissue will require a minimum of 10–20 times the volume of fixative.

Davidson's solution

Davidson's solution[12,23] is similar to Dietrich's,[7,38] although it has higher concentrations of the formaldehyde and glacial acetic acid, but both of these can be used for the same organisms. Davidson's I is recommended for crustaceans and Davidson's II has been used for bivalves.[37,40] Both decalcify, as well as fix, the tissues; however, secretions and granules might not be well preserved. Mix the ingredients in order:

	Davidson's I (ml)	Davidson's II (ml)	Dietrich's (ml)
Glycerin		100	
Formaldehyde (37–40%) (CAS No. 50–000)	225	200	100
95% undenatured ethanol (CAS No. 64-17-5)	310	300	290
Tap or distilled water	345	300[a]	590
Glacial acetic acid (CAS No. 64-19-7)	120	100	20

[a] Filtered ambient seawater for Davidson's II will appear cloudy when made with high salinity seawater.

Helly's fixative

Replacing the original mercuric chloride with zinc chloride reduced the risks of mercury exposure and no precipitate forms in the tissues[39]; however, zinc chloride must be handled carefully as it is hygroscopic and can cause burns.

Zinc chloride (CAS No. 7646-85-7)	50 g
Potassium dichromate (CAS No. 7778-50-9)	25 g
Water	1000 ml (distilled, deionized, or seawater)

Add 50 ml of 37–40% formaldehyde (CAS No. 000-050-000) just before placing animals in this solution. It will not work 24 h after the formaldehyde is added, and specimens should not be left in the solution more than 16–20 h or they will become brittle.

10% seawater formalin

For field use, this is the simplest fixative solution for marine organisms:

Formaldehyde (37–40%) (CAS No. 50-000)	100 ml
Seawater	900 ml (filter to remove debris, if possible)

This EDTA-based decalcifying solution is useful for fragile tissues or when IHC is to be performed on the tissue sections:

10% neutral EDTA

Disodium EDTA ($Na_2ClOH_{14}O_8N_2 \cdot 2H_2O$)	300 g
Distilled or deionized water	3000 ml

Place a large glass flask or beaker on a magnetic stirrer and add a large stir bar. Turn on the stirrer and add the EDTA to the water while stirring. Then add solid NaOH pellets to the solution while titrating to bring the pH to 7.0 (approximately 29 g). The NaOH will speed up the dissolution of the EDTA.

The following staining solutions are useful when working with invertebrates:

Cason's trichrome solution

Dissolve each chemical in order:

Distilled or deionized water	200 ml
Phosphotungstic acid (CAS No. 12067-99-1)	1.0 g
Orange G (CI 16230) (CAS No. 1936-15-8)	2.0 g
Aniline blue, WS (CI 42780) (CAS No. 28631-66-5)	0.5[a] g
Acid fuchsin (CI 42685) (CAS No. 3244-88-0)	6.0[b] g

[a] Adapted from Reference 16, modified amount from original procedure of 1.0 g.

[b] Modified amount from original procedure of 3.0 g.

Alcian blue solution

Combine:

Alcian blue	1 g
Deionized water	100 ml
Glacial acetic acid	1 ml

Solution can be saved and reused many times.

Alkaline alcohol solution

Combine:

Ammonium hydroxide	10 ml
95% undenatured ethanol	100 ml

Mix and store in amber glass bottle.

Wiegert's iron hematoxylin

Prepare two solutions (A and B) and mix them together just prior to staining. Discard mixed solution after staining each rack of slides.

Solution A	
Hematoxylin crystals	1 g
95% undenatured ethanol	100 ml
Solution B	
29% aqueous ferric chloride	4 ml
Deionized water	95 ml
Concentrated hydrochloric acid	1 ml
Woodstain scarlet solution: Combine	
Woodstain scarlet N.S. conc.	0.1 g
Deionized water	95.5 ml
Glacial acetic acid	0.5 ml

Should be saved and reused. If you cannot find woodstain scarlet, prepare crocein scarlet-acid fuchsin working solution.[10] Discard this solution after each use.

Aqueous glacial acetic acid solution

Mix:

Glacial acetic acid	0.5 ml
Deionized water	99.5 ml

This can be made up ahead of time, but do not reuse after staining.

5% aqueous phosphotungstic acid solution

Combine in a 100-ml volumetric flask:

Phosphotungstic acid	5 g
Deionized water to fill the flask to the 100-ml mark	

This can be made up ahead of time, but do not reuse after staining.

Alcoholic saffron solution

Combine as directed:

Saffron (stain)	6 g
100% (absolute) ethanol	100 ml

Place in an airtight, stoppered glass container to prevent dehydration. Extract the saffron by placing the solution in an oven at 58°C for 48 h. May be reused many times, store in a tightly sealed bottle (saffron is expensive!).

Crystal violet

Crystal violet	1 g
25% ethanol	200 ml

Gram's iodine

Place in small clean glass vial with stopper:

Iodine	1 g
Potassium iodide	2 g
Distilled water	10 ml

Shake or grind until dissolved. Transfer solution to a 300-ml volumetric flask and fill to the mark with distilled water. Stopper and shake to mix.

Twort's stain

For stock solution, mix the following together and store in a screw-capped bottle. It is stable for at least a year:

0.36 g neutral red (CI 50040) dissolved in 180 ml 95% ethanol
0.02 g fast green FCF (CI 42053) dissolved in 20 ml of 95% ethanol

Working solution

Mix this immediately prior to use.

Stock solution	48 ml
Distilled water	144 ml

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chapter thirty-seven

Isolation of genes in aquatic animals using reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends

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Contents

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Introduction

Aquatic environments are receiving areas for significant levels of chemical contaminants. Such contaminants have been implicated in specific disease endpoints in aquatic organisms. Our laboratory has been using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) in studies to identify and characterize the genes involved in the responses of aquatic organisms to toxicants. We have been particularly interested in the role of specific cancer genes, oncogenes, and tumor suppressor genes, in contaminant-induced tumor development in aquaria and feral populations of fish. For example, we have characterized the normal retinoblastoma (Rb) cDNA sequence from medaka (*Oryzias latipes*)[1] and proceeded to analyze it for the presence of chemically induced damage in hepatic tumor tissue using SSCP mutation detection techniques.[2,3] We have developed a protocol that works well with many species of fish, as well as invertebrates, for a variety of cancer genes provided care is taken in the primer design stage. The approach can be adapted to investigate virtually any gene provided some knowledge regarding sequence homology is available in the literature. This protocol is divided into five sections:

1. Isolation of RNA
2. Treatment of RNA with DNase I to remove genomic DNA contamination
3. Production of first strand cDNAs by reverse transcription
4. Isolation of an internal fragment of the targeted gene using PCR and degenerate primers, sub-cloning and sequence characterization
5. Isolation of the 5′ and 3′′ cDNA ends using gene-specific primers (GSPs)

Materials required

Chemicals from specific suppliers that have worked well in our laboratory are noted by the accompanying catalog number. All other chemicals can be obtained from any reputable supplier, such as Invitrogen/Gibco.

Total RNA isolation

Equipment required:

- Tissue homogenizer, if preparations are from small (<100 mg) tissue samples

Reagents required:

- RNeasy Total RNA isolation kit (Qiagen, #74104), which includes mini spin columns, RNase-free Microfuge tubes, lysis buffer (RLT), two wash buffers RW1 and RPE, and RNase-free dH_2O

- 70% ethanol
- β-Mercaptoethanol
- RNaseAWAY (Invitrogen, #10328-011)

DNase I treatment

Equipment required:

- 37°C water bath or thermocycler

Reagents required:

- DNase I — RNase-free, 1 unit/μl (Promega, #M6101)
- 10× DNase buffer (400 m*M* Tris–HCl [pH 8.0 at 25°C], 100 m*M* $MgSO_4$, 10 m*M* $CaCl_2$). Supplied with enzyme
- Stop buffer (20 m*M* EGTA, pH 8.0 at 25°C). Supplied with enzyme
- Ethanol
- 3 *M* sodium acetate, pH 5.2
- Diethylpyrocarbonate (depc)-dH_2O

First strand synthesis

Equipment required:

- Thermocycler
- 37°C, 42°C, 65°C, and 70°C water baths or heat blocks

Reagents required:

- Superscript™ First-strand synthesis system for RT-PCR (Invitrogen, #11904-018), which includes: 10× buffer (200 m*M* Tris–HCl [pH 8.4], 500 m*M* KCl), Superscript II reverse transcriptase (50 units/μl), oligo (dT)20 primer (0.5 μg/μl), 25 m*M* $MgCl_2$, 0.1 *M* dithiothreitol (DTT), 10 m*M* deoxyribonucleotides (dNTPs), RNase-OUT (40 units/μl), *E. coli* RNase H (2 units/μl), depc-dH_2O.

PCR of internal fragment, sub-cloning and sequence characterization

Equipment required:

- Thermocycler
- Water bath at 42°C
- Shaking and static incubators at 37°C
- Standard horizontal agarose gel electrophoresis apparatus (e.g., BioRad, #170-4487)

Reagents required:

- Taq DNA polymerase, such as Platinum™ Taq DNA polymerase (Invitrogen, #10966-018) (without proofreading capacity, i.e., *not* Pfu, Pfx, or Vent). Supplied with 10× buffer and 50 m*M* $MgSO_4$.

- 10 m*M* dNTP mix.
- Two (degenerate) amplification primers (forward and reverse) specific for your target mRNA.
- Sub-cloning reagents (Invitrogen, #K2040-01), which includes: T4 DNA ligase, 10 × ligation buffer (60 m*M* Tris–HCl [pH 7.5], 60 m*M* $MgCl_2$, 50 m*M* NaCl, 1 mg/ml bovine serum albumin, 1 m*M* ATP, 20 m*M* DTT, 10 m*M* spermidine), cloning vector, SOC medium (2% tryptone, 0.5% yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $MgSO_4$, 20 m*M* glucose), and competent cells.
- Plasmid preparation reagents (Promega, #A7100), which includes: cell resuspension solution (50 m*M* Tris–HCl [pH 7.5], 10 m*M* EDTA, 100 μg/ml RNase A), cell lysis solution (0.2 *M* NaOH, 1% SDS), neutralization solution (1.32 *M* potassium acetate), Wizard® Minipreps DNA purification resin, column wash solution (80 m*M* potassium acetate, 8.3 m*M* Tris–HCl [pH 7.5], 40 μ*M* EDTA), and syringe barrels. A vacuum manifold device (Promega, #A7231) simplifies the procedure but is not compulsory.

RACE

Equipment required:

- Thermocycler
- Standard horizontal agarose gel electrophoresis apparatus (e.g. BioRad, #170-4487)
- Water bath at 42°C
- Shaking and static incubators at 37°C

Reagents required:

- SMART™ RACE cDNA amplification reagents (BD Biosciences, #K1811-1), which includes: SMART oligonucleotide (10 μ*M*), 3′-RACE and 5′-RACE cDNA synthesis (CDS) primers (10 μ*M*) Powerscript™ reverse transcriptase, 5× buffer (250 m*M* Tris–HCl [pH 8.3], 375 m*M* KCl, 30 m*M* $MgCl_2$), 20 m*M* DTT, 10× universal primer mix (UPM): 0.4 μ*M* of long primer and 0.2 μ*M* of short primer, 10 m*M* dNTPs, tricine–EDTA buffer (10 m*M* tricine–KOH [pH 8.5], 1 m*M* EDTA).
- Advantage 2 PCR reagents for PCR reactions (BD Biosciences, #K1910-y, free if bought with SMART reagents), which includes: Taq DNA polymerase, 10× buffer, 10 m*M* dNTPs, depc-dH_2O.
- Two GSPs.
- Sub-cloning reagents (Invitrogen, # K2040-01) as above.

Optional: SSCP-PCR analysis

Equipment required:

- Thermocycler
- PhastSystem gel electrophoresis and gel development apparatus (Amersham Biosciences, #18-1018-24)

Reagents required:

- Taq DNA polymerase, such as Platinum Taq (Invitrogen, #10966-018). Supplied with 10× buffer and 50 m*M* $MgSO_4$
- 10 m*M* dNTP mix
- Gene-specific amplification primers (forward and reverse) that span your target cDNA
- Phastgel homogeneous 12.5 (Amersham Biosciences, #17-0623-01)
- Phastgel DNA silver staining reagents (Amersham Biosciences, #17-1596-01)
- DNA buffer strips (Amersham Biosciences, #17-1599-01)
- Stop solution/gel loading dye (95% formamide, 10 m*M* NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol)

Procedures

Figure 37.1 is a schematic overview of the procedures to be described, as well as potential applications for the normal cDNA sequence derived from RT-PCR/RACE. The specific protocols detailed herein are total RNA isolation, DNase I treatment, first strand synthesis, PCR of an internal target cDNA fragment, and RACE.

Total RNA isolation

Isolation of total RNA can be conducted using a variety of techniques, but those based on the method of Chomcynski and Saachi[4] have been successfully employed for many tissues types taken from a range of species. Commercial products, such as the RNeasy reagents from Qiagen, exploit silica-gel-based membrane binding and spin column technology to speed up the method still further. The latter technique avoids the use of toxic (phenol) reagents, and also works well with a variety of tissues. The resulting total RNA is suitable for RT-PCR, RACE, and Northern hybridization.

Total RNA isolation protocol

The tissue sample should be fresh or snap frozen in liquid nitrogen and stored at −70°C. The homogenizer should be wiped with RNaseAWAY to reduce possible contamination with RNases. Ethanol must be added to wash buffer RPE before first use. β-Mercaptoethanol should be added to the lysis buffer (RLT) just before use. The general protocol is as recommended by the reagent's supplier with the following changes that have been optimized for small fish (eye and liver) samples.

- Drop ~20 mg of tissue into 350 μl of RLT inside a Microfuge tube and quickly homogenize using three return (up and down) strokes. Check for large tissue pieces and repeat if necessary. Keep the homogenate on ice.
- Spin for 3 min at high speed in a microcentrifuge and extract supernatant to a clean tube.
- Add 350 μl of 70% ethanol and mix by pipetting up/down.
- Add 700 μl of sample to a minicolumn within a collection tube, spin for 15 s at 10,000 rpm. Repeat until the sample is loaded onto the column.
- Add 700 μl of wash buffer RW1, and spin for 15 s at 10,000 rpm.

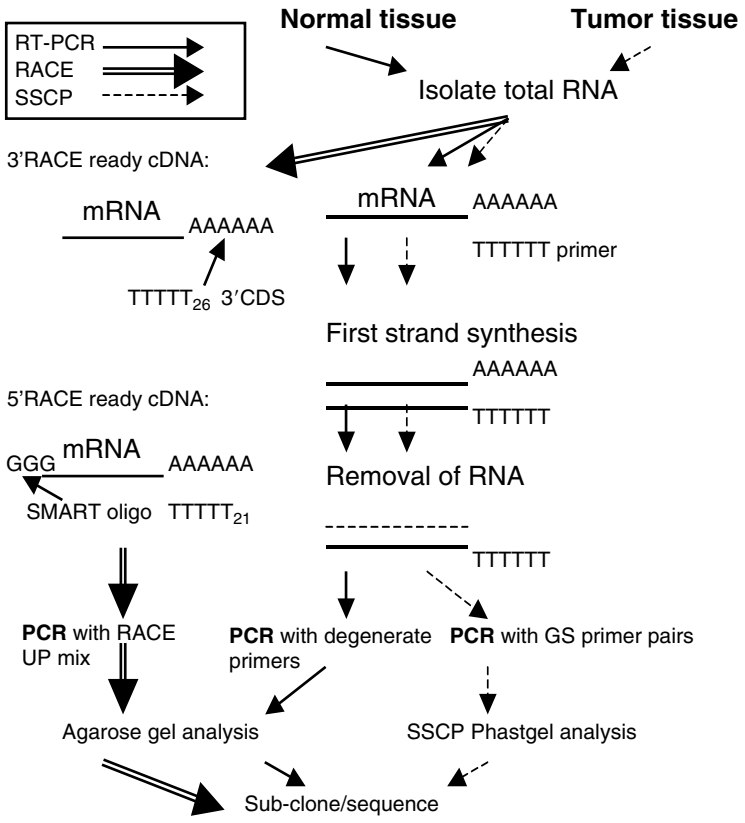

Figure 37.1 A schematic overview of the RT-PCR and RACE procedure. The first step, RT-PCR to isolate an internal fragment of the desired cDNA, and its methodological pathway, is shown by a regular arrow. Subsequently, RACE analysis and its methodological pathway are represented by an arrow with double lines. The last step, using the sequence derived for the normal cDNA to screen for DNA damage and its methodological pathway, is represented by an arrow with a gapped line. Abbreviations: GS, gene-specific; UP, universal primer.

- Place the spin column in a clean collection tube, add 500 μl of buffer RPE, and spin for 15 s at 10,000 rpm. Empty the collection tube.
- Add another 500 μl of buffer RPE, and spin for 2 min at maximum speed. Empty the collection tube.
- Place the spin column in a clean Microfuge tube and add 30 μl of RNase-free dH_2O. Wait for 5 min at room temperature and spin for 1 min at 10,000 rpm.
- Measure absorbance at 260 nm to estimate concentration of nucleic acid.
- Store the resulting total RNA preparation on ice if proceeding to the DNase treatment or at −20°C.

DNase I treatment

It is very likely that the total RNA preparation contains some contaminating genomic DNA that may serve as an inappropriate template during PCR.

DNase I treatment protocol

- In a Microfuge tube, combine approximately 20 μg of total RNA, 10 μl of 10× DNase buffer, 10 μl (10 units) of DNase, and depc-treated dH_2O to a final volume of 100 μl.
- Incubate for 1 h at 37°C.
- Repeat RNA isolation protocol above using 350 μl of lysis buffer RLT in the first step.
- Measure absorbance at 260 nm to estimate concentration of RNA.
- Store the RNA at −20°C or −70°C for the longer term.

First strand synthesis

In this step, the RNA is used as a template to produce a cDNA strand. The enzyme used is reverse transcriptase that uses RNA (or single strand DNA) as a template, provided a suitable primer is present. A first strand cDNA synthesis reaction can be primed using three different methods that exploit different primers: random hexamers, oligo (dT), and GSPs. The protocol described uses oligo (dT) primers, which hybridize to the 3′ poly (A) tails that are located on most eukaryotic mRNAs. The resulting product is an RNA–cDNA hybrid that is subsequently digested with RNase H to remove the RNA template.

First strand synthesis protocol

- The total reaction volume is 20 μl.
- Prepare two tubes as follows:

	Sample (label RT+)	Control (label RT–)
RNA (~2.5 μg)	X μl	X μl
10 m*M* dNTP mix	1 μl	1 μl
Oligo (dT) (0.5 μg/μl)	1 μl	1 μl
Depc-treated dH_2O	To 10 μl	To 10 μl

- Incubate each tube at 65°C for 5 min, and then place on ice for at least 1 min.
- Prepare the following reaction mixture (for n samples plus 1 RT-control, prepare reaction mix for $n + 3$ reactions):

	Per each reaction (μl)
10× RT buffer	2
25 m*M* $MgCl_2$	4
0.1 *M* DTT	2
RNaseOUT	1

- Add 9 μl of reaction mixture to each RNA/primer mixture, mix and spin gently.
- Incubate at 42°C for 2 min.

- Add 1 μl of reverse transcriptase to each tube (except the ''RT–'' tube), mix and incubate at 42°C for 50 min.
- Stop the reactions by incubating at 70°C for 15 min. Place on ice.
- Briefly spin the tubes before adding 1 μl of RNase H to each and incubate at 37°C for 20 min.
- Place on ice if proceeding to the next step, or at −20°C.

PCR of internal fragment, sub-cloning and sequence characterization

The first strand cDNAs can be used to amplify the target cDNA directly using PCR. Any *Taq* DNA polymerase can be used but, if the resulting products are to be sub-cloned, a *Taq* that adds adenine overhangs is desirable. The design of the PCR primers is critical in obtaining a target cDNA. The primers degeneracy, length, GC content, and subsequent melting temperature will affect the results achieved. Those primers with high degeneracy, short length, and low melting temperature will give rise to the production of many nonspecific PCR products. For guidance on how to design degenerate primers, the papers of Rotchell et al.[1,2] and Rotchell and Ostrander,[3] which include primer designs based on homology alignments for the fish *ras* genes and vitellin/aromatase genes, respectively, may be of help.

Once a PCR product of the expected size has been obtained, it is then possible, exploiting the adenine overhangs, to sub-clone into a cloning vector that possesses complimentary thymine overhangs. It is possible to sequence PCR products directly, but in our experience it is rare to amplify a single PCR product of the expected size using degenerate primers, and this creates sequencing results that are hard to interpret due to the presence of extra templates. The preferred technique in our lab is to excise the desired PCR product from an agarose gel, purify it and then ligate into a vector using a 1:1 ratio (vector/insert). The size of the insert is important in determining the amount of vector to use (and it is important to refer to the kit instructions for further guidance). The vector is then transformed into competent cells and plated to obtain single colonies. The LB plates used would have been prepared the preceding day. The overnight culture derived from the single colony can then be used for a plasmid preparation that is subsequently sequenced to characterize the PCR product obtained.

PCR protocol

The following PCR protocol serves as a starting point only, it is usually necessary to optimize several of the parameters (e.g., the Mg concentration, the cDNA concentration, the use of bovine serum albumin, the cycling conditions, and the number of cycles) before obtaining the desired PCR product.

- The final reaction volume is 50 μl.
- Add the following to a 0.2-ml thin walled PCR tube (the following is for one reaction):

10× PCR buffer	5 μl
50 m*M* $MgCl_2$	3 μl
10 m*M* dNTP mix	1 μl
10 μ*M* forward primer	1 μl

10 μ*M* Reverse primer	1 μl
Taq DNA polymerase	0.5 (1.25 units)
First strand cDNA	3 μl
depc-treated dH_2O	To a final volume of 50 μl

- Mix the components and spin briefly.
- Add mineral oil if your thermocycler does not have a heated lid.
- Perform 20–40 cycles of PCR with optimized conditions for your target cDNA. The following is a starting point for a 1-kb target cDNA — 1 cycle at 94°C for 4 min, followed by 30 cycles of 94°C for 45 s (denaturation), 50°C for 50 s (annealing), 72°C for 1 min (extension).
- Analyze 10 μl of amplified products using agarose gel electrophoresis.

Sub-cloning and sequence characterization protocol

PCR products generated with *Taq* DNA polymerase have adenine overhangs at the 3′ end, and this can be exploited using the TA cloning technique. Cloning is achieved in two steps: ligation and transformation.

- Combine the following ligation reagents in a 200-μl PCR tube on ice:

Fresh PCR product	X μl to give a 1:1 (vector/insert) ratio
10× ligation buffer	1 μl
Vector (25 ng/μl)	2 μl
Sterile dH_2O to a final volume	9 μl
T4 DNA ligase (4 Weiss units)	1 μl

- Incubate the reaction at 14°C for at least 4 h.
- Keep on ice or store at −20°C until ready to transform.
- Prepare a water bath at 42°C.
- Warm SOC medium to room temperature, prepare LB plates (the day before use) containing 50 μg/ml of either kanamycin or ampicillin and warm to room temperature. Spread each plate with 40 μl of 40 mg/ml X-Gal.
- Thaw on ice one tube containing a 50-μl aliquot of competent cells per ligation reaction.
- Add 2 μl of ligation reaction and stir gently using the pipette tip.
- Incubate on ice for 30 min.
- Heat shock at 42°C for exactly 30 s and return to ice.
- Add 250 μl of SOC medium and shake tubes at 37°C for 1 h at 225 rpm.
- Spread 10–200 μl from each transformation tube on a separate LB agar plate that contains the X-Gal and antibiotic. Plating two different volumes should ensure that you obtain at least one plate from which it is possible to pick a single colony later.
- Incubate at 37°C overnight. In the morning, place the plates at 4°C for 3 h to allow color development.
- To determine the presence of the desired insert, pick at least 10 colonies for plasmid preparation and restriction digest analysis. Pick each colony into 10 ml of LB broth containing 50 μg/ml of either kanamycin or ampicillin and grow overnight at 37°C in a shaking incubator.

Plasmid preparation:

- Prepare a 3-ml pellet from the overnight culture by spinning cells (in two 1.5-ml batches, emptying the supernatant after each spin) for 2 min at low speed (~10,000*g*) in a Microfuge. Alternatively, prepare a 3-ml pellet using a larger collection tube and a tabletop centrifuge.
- Resuspend cells using 200 μl of cell resuspension solution.
- Add 200 μl of cell lysis solution and invert tube several times to mix.
- Add 200 μl of neutralization solution and invert to mix.
- Spin at low speed in a Microfuge for 5 min.
- Decant the clear supernatant into 1 ml of resin and load both into a barrel of the minicolumn/syringe assembly.
- Apply a vacuum to pass the sample/resin through the minicolumn.
- Wash the minicolumn with 2 ml of wash solution using the vacuum.
- Disassemble the minicolumn/syringe, placing the minicolumn in a Microfuge tube and then spin at low speed to remove residual wash solution.
- Place the minicolumn in a clean Microfuge tube and add 50 μl of depc-dH_2O. Wait for 1 min and then elute the DNA from the column by spinning for 20 s at low speed. Elution using TE buffer is not recommended for several methods of sequencing.
- Store at −20°C.

Sequencing: We have found that sending 1 μg of plasmid DNA to commercial sequencing companies (such as MGWBiotech — www.mwg-biotech.com) is faster and more cost-effective than carrying out the reaction in your own laboratory, especially if you only have a small number of samples to process. Alternatively, use an onsite sequencing core facility if available.

RACE

Once an internal fragment of your target cDNA has been sequenced, it is then possible to design GSPs and use the RACE technique to isolate the entire cDNA. It is possible to obtain full-length cDNAs without having to construct or screen a cDNA library. If the gene of interest is remarkably well conserved across species and phyla, it may, in theory, be possible to use degenerate primers, omitting the previous internal fragment PCR step, to isolate the target cDNA. In practice, we have only had success with GSPs.

The reverse transcriptase employed has terminal transferase activity, adding cytosine residues to the 3′ end of the first strand cDNAs. This, in turn, provides a terminal stretch to which the SMART oligo provided can anneal, serving as an extended template for reverse transcription. A complete cDNA copy of the mRNA molecule is synthesized, which contains a SMART sequence at the end, and can then be used in 5′ and 3′ PCR reactions.

RACE protocol

For the RACE technique, at least two GSPs are required. In terms of the primer design, the two GSPs should be 23–28 nucleotides in length, 50–70% GC content, and have a melting temperature of at least 60°C (70°C is better). The location of the primers can also affect results, those that will produce a PCR product of 2 kb or less is optimal.

First strand synthesis:

- The final reaction volume is 10 μl.
- To prepare the first strand cDNAs, combine the following in separate 200-μl tubes.

(a) For the preparation of 5′-RACE ready cDNA:

X μl of total RNA (~1 μg)
1 μl of 5′-CDS primer
1 μl of SMART oligo
X μl of depc-dH_2O to a final volume of 5 μl

(b) For the preparation of 3′-RACE ready cDNA:

X μl of total RNA (~1 μg)
1 μl of 3′′-CDS primer A
X μl of depc-dH_2O to a final volume of 5 μl

- Mix the contents, spin briefly, and incubate at 70°C for 2 min.
- Cool the tubes on ice for 2 min and then spin briefly to collect the contents at the bottom of the tube.
- Add the following components to each reaction tube:

5× first strand buffer	2 μl
DTT (20 m*M*)	1 μl
dNTP mix (10 m*M*)	1 μl
Powerscript reverse transcriptase	1 μl

- Mix by pipetting up and down, spin briefly and incubate at 42°C for 1.5 h.
- Dilute with 250 μl of tricine–EDTA buffer.
- Incubate at 70°C for 7 min.
- Store samples at −20°C.

Before performing PCR it is advisable to carry out the control reactions supplied by the manufacturer to ensure that all components are working correctly. Once confirmed, the 5′ and 3′ PCR reactions can be performed as follows (using the advantage reagents listed above).

- Prepare enough PCR master mix for each reaction plus one extra by combining the following (1 reaction mix) —

Depc-dH_2O	34.5 μl
10× PCR buffer	5 μl
dNTP (10 m*M*)	1 μl
50× polymerase mix	1 μl
Total reaction volume	50 μl

- Mix and spin briefly, keep on ice.
- Combine the following in separate tubes:

For the 5′-RACE PCR:

Tube number	1	2	3	4
5′-RACE-ready cDNA (μl)	2.5	2.5	2.5	2.5
Universal primer mix (μl)	5	0	5	0
GSP 1 (10 μ*M*) (μl)	1	1	0	1
GSP 2 (10 μ*M*) (μl)	0	1	0	0
Depc-dH_2O (μl)	0	4	1	5
Master mix (μl)	41.5	41.5	41.5	41.5
Total reaction volume (μl)	50	50	50	50

For the 3′-RACE PCR

Tube number	5	6	7	8
3′-RACE-ready cDNA (μl)	2.5	2.5	2.5	2.5
Universal primer mix (μl)	5	0	5	0
GSP 1 (10 μ*M*) (μl)	0	1	0	0
GSP 2 (10 μ*M*) (μl)	1	1	0	1
Depc-dH_2O (μl)	0	4	1	5
Master mix (μl)	41.5	41.5	41.5	41.5
Total reaction volume (μl)	50	50	50	50

- Mix all tubes and spin briefly.
- Amplify using the following program:

5 cycles	94°C	5 s
	72°C	3 min
5 cycles	94°C	5 s
	70°C	10 s
	72°C	3 min
25 cycles	94°C	5 s
	68°C	10 s
	72°C	3 min

- Analyze 10 μl of amplified products using agarose gel electrophoresis.
- Sub-clone PCR product of desired length and sequence.

Optional application: SSCP analysis for the detection of mutations in tumor tissue samples

Having determined the normal sequence of a cDNA, such as that for a proto-oncogene or tumor suppressor gene, it is then a relatively simple task to screen tumor tissue samples for the presence of mutational damage that may have been an early cause of that

condition. In a number of studies, investigators have also built mutational profiles that relate specific chemical exposure to mutational ''hot spots'' of DNA damage within critical genes, such as the *ras* gene.[2]

The technique that we routinely use to screen for the presence of mutations is SSCP analysis of PCR products, followed by sequencing to characterize the actual nature of any DNA damage detected. The screening procedure can be completed in a day using the PhastSystem detailed in the section ''Materials required.'' Alternatively, it is possible to use sequencing gel electrophoresis apparatus (a less expensive equipment option) to conduct SSCP analysis, but this method usually requires long gel running times (overnight), radioisotope labeling, and autoradiography (overnight).

SSCP protocol:

- Design *overlapping* GSPs that span your target cDNA and generate PCR products of approximately 200 bp in size (see Figure 37.2 as an example).
- Using the protocols detailed earlier, isolate total RNAs and construct first strand cDNAs from your normal (control) and tumor tissue samples.
- Add the following to a 0.2-ml thin walled PCR tube. (The following mix is for one reaction only. Set up one reaction for each primer pair that spans your target cDNA, for each normal/tumor sample to be analyzed. For instance, if your target cDNA is divided into 10 overlapping PCR primer pairs and you wish to analyze two normal and two tumor tissue samples, you would set up 10 × 4 reactions.)

10× PCR buffer	5 μl
50 m*M* $MgCl_2$	3 μl
10 m*M* dNTP mix	1 μl
10 μ*M* forward primer	1 μl
10 μ*M* reverse primer	1 μl
Taq DNA polymerase	0.5 (1.25 units)
First strand cDNA	3 μl
Depc-treated dH_2O	To a final volume of 50 μl

- Mix the components and spin briefly.
- Add mineral oil if your thermocycler does not have a heated lid.
- Perform 20–40 cycles of PCR with optimized conditions for your target cDNA. The following is a starting point for a 200-bp target cDNA − 1 cycle at 94°C for 4 min, followed by 30 cycles of 94°C for 45 s (denaturation), 60°C for 50 s (annealing), 72°C for 30 s (extension).

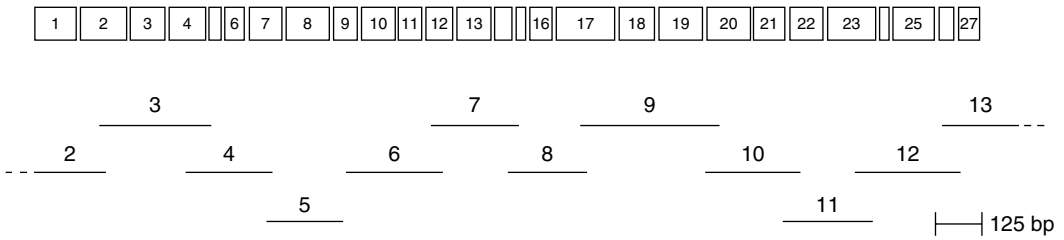

Figure 37.2 A map of the Rb cDNA and the location of overlapping PCR products obtained using the primer pairs (PPs) designed. To facilitate SSCP analysis, the PCR primer pairs are designed to produce amplification products that are approximately 200 bp in length.

- Mix 5 μl of PCR product with 5 μl of stop solution, denature at 98°C for 3 min and cool on ice.
- Pre-run gels at 400 V, 5 mA, 1 W for 10-volt hours (Vh).
- Analyze 1 μl of ''PCR products/stop solution'' mix using Phastgel electrophoresis.
- Stain the gel using the protocol supplied with the silver stain reagents.

Results and discussion

Anticipated results

The desired outcome of RT-PCR and RACE is the isolation and subsequent characterization of the desired target cDNA. The protocol detailed here will allow the investigator to isolate, firstly, an internal fragment of the target cDNA to serve as a starting point for the amplification of the two remaining cDNA ends. By sequencing the internal fragment of this target cDNA, it is possible to design GSPs that can be used in parallel with RACE primers and RACE-ready first strand template. Following amplification with such primers, it is possible that more than one 3′-RACE product, in particular, may be obtained. Additional products may represent truncated versions of the desired cDNA or closely related members of that gene family. In any case, it is important to sequence the product to confirm its identity as the desired target cDNA. An example from our work, involving the isolation of the Rb cDNA from medaka, can help to illustrate some of the typical features of a RACE result. Figure 37.3 displays an agarose gel of the 5′-RACE and 3′-RACE results using medaka total RNA and GSPs for the Rb cDNA. In the 5′ reaction, there is a single amplified fragment of approximately 2.8 kb, which was the expected size in this instance. For the 3′ reaction, at least three amplified products of approximately 1.9 kb, 1.6 kb, and 750 bp were visible. The expected size in this instance

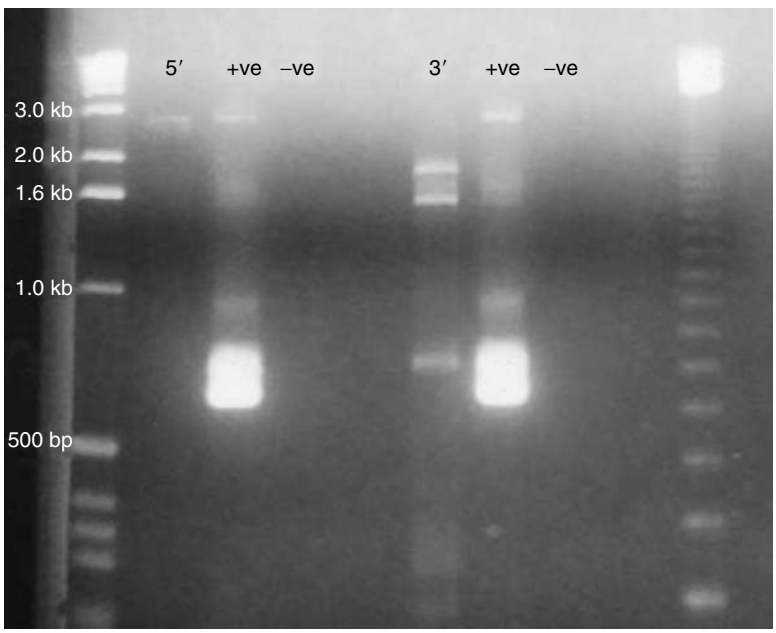

Figure 37.3 A representative agarose gel result of the 5′-RACE and 3′-RACE reactions from the medaka Rb cDNA.

was 1.7 kb, so the two larger fragments were purified from the agarose, sub-cloned, and sequenced. Once the sequence had been characterized, the smaller of the two fragments aligned well with the Rb cDNA sequences already published in the literature. This figure demonstrates a commonly observed feature of RACE results in that more than one amplified product is obtained and these may represent the presence of truncated forms or related family members of the cDNA of interest or simply be artifacts.

A similar RACE strategy has been adopted by investigators in the characterization of a variety of genes involved in aquatic toxicology applications. Recent examples include the characterization of fish alpha estrogen receptors,[5,6] an invertebrate vitellogenin cDNA,[7] and a fish cytochrome P450 2X1 detoxification enzyme cDNA.[8]

SSCP application: anticipated results

Laboratory studies have demonstrated that a variety of tumors, such as hepatocellular tumors, can be induced experimentally in aquaria fish using a variety of compounds.[9,10] The histopathology of the induced tumors in fish often corresponds broadly with the human conditions, and it is anticipated that the molecular etiology may correspond similarly. Furthermore, understanding the underlying cause of experimentally induced tumors should help elucidate the cause of environmentally induced tumors.

In our laboratory we have been interested in medaka fish tumors in particular, and exploiting the unique opportunity that existed to examine the role of the Rb gene in malignancy using a fish model. Using the RACE protocol and SSCP application described herein, the mutational screening of the Rb cDNA from normal liver and eye, as well as chemically induced medaka eye and liver tumor samples, were conducted.[1,11] Figure 37.4

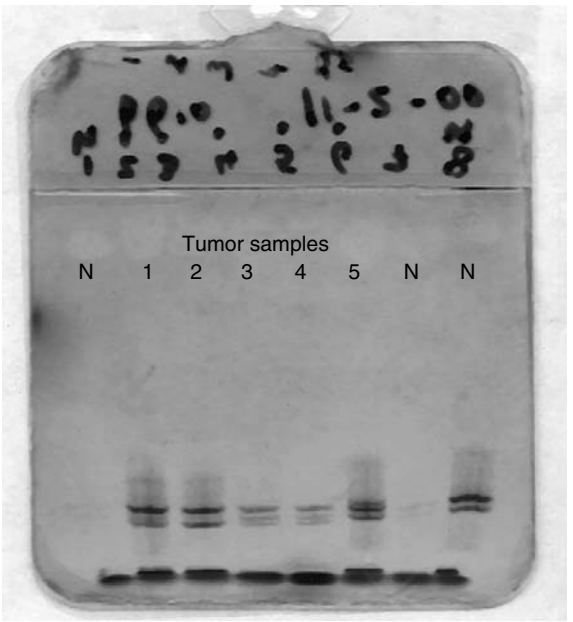

Figure 37.4 An example of PCR-SSCP analysis of normal medaka eye samples (N) and eye tumor samples (T1–T5) with Rb-specific primers PP10 (spanning exons 21–23). Abnormal electrophoretic patterns are observed using primers PP10 with samples T1, T3, T4, and T5.

displays a typical SSCP gel result of, in this instance, an analysis of eye normal and chemically induced tumor samples. In this investigation, abnormal electrophoretic patterns were observed in four samples (Figure 37.4: samples T1, T3, T4, and T5). Sequence analysis of these PCR products revealed point mutations and two deletions within the Rb cDNA sequence. No mutations were detected in the normal eye samples. However, it is possible to detect changes using normal/control samples in the sequence of the Rb cDNA that represent polymorphic variation.

Summary

In summary, RT-PCR combined with RACE is an effective method to isolate and characterize genes that are involved in the responses of aquatic organisms to toxicants. Having a limited amount of prior sequence information from related species, usually obtainable from the Genbank database, is sufficient to enable an investigator to design degenerate primers for their targeted gene. Inversely, having too limited sequence information available can be the major drawback of this technique. In such instances, the alternative approach of constructing and screening a cDNA library for the desired gene (though more time consuming) may be more successful. As more relevant cDNA sequences (of critical growth controlling proteins and detoxification enzymes, for instance) become available for a greater variety of aquatic organisms, it will become a much simpler task to isolate such genes using cross-species degenerate primers in a chosen species of interest using the RT-PCR and RACE methods.

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chapter thirty-eight

Analysis of mutations in λ transgenic medaka using the cII *mutation assay*

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Contents

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Introduction

The ability to identify induced mutations and to understand their roles in a variety of disease processes within whole animals pose significant challenges. The basis of these challenges is primarily a sampling problem. Mutations in genes, even those induced following exposure to potent chemical or physical mutagens, typically occur at low frequencies (e.g. ~1 spontaneous mutation/10^{-5} to 10^{-7} loci). Consequently, to distinguish mutant genes among a very large number of nonmutant genes, the gene carrying the mutation must be identified accurately and recovered efficiently. Until recently, routine *in vivo* mutation analyses have been hampered by the lack of methods capable of meeting these requirements. For example, although a variety of *in vivo* genotoxicity assays provide information related to the ability of the chemical to alter DNA or the structure of a chromosome by using such endpoints as the induction of DNA adducts, DNA repair, DNA strand breaks, and chromosomal damage, these endpoints are limited in that they are not direct measures of induced gene mutations. Other assays using endogenous genes are either insensitive, or are limited to specific tissues or developmental stages,[1–3] or unavailable in such organisms as fish.

Mutation assays based on transgenic animals are meeting many of the fundamental challenges confronting studies of *in vivo* mutagenesis. Developed originally in rodent models,[4–8] and now in fish,[9–11] these transgenic mutation assays share a similar general approach. The transgenic animals have been produced that carry prokaryotic vectors that harbor a specific gene that serves as a mutational target for quantifying spontaneous and induced mutations. After treatment with a mutagen, and allowing sufficient time for the mutations to manifest, genomic DNA is isolated from target tissues. The vectors are then separated from the animal's genomic DNA and shuttled into specialized indicator bacteria that facilitate distinguishing mutant and nonmutant genes.

In addition to a common general approach, transgenic mutation assays share numerous benefits for *in vivo* mutation analyses. These assays permit the screening of a large number of copies of a locus rapidly, thereby providing statistically reliable information on the frequency of mutations in virtually any tissue in the animal, while reducing the need for large numbers of animals. Mutations are quantified directly at the level of single genes, the endpoint of DNA damage and repair. The mutation target transgenes are genetically neutral affording the persistence and accumulation of mutations over time without being subjected to selection in the animal.[12–14] In addition, the recovered mutant gene can be sequenced to characterize the mutation at the molecular level and to assist in identifying the mutagen's possible mechanism of action.

In this chapter, we describe features of, and procedures for, conducting mutation analyses using the *cII* mutation assay based on the λ transgenic medaka.[10] The λ transgenic medaka carry multiple copies of the bacteriophage λLIZ vector, which harbors the *lacI* and *cII* bacterial genes that serve as mutational targets. The most widely used transgenic mutation assay in rodents is also based on this bacteriophage vector,[5] providing ample opportunities for studies of comparative mutagenesis in identical mutation target genes carried by different species. Mutation analyses are feasible in either the *lacI* or *cII* target genes by using different assay procedures. Here, we focus on a description of the *cII* mutation assay, a positive-selection assay that uses the *cII* target gene as a logistically simple and cost-effective alternative to the *lacI* mutation assay.[15] Following the descriptions of procedures, we summarize specific examples of analyses of induced mutations detected in the *cII* target gene recovered from λ transgenic medaka exposed to four chemical mutagens. These examples highlight many features of the λ transgenic medaka and illustrate some of the more important factors to be considered when designing and conducting *in vivo* mutation studies.

Overview of procedures for mutation analyses using λ transgenic medaka

1. Treat animals with chemical or physical mutagens
2. Isolate genomic DNA
3. Prepare G1250 plating culture
4. Perform packaging reaction and plate the packaged DNA samples
5. Examine titer plates and mutant screening plates
6. Verify putative λ *cII*− mutants
7. Characterize λ *cII*− mutants by sequencing

Materials required

Animals

The λ transgenic medaka, designated lineage λ310, were generated using the orange-red strain (Himedaka), strain that originated from the Gulf Coast Research Laboratories (Gulf Coast, MS). The λ310 lineage carries ~75 copies per haploid genome of the bacteriophage λLIZ vector (~45 kb) harboring the *lacI* and *cII* bacterial genes that are used as mutational targets.[10] The animals used in these studies were obtained from in-house stocks maintained at the Aquatic Biotechnology and Environmental Laboratory (ABEL) at the University of Georgia. Medaka was selected for this application because it is widely used in environmental toxicology and is the fish species of choice in carcinogenesis bioassays and

germ-cell mutagenesis studies. The small size, sensitivity, well-characterized histopathology, short generation time, and cost-effective husbandry contribute to the utility of this species in routine toxicity testing.

Tissue dissection and DNA isolation

Equipment

Equipment required for tissue dissection and DNA isolation is listed in Table 38.1.

Supplies and reagents

Supplies and reagents required for tissue dissection and DNA isolation are listed in Table 38.1.

Solutions:

- Phenol: chloroform (0.5 ml/sample)

In a sterile 50 ml polypropylene centrifuge tube, mix equal volumes of saturated phenol with chloroform. Prepare on day of use and equilibrate at room temperature.

- 20 mg/ml proteinase K (15 μl/sample)

Prepare on day of use.

- 1× SSC stock solution (1 l)
  8.77 g NaCl
  4.41 g sodium citrate

Dissolve salts in 800 ml of water. Adjust the pH to 7.0. Bring the volume up to 1000 ml. Sterilize by autoclaving at 121°C for 30 min.

Table 38.1 Materials required for tissue dissection and DNA extraction

Refrigerated Microfuge w/rotor for 1.5-ml tubes
37°C incubator
Rotating platform
Pipetters 20–100 μl
1.5-ml sterile Microfuge tubes
50-ml sterile polypropylene centrifuge tubes
2-ml glass Pasteur pipettes
Wide bore pipette tips 1–1000 μl
Pipette tips for pipetters
Saturated phenol (Fisher biotech grade)/0.5 ml
Chloroform (reagent grade)/1 ml
100% ethanol/1 ml
8 *M* potassium acetate (pH not adjusted)/50 μl
Proteinase K (Invitrogen, #25530-03)/10 mg
Sodium chloride, 100 g
Sodium citrate, 100 g
Sodium dodecyl sulfate (99% pure)/20 g

- 20% SDS (w/v) stock solution (100 ml)
  20.0 g SDS

Dissolve 20 g of SDS in water to obtain a final volume of 100 ml.

- Homogenization buffer (10 ml)
  10 ml 1× SSC
  625 μl 20% SDS

Add SDS solution to the 1× SSC in a sterile tube. Prepare for each sample to be extracted.

- TE, pH 7.5
  10 m*M* Tris, pH 7.5
  1 m*M* EDTA, pH 8.0

λ *cII mutation assay*

Equipment

Equipment required for the λ *cII* mutation assay is listed in Table 38.2.

Supplies and reagents

Supplies and reagents required for the λ *cII* mutation assay are listed in Table 38.3. Table 38.4 lists the amounts necessary for the analysis of one DNA sample.

In vitro packaging extracts:

λ bacteriophage *in vitro* packaging extracts (see Appendix I for protocol for preparation, or use commercially available reagents from Stratagene, La Jolla, CA).

Table 38.2 Equipment required for the λ *cII* mutation assay

37°C incubator
24°C incubator (± 0.5°C)
30°C shaking incubator
30°C water bath
55°C water bath
Refrigerated centrifuge (50-ml centrifuge tubes)
Temperature data-logger, accurate to 0.1°C
Autoclave
UV/Vis spectrophotometer
Microwave
Light box
Vortex
Electric pipetters
Pipetters 20–100 μl

Table 38.3 Supplies and reagents required for the λ *cII* mutation assay

Glass culture tubes and lids (12 × 75 mm)	Casein digest (Difco #211610/0116-17)
Petri dishes (100 × 15 mm plastic)	Thiamine hydrochloride (Invitrogen #13540-018)
Latex gloves	Gelatin (Sigma #G-2500)
10 ml sterile disposable serological pipettes	Magnesium sulfate 7-hydrate crystal
1.5 ml sterile Microfuge tubes	Kanamycin powder
Pipetters 20–100 μl	Maltose (Difco #216830/0168-17)
Wide bore pipette tips for 0–200 μl	Trizma base (Sigma #T1503)
Sterile loop for cell culture	Hydrochloric acid
Agar (Invitrogen Select Agar #30391-049)	

Table 38.4 Quantities of reagents used for analysis of one DNA sample using the λ *cII* mutation assay

Reagents	Quantity
TB-1 agar plates	13
10 m*M* $MgSO_4$	100 ml
SM buffer	100 ml
20% maltose-1 *M* $MgSo_4$	100 μl
TB-1 liquid media	10 ml
TB-1 Top agar	40 ml
TB-1/Kan+ plates	1

Solutions:

- 20% (w/v) maltose–1 *M* $MgSO_4$ (100 ml)
  20 g maltose
  1.6 g $MgSO_4 \cdot 7H_2O$

Dissolve in water and bring to a final volume of 100 ml. Filter sterilize and store at 4°C for 6 months.

- 10 m*M* $MgSO_4$ (1 l)
  2.46 g $MgSO_4 \cdot 7H_2O$

Dissolve in water and bring to a final volume of 1000 ml. Sterilize in an autoclave for 30 min at 121°C and store at room temperature for up to 1 year.

- 1 *M* Tris buffer, pH 7.5 (1 l)
  121.1 g Trizma® base

Dissolve in 800 ml of water and add approximately 65 ml of hydrochloric acid to adjust the pH to 7.5. It is important that the pH is measured at room temperature using a pH probe that will accurately measure the pH of Tris buffers. Adjust the final volume to 1000 ml. Sterilize in an autoclave for 30 min at 121°C and store at room temperature for up to 1 year.

- SM buffer (1 l)
  5.8 g NaCl
  2 g $MgSO_4 \cdot 7H_2O$
  50 ml 1 *M* Tris buffer, pH 7.5
  5 ml 2% (w/v) gelatin

Dissolve in water and bring the final volume to 1000 ml. Sterilize in an autoclave for 30 min at 121°C and store at room temperature for up to 1 year.

Media preparation:

All media should be prepared at least 24 h in advance of conducting the assay. The selection of casein digest and agar used to prepare the media is important to the success of the mutation assay. It is critically important that Difco brand casein digest is used. Other brands have been shown to alter mutant selection or quality of the mutant screening plates. Select agar obtained from Invitrogen is also recommended for preparation of the media.

- TB-1/Kan+ plates (250 ml = 6 plates)
  1.25 g NaCl
  2.5 g casein digest
  3.0 g agar
  250 μl 0.1% thiamine HCl

Dissolve in approximately 200 ml of water and adjust the pH to 7.0. Adjust the final volume to 250 ml. Sterilize in autoclave for 30 min at 121°C. Cool to 55°C.

  12.5 mg kanamycin

Add the kanamycin after cooling media. Mix on stir plate, then pour 40 ml into each Petri dish. Store plates in plastic sleeves at 4°C for up to 2 weeks.

- TB-1 agar plates (1 l = 25 plates)
  5 g NaCl
  10 g casein digest
  12 g agar
  1 ml 0.1% thiamine HCl

Dissolve in approximately 800 ml of water. Adjust the pH to 7.0. Adjust the final volume to 1000 ml. Sterilize in an autoclave for 30 min at 121°C. Cool to 55°C and pour approximately 40 ml/plate. Store plates upside down in plastic sleeves at 4°C for up to 2 weeks.

- TB-1 liquid media (1 l)
  5 g NaCl
  10 g casein digest
  1 ml 0.1% thiamine HCl

Dissolve in approximately 800 ml of water. Adjust pH to 7.0. Adjust the final volume to 1000 ml. Prepare 100-ml aliquots into small glass bottles. Sterilize in an autoclave for 30 min at 121°C. Store at room temperature for up to 3 months.

- TB-1 Top agar (1 l = 25 packaged DNA samples)
  5 g NaCl
  10 g casein digest
  7 g agar
  1 ml 0.1% thiamine HCl

Dissolve in approximately 800 ml of water. Adjust the pH to 7.0 before heating. Adjust the final volume to 1000 ml and heat on a stir plate to form a homogenous suspension. Prepare 100 ml aliquots using small glass bottles. Sterilize in an autoclave for 30 min at 121°C. Melt completely in microwave before using. Each DNA sample uses 40 ml of top agar (~2.5 ml/plate). Store at room temperature for up to 3 months.

Sequencing of λ cII– mutants

Equipment

- Thermocycler
- Gel electrophoresis system
- Microfuge

Supplies and reagents

Supplies and reagents required for sequencing are listed in Table 38.5.

Procedures

Overview of λ cII mutation assay procedures

The *cII* mutation assay is based on the role the *cII* protein plays in the commitment of bacteriophage λ to the lysogenic cycle in *Escherichia coli* host cells (Figure 38.1). To select the mutant λ *cII–*, a specialized strain of *E. coli* (*hfl–*) is used to extend the longevity of the *cII* product. After isolation of fish genomic DNA, *in vitro* packaging procedures simultaneously excise and package the vector into viable phage particles. To determine the total number of packaged phage, a sub-sample of a package of DNA solution is mixed with *E. coli* cells, mixed with top agar and incubated on titer plates at 37°C overnight. To select *cII* mutants, the remaining packaged phage are mixed with *E. coli* host cells, plated, and incubated at 24°C for 40 h. The phage with wild-type *cII* produce lysogens and are indistinguishable in the *E. coli* lawn, whereas phage that carry a mutation in *cII* form plaques on the bacterial lawn when incubated at 24°C. Mutant frequencies are calculated by dividing the total number of *cII* mutant plaque forming units (PFUs) on the selective mutant screening plates by the estimated total λ+ and *cII* assayed on the titer plates.

Table 38.5 Supplies and reagents required for sequencing of λ *cII* mutants

Wide bore pipette tips	TB-1/Kan+ plates, 1
Glass culture tubes and lids (12 × 75 mm)	TB-1 liquid media, 100 ml
PCR nucleotide mix (Roche #1814362)	Dimethyl sulfoxide (DMSO)
Vent DNA polymerase (New England Biolabs)	SM buffer
TB-1 plates, 1 per sequence	

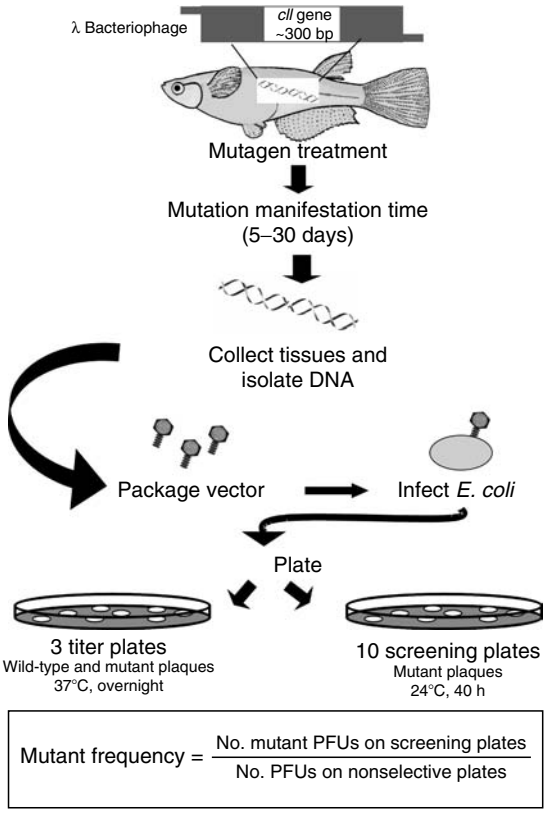

Figure 38.1 Overview of the bacteriophage λ-based transgenic medaka mutation assay using the *cII* gene (~300 bp) as a mutational target. After mutagen treatment and allowing sufficient time for mutations to manifest (~5–30 days), tissues are collected, and genomic DNA is isolated. The λ vector (~45 kb) is excised and packaged using *in vitro* packaging procedures. Individually packaged phage infect the G1250 *E. coli* host extending the longevity of the *cII* product to facilitate selection of mutant *cII* incubated at 24°C. λ Phage with a mutation in *cII* are selected by forming plaques while phage containing wild-type *cII* produce lysogens and are indistinguishable in the *E. coli* lawn. To determine the total number of plaques screened, a dilution of the infected *E. coli* is incubated at 37°C. The *cII*–mutant frequency is the ratio of the number of mutant λ *cII*– plaques selected to the total number of plaques screened.

Mutagen treatment protocols

A variety of methods, including static, single-pulse exposures, multiple-pulse exposures, and sophisticated chronic exposures using flow-through exposure systems used commonly for testing carcinogenicity in small fish species, may be appropriate for exposing λ transgenic medaka to chemical mutagens. See the section ''Results and discussion'' for additional comments about this subject.

Dissection of tissues

Following euthanization, standard procedures commonly used to dissect tissues from fish may be used. However, care should be taken to reduce potential DNA degradation by quickly removing tissues/organs and immediately placing samples on ice for immediate DNA isolation, or preferably, flash freezing in liquid nitrogen.

Isolation of high molecular weight DNA from dissected tissues

Isolation of high molecular weight DNA is essential for efficient *in vitro* packaging of the bacteriophage, and the ultimate success of the mutation assay. The following protocol has been optimized to isolate high molecular weight DNA from various fish tissues, including liver, eyes, skin, testes, and fins, thereby permitting mutation analyses from single tissues without pooling multiple samples from several individuals. Other DNA extraction procedures may not provide DNA of sufficient quality and quantity for this application. The authors may be contacted for procedures required to prepare DNA from whole fish.

1. Add tissue to a 1.5-ml Microfuge tube containing 500 μl of homogenization buffer, then add 15 μl of cold proteinase K solution to sample. Vortex briefly to mix.
2. Using small scissors finely mince each tissue into several pieces. Alternatively, use sterile pestles for 1.5-ml tubes to homogenize the tissue.
3. Immediately place samples into 37°C incubator and digest on rotating platform for 15–30 min or until tissue has digested.
4. Add 500 μl phenol/chloroform to digested tissue and invert gently 5 times.
5. Centrifuge samples at 1300 *g* at 4°C for 10 min.
6. Remove supernatant using a wide bore pipette tip and place it into a clean Microfuge tube.
7. Add 50 μl of 8 *M* potassium acetate to supernatant. Gently mix by inversion, then add an equal volume of chloroform and gently invert 5 times to mix.
8. Centrifuge samples at 1300 *g* at 4°C for 10 min.
9. Remove supernatant using a wide bore tip and place it into a clean Microfuge tube. Add 1 ml 100% ethanol.
10. Invert gently approximately 10 times or until the DNA has precipitated completely.
11. If the DNA is visible, collect by spooling with a flame-sealed Pasteur pipette. Carefully remove excess ethanol from the pipette using a paper tissue (e.g., Kimwipe) without touching the DNA.
12. Resuspend DNA in 25–50 μl TE (pH 7.5) in a 1.5-ml Microfuge tube. Resuspension volume should be sufficiently low as to maintain DNA in a highly viscous solution.
13. Alternatively, if the DNA is not visible, spin sample at 1300 *g* at 4°C for 10 min. Decant ethanol, dry tube around pellet. Do not over dry the pellet. Resuspend in 10 μl TE and, if necessary, make only minor adjustments to resuspension volumes to avoid over diluting the DNA.
14. Samples may remain on bench-top overnight to resuspend. Do not pipette the DNA for at least 12 h. Mix the samples the next day by pipetting up and down several times with a wide bore tip. Standard methods used to quantify the DNA concentration are not necessary, as they do not typically provide reliable predictors of potential success in the efficiency of packaging the DNA for this assay. Store the DNA at 4°C until use. Do not freeze the DNA.

λ *cII mutation assay procedures*

Schematic overview of procedures

- Day 1: streak TB-1/Kan+ plate with G1250 *E. coli* host strain

- Day 2: prepare G1250 liquid culture
- Day 3: package DNA samples and plate
- Day 4: count plaques on titer plates; calculate recoveries
- Day 5: count plaques on mutant screening plates; calculate mutant frequencies

It is recommended that 2–14 samples be analyzed at a time. An assay should ideally consist of a blocked design for each analysis, in which at least a sample from each treatment is analyzed simultaneously to account for potential variability in assay procedures (i.e., samples from all controls or all treatments should not be analyzed separately).

Day 1, preparation of the G1250 streak plate:

1. Using a sterile loop, streak a TB-1/Kan+ plate with frozen (−80°C) G1250 stock cells.
2. Incubate the streak plate upside down at 30°C for 24 h. Following incubation, wrap the G1250 streak plate in parafilm and store upside down in refrigerator. If properly stored, a streaked plate can be used for up to 1 week.

Day 2, preparation of G1250 liquid culture:

1. Add 10 ml TB-1 liquid media and 100 μl 20% maltose–1 *M* $MgSO_4$ to a sterile 50-ml conical tube.
2. Transfer 3 G1250 colonies from streak plate into the tube using a sterile pipette tip (be sure lids on the cultures are loose, so that air exchange will occur).
3. Shake culture at 265 rpm in a 30°C incubator for 16–21 h.

Prepare G1250 liquid culture 16–21 h before plating (e.g., 5:00 p.m.). Prepare one 10-ml culture for every four DNA samples (e.g., if plating 12 DNA samples, make 3 10-ml cultures).

Day 3, packaging and plating the DNA sample: Procedures are described in the following for using packaging extracts that are prepared in the laboratory. See Appendix I for procedures. Alternatively, follow the manufacturer's recommendations for using commercially available extracts.

1. Adjust water bath temperature to 30°C. Monitor the water bath to ensure it has stabilized at 30°C prior to the following steps.
2. Prior to performing the first packaging reaction, remove 13 TB-1 plates per sample from the refrigerator, shake out excess water, and lay out (upside down) to warm and dry.
3. Turn on 55°C water bath.
4. Label and lay out plates consisting of 3 titer plates (e.g., T-20 titer plates) and 10 mutant screening plates per sample (Table 38.6).
5. Set up a rack with 13 sterile glass culture tubes per sample, and label the same as the 13 plates for each sample.

Part 1, packaging reaction:

1. Using a wide bore tip, mix DNA samples to be plated by pipetting up and down 5–6 times. Aliquot 10 μl of DNA from each sample into a new Microfuge tube.

Table 38.6 Summary of materials and conditions for plating a packaged DNA sample

Tube labels	G1250 plating culture (μl)	1:100 dilution of packaged DNA	Undiluted packaged DNA (μl)	Corresponding TB-1 plates	Incubation temperature (°C)	Incubation time
T-20 A	200	20 μl of rep. A	—	T-20 A	37	O/n
T-20 B	200	20 μl of rep. B	—	T-20 B	37	O/n
T-20 C	200	20 μl of rep. C	—	T-20 C	37	O/n
Screening 1–10	200	—	100	Screening 1–10	24	40 h

2. At approximately 12:00 noon (13–18 of the G1250 liquid culture), begin the packaging reaction by removing the packaging extracts from the −80°C freezer. 1 Tube A and 1 Tube B will be needed for every 2 samples. Thaw the tubes quickly, centrifuge briefly to collect contents and, using a wide bore pipette tip, combine contents of Tube A (40 μl) and Tube B (60 μl). Mix by pipetting up and down 6 times. One tube of combined packaging extracts will package 2 DNA samples.
3. Using a wide bore pipette tip, pipette 50 μl of the combined packaging extracts into each DNA sample and mix by pipetting up and down 6 times. It is very important not to introduce bubbles into the mixtures, as these will reduce packaging efficiency.
4. Incubate at 30°C for 1.5 h.
5. Following the 1.5-h incubation period, continue the packaging reaction by repeating steps 2 and 3 above except using a standard pipette tip instead of a wide bore tip.
6. Melt top agar in microwave oven. Place melted top agar in 55°C water bath. It is very important that the agar be completely melted. Swirl and inspect to verify no unmelted pieces of agar are present.
7. 1 h following step 5 (above) begin preparation of the G1250 plating culture by centrifuging the tubes of G1250 overnight culture at 3200 *g* for 10 min at 4°C.
8. Decant supernatant and resuspend pellet in 10 ml of 10 m*M* $MgSO_4$.
9. Using a spectrophotometer, measure absorbance ($OD_{600\,nm}$) by adding 100 μl of cells to 900 μl of 10 m*M* $MgSO_4$. Multiply absorbance by 10 for OD value.
10. Dilute cell suspension to OD = 0.5.
11. Place cells on ice until ready for use. This is the G1250 plating culture.
12. Following the second 1.5-h incubation, add 990 μl sterile SM buffer to the samples.
13. Vortex for 10 s.
14. Place samples on ice. These are referred to as the packaged DNA samples.

Part 2, plating of λ *cII*:

1. For the first DNA sample, add 200 μl of the G1250 plating culture into each of the 13 sterile glass culture tubes for the packaged DNA sample.
2. Label 3 Microfuge Tubes A, B, and C.
3. Add 990 μl SM buffer to each one.
4. Add 10 μl of packaged DNA to Tubes A, B, and C and vortex to mix.
5. Add 20 μl from Tube A to 1 of the 3 culture tubes representing the T-20 titer plates. Repeat with Tubes B and C.

4. Add 100 μl of the undiluted packaged DNA sample to each of the 10 remaining culture tubes for the 10 mutant screening plates.
5. Incubate at room temperature for 30 min.
6. Repeat steps 1–5 above for each of the remaining DNA samples. Set timers such that all samples are incubated for exactly 30 min. It is important to finish these steps for all DNA samples before the 30-min incubation is completed for the first sample.
7. Following the 30-min incubation, add 2.5 ml of top agar to each culture tube, mix, and immediately pour onto an appropriately labeled TB-1 plate while swirling gently to evenly distribute the top agar.
8. Repeat for remaining samples. Allow at least 5 min for the agar to solidify on each plate.
9. Transfer plates to appropriate incubators within 30 min of plating. Incubate the titer plates upside down at 37°C overnight. Incubate the mutant screening plates upside down at 24°C for 40 h. Monitor the temperature in this incubator using a data-logger. Internal incubator temperature should vary no more than ± 0.5°C (Table 38.6).

Day 4, count titer plates and calculate recovery:

1. Following the overnight 37°C incubation, count PFUs on titer plates to determine the total number of λ bacteriophage recovered from the fish genomic DNA. A light box partially covered with black paper is helpful to assist in the counting. PFUs are easiest to see in the contrast between the light and black background. Use the following to determine the recovery for each sample:

$$\text{recovery} = \frac{(\text{mean No. plaques/titer plate})}{(\text{No. } \mu\text{l of dilution/titer plate})} \times \text{ dilution} \times (100\,\mu\text{l/plate}) \times 10 \text{ plates}$$

   Example: Using a mean number of 161 PFUs counted per plate in the T-20 titer plate group, and 20 μl of a 100-fold dilution plated on each T-20 titer plate, the total number of PFUs recovered is 161/20 μl of diluted phage per titer plate × 100 (the dilution factor) × 100 μl of undiluted phage per mutant screening plate × 10 mutant screening plates = 805,000 total plaques screened.

2. After counting PFUs, plates may be discarded. Sterilize plates (original TB-1/Kan+ plate, titer plates, and unwanted mutant screening plates) in an autoclave at 121°C for 30 min prior to discarding.

Day 5, count PFUs on mutant screening plates and calculate cII– mutant frequency:

1. Count PFUs on mutant screening plates at 40 h. This can be best achieved by holding the plates next to a lamp. Individual PFU may vary in size. Be careful not to count plating artifacts (e.g., bubbles). The *cII* mutant frequency is calculated as follows:

$$\text{mutation frequency} = \frac{\text{total No. PFUs on screening plates}}{\text{total No. PFUs recovered}}$$

Example: Using a total of 23 PFUs counted on the 10 mutant screening plates, and a total recovery of 805,000 PFUs for the sample, the mutation frequency is $23/805{,}000 = 2.8 \times 10^{-5}$.

2. Mutant screening plates should be saved for later coring, mutant verification, and sequencing procedures if desired. Store plates by placing upside down in Petri dish bags at 4°C.

Sequencing of λ cII– mutants

Schematic overview of procedures

1. Core mutant plaques and elute phage
2. Re-plate eluted phage and incubate plates at 24°C for 40 h
3. Perform PCR amplification of re-plated mutants
4. Sequence samples following the appropriate protocol for available sequencing system

Day 1:

1. Determine percentage of plaques to be cored from each plate.
2. Add 500 μl sterile SM buffer into a sterile Microfuge tube for each core.
3. Core an individual plaque from a plate using a 1000-μl wide bore pipette tip by stabbing the tip into the plate.
4. Place entire agar plug containing the plaque into a microcentrifuge tube containing SM buffer.
5. Repeat for remaining samples.
6. Elute phage at 4°C overnight (for long-term storage, transfer an aliquot of the eluted phage to a clean microcentrifuge tube, add DMSO to a final concentration of 7% [v/v], and store at −80°C).
7. Prepare one 10-ml G1250 liquid culture for every 50 plaques re-plated (see Day 2 of the λ *cII* mutation assay).

Day 2:

1. Prepare a G1250 plating culture (see steps 7–11 on Day 3 of the λ *cII* mutation assay).
2. Add 200 μl of the G1250 plating culture to each culture tube.
3. Add 1 μl of eluted phage solution to culture tube, mix, and incubate for 30 min at room temperature.
4. Following the 30-min incubation, add 2.5 ml top agar to each culture tube, pour onto plate, and swirl to distribute evenly.
5. Incubate the plates upside down at 24°C for 40 h.

Phage isolation and PCR amplification for sequencing:

1. Determine number of samples to be sequenced.
2. Pick an individual plaque from a plate containing the re-plated phage (verification plate) using a 200-μl wide bore tip and place into 25 μl sterile water in a

screw-top tube. It is very important to scrape only the top layer of the plate containing the plaque. Avoid taking any agar, as it will inhibit the PCR amplification.

3. Repeat for remaining samples to be sequenced.
4. Prepare a master mix for the PCR reaction to obtain the following concentration of reagents in a final 25-μl volume:
   - 1× PCR buffer
   - 200 μ*M* dNTPs
   - 0.5 μ*M* primers (*cII* primer 1: 5′ to 3′ AAA AAG GGC ATC AAA TTA AAC C; *cII* primer 2: 5′ to 3′ CCG AAG TTG AGT ATT TTT GCT GT)
   - 1 unit of DNA polymerase
5. Place tubes containing the scraped phage into boiling water for 5 min.
6. Centrifuge tubes for 3 min at maximum speed.
7. Immediately pipette 2.5 μl of supernatant into the PCR reaction.
8. Perform the PCR reaction using the following amplification conditions:
   - 95° for 3 min
   - 30 cycles of:
   - 95° for 1 min
   - 55° for 1 min
   - 72° for 1 min
   - followed by 72° for 10 min
   - 4°C storage

Results and discussion

Considerations for conducting mutation analyses

A number of factors that may influence the induction of mutations in animals must be considered when designing and conducting an assessment of mutations using the λ transgenic medaka (Figure 38.2). Genotoxicity will be a function of uptake, distribution, detoxification, metabolic activation, DNA repair, and the types of DNA adducts formed. These factors may be modified by, for example, age of animals at the time of exposure, concentration of chemical at exposure, duration of exposure, and the time of sampling after exposure. Whereas some of the variables that influence the induction of mutation are specific to detecting mutations using the λ *cII* mutation assay, others (e.g., routes of exposure, duration of exposure, chemical concentrations, and characteristics of the chemical) are similarly considered when designing and conducting toxicity assessments using different endpoints. The following describes some of the more important factors related to performing mutation analysis using the λ transgenic medaka. Many of the features of detecting mutations in the λ transgenic medaka are shared with the transgenic rodent mutation assays. Consequently, additional guidance to addressing these mutation analyses in fish may be obtained from the transgenic rodent literature.[16]

Mutagen treatment regimen

Small laboratory fish species, including the λ transgenic medaka, provide significant flexibility in their use in toxicity assessments. Depending on the characteristics of the test compound (e.g., potent mutagens) and research aims (e.g., screening potential mutagenicity of a chemical), short-term chemical treatments conducted over one to several hours may be sufficient to achieve significant inductions of mutations. However, some

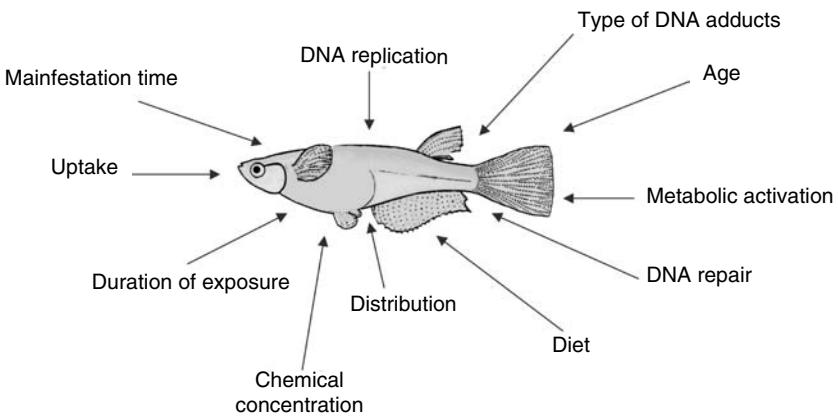

Figure 38.2 Schematic overview of important factors that affect or modify the induction of mutations to be considered in the design and conduct of *in vivo* mutation analyses.

chemicals may, in addition to producing DNA adducts, induce cell proliferation. Mutations in transgenic mutation assays are detected using genetically neutral targets, thereby avoiding the potential selective pressures on the mutant frequency *in vivo* and allowing the accumulation and persistence of mutations.[12–14] As a consequence of the accumulation of mutations in these loci over time, the use of repeated or chronic chemical treatments will increase the sensitivity of the mutation assay. A chemical exposure regimen consisting of repeated treatments will facilitate the administration of the chemical during those periods, in which cell proliferation may have been induced by an earlier exposure. Consequently, chemically induced mutations should be studied ideally under conditions of sub-chronic or chronic administration if resources are available. Alternatively, short-term multiple doses protocols, for example, at a minimum of 3 days, and up to 2–3 weeks, to allow chemical administration in the presence of chemically induced cell turnover, could be considered. Thus far, data from studies on the transgenic medaka indicate that mutations accumulate linearly using daily chemical treatments, including exposures extending greater than 9 months (unpublished data).

The selection of the concentrations of the chemicals to be administered should be determined from standard range finding tests of toxicity. However, in considering the potential adverse toxic effects of compounds administered at high concentrations, and the possible need to increase number of treatments when exposing at low chemical concentrations, a compromise between the number of treatments and the exposure concentration may have to be made.

Numbers of animals per treatment

The efficient recovery of the λ bacteriophage from the λ transgenic medaka genomic DNA (typically >300,000 PFUs/sample) and, the low variability in mutational responses observed among animals within treatments, contribute to the ability to use relatively small numbers of animals per treatment. Statistical analysis revealed that as few as 6–7 animals may be required to detect a 50% induction of mutations above the spontaneous *cII* mutant frequency (e.g., $2–5 \times 10^{-5}$ for most tissues), and 2–3 animals may be required

to detect a 100% induction of mutations.[10] As a general rule, 10–15 fish per treatment should provide sufficient material for analyses. Additional tissues can be held frozen for further analyses as needed.

Mutation manifestation time

The interval between mutagen treatment and sample processing, termed mutation manifestation time, is an important factor to consider in the design and interpretation of mutation studies.[17–19] The mutation manifestation time is important because DNA replication is necessary for genetic damage to become fixed as a mutation. Consequently, mutation manifestation time will directly influence the frequency of mutations observed. The time for mutations to manifest is affected by several variables, including tissue/cell type (e.g., rate of cell turnover), mutagen (e.g., potent or weak), and mutagen treatment regimen (e.g., number of treatments). For example, a tissue with a rapid cell turnover, such as intestinal epithelium, sampled within several days of a short-term exposure to a potent mutagen would likely exhibit a significant induction of mutations. In contrast, when using a weakly mutagenic compound and/or when using a target tissue with slower cell turnover, such as liver, a manifestation time greater than 30 days may be required. Sampling times may have to be determined on a case-by-case basis depending on the variables. As a guide, when collecting multiple tissues, all tissues should be sampled at the time appropriate for the tissue with the slowest cell turnover. Thus far, results indicate that a mutation manifestation time of 15 days may be sufficient time to detect a significant fold induction of mutations over a spontaneous frequency in most fish tissues, although a mutation manifestation time greater than 30 days may be required to detect induction by weak mutagens, and a 5-day sampling time is probably suitable only for the most potent mutagens.

Target tissues/organs

As with other toxicity assessments, the choice of target tissue/organ to be examined in the *cII* mutation assay will be dictated by the experimental questions. The liver is among the more widely studied target organs in a variety of toxicological assessments. Analyses of mutations in the liver of λ transgenic medaka have proven valuable in demonstrating that many aspects of *in vivo* mutagenesis, including the modes of mutagen action of several chemicals, are shared with transgenic rodent models.[10,11]

The efficiency with which the λ bacteriophage is recovered from the fish genomic DNA is one of the most important factors that can influence the ability to perform the *cII* mutation assay. DNA extraction procedures and other protocols described here have been optimized to assist in isolating high molecular weight DNA required for λ bacteriophage *in vitro* packaging from virtually any medaka tissue, including liver, testes, eye, fin, blood, skin, embryos, and fry, without the need to pool DNA from multiple samples.

Number of PFUs needed to determine mutant frequency

As the number of PFUs recovered from the fish genomic DNA approaches ~300,000 PFUs/sample, the average spontaneous mean frequency will become less variable. Consequently, based on experience, at least 300,000 PFUs from each tissue should be analyzed to calculate the spontaneous or induced mutant frequency.

Spontaneous mutant frequency

The measure of the frequency of spontaneous *cII*– mutants provides the basis for comparing mutations induced after mutagen exposure. The sensitivity of a mutation assay is

defined by the magnitude of the induced mutational response compared to the frequency of spontaneous mutations. Spontaneous mutant frequencies will vary among different tissues with the lowest mean mutant frequencies exhibited by testes ($1–2 \times 10^{-5}$), followed by whole fish ($3–4 \times 10^{-5}$), and then liver ($4–6 \times 10^{-5}$).

Statistics

Application of statistics to data obtained from transgenic mutation assays is consistent with statistical approaches used commonly in toxicity studies. Nonparametric tests, such as the generalized Cochran–Armitage test, are generally used with data from these assays.[16] Typically, a positive response will be indicated if the mean mutant frequency of a treatment group exceeds at least 2 times that of an untreated and/or historical control.

Sequencing of mutants

One of the strengths of the *cII* mutation assay is the ability to obtain information about the types of mutations induced. The small size of the *cII* target gene (~300 base pairs, bp) facilitates efficient characterization of the mutations by direct sequencing of the entire gene. Mutations detected in λ-based systems consist primarily of point mutations, base pair substitutions, with some frameshifts, and small insertions and deletions. Although sequencing data may not be required in all cases, sequencing can be very informative. In addition to the importance of using sequencing to verify a λ *cII*– mutant, sequencing may provide important information on the spectra of mutations that may be highly characteristic of specific compounds, as well as may disclose possible mechanisms of mutagen action. When only marginal increases of mutations may be observed, sequencing may disclose actual mutation induction. In this instance, an induction of a specific class may be too small in absolute numbers to be apparent in the overall mutant frequency, but sequencing may reveal it. Similarly, sequencing may be particularly useful in examining whether small increases in mutant frequencies after exposure to chemicals at low environmental concentrations are accompanied by shifts in mutational spectra. Sequencing will also aid in reducing the variability in mutant frequencies by identifying and removing clusters or ''jackpot'' mutants that have arisen from a single mutation.

Examples of studies of chemical mutagenesis

The following are examples of studies of induced mutagenesis in the λ transgenic medaka using four chemicals, ethylnitrosourea (ENU), dimethylnitrosamine (DMN), diethylnitrosamine (DEN), and benzo(*a*)pyrene (BaP). These studies illustrate many of the features of the *cII* mutation assay based on medaka, and highlight some of the important issues to be considered when conducting studies of *in vivo* mutagenesis.

Ethylnitrosourea

The feasibility of the *cII* mutation assay in detecting induced mutations in the λ transgenic medaka was first demonstrated by using the model mutagen, ENU.[10] ENU is a well-characterized, and widely used direct-acting alkylating agent, and potent germ-cell mutagen. Using a static exposure regimen, fish (6–10 hemizygous, 2- to 6-month old transgenic fish/treatment) were immersed 1 h in water containing 0, 60, or 120 mg/l of ENU. Fish were rinsed, transferred to clean water, and held for 5, 15, 20, or 30 days until euthanized with an overdose of MS222. Fish treated similarly without mutagen exposure

served as controls. Whole fish, or dissected tissues (liver, testes), were flash frozen in liquid nitrogen and stored at −80°C until processed for DNA isolation.

The *cII* target gene was highly responsive to ENU treatment, exhibiting responses that were dependent on chemical exposure concentration. In whole fish, sampled 15 days after exposure, *cII* mutant frequencies were induced significantly, 2.7-fold ($8.0 \pm 1.0 \times 10^{-5}$), and 4-fold ($12.0 \pm 1.9 \times 10^{-5}$), over untreated fish ($3.0 \pm 0.3 \times 10^{-5}$) at 60 and 120 mg/l, respectively (Table 38.7). The time between mutagen exposure and tissue sampling, or mutation manifestation time, was shown to have a significant influence on the mutant frequencies, and the influence on mutant frequencies was tissue-specific. Mutant frequencies in liver of treated fish did not increase significantly above that of untreated fish ($4.4 \pm 0.7 \times 10^{-5}$) at 5 days ($5.1 \times 10^{-5}$), but increased significantly 3.5-fold (15.6×10^{-5}) at 15 days, 5.7-fold (25.1×10^{-5}) at 20 days, and 6.7-fold by 30 days after mutagen treatment. In testes, the mutant frequencies were elevated 5.2-fold above that of the untreated fish within 5 days after mutagen exposure, reaching a peak or threshold 10-fold induction at 15 days (Table 38.8). The relatively higher magnitude of mutation inductions and shorter mutation manifestation time observed in testes compared with liver reflect apparent differences in cell proliferation rates and/or mutagen action in these tissues. The different frequencies of mutations exhibited by these tissues at equivalent mutagen treatments illustrate the significant utility of this mutation assay for examining tissue-specific mutagenesis.

To characterize the spectra of spontaneous and mutagen-induced mutations, the phenotype of individual *cII* mutant phage obtained from the mutant screening plates was first verified using selective plating conditions. A 446-bp product, including the entire *cII* gene (~300 bp), was then sequenced for each of the isolated mutants. Sequence analyses revealed that single base substitutions comprised the majority of spontaneous and ENU-induced mutations in fish, with the large percentage of G:C to A:T mutations at CpG sites (Table 38.9). ENU is a direct acting mutagenic agent producing 0^6-ethylguanine, 0^4-ethylthymidine, and 0^2-ethylthymidine in DNA, promoting G:C to A:T and A:T to G:C transitions, as well as A:T to T:A transversions. The proportion of mutations at A:T base

Table 38.7 Frequencies of mutants recovered from whole fish treated with ENU[10]

Treatment	Mean mutant frequency $\times 10^{-5} \pm$ SEM (n = 6–9)
Control	3.0 ± 0.3
60 mg/l	8.0 ± 1.0
120 mg/l	12.0 ± 1.9

Table 38.8 Frequencies of mutants from medaka sampled at different times after exposure to 120 mg/l ENU[10]

	Mutant frequency $\times 10^{-5} \pm$ SEM			
	5 days (n)	15 days (n)	20 days (n)	30 days (n)
Control liver	3.8 ± 0.7 (9)	5.2 ± 0.6 (6)	4.8 ± 1.9 (4)	3.6 ± 0.5 (5)
Exposed liver	5.1 ± 0.8 (9)	15.6 ± 1.8 (6)	25.1 ± 2.3 (6)	29.8 ± 2.0 (5)
Control testes	2.1 ± 0.5 (5)	2.2 ± 0.3 (6)	2.9 ± 0.8 (5)	1.3 ± 0.1 (6)
Exposed testes	10.3 ± 1.1 (8)	19.6 ± 1.2 (8)	17.5 ± 1.6 (7)	16.4 ± 2.1 (9)

Table 38.9 Comparison of mutational spectra from untreated and mutagen-treated λ transgenic medaka

	Spontaneous	ENU 120 mg/l	DMN 600 mg/l	BaP 50 μg/l
Total mutations	89	39	44	98
Mutations outside of *cII*	5	3	6	5
Independent mutations[a]	74	36	34	81
	Percentage (*n*)	Percentage (*n*)	Percentage (*n*)	Percentage (*n*)
Transitions				
G:C to A:T	20 (15)	28 (10)	73 (25)	6 (5)
At CpG sites[b]	47 (7)	30 (3)	12 (3)	40 (2)
A:T to C:G	12 (9)	25 (9)	3 (1)	1 (1)
Transversions				
G:C to T:A	20 (15)	6 (2)	0 (0)	59 (48)
G:C to C:G	11 (8)	6 (2)	6 (2)	22 (18)
A:T to T:A	4 (3)	28 (10)	3 (1)	1 (1)
A:T to C:G	7 (5)	3 (1)	0 (0)	1 (1)
Frameshift				
+1	14 (10)	3 (1)	3 (1)	3 (2)
−1	11 (8)	0 (0)	9 (3)	3 (2)
Other	1 (1)	3 (1)	3 (1)	4 (3)

[a] Clonal mutations were subtracted.

[b] Percent occurring at transitions [e.g., 7 of the 15 G:C to A:T transitions (47%) were at CpG sites for the spontaneous mutations].

pairs increased from 31% in untreated fish to 59% in ENU-treated livers, with the bulk of the increase being A:T to T:A transversions. This shift in the frequency of mutations is characteristic of the greater mutagenic effect of ENU at A:T base pairs.[20,21] Also consistent with the known mode of action of ENU was the reduction in the frequency of frameshift mutations observed in ENU-exposed fish compared to that of the untreated animals. ENU has been shown to not induce high numbers of frameshift mutations.[22]

Dimethylnitrosamine

Induction of mutations in the λ transgenic medaka was examined after exposure to DMN, a potent liver carcinogen used extensively as a model mutagen in carcinogenesis studies.[11] DMN is among a class of nitrosamines that induced hepatic carcinogenesis in fish with progressive stages similar to those characterized in rodent hepatic neoplasia.[23] Studies in rodents demonstrated that cell proliferation is a requisite for DMN-produced methyl DNA adducts to become fixed as mutations.[24] The results demonstrate the potent mutagenicity of DMN in medaka and illustrate the importance of considering cell proliferation in the design of *in vivo* mutagenesis studies. Adult fish were exposed to DMN at 0, 300, or 600 mg/l administered in a 96-h static renewal exposure regimen. *cII* mutants were induced 7.1-fold ($16.7 \pm 2.8 \times 10^{-5}$), and more than 16-fold ($38.4 \pm 6.6 \times 10^{-5}$) above untreated fish ($2.1 \pm 0.25 \times 10^{-5}$) 15 days after exposure to DMN at 300 and 600 mg/l, respectively (Table 38.10).

Sequencing disclosed a different spectra of DMN-induced mutations compared to that induced by ENU, revealing different modes of action of these mutagens. Most notably, single-base substitutions were the most frequent mutations in liver of treated

Table 38.10 Frequencies of mutants recovered from medaka liver treated with DMN[10]

Treatment	Mean mutant frequency $\times 10^{-5} \pm$ SEM (n = 6–9)
Control	2.1 ± 0.25
300 mg/l	16.7 ± 2.8
600 mg/l	38.4 ± 6.6

fish, with a large percentage of G:C to A:T transitions. The higher frequency of G:C to A:T transitions compared to transversions is characteristic of DMN exposure (Table 38.9). These mutations are attributed to the mispairing of 0^6-methylguanine with thymine, leading to G:C to A:T transitions during replication.[25]

Diethylnitrosamine

DEN is a potent genotoxic carcinogen, and is one of the most widely used compounds to investigate the mechanisms of carcinogenesis in medaka having been shown to be a strong inducer of hepatic neoplasms.[26,27] To examine the potential mutagenicity of this compound, adult fish (3 months) were held in water containing 100 mg/l DEN for 48 h and then transferred to toxicant-free water for a 30-day mutation manifestation time. The potency of DEN as a mutagen was shown clearly in the livers of the treated fish, which exhibited a mean mutant frequency induced 65-fold ($136.4 \pm 18.24 \times 10^{-5}$) above that of untreated fish ($2.1 \pm 0.33 \times 10^{-5}$) (Table 38.11). DEN has been shown to require only a brief and relatively low concentration to initiate carcinogenesis in the medaka.[28] Considering the proven utility of medaka in carcinogenesis research, and the extensive use of DEN as a model compound for inducing hepatic neoplasms, the mutation data presented here illustrate the potential value of combining the use of λ transgenic medaka and *cII* mutation assay with the assessment of histopathological, and other endpoints in the fish,

Table 38.11 Mutant frequencies in liver of medaka exposed to DEN (48 h, 30-day mutation manifestation)

Treatment	Concentration (mg/l)	Total PFU	Mutant PFU	MF $\times 10^{-5}$	Group mean MF $\times 10^{-5} \pm$ SEM
Control	0	1,225,000	29	2.4	$x = 2.1 \pm 0.33$
		2,560,000	77	3.0	
		2,160,000	48	2.2	
		1,090,000	9	0.8	
		4,505,000	70	1.6	
		4,085,000	35	0.9	
		2,180,000	73	3.4	
		820,000	19	2.3	
DEN	100	3,775,000	6,249	165.5	$x = 136.4 \pm 18.24$
		3,065,000	3,930	128.2	
		2,480,000	2,022	81.5	
		1,490,000	2,543	170.7	
		2,810,000	5216	185.6	
		1,520,000	1319	86.8	

to enhance understanding of the initiation, promotion, and progression of hepatic carcinogenesis.

Benzo(a)pyrene

The responsiveness of the *cII* target to mutagen exposure was further examined using a mutagen that requires metabolic activation, BaP, a persistent contaminant in aquatic systems, and a widely used model compound in laboratory fish carcinogenesis studies[29] and transgenic rodent assays.[21,30] BaP induces the formation of DNA adducts following the metabolism by CYP1A to its ultimate carcinogenic metabolite, BaP 7,8-dihydrodiol-9, 10-epoxide (BPDE). In a simple static-renewal exposure regimen, medaka adults were treated with water containing acetone as a vehicle and 0 or 50 μg/l BaP over 96 h (6 h/day). The fish were then allowed a 15-day mutation manifestation time. Mean mutant frequencies in the liver of fish treated with BaP were induced nearly 5-fold ($14.1 \pm 4.69 \times 10^{-5}$) above that of the controls ($2.97 \pm 0.30 \times 10^{-5}$) (Table 38.12).

Sequence analysis revealed that single base substitutions comprise the majority of spontaneous and BaP-induced mutations in fish (Table 38.9). The majority of mutations at G:C base pairs in BaP-treated fish were G:C to T:A transversions (59%), a spectral shift consistent with the greater mutagenic effect of BaP at G:C base pairs.[21] The BaP metabolite, BPDE, forms covalent bonds at the N2 position of guanine, thereby resulting in point mutations at G:C base pairs, which can lead to oncogene activation. Similar significant increases in G:C to T:A transversions in BaP-treated transgenic mice compared controls have also been observed.[31] Also consistent with previous reports in rodent models, a large proportion of the mutations in the fish were located at CpG sites.

Summary

Studies to date support the continued use of the λ transgenic medaka and the associated *cII* mutation assay for assessing spontaneous and induced *in vivo* mutagenesis. The λ transgenic medaka exhibits chemical concentration-dependent, and tissue-specific

Table 38.12 Mutant frequencies in liver of medaka exposed to BaP (96 h, 15-day mutation manifestation)

Treatment	Concentration (μg/l)	Total PFU	Mutant PFU	MF $\times 10^{-5}$	Group mean MF $\times 10^{-5} \pm$ SEM
Control	0	380,000	12	3.1	$x = 2.9 \pm 0.3$
		1,560,000	61	3.9	
		1,930,000	57	2.9	
		1,518,000	59	3.8	
		936,000	23	2.4	
		1,603,000	28	1.7	
BaP	50	1,238,000	275	22.2	$x = 14.1 \pm 2.1$
		2,460,000	336	13.6	
		1,030,000	105	10.2	
		545,000	66	12.1	
		323,000	40	12.4	

responses to mutagen exposures reflecting known modes of mutagen action. The mutation analyses, when used in combination with measures of other toxicological endpoints, promise to be useful in more fully characterizing health risks associated with chemical exposures in the environment. In addition, the practical benefits afforded by this mutation assay and the medaka as an animal model should facilitate in-depth investigations into the mechanisms of mutagenesis and carcinogenesis.

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Appendix I. Preparation of λ packaging extracts

The *in vitro* packaging materials are essential to the simultaneous excision of the λ bacteriophage from the transgenic fish genomic DNA, and packaging of individual λ bacteriophage. The following protocol consists of a series of steps that may be completed over 5 days. Note that two different cell extracts are prepared in these procedures. Dr. Peter Glazer (Yale University) kindly provided the cell stocks and the original protocols adapted here for preparing these packaging extracts. Dr. Glazer should be contacted to obtain these materials.

Day 1: Prepare LB plates.
Day 2: Streak LB plates with BHB 2688 and NM 759 stock cells
Prepare solutions and NZY media
Autoclave glassware and centrifuge bottles
Day 3: BHB extracts (Tube A), inoculate NZY media with BHB colony.
Day 4: Perform BHB freeze–thaw preparation.
Day 5: Perform ultracentrifugation of frozen BHB cell mixture from Day 4
Freeze BHB cell extracts
Day 6: NM extracts (Tube B), inoculate NZY media with NM colony.
Day 7: Make and freeze NM cell extracts.

Materials required

Equipment

Equipment required for preparation of λ packaging extracts is listed in Table 38.A1.

Supplies and reagents

Supplies and reagents required for preparation of λ packaging extracts are listed in Table 38.A2. Table 38.A3 lists the amounts necessary for preparation of approximately 70 extracts.

Table 38.A1 Equipment required for preparation of λ packaging extracts

30°C incubator
42°C incubator
30°C shaking incubator
32°C shaking incubator
Sonicator (Fisher Scientific Sonic Dismembrator Model 100, setting 3)
Refrigerated centrifuge with rotor capable of spinning 250 ml bottles at 3200 *g*
Ultracentrifuge with Beckman 75 TI rotor or equivalent
65°C water bath
45°C water bath
Vortex
Spectrophotometer

Solutions:

- 1 *M* TrisCl (250 ml)
  30.27 g Tris-base

Dissolve in 100 ml water. Adjust pH to 8.0 using a pH probe that will accurately measure Tris solutions. Adjust the final volume to 250 ml. Sterilize in an autoclave for 30 min at 121°C and store at room temperature for up to 1 year.

- 1 *M* $MgCl_2$ (250 ml)
  50.8 g $MgCl_2$
  Filter-sterilize
  Store at room temperature for 1 year

- 0.1 *M* ATP (1 ml)
  0.06 g ATP
  1 ml H_2O
  Filter-sterilize

- 10% Sucrose solution (10 ml)
  1 g sucrose
  500 μl 1 *M* TrisCl, pH 8.0
  Filter-sterilize; store at 4°C for 3 months

- Lysozyme solution (10 ml)
  0.02 g lysozyme
  100 μl 1 *M* TrisCl, pH 8.0
  Filter-sterilize; store at 4°C; make fresh every time

- Sonication buffer (10 ml)
  200 μl 1 *M* TrisCl, pH 8.0
  20 μl 0.5 *M* EDTA
  3.5 μl B-mercaptoethanol
  Filter-sterilize; store at 4°C for 3 months

Table 38.A2 Supplies and reagents required for preparation of λ packaging extracts

Sterile 1.5-ml centrifuge tubes
Sterile 50-ml centrifuge tubes
Sterile 250-ml centrifuge bottles (6–8)
250-ml Erlenmeyer flasks (2)
1-l Erlenmeyer flasks (4)
5-ml serological pipettes
25-ml serological pipettes
Sterile foam stoppers
Chloroform
Timers
Thermometer
Liquid nitrogen
Loop for cell culture
16 × 76 mm tubes for ultracentrifuge (Nalgene #3425-1613)
Casein digest (Difco #211610/0116-17)
Agar (Invitrogen Select Agar #30391-049)
Yeast extract (Invitrogen #30393-029)
NaCl
$MgCl_2 \cdot 6H_2O$
ATP (Roche #519987)
β-mercaptoethanol
Sucrose
Lysozyme
Spermadine
Putrescine

- Packaging buffer (2.5 ml)
  - 15 μl 1 *M* TrisCl, pH 8.0
  - 0.032 g spermadine
  - 0.02 g putrescine
  - 50 μl 1 *M* $MgCl_2$
  - 5.25 μl B-mercaptoethanol
  - 750 μl 0.1 *M* ATP, pH 7.0
  - Store at 4°C for 3 months

Media preparation: It has been found that the media used for growing the cells is a very important factor in the efficiency of the extracts. It is recommended to stick to the exact brands of agar, casein digest, and yeast to optimize the production of the extracts. Once optimized, any changes should be tested systematically.

- LB plates (0.5 l)
  - 5.0 g casein digest
  - 2.5 g yeast extract
  - 5.0 g NaCl
  - 7.5 g agar

Autoclave for 30 min at 121°C; mix well and cool to 55°C; pour into sterile Petri plates (~40 ml/plate).

Table 38.A3 Quantities[a] of reagents used for preparation of λ packaging extracts

Reagents	Quantity	Reagents	Quantity
LB plates	2/cell line	0.1 *M* ATP (−70°C)	750 µl
NZY media	2 l	β-mercaptoethanol	9 µl
10% sucrose solution (4°C)	3 ml	1 *M* $MgCl_2$	100 µl
Packaging buffer (4°C)	3 ml	Spermadine (−20°C)	0.032 g
Sonication buffer (4°C)	2 ml	Putrescine	0.02 g
1 *M* Tris buffer	900 µl		
Lysozyme solution (4°C) (make fresh on day of use)	300 µl		

[a] These quantities will produce BHB (Tube A) and NM (Tube B) extracts for ~ 70 samples.

- NZY media (1 l)
  10 g casein digest
  5 g yeast
  5 g NaCl
  2.01 g $MgCl_2 \cdot 6\ H_2O$
  pH 7.0, autoclave for 30 min at 121°C

Procedures

Packaging extract A (BHB 2688 cell stock)

Day 1:

1. Using a sterile loop, streak 2-LB plates with frozen BHB 2688 stock cells (−80°C).
2. Incubate the plates overnight, one at 30°C and the other at 42°C.

Use the plate grown at 30°C for inoculation. This plate may be stored at 4°C and used for 1 week. Check the plate grown at 42°C to ensure no growth has occurred. If it has, discard stock cells and re-streak plates with new stock cells.

Day 2:

1. Inoculate 80 ml of NZY broth with a colony of BHB 2688 (30°C plate).
2. Grow overnight in a shaking incubator at 30°C and 265 rpm.

Day 3:

1. Inoculate three 1-l flasks (each containing 500 ml sterile NZY media) with 25 ml each of the BHB 2688 overnight culture. Cap flasks with sterile foam stoppers.
2. Grow the culture in a shaking incubator at 32°C and 175 rpm until the optical density at wavelength 600 nm (OD_{600}) reaches 0.6 (~2.5 h). It is important not to overgrow the culture.
3. Transfer the flasks to a 65°C water bath. Swirl the flasks and monitor the temperature in flasks until they reach 45°C (~5 min).
4. Transfer the flasks to a 45°C water bath for 15 min, swirling the flasks every 5 min.

5. Return the flasks to the shaking incubator now set at 38–39°C, and shake for 2–3 h (do not exceed 40°C).
6. After approximately 2.5 h of incubation time, remove 2 ml of the cell suspension from a flask and aliquot 1 ml into each of 2 culture tubes. Add 3–4 drops of chloroform to one and see if it clears (compared to other tube) when incubated at 37°C for 2–3 min.
7. When the chloroform/cell suspension clears, aliquot flask contents into six 250-ml centrifuge bottles and centrifuge at 3200 *g* at 4°C for 15 min.
8. Immediately decant the supernatant, place the bottles on ice, and dry the inside of the bottles with a lint-free towel. Avoid touching the pellet at the bottom of the bottle.
9. Vortex each bottle for a few seconds.
10. Pipette 3 ml of 10% sucrose solution into one bottle and vortex to resuspend the pellet.
11. Using a 5-ml serological pipette, transfer the resuspended cells/sucrose solution to the next bottle and vortex again. Repeat this process until all six pellets are resuspended together. It is important to avoid introducing air into the solution at this stage.
12. Aliquot 500 μl of the solution into Microfuge tubes (~10 tubes).
13. Add 25 μl of lysozyme solution. Gently mix.
14. Flash-freeze the tubes in liquid nitrogen and store at −80°C.

Day 4:

1. Thaw tubes from the day before on ice for at least 1 h.
2. Add 25 μl of packaging buffer to each tube.
3. Use a small spatula to scoop the material into a centrifuge tube. The material will be highly viscous.
4. Spin in ultracentrifuge at 45,000 rpm for 90 min at 4°C.
5. Remove supernatant using a serological pipette and add it into a chilled 50-ml tube.
6. Aliquot 45 μl into each Microfuge tube (~70 tubes) and freeze in liquid nitrogen. Store at −80°C.

Packaging extract B (NM 759 cell stock)

Day 1:

1. Using a sterile loop, streak 2-LB plates with frozen NM 759 stock cells (−80°C).
2. Incubate the plates overnight, one at 30°C and the other at 42°C.

Use the plate grown at 30°C for inoculation. This plate may be stored at 4°C and used for up to 1 week.

Check the plate grown at 42°C to ensure no growth has occurred. If it has, discard stock cells and re-streak plates with new stock cells.

Day 2:

1. Inoculate 30 ml of NZY media with a colony of NM 759 (30°C plate).
2. Grow overnight in a shaking incubator at 30°C and 265 rpm.

Day 3:

1. Inoculate one 1-l flask containing 500 ml of NZY media with 25 ml of the NM 759 overnight culture. Cap flasks with sterile foam stoppers.
2. Grow the culture in a shaking incubator at 32°C and 175 rpm until the optical density at wavelength 600 nm (OD_{600}) reaches 0.3 (~2.5–3 h).
3. Transfer the flask to a 65°C water bath. Swirl the flask and monitor the temperature in the flask until it reaches 45°C (~5 min).
4. Transfer the flask to a 45°C water bath for 15 min, swirling every 5 min.
5. Return the flask to the shaking incubator now set at 38–39°C, and shake for 2–3 h (do not exceed 40°C).
6. After approximately 2.5 h of incubation time, remove 2 ml of the cell suspension from the flask and aliquot 1 ml into each of 2 culture tubes. Add 3–4 drops of chloroform to one and see if it clears (compared to other tube) when incubated at 37°C for 2–3 min.
7. When the chloroform/cell suspension clears, aliquot flask contents into two 250-ml centrifuge bottles and centrifuge 3200 *g* at 4°C for 15 min.
8. Immediately decant supernatant, place bottles on ice, and dry inside of bottles with a lint-free towel. Avoid touching the pellet at the bottom of the bottle.
9. Vortex each bottle for a few seconds.
10. Pipette 3.6 ml sonication buffer into one bottle and vortex to resuspend pellet.
11. Using a 5-ml serological pipette, transfer the resuspended cells/sonication solution to the second bottle and vortex again.
12. Aliquot mixture into sterile Microfuge tubes (~4 tubes).
13. Adjust sonic dismembrator to setting 3 and sonicate each tube 8 times in 2-s bursts. Keep tubes on ice between bursts for at least 30 s. Samples should decrease in viscosity and foam. Air bubbles are acceptable at this stage.
14. Centrifuge tubes at 8000 *g* for 10 min at 4°C.
15. Transfer supernatant using a wide bore tip to a cooled 15-ml tube.
16. Add 1/2 volume of sonication buffer to supernatant.
17. Add 1/6 volume (original) of packaging buffer to supernatant.
18. Invert tube to mix and aliquot 60 μl of mixture into sterile Microfuge tubes (~90 tubes) using a wide bore tip.
19. Flash-freeze tubes in liquid nitrogen and store at −80°C.

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chapter thirty-nine

Improved methods of conducting microalgal bioassays using flow cytometry

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Contents

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Introduction

Microalgal toxicity tests have been widely used to evaluate the potential impact of contaminants and nutrient inputs in marine and freshwater ecosystems. Standard tests use population growth and measure chronic toxicity, i.e., the inhibition of cell division rate (growth rate) or cell yield over 48–96 h. To avoid losses of contaminants to the test containers or high cell numbers that may occur over this period, shorter tests that measure acute toxicity, e.g., enzyme inhibition, have also been developed. Tests based on inhibition of esterases, enzymes essential for phospholipid turnover in cell membranes, have been shown to be good surrogates for metabolic activity and cell viability.[1,2–4] However, most conventional techniques used to count and analyze cells in growth rate and enzyme inhibition tests suffer from several limitations.[5–7]

1. They use environmentally unrealistic high cell densities in order to obtain a measurable response, which may alter contaminant speciation, bioavailability, and toxicity.
2. They measure only one effect parameter (e.g., growth or enzyme activity at a time).
3. It is difficult to distinguish between live and dead cells.
4. Cells cannot be counted in the presence of particulates (i.e., the samples require filtering which may remove toxicants).
5. They are single species tests, lacking in environmental realism.

One technique that may be used to overcome these limitations, resulting in more environmentally relevant tests, is flow cytometry. Flow cytometry is a rapid method for the quantitative measurement of individual cells in a moving fluid. Thousands of cells are passed through a light source (usually a laser) and measurements of cell density, light scatter, and fluorescence are collected simultaneously. Although this technique has been widely applied to biomedical and oceanographic studies, flow cytometry has only recently been applied to ecotoxicology.[6–8] Microalgae are ideally suited to flow cytometric analysis as they contain photosynthetic pigments, such as chlorophyll *a*, which autofluoresce when exited by blue light. In addition, specific fluorescent dyes can be used and detected by flow cytometry to provide information about the physiological status of algal cells in response to toxicants.

Tests with both marine and freshwater microalgae have been developed using flow cytometry and applied to testing wastewaters, chemicals, and sediments.[8,9–13] In this chapter, we describe a chronic growth rate inhibition test using flow cytometry to count low algal cell densities and an acute enzyme (esterase) inhibition test.

Materials required

Test species

Any nonchain forming marine or freshwater microalga can be used as the test species. In this chapter, we describe two freshwater tests with the chlorophytes (green algae)

Pseudokirchneriella subcapitata (Korschikov) Hindák, 1990 (previously called *Selenastrum capricornutum*) and a tropical *Chlorella* sp. *P. subcapitata* was obtained from the American Type Culture Collection (ATCC-22662), Maryland, USA. *Chlorella* sp. was isolated from Lake Aesake, Strickland River, Papua New Guinea. This species is available from the Centre for Environmental Contaminants Research, CSIRO Energy Technology, Australia (contact: Jenny.Stauber@csiro.au).

Tests are also described for two marine algae (both pennate diatoms): a planktonic species *Phaeodactylum tricornutum* Bohlin and a benthic species *Entomoneis* cf. *punctulata* Osada and Kobayashi. *P. tricornutum* was obtained from the National Research Centre Istituto di Biofisica, Pisa, Italy, while *E.* cf. *punctulata* was isolated from Little Swanport, Tasmania, Australia, and provided by CSIRO Marine Research (strain No. CS-426).

Equipment:

- Flow cytometer (e.g., Becton Dickinson FACSCalibur TM, BD BioSciences, San Jose, CA, USA)
- Environmental chamber/incubator with built-in light and temperature controls and mechanical shaking platform
- Milli-Q® (Millipore) water purification system or equivalent
- Centrifuge (benchtop), 4 × 30 ml capacity with swing out buckets
- Refrigerator/freezer for storing stock solutions and reagents
- Biohazard cupboard or laminar flow cabinet for aseptic algal culturing
- Analytical balance
- Autoclave
- Adjustable pipettes (5 μl to 5 ml)
- Vortex mixer
- pH meter and buffers
- Light meter
- Conductivity meter
- Thermometer
- Filter apparatus, 47 mm filter holder, 1-l flask, vacuum pump, and tubing
- Magnetic stirrer

Supplies:

- Microalgae
- Fluorescent calibration beads for flow cytometer (e.g., CaliBRITE™ beads, BD BioSciences)
- TruCount™ tubes or equivalent flow cytometer counting beads
- Glass tissue homogenizer (hand-held, 15 ml) with Teflon pestle
- Glass centrifuge tubes, 30 ml capacity
- Glass Erlenmeyer flasks (200 or 250 ml) with loose-fitting glass caps
- Glass scintillation minivials (20–30 ml capacity) with plastic screw-on lids
- Glass volumetric flasks
- Glass beakers
- Glass graduated measuring cylinders
- Centrifuge tube rack
- Weighing trays and spatula
- Magnetic stirrer bars

- Polyethylene wash bottles and storage containers (1–10 l)
- Graduated glass (or sterile disposable serological pipettes) 2 ml
- Disposable glass Pasteur pipettes
- Disposable pipette tips
- Disposable plastic counting cups
- Membrane filter papers, 0.45-μm pore size
- Parafilm or equivalent laboratory sealing film
- Coatasil (AJAX) for silanizing glassware
- Disposable plastic counting tubes (12 × 72 mm Falcon tube or equivalent)
- Polycarbonate vials (50 ml capacity) used for chemical analysis
- Gloves
- Timer
- Seawater (filtered through an acid-washed Millipore HA 0.45-μm membrane filter and stored in polyethylene or glass containers at 4°C)

Chemicals and reagents:

- Fluorescein diacetate (FDA) (Sigma F-7378, dissolved in acetone)
- Propidium iodide (PI) (Sigma P-4170, dissolved in Milli-Q water)
- Salts for algal culture media and synthetic water (AR grade) (Tables 39.1 and 39.2)
- Acids and bases for pH adjustment (HCl, NaOH)
- Acetone (AR grade)
- $CuSO_4 \cdot 5H_2O$ (AJAX Chemicals)
- Nonphosphate detergent and HNO_3 for glassware washing

Procedures

All glassware used exclusively for toxicity testing was soaked in 10% (v/v) nitric acid (HNO_3) overnight and was thoroughly rinsed with Milli-Q water. All polycarbonate vials used for chemical analysis were acid-washed and rinsed in the same manner. General laboratory glassware was washed in a laboratory dishwasher (Lab999, Gallay) with a phosphate free detergent, followed by nitric acid (1%) and six rinses with Milli-Q water.

Maintenance of algal cultures

Freshwater algae

P. subcapitata stock cultures were maintained in clean 250-ml glass Erlenmeyer flasks in 100 ml USEPA medium with EDTA (Table 39.1).[14] Medium was prepared using Milli-Q water as the base and sterilized by filtering through a 0.45-μm sterile membrane filter. Stock cultures were maintained in a controlled environmental chamber on a 24-h light cycle (65 ± 5 μmol photons/m^2/s, cool white fluorescent lighting) at 24°C. Each week, fresh medium was inoculated with about 1 ml of algal stock culture under aseptic conditions in a biohazard cupboard. These cells were in late exponential phase growth and resulted in an algal cell density of the new stock of approximately 2–4 × 10^4 cells/ml for all species.

Chlorella sp. was cultured in one-fifth strength modified Jaworki's medium[15] (Table 39.1). The medium (in a Milli-Q water base) was sterilized by autoclaving (50 ml in 250-ml Erlenmeyer flasks, with loose-fitting glass lids). Stock cultures were maintained on a 12:12 h light/dark cycle (75 ± 5 μmol photons/m^2/s, cool white fluorescent lighting) at 27°C. Algae were subcultured weekly using the same procedure as for *P. subcapitata*.

Table 39.1 Algal culture medium for marine and freshwater toxicity tests

Chemical	EPA[a]	JM/5[b]	f/2[c]
Macronutrients (mg/l)			
$NaNO_3$	25.5	18	75
$MgCl_2 \cdot 6H_2O$	12.2	–	–
$CaCl_2 \cdot 2H_2O$	4.41	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	–	2.8	–
$MgSO_4 \cdot 7H_2O$	14.7	10	–
KH_2PO_4	1.04	2.5	–
K_2HPO_4	–	3.5	–
$NaHCO_3$	15.0	3.2	–
$NaH_2PO_4 \cdot 2H_2O$	–	–	5
$Na_2SiO_3 \cdot 5H_2O$	–	–	13
$C_6H_5O_7Fe \cdot 5H_2O$[d]	–	–	2.3
$C_6H_8O_7 \cdot H_2O$[e]	–	–	2.3
Micronutrients (μg/l)			
H_3BO_3	185	496	–
$MnCl_2 \cdot 4H_2O$	416	278	90
$ZnCl_2$	3.27	–	–
$CoCl_2 \cdot 6H_2O$	1.43	–	5
$CuCl_2 \cdot 2H_2O$	0.012	–	–
$Na_2MoO_4 \cdot 2H_2O$	7.26	–	–
$(NH_4)6MoO_4 \cdot 4H_2O$	–	200	–
$FeCl_3 \cdot 6H_2O$	160	–	–
$Na_2EDTA \cdot 2H_2O$	300	450	–
$FeNaEDTA \cdot 2H_2O$	–	450	–
$CuSO_4 \cdot 5H_2O$	–	–	4.5
$ZnSO_4 \cdot 7H_2O$	–	–	11
Thiamine HCl	–	8	100
Biotin	–	8	0.5
Vitamin B_{12}	–	8	0.5
pH	7.5 ± 0.1	7.3 ± 0.1	8.0 ± 0.2

[a] USEPA medium for *Pseudokirchneriella subcapitata*.

[b] One-fifth strength modified Jaworki's medium for *Chlorella* sp.

[c] Half-strength f media for *Entomoneis* cf. *punctulata* and *Phaeodactylum tricornutum*. Natural filtered seawater was used as the test medium base. Concentrations of ambient phosphate and nitrate in seawater <5 μg PO_4/l and <100 μg NO_3/l.

[d] Ferric citrate.

[e] Citric acid.

Marine algae

Both species were maintained in half-strength f medium (f/2).[16] Medium was prepared by adding all stock solutions, except phosphate, to 100 ml of 0.45 μm filtered clean seawater and sterilized by autoclaving. After cooling overnight, phosphate was added to give the final concentration shown in Table 39.1. A 1-ml algal inoculum was added to 100 ml of medium under aseptic conditions. The algae were subcultured weekly and maintained on a 12:12 h light/dark cycle (daylight, 65 ± 5 and 40 ± 5 μmol photons/m^2/s for *P. tricornutum* and *E.* cf. *punctulata*, respectively) at 21°C.

Table 39.2 Final concentration of inorganic components in *Chlorella* sp. synthetic softwater (80–90 mg $CaCO_3$/l) used for toxicity testing (U.S. EPA)[14]

Element	Concentration (mg/l)
Na	26
Ca	14
S	25
Mg	11
K	2.1
Cl	1.9
C	14

Preparation of algae for toxicity tests

Immediately prior to the test, the algal inoculum was prepared and used within 2 h. An exponentially growing stock culture (usually 4–5 days old) was decanted into two glass centrifuge tubes (about 25 ml in each) and centrifuged at low speed (~2500 rpm) for 7 min. The supernatant in each tube was poured off and the algal cell pellet gently resuspended in about 25 ml of Milli-Q water (freshwater algae) or filtered seawater (marine algae). The suspensions were mixed with a vortex mixer for several seconds and then centrifuged again. The centrifuging and rinsing process was repeated three times, resulting in a concentrated algal suspension that was diluted in about 15 ml of Milli-Q water or seawater, ready for counting and inoculating into the toxicity test containers.

Test sample preparation

Although toxicity tests described in this chapter were conducted with individual chemicals, some guidance on the procedure for handling and testing effluents, natural waters, groundwaters, and leachates is also given in the following.

Chemicals

Copper stock solutions were prepared by dissolving the metal salt in Milli-Q water and acidified to pH <2 by the addition of 10 ml HCl (Suprapur grade, Merck) per liter to enable storage and to reduce metal losses to the container walls. Neutralization was carried out by the addition of appropriate amounts of dilute NaOH when the pH of the test medium changed by more than 0.1 unit with the addition of the metals. Subsamples (5 ml) were immediately taken from each flask and replicates combined to give a total volume of 15 ml, which was acidified with 30 fl concentrated HNO_3 (Normatom). Samples were analyzed for total dissolved metals using appropriate analytical methods [e.g., graphite furnace atomic absorption spectrometry (GFAAS) and inductively coupled plasma atomic emission spectrometry (ICPAES)]. Measured, rather than nominal, metal concentrations were used to calculate toxicity indices.

For other chemicals, particularly those that degrade rapidly in aqueous solutions, stock solutions should be prepared fresh on the day of testing. For poorly water-soluble chemicals, mild heating, stirring, or ultrasonic dispersion may be sufficient. If carrier

solvents are necessary to dissolve chemicals that are poorly soluble in water, several additional steps are required. Firstly, the prepared stock solution must be diluted in solvent to give working stock solutions at each test concentration. Exactly the same volumes can then be taken from each of the working stocks and spiked into the appropriate test vessels, ensuring that the final concentration of solvent in the test solutions is less than the no-observable-effect concentration (NOEC) for that particular solvent and test species (e.g., 0.3% for ethanol for *P. subcapitata*). Secondly, a set of solvent controls must be included in the bioassay to account for any toxicity caused by the solvent alone. These are prepared by adding the same volume of solvent to diluent water as was added to the test solutions.

Effluents and natural waters

Complex effluents, natural waters, groundwaters, and leachates should be collected and/or extracted according to standard methods to ensure that the sample is representative. For complex effluents, either grab samples or 24-h composite samples are usually collected into prerinsed cleaned containers, preferably glass or polyethylene, filled with no headspace and transported at 4°C (not frozen). About 1 l of sample is sufficient for either the algal growth or esterase inhibition toxicity test. Effluents should be tested within 3 days of sample collection to avoid chemical and biological changes associated with effluent aging and storage.

On receipt of the sample, physicochemical measurements, including pH, conductivity/salinity, temperature, and dissolved oxygen, should be taken immediately. If the sample pH is outside the optimal pH range for the particular algal test species, it can be adjusted or left unadjusted, depending on the purposes of the test. If the conductivity of the sample is greater than 2000 μS and freshwater test species such as *P. subcapitata* are to be used, then the sample may need to be diluted with diluent (control) water. Alternatively, an additional set of controls, adjusted to the same conductivity/salinity as the sample, can be prepared and tested alongside the standard controls.

The sample may be filtered through either a GF/F filter (approximate pore size 0.7 μm) or a 0.45 μm cellulose acetate filter. Once filtered, the physicochemical parameters of the sample should be measured again. However, the advantage of the flow cytometry-based toxicity tests are that the samples do not have to be filtered prior to testing, Testing of unfiltered samples allows detection of toxicants associated with particulate material. However, while bacteria and other algae in the sample may be distinguished from the algal test species, they may alter the growth of the test algae and affect the toxic response. Careful interpretation of results from unfiltered samples is therefore required.

Preparation of test solution

All toxicity tests were conducted in 250-ml borosilicate glass Erlenmeyer flasks that had been precoated with a silanizing solution (Coatasil, AJAX) to reduce adsorption of contaminants, particularly metals, to the vessel walls. Alternatively, tests may be carried out in 30-ml glass minivials (scintillation vials) with plastic screw-on lids if only small contaminant volumes are available or due to space limitations (see the section ''Modification to standard flask bioassays using minivials'' in the following).

All marine toxicity tests were conducted in filtered seawater at a pH of 8.0 $\pm$ 0.2 and salinity of 34%. Fifty milliliters of seawater were added to each flask and supplemented with 0.5 ml of 26 m*M* sodium nitrate (15 mg NO_3^-/l) and 0.5 ml of 1.3 m*M* potassium

dihydrogen phosphate (1.5 mg PO_4^{3-}/l) in order to maintain exponential growth over 72 h.

P. subcapitata test medium (50 ml) consisted of the standard USEPA media (Table 39.1) without EDTA in order to reduce the possibility of metal complexation. However, without EDTA, iron in the medium was found to precipitate, leading to a minor decrease in dissolved copper in the test medium (NB: negligible losses at copper concentrations >20 μg/l). This medium had an alkalinity of 9 mg $CaCO_3$/l and water hardness of 15 mg $CaCO_3$/l.

For *Chlorella* sp., tests were conducted in a synthetic softwater[14] having a water hardness of 80–90 mg $CaCO_3$/l and an alkalinity of 54 mg $CaCO_3$/l. The synthetic softwater was prepared in 5-l volumes by weighing 0.30 g of $CaSO_4{\cdot}2H_2O$, 0.48 g of $NaHCO_3$, 0.30 g $MgSO_4{\cdot}H_2O$, and 0.02 g of KCl into a beaker of Milli-Q water. The solution was left overnight and then made up volumetrically to 5 l before filtering through a Millipore HA 0.45 μm membrane filter. The pH was adjusted to 7.5 ± 0.1 with 0.02 *M* NaOH or HCl. An aged and acid-washed 5-l plastic container was used to store the water at 4°C for no longer than 4 weeks before use. The final concentrations of inorganic components in the synthetic softwater are shown in Table 39.2. Fifty milliliters of synthetic softwater were supplemented with 0.5 ml of 26 m*M* sodium nitrate (15 mg NO_3^-/l) and 0.05 ml of 1.3 m*M* potassium dihydrogen phosphate (0.15 mg PO_4^{3-}/l).

All toxicity tests consisted of triplicate controls, together with at least five toxicant concentrations (also in triplicate). Test concentrations were chosen with the aim of encompassing a range of responses from 0% to 90–100% growth inhibition (or esterase activity) and were in a geometric series with a dilution factor of 2 (i.e., 2.5, 5, 10, 20, and 40 μg/l). Range-finding tests were initially performed for all species using widely separated concentrations of each metal to broadly estimate the IC_{50} for later definitive tests. All definitive tests were repeated at least three times.

Modification to standard flask bioassays using minivials

In our laboratory, we have conducted toxicity tests in both flasks and glass minivials (20–30 ml capacity). Minivials have the advantage that only small sample volumes are required, making it an ideal method for testing complex environmental samples (e.g., industrial effluents) that often need a time-consuming filtering step prior to testing. Due to the reduced size of the test vessel, the minivial bioassay also requires much less incubator space, allowing multiple tests to be conducted at the same time.

Test solutions for the minivial bioassay are prepared as mentioned above but with the following modifications:

- The test volume is reduced to 6 ml.
- Additional minivials are prepared (controls and diluent water) for physicochemical measurements at the beginning and end of the test and for chemical analysis, e.g., number of replicate vials/concentration = 6 vials (3 vials for daily cell counts, 2 vials for pH measurement, 1 vial for chemical analysis).

For chemical testing, the chemical is spiked directly into each test vial, ensuring that the spike volume is less than 60 μl.

Flow cytometric analysis

A variety of flow cytometers are currently on the market and suitable for analyzing microalgae. In this chapter, we describe the use of the BD-FACSCalibur (Becton Dickinson

BioSciences, San Jose, CA, USA) flow cytometer. It should be noted that between different flow cytometers the instruments settings and methods of analysis may vary.

The BD-FACSCalibur flow cytometer is a four-color, dual-laser benchtop instrument capable of both cell analysis and cell sorting. It is equipped with an air-cooled argon-ion laser providing 15 mW at an excitation wavelength of 488 nm (blue light) and with standard filter set up. Dual excitation is possible as it also has a diode capable of excitation in the red region of the spectrum.

The instrument has two light-scatter detectors, which serve to identify the morphology of the cell. The forward angle light scatter (FSC < 15°) detector provides information on cell size, while the side angle light scatter (SSC, 15–85°) detector provides information on internal cell complexity/granularity. Fluorescence is collected at a range of wavelengths by photomultiplier tubes (PMTs) with different fluorescence emission filters. Chlorophyll *a* or autofluorescence (present in all algae) is detected as red fluorescence in greater than 600-nm-long pass filter band (FL3). Green fluorescence from cells stained with FDA is collected in the FL1 channel (530 $\pm$ 15 nm) and orange fluorescence from cells stained with PI is collected in the FL2 channel. Cells are presented to the flow cytometer and are hydrodynamically focused in a sheath fluid as they pass through the fluorescence and light-scatter detectors. Sheath fluid was high purity Milli-Q water.

Data were displayed and analyzed using the flow cytometric software package CellQuest™ Pro in one-dimensional histograms (256 channels) and two-dimensional dot plots (comprising 64 $\times$ 64 channels) based on a combination of fluorescence and light-scatter signals. Although flow cytometry is able to count cells at very low cell densities, a minimum of 1000 events (cells) per sample should typically be analyzed to achieve a CV of less than 10%.[17] For bioassays using cell inocula of 1×10^4 cells/ml, a preset acquisition time of 120 s was used to obtain sufficient cell numbers. However, at recommended low initial cell densities of 10^2 to 10^3 cells/ml, longer acquisition times (e.g., 300 s) were required. To avoid unnecessarily long counting times when cell numbers have increased over the course of the bioassay, a feature of this flow cytometer allowed data acquisition to be stopped when the number of cells (events) in a specified region (e.g., *Chlorella* sp. cells) reaches a value greater than 1000. All parameters were collected as logarithmic signals and analysis performed at a high flow rate (60 µl/min).

As a quality control procedure, the flow cytometer was calibrated daily using flow cytometer standardization CaliBRITE™ 3 beads (BD BioSciences) or equivalent, to verify instrument performance. Because CaliBRITE beads set up the cytometer for human cell analysis, it was necessary to optimize (adjust) the instrument settings for analyzing microalgae. Typical instrument settings used for analyzing the marine and freshwater microalgae presented in this chapter are listed in Table 39.3. A detailed description of the initial set-up, calibration, and analysis of cells using flow cytometry can be found in the FACSCalibur instrument manual and CellQuest Pro and FACSComp™ software manuals (BD Bioscience).

Protocol 1: Chronic growth rate inhibition test

The growth rate inhibition test measures the decrease in algal growth rate over 72 h. Exponentially growing cells are exposed to various concentrations of the toxicant over several generations under defined conditions. A comparison of growth rates in the controls and the test-exposed algae enables typical toxicity test endpoints, such as the IC_{50}, NOEC, and lowest-observable-effect concentration (LOEC), to be determined. One unique feature

Table 39.3 Flow cytometry instrument settings for the analysis of microalgae

Parameter	Setting			
Threshold				
Primary parameter	LS2	Value	200[a]	
Secondary parameter	None	Value	—	
Compensation				
FL1	0.0% of FL2			
FL2	0.0% of FL1			
FL2	0.0% of FL3			
FL3	0.0% of FL2			
Detectors/amps		Voltage	AmpGain	Mode
P1	FSC	E-1	3.93	Log
P2	SSC	320[b]	1.00	Log
P3	FL1	470[b]	1.00	Log
P4	FL2	470[b]	1.00	Log
P5	FL3	370[b]	1.00	Log
P6	FL1-A	—	1.00	Lin
P7	FL1-W	—	1.00	Lin
Four color	OFF			

[a] The threshold must remain on the left of the distribution of algal cells to ensure that all algal cells are captured for analysis.

[b] These values are a guide only. The operator can adjust these values to alter the position of the algal distribution along the FSC, SSC, FL1, FL2, and FL3 axis. Analyzing healthy cells harvested from a stock culture is the best way to gauge this movement. Generally, algal populations are positioned in the center of the FSC, SSC, FL2, and FL3 axis so that shifts (both increase and decrease in intensity) can be observed. For the FL1 axis it is necessary to position the algal distribution (unstained cells) in the first decade of the logarithmic scale so that high fluorescence intensities can be observed when cells are stained with FDA.

of this bioassay is that the initial inoculum can be lowered to 100 or 1000 cells/ml using the technique of flow cytometry, which is more representative of cell numbers in aquatic systems. This prevents changes in toxicant speciation and subsequent bioavailability, which can lead to an underestimation of toxicity in standard bioassays.

Day 0 (i.e., starting day of test $t = 0$ h):

1. The concentrated algal inoculum was prepared as described earlier.
2. For those algal species prone to clumping (e.g., *P. tricornutum* and *E.* cf. *punctulata*), a hand-held glass tissue grinder with Teflon pestle was used to separate clumps in the resuspended cells. Preliminary studies have shown that this does not cause any rupture or damage to these species (checked microscopically). The cell density of the algal suspension was determined by adding a known volume (x μl) to a flask containing 50 ml of the appropriate test medium and counted by flow cytometry (described in the following). From this ''counting flask,'' the volume of algal inoculum required to give an initial cell density of 10,000 cells/ml (standard test) or 1000 cells/ml in low cell density test was determined.
3. Test solutions were prepared as described earlier and left at room temperature.
4. Test flasks were inoculated with the appropriate amount of prewashed algal cells (see the previous section ''Preparation of algae for toxicity testing'') ($2–4 \times 10^3$ cells/ml recommended for low cell density test). To ensure that the suspension remained homogenous, the concentrated algal suspension was vortexed between

every 3 and 4 inoculations. The volume of test inoculum added to each vessel did not exceed 0.5% of the total volume in the vessel.

5. The flasks were placed randomly in an environmental cabinet at the specified test conditions for each species (Table 39.4).

NB: For the minivial growth inhibition bioassay, minivials are placed in racks on an electronic shaker in an environmental cabinet set at the conditions defined in Table 39.4 and left shaking for 72 h at 100 rpm.

Days 1–3:

1. Each flask was gently agitated by hand twice daily throughout the test to avoid gas limitation. This was done by swirling the solution approximately six times in the clockwise direction and six times in the anticlockwise direction.
2. A subsample (1.0 ml) from each flask was taken for counting by flow cytometry at 24, 48, and 72 h after test commencement. Cell counts recorded at the end of the 48-h period were designated as Day 2 and at the end of the 72-h period as Day 3 observations. The pH, temperature, and conductivity of one replicate flask at each test concentration were measured and recorded at the end of the test (Day 3).

Determination of cell counts by flow cytometry

For most commercially available flow cytometers, absolute cell counts are obtained by adding a known amount of reference beads into the sample (ratiometric counting). By comparing the algal cell count with the bead count, the cell concentration can be calculated. This approach has been shown to be accurate, particularly when using primary reference bead solutions (i.e., Becton Dickinson TruCount tubes, described in the following) and also provides an internal standard that can be used to assess the performance of

Table 39.4 Culture conditions for algal stock culture maintenance and toxicity tests

Alga	Temperature (°C)	Light[a] (μmol photons/m^2/s)	Light/dark cycle (h)	Culture medium
Stock maintenance				
Chlorella sp.	27	75	12:12	JM_5
P. subcapitata	24	65	24:0	U.S. EPA
P. tricornutum	21	65	12:12	f_2
E. cf. *punctulata*	21	40	12:12	f_2
Toxicity tests[b]				
Chlorella sp.	27	140	12:12	Synthetic softwater + 15 mg/l NO_3 + 0.15 mg/l PO_4
P. subcapitata	24	65	24:0	U.S. EPA medium (without EDTA)
P. tricornutum	21	140	12:12	Filtered seawater + 15 mg/l NO_3 + 1.5 mg/l PO_4
E. cf. *punctulata*	21	140[c]	12:12	Filtered seawater + 15 mg/l NO_3 + 1.5 mg/l PO_4

[a] Philips TL 40 W cool white fluorescent lighting.

[b] Suitable pH ranges for growth inhibition bioassays with each species are: pH 6.0–8.0 for *Chlorella* sp; pH 5.3–8.7 for *P. subcapitata*; pH 7.5–8.5 for *E.* cf. *punctulata*; pH 7.0–9.0 for *P. tricornutum*.

[c] Acute esterase inhibition bioassay conducted at a reduced light intensity of 1–4 μmol photons/m^2/s.

the instrument (e.g., standardization of light scatter and fluorescence). Unfortunately, this counting method adds additional cost in consumables (e.g., fluorescent beads) and requires extra manipulations/calculations. In our laboratory, we have also used a Bio-Rad Bryte HS flow cytometer, which is particularly simple to operate and inexpensive to count as the instrument takes a known sample volume, so direct algal counts are possible without the need for internal calibration with beads. Unfortunately, this instrument has been discontinued, so protocols with this instrument were not described in this chapter. Alternatively, the flow rate of the instrument could be calibrated daily; however, this approach would require a very stable instrument and the accuracy of this method would need to be verified.

Before daily counting, the test flasks were well mixed. For bioassays with *P. tricornutum* and *E. punctulata*, a disposable pipette tip was used to scratch the bottom of the flasks to remove cells that had adhered to the test vessel. An aliquot of cells (1 ml) was immediately taken and placed directly into a TruCount tube.* The tube was mixed well and checked for the presence of air bubbles under the metal retainer, which can interfere with the analysis (air bubbles are removed by gentle tapping on the tube). To ensure that accurate counts were obtained, all pipettes used for dispensing solutions were calibrated prior to use.

The sample was acquired on the flow cytometer using the appropriate instrument settings for each species (see Table 39.3). The data were analyzed by drawing a gate around the TruCount bead population (see Figure 39.1A) from a dot plot of FL1 versus FL2. This population was then removed (i.e., gated out) from a new plot of FL3 versus SSC to obtain the algal population alone (Figure 39.1B). The region statistics (Figure 39.1) were used to determine the number of bead and algal events within each region.

The absolute number of algal cells in the sample was calculated using the following equation:

$$\frac{\text{No. events in region containing cell population}}{\text{No. events in bead region}} \times \frac{\text{No. beads per test}}{\text{test volume}} = \text{concentration of algal population} \tag{39.1}$$

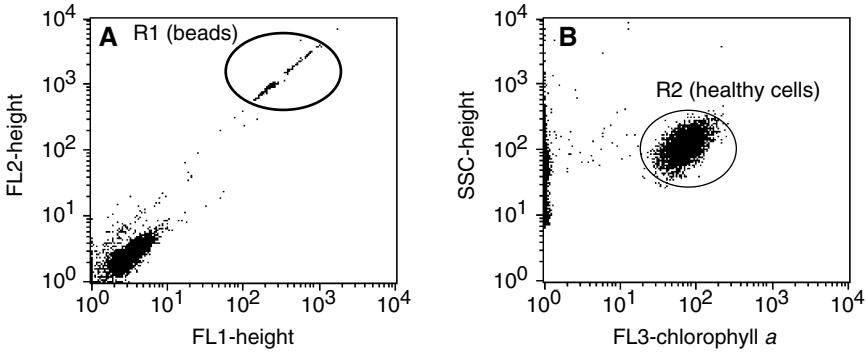
(The number of beads in each pellet (beads per test) varies among lots and is printed on the foil pouch.)

Modification of the counting method using a diluted TruCount tube bead stock

To help reduce costs associated with running the bioassay, we have included a modification to the method of counting cells based on diluting TruCount tubes, e.g., rather than using a new TruCount tube for every sample, the TruCount tube becomes a bead stock solution, which is then added to the algal sample to be counted.

The fluorescent bead stock solution is prepared by pipetting 1 ml of Milli-Q water or filtered seawater directly into a TruCount tube containing a known quantity of fluorescent beads. The tube is then mixed thoroughly and 200 μl of bead stock added to a sample tube (12 × 72 mm Falcon tube or equivalent) containing an appropriate aliquot of algal sample (e.g., 0.5 ml). It is recommended that the pipette tip be rinsed with the sample

* Becton Dickinson TruCount Absolute Count Tubes contain a lyophilized pellet of 4.2-μm fluorescent-dyed beads. The pellet is restrained in the bottom of the tube by a stainless-steel retainer. Store tubes in the foil pouch at room temperature and use within 1 h after removal from the foil pouch. Reseal foil pouches immediately after each use. Once the pouch has been opened, the tubes are stable for 30 days.



x parameter: FL1-height (log)
y parameter: FL2-height (log)

region	events
beads (R1)	1283

x parameter: FL3-chlorophyll *a* (log)
y parameter: SSC-height (log)

region	events
beads (R1)	0
algal cells	5993

Cell density calculation:

$$\frac{5993}{1283} \times \frac{10{,}489}{0.7} \times 1.29 \text{ (dilution factor)} \div 10{,}000 = \mathbf{9.0 \times 10^4 \text{ cells/ml}}$$

Figure 39.1 Determination of absolute cell counts of *Chlorella* sp. using diluted TruCount tubes (200 μl spike). (A) Dot plot of FL1 versus FL2 fluorescence identifying bead population (R1). (B) Dot plot of FL3 (chlorophyll *a*) versus SSC (side-angle light scatter/cell complexity) identifying healthy algal population.

solution by taking up and dispensing the solution several times. It should be noted that the high precision and accuracy of this counting method is limited only to the microalgae and bead pipetting step. Absolute cell counts are determined using Equation (39.1), with the exception that the number of beads per test is now the number of beads in 200 μl of the bead stock solution (e.g., 52,445 beads in 1 ml = 10,489 beads in 200 μl), and the test volume is increased to include the total volume of the sample (e.g., 0.5 ml algae + 0.2 ml bead spike). A dilution factor is also required to account for the bead volume. At least 1000 bead events must be acquired to ensure the accuracy of this counting method.

Statistical analysis of test data

The growth rate (cell division rate) of algae in each flask over 72 h was calculated using linear regression analysis. A regression line was fitted to a plot of the $\log_{10}$ cell density for each replicate versus time (h). The slope of the regression line is equivalent to the cell division rate per hour (μ) for each treatment. Daily doubling times were calculated by multiplying this value $\mu \times 24 \times 3.32$ (constant). Growth rates of the treated flasks were presented as a percentage of the control growth rate. A concentration–response curve was obtained by plotting the percentage control growth rates versus the measured toxicant concentrations.

The endpoints of the algal growth inhibition test were the 72 h IC_{50}, NOEC, and LOEC. The 72 h IC_{50} is the inhibitory toxicant concentration that gives 50% reduction in algal growth rate over 72 h compared to the controls. IC_{50} values were calculated using a computer program, NYHOLM-3 (modified by Yuri Tsvetnenko from Nyholm et al.[18]).

Calculations were carried out assuming that the concentration–response can be described by the probit function and were performed using weighted linear regression analysis on probit transformed data. Alternatively, the IC_{50} value can be calculated by linear interpolation using commercially available software, such as ToxCalc (Tidepool Software).

A hypothesis-testing approach was used to determine which metal-exposed treatments were significantly different from the controls. Data were initially tested for normality and homogeneity of variance, and Dunnett's Multiple Comparison test (ToxCalc Version 5.0.14, Tidepool Software) was used to estimate the NOEC and LOEC values. The NOEC is the highest tested metal concentration that yielded no statistically significant deviation from the control. The LOEC is the lowest tested metal concentration to yield a statistically significant deviation from the control.

Acceptability of the test

The data were considered acceptable if the growth rate of the control algae increased by a factor of 16 after 72 h, corresponding to a specific growth rate of 0.9/day. In addition, variability in the growth rate of controls should not exceed 10%, and the recorded temperature and water quality parameters throughout the test must be within acceptable limits, with a pH change of not more than 1.5 pH units. For metal toxicants, pH changes of less than 0.5 pH units are desirable. Each test should also include a reference toxicant, e.g., copper, to ensure that the algae are responding in a reproducible way to a known toxicant (i.e., IC_{50} within 2 SD of the mean IC_{50}).

Protocol 2: Acute esterase inhibition tests with P. subcapitata *and* E. *cf.* punctulata

This acute enzyme toxicity test uses flow cytometry to measure inhibition of the enzyme esterase after a 3- or 24-h exposure to toxicant. After this time, the substrate FDA is added, and the cells incubated for 5 min, followed by flow cytometric analysis. Healthy control cells take up FDA, which is cleaved by esterases, releasing a fluorescent product fluorescein that is retained in the cells. This is measured as an increase in algal cellular fluorescence in the green region of the spectrum. Toxicants decrease FDA cleavage by esterases and subsequent green fluorescence, and this is measured as a shift in fluorescence from the healthy control region towards the unhealthy (dead) cell region. This shift, detectable by flow cytometry, is quantified and the percentage of shift out of the control region is calculated and used for determination of EC_{50}, NOEC, and LOEC values.

The test set up and exposure conditions for the acute algal esterase tests was similar to the growth rate bioassay protocol with two exceptions. For toxicity tests with *P. subcapitata*, the starting inoculum used was 1×10^4 cells/ml ($\pm$ 10%), and the test media was adjusted to a pH of 7.8–8.5 prior to use. Acute tests using the benthic marine alga *E.* cf. *punctulata* had a starting inoculum of 2–4 $\times$ 10^4 cells/ml and were conducted at a low light intensity of 1–4 μmol photons/m^2/s throughout the 3- and 24-h exposure period.

Analysis of cellular esterase activity

A 1-m*M* stock solution was prepared daily by weighing 0.0104 g of FDA into a small glass weighboat and rinsed into a 25-ml volumetric flask with acetone. The dissolved FDA solution was made up to volume with acetone and stored at 4°C.

After a 3- and 24-h exposure, a subsample (4.88 ml) from each flask (control and test concentrations) was transferred into 20-ml glass scintillation vials. An aliquot of the FDA

stock solution (125 μl) was added to each vial to give a final FDA concentration of 25 μM and incubated for 5 min prior to flow cytometric analysis for 120 s.

Flow cytometric analysis was similar to that used for the algal growth rate bioassay. After identifying and capturing the algal population on the FL3 versus SSC cytogram, shifts in algal esterase activity were determined by using the histogram of cell number versus FL1 fluorescence gated on the algal population. For each toxicant concentration, the data were expressed as the difference in the percentage of cells falling into three defined metabolic activity states (S1, S2, S3), obtained by merging reference histograms of FDA-stained control and heat-treated samples (see the section ''Negative controls'' in the following). Region S2 was defined manually around the normal distribution of control healthy cells (stained with FDA) and incorporated greater than 90% of the cells. The percentage of cells falling into regions S1 (decreased FL1 fluorescence) and S3 (enhanced FL1 fluorescence) were recorded and expressed as a percentage decrease in S2 compared to a control according to the following equation:

$$(100 - \%S1_t) \div (100 - \%S1_c) \times 100 = \%\ \text{control} \tag{39.2}$$

where $\%S1_t$ is the percentage of treated cells in S1 and $\%S1_c$ is the percentage of control (untreated) cells in S1.

Negative controls

Healthy unstained cells and FDA-stained deactivated cells were included as negative controls. The esterase enzyme in algal cells was deactivated by heat treatment (10 min in a water bath at 100°C) or by the addition of formalin (final concentration of 4% v/v). The FL1 (FDA) fluorescence intensity of the negative controls should fall in the S1 region of the histogram (ideally in the first decade of the log scale). To ensure that shifts in FL1 fluorescence intensity and hence changes in cellular esterase activity could be observed, the algal distribution of the FDA-stained controls and negative controls should not overlap by more than 10%.

Statistical analysis of test data

The percentage of control data was used for all calculations. The EC_{50} value (concentration to cause a 50% inhibition in esterase activity), NOEC, and LOEC values were calculated using the same statistical methods used for the growth rate inhibition test.

Modification of esterase test set up for a minivial assay

Each test concentration was prepared by dispensing 100 ml of test media (Table 39.4) into 120-ml polycarbonate vials and spiked with the copper stock solution to give test concentrations of 5–300 μg Cu/l. The pH of each test concentration was measured and adjusted, if necessary, with NaOH or HCl to within the pH range specified in Table 39.5. Ten milliliters of each solution were dispensed into 20-ml glass scintillation vials coated with a silanizing solution, in triplicate. Controls consisting of test media were also included in triplicate. Two vials were prepared for each replicate, one vial for 3-h FDA analysis and one vial for 24-h analysis. One additional vial per test concentration was prepared for pH measurements throughout the test. Each vial was inoculated with a washed algal inoculum to give a final cell density of 1×10^4 cells/ml ($\pm$ 10%) and 2–4 $\times$ 10^4 cells/ml for *P. subcapitata* and *E.* cf. *punctulata*, respectively. The vials were loosely capped and incubated under standard conditions specified in Table 39.5.

Table 39.5 Test conditions for esterase inhibition bioassays with *Pseudokirchneriella subcapitata* and *Entomoneis* cf. *punctulata*

Parameter	*P. subcapitata*	*E.* cf. *punctulata*
Test media	USEPA media (without EDTA)	Filtered seawater + 15 mg/l NO_3 + 1.5 mg/l PO_4
Acceptable pH range	7.8–8.5	6.5–8.5
Acceptable salinity range	NA[a]	15–35%
Initial cell density	1×10^4 cells/ml ± 10%	2–4 $\times 10^4$ cells/ml
Exposure conditions	24°C, cool-white continuous lighting 65 μmol photons/m^2/s	21°C, daylight fluorescence lighting, 1–4 μmol photons/m^2/s 12 h light:12 h dark

[a] Not applicable.

Propidium iodide staining

To ensure that a decrease in FDA fluorescence was due to an effect on esterases within the cell, and not due to membrane disruption (i.e., reduced uptake of the dye/reduced retention inside the cell), the nucleotide-binding stain PI was used to assess membrane integrity.[10] A fully intact membrane is impermeable to PI, and DNA will only be stained in cells that are dead or that have compromised membranes. After a 3- to 24-h exposure, a separate sample of toxicant-exposed cells was therefore analyzed for changes in membrane permeability using PI and flow cytometry. Dual staining (FDA and PI in the same sample at the same time) was not performed in this bioassay but is possible when fluorescence compensation is used to eliminate spectral overlap in the dyes.

A stock solution of 100 μ*M* PI (Sigma P-4170 in Milli-Q water) was added to 5 ml aliquots of cells, to give final concentrations 7.5 and 120 μ*M* PI, suitable for *P. subcapitata* and *E.* cf. *punctulata*, respectively. After a 5-min incubation time, the sample was analyzed by flow cytometry, with PI detected as orange fluorescence emission (FL2). Cells with compromised membranes have a higher orange fluorescence than healthy intact cells, and this is seen as a shift to the right on a histogram plot of count versus FL2. If no cells have higher FL2 fluorescence, then any decrease in FDA fluorescence in FL1 is due to true inhibition of esterase activity. Cells killed by heat treatment (100°C for 10 min) or formaldehyde treatment (4% for 24 h) should be included in each experiment as negative controls.

Results and discussion

We have used flow cytometry-based protocols described in this chapter to examine the toxicity of copper to four species of microalgae. Through the ability to gate on chlorophyll *a* fluorescence (FL3), dead cells could easily be distinguished from live cells, so that only healthy cells were included in cell counts used to determine inhibition of cell division rate. This technique is therefore more accurate than automatic particle counters (e.g., Coulter counters) routinely used for algal growth tests, which often include dead cells and particulate matter in cell counts.

A major advantage of flow cytometry for growth inhibition tests is the ability to analyze and count low algal cell densities, more typical of algal concentrations in natural aquatic systems. Most standard test protocols use high initial cell densities of 10^4 to 10^5 cells/ml in order to obtain a measurable algal response. However, at these cell densities,

significant adsorption of the test substance to the rapidly growing algal biomass may occur.[19,20] Algal metabolism at high cell densities can also cause drifts in pH due to CO_2 depletion and subsequent chemical alteration of the test substance.[21] Increased exudate production from an increasing number of cells may also influence chemical speciation through the formation of nontoxic metal–exudate complexes.[22] With the move towards miniscale tests to reduce costs,[23,24] surface adsorption losses and use of high cell densities may be even more problematic.

The effect of increasing initial cell density from 10^2 to 10^5 cells/ml on the toxicity of copper to *Chlorella* sp. and *P. subcapitata* is shown in Figure 39.2. As the initial cell density increased, the toxicity of copper to each species decreased (e.g., the curve shifts to the right). This corresponded to a significant decrease ($p < 0.05$) in the 72-h IC_{50} values for copper at initial cell densities of 10^4 and 10^5 cells/ml, compared to those at 10^2 and 10^3 cells/ml (Table 39.6). For *Chlorella* sp., the NOEC values at high initial cell densities (10^4 and 10^5 cells/ml) (4.7 and 9.0 μg Cu/l, respectively) were higher than the 72-h IC_{50} values obtained at the lower initial cell densities (4.4 and 4.6 μg Cu/l for 10^2 and 10^3 cells/ml, respectively).

Similar effects of reduced toxicity with increasing algal cell densities have been reported for a number of contaminants, including copper, cadmium, and lead.[25,26] Measured concentrations of extracellular and intracellular copper at 10^3, 10^4, and 10^5 cells/ml were determined by washing cells in dilute EDTA to remove extracellular copper.[9,27] As cell density increased, less copper was bound to the cells, leading to less copper uptake into the cell and consequently less disruption of cell division. Decreased copper toxicity at higher cell densities was primarily due to greater copper adsorption by algal cells and exudates, resulting in depletion of dissolved copper in solution.[9]

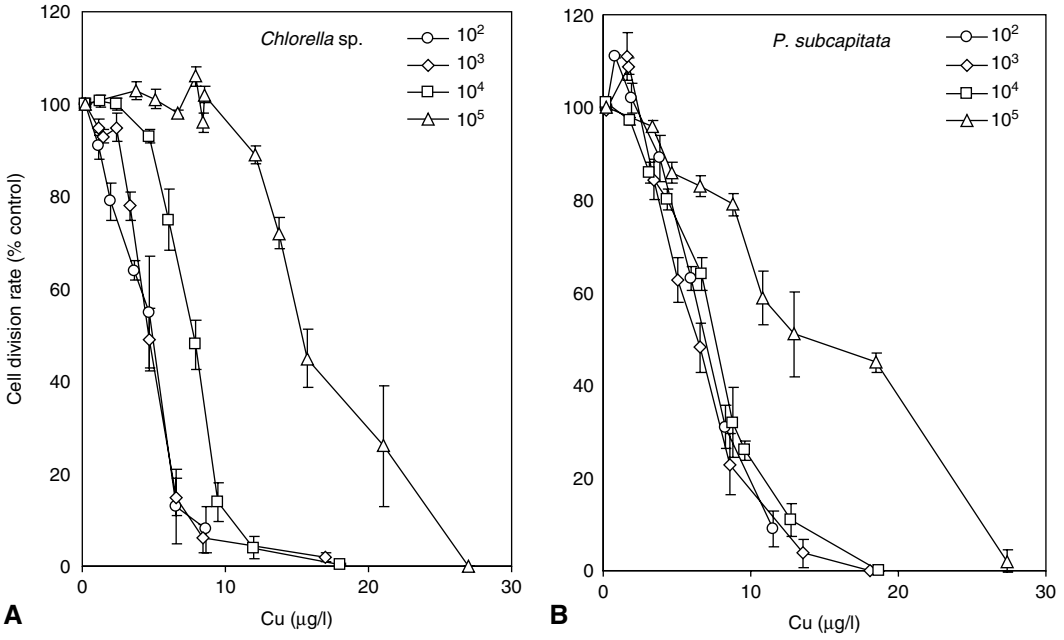


Figure 39.2 Growth inhibition of *Chlorella* sp. and *Pseudokirchneriella subcapitata* exposed to copper for 72 h at initial cell densities of 10^2, 10^3, 10^4, and 10^5 cells/ml. (From Franklin, N.M., Stauber, J.L., Apte, S.C. and Lim, R.P., *Environ. Toxicol. Chem.*, 21, 742–751, 2002.)

Table 39.6 Effect of initial cell density on the toxicity of copper to *Chlorella* sp. and *Pseudokirchneriella subcapitata* after a 72-h exposure. (Modified from Franklin, N.M., Stauber, J.L., Apte, S.C. and Lim, R.P., *Environ. Toxicol. Chem.*, 21, 742–751, 2002.)

Initial cell density (cells/ml)	*Chlorella* sp.			*P. subcapitata*		
	NOEC (μg/l)	LOEC (μg/l)	IC_{50} (μg/l)	NOEC (μg/l)	LOEC (μg/l)	IC_{50} (μg/l)
10^2	1.1	2.0	4.6[a] (3.5–6.0)	1.9	3.8	6.6[de] (5.9–7.3)
10^3	2.4	3.3	4.4[a] (3.9–5.0)	3.4	4.8	6.2[d] (5.5–6.9)
10^4	4.7	6.0	7.3[b] (6.7–8.0)	1.8	3.2	7.5[e] (6.8–8.2)
10^5	9.0	12	16[c] (14–18)	4.6	6.6	17[f] (14–20)

Superscript letters ''a–f'' denote whether IC_{50} values are significantly ($p < 0.05$) different from each other, i.e., same superscript means no significant ($p > 0.05$) difference.

The finding of decreased toxicity with increasing initial cell densities has important ramifications for static laboratory bioassays with microalgae, suggesting that the algal biomass itself may alter the concentration and speciation of copper in the test solution, leading to an underestimation of copper toxicity. While our research has focused on copper, the same limitations may apply to algal bioassays with other metals. It is therefore a key recommendation that initial cell densities used in standard growth bioassays do not exceed 2 to 4×10^3 cells/ml. In this way, metal bioavailability determined in laboratory algal bioassays should more closely estimate metal bioavailability in natural aquatic systems.

In addition to counting algal cells, the ability of flow cytometry to analyze algal light scatter and fluorescence properties can be exploited as alternative endpoints for acute and chronic toxicity tests.[6,10] Although chronic test endpoints are preferred due to their sensitivity and direct relevance to ecosystems, acute tests are often useful due to their rapid nature and low cost. In particular, chronic algal bioassays can suffer from changing contaminant concentrations over time (as described earlier) making them difficult to interpret.[28,29] By limiting exposure to only a few hours in short-term toxicity tests, such as the enzyme inhibition bioassay described in this chapter, these test limitations can be largely overcome. The esterases involved in the FDA bioassay turn over on a time frame of several hours,[30] making the technique suitable for monitoring short-term algal responses to environmental contaminants.[31]

Esterase activity, measured using FDA and flow cytometry, was a sensitive sublethal indicator of copper toxicity in *P. subcapitata* and *E.* cf. *punctulata* (Table 39.7). As copper concentrations increased, esterase activity decreased in a concentration-dependent manner, and this was seen as a shift in cells from S2 (control region) to S1 on a histogram of log fluorescein fluorescence (Figure 39.3). For *E. punctulata*, greater than 50% of cells had shifted into the lower esterase state (S1) after 24-h at a copper concentration of 85 μg/l, corresponding to an EC_{50} value of 63 $\pm$ 30 μg/l (Figure 39.3; Table 39.7). As little as 40 μg Cu/l caused a significant reduction in FDA fluorescence over the same exposure time (i.e., NOEC = 21 μg/l). The freshwater alga, *P. subcapitata*, showed similar sensitivity to copper with a 24-h EC_{50} value of 51 μg/l. PI staining for both species showed that high concentrations of copper did not significantly alter membrane integrity over 3- to 24-h copper exposures, indicating that the decreased FDA fluorescence was due to true inhibition of intracellular esterases.

Table 39.7 Inhibitory effect of copper on cell division rate and esterase activity (FDA fluorescence) in marine and freshwater algae using flow cytometry. (The data in this table is a compilation of published data from Franklin, N.M., Stauber, J.L. and Lim, R.P., *Environ. Toxicol. Chem.*, 20, 160–170, 2001a; Franklin, N.M., Adams, M.S., Stauber, J.L. and Lim, R.P., *Arch. Environ. Contam. Toxicol.*, 40, 469–480, 2001b; except the esterase data for *Entomoneis* cf. *punctulata*, which are unpublished.)

Test endpoint	Exposure time (h)	I/EC_{50}[a] (μg/l)			
		Chorella sp.	*P. subcapitata*	*E. punctulata*	*P. tricornutum*
Cell division rate[b]	48	6 ± 2[c]	6 ± 2	17 (15–20)	9 ± 3
	72	8 ± 2	8 ± 2	10 ± 4	10 ± 4
Esterase activity (FDA fluorescence inhibition)	3	—	112 (88–143)[d]	173 ± 80	—
	24	—	51 (38–70)	63 ± 30	—

[a] Inhibitory or effective concentration giving 50% effect or affecting 50% of cells.

[b] Tests conducted with initial cell density of 2 to 4 × 10^4 cells/ml.

[c] Mean ± SD (n = 3–13).

[d] 95% confidence limit (n = 6–30).

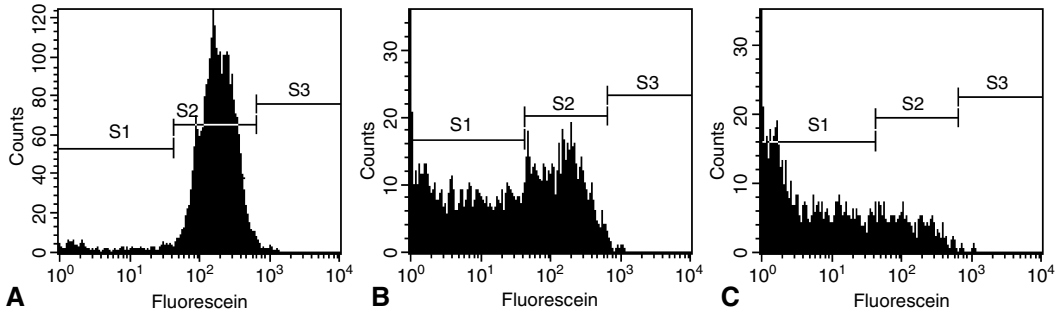


Figure 39.3 Flow cytometric histogram showing shifts in esterase activity (FL1 fluorescence) of *E.* cf. *punctulata* at copper concentrations of 0, 85, and 250 μg/l after a 24-h exposure.

For *E.* cf. *punctulata* and *P. subcapitata*, the FDA inhibition bioassay was less sensitive (~2-fold) after only a 3-h exposure and considerably less sensitive than chronic cell division rate over 48–72 h (Table 39.7). Gala and Giesy,[2] in assessing the toxicity of the surfactant SDS, also found that the 24-h EC_{50} value for cell viability (using FDA) was significantly greater than the 96-h EC_{50} value for specific growth rate. This is not unexpected as this is comparing an acute effect with a chronic effect, and EC_{50} values typically decrease with increasing exposure time. One area where acute enzyme endpoints are particularly useful is assessing the sensitivity of contaminants in sediments. In this situation, growth is often stimulated by ammonia release from sediments, which can mask the toxic effects of contaminants. By contrast, FDA fluorescence is rarely stimulated and is therefore a more suitable endpoint for whole sediment and porewater tests than growth rate.[8]

Analysis of fluorescein fluorescence by flow cytometry, rather than fluorescence microscopy or fluorimetry, has improved the FDA method by providing a rapid and highly precise means of detecting the fluorescence of individual cells.[32] The cell-level

perspective provided by flow cytometry offers more detailed information on cell physiology, which may be obscured if only the average characteristics of samples are measured (population-based endpoints assume that each cell behaves in an identical manner). With flow cytometry, cell functions are determined at conditions close to the *in vivo* state, without prolonged exposure to unusual light levels, and without the need for extractions of fluorescent products from cells in solution. In contrast to fluorescence microscopy, flow cytometry can not only distinguish labeled from nonlabeled cells but also estimate the fraction of the population with a positive reaction to the substrate. It can also provide a quantitative measure, in terms of relative fluorescence, of the rate at which the cells process the substrate (i.e., kinetics of reactions).[31] These advantages also apply to measurement of chlorophyll *a* or other fluorescence properties, not just FDA fluorescence.

Although not described in this chapter, another example of how flow cytometry is being used to improve the environmental relevance of laboratory toxicity testing is the development of multispecies algal bioassays. In aquatic systems, algae rarely occur in single species populations (except in algal blooms) but as mixtures of different species. In our laboratory, multispecies tests have recently been developed using mixtures of three freshwater (*Microcystis aeruginosa*, *P. subcapitata*, and *Trachelomonas* sp.) and three marine (*Micromonas pusilla*, *P. tricornutum*, and *Heterocapsa niei*) microalgae. Flow cytometry enabled separation of each algal population on the basis of their characteristic size and pigment fluorescence, so that the effects of copper in single species, versus multispecies tests could be assessed. Single species freshwater bioassays were shown to underestimate the toxicity of copper, whereas the marine single species tests overestimated copper toxicity.[7] Such multispecies bioassays open a new approach to water quality management, and flow cytometry offers great potential for further development in this area. The ability of some flow cytometers to physically separate different cell populations (cell sorting) will also enable wider application of flow cytometry in ecotoxicology in future.

Despite its many advantages, the expense of acquiring and maintaining a flow cytometer may be a potential factor currently limiting its routine use in ecotoxicology. Flow cytometers require considerable investment in equipment, together with skilled operators. However, once the individual test protocols are established, it is relatively easy to train operators in their routine use. Development of flow cytometric methods has predominantly been directed towards medical research and diagnostics involving the analysis of mammalian cells, particularly red and white blood cells. However, with increasing interest in the ecotoxicological applications of flow cytometry, particularly in the area of microbial research, costs will continue to be reduced and instruments will increasingly be tailored to these purposes. Use of flow cytometers in a multiuser central facility or university laboratory may be a suitable option to reduce costs.

Conclusions

The application of flow cytometry to ecotoxicology is enabling the development of more environmentally relevant aquatic toxicity tests with microalgae. Multiparameter, multispecies toxicity tests at low cell densities are now available to better assess the bioavailability of contaminants in aquatic systems. These test protocols are suitable for assessing the toxicity of effluents, sediments, and marine and freshwaters. Such toxicity tests are an important part of an integrated approach to water quality management, combining chemical measurement techniques, geochemical speciation modeling, bioassays, and

ecological monitoring, to better understand ecological processes, metal bioavailability, and mechanisms of toxicity to microorganisms.

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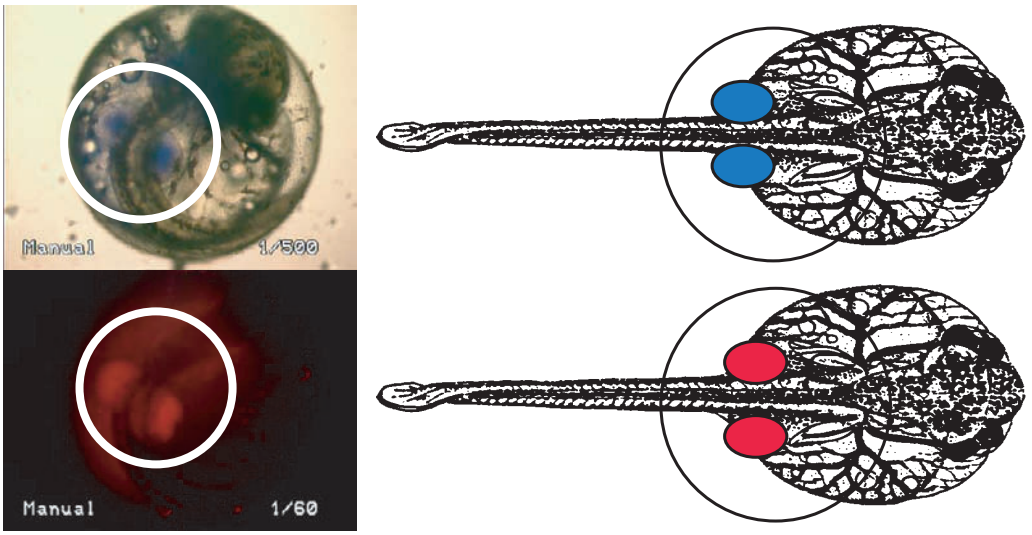

Figure 11.4 Bilobed bladder in *Fundulus heteroclitus* embryo (10 days post-fertilization) fluoresces blue (420 nm) under UV excitation (365 nm); bladders in embryos exposed to aryl hydrocarbon agonists and the EROD substrate (ethoxyresorufin) accumulate resorufin and fluoresce red (590 nm) under green (510 nm) excitation.

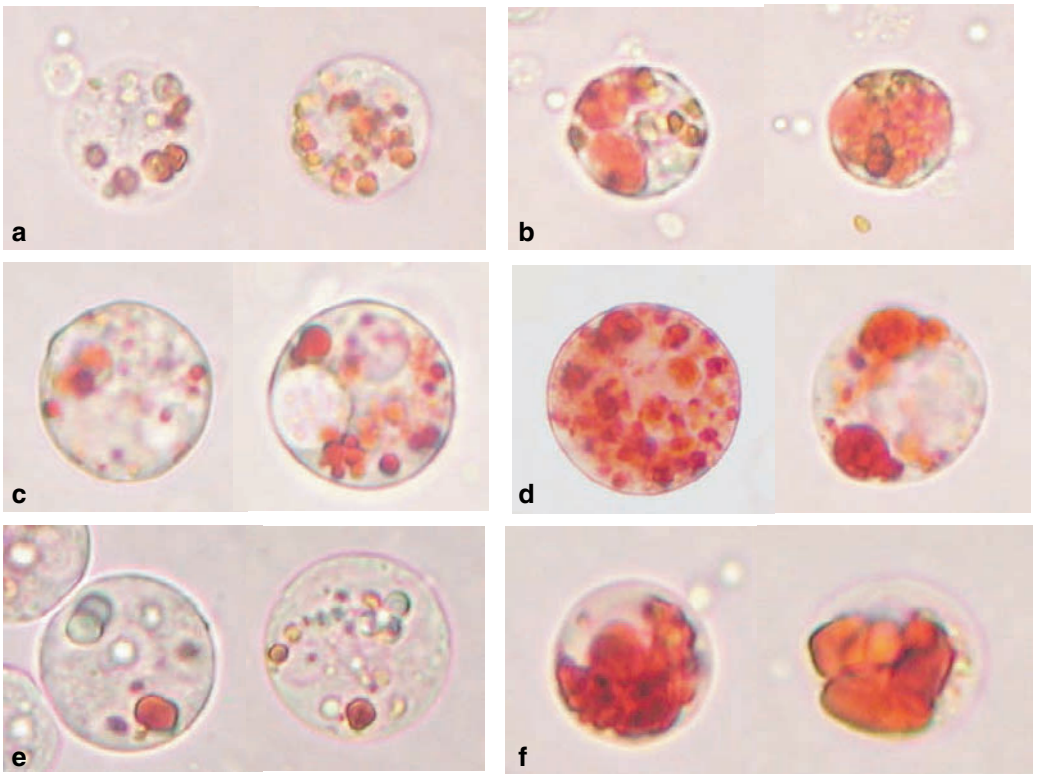

Figure 16.2 Photographs of examples of hepatic cells with stable and destabilized lysosomes for oysters, grass shrimp, and mummichogs: (a) oyster hepatopancreas cells scored as stable; (b) oyster hepatopancreas cells scored as destabilized; (c) grass shrimp hepatopancreas cells scored as stable; (d) grass shrimp hepatopancreas cells scored as destabilized; (e) fish liver cells scored as stable; (f) fish liver cells scored as destabilized.

Figure 19.1 Cross-section of coral colony with pink eggs and white spermaries.

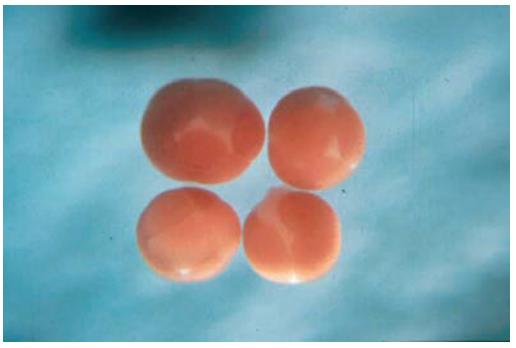

Figure 19.2 Combined egg–sperm clusters.

Figure 19.3 Floating gamete bundles collected in sieves.

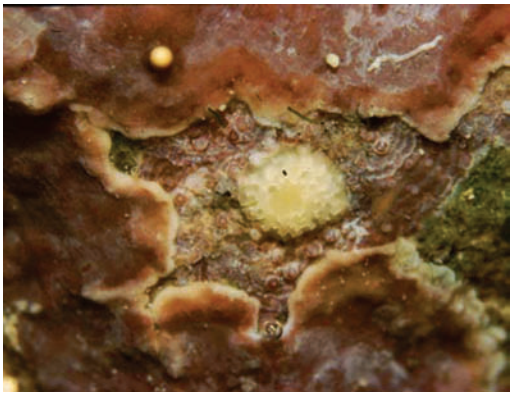

Figure 19.4 Coral larval recruit, settled and metamorphosed.

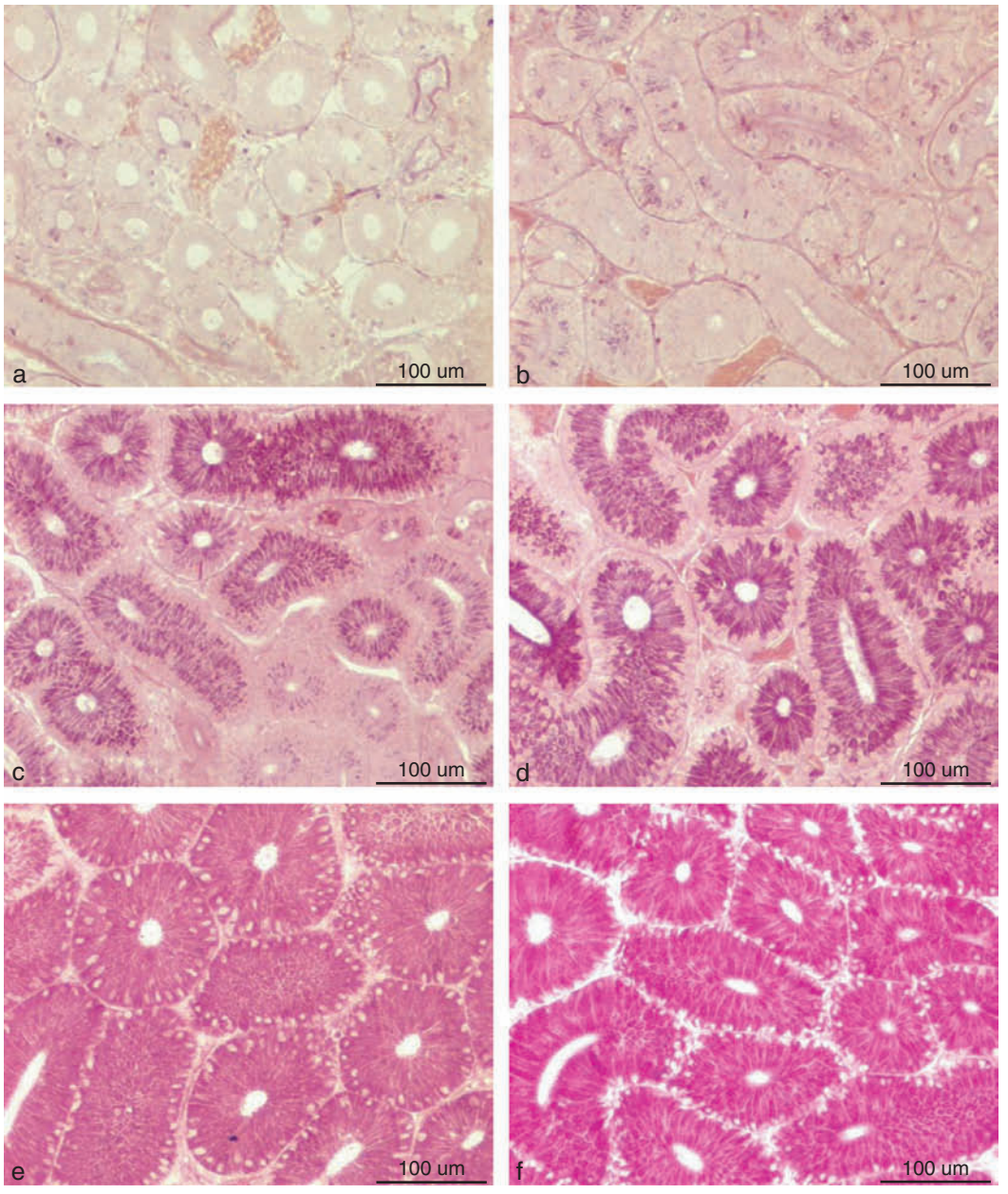

Figure 20.2 Renal histology of 17α-MT-treated female sticklebacks (a–e) and a naturally breeding male (f). Plates are at the same power and stained with periodic acid-Schiff (PAS). (a) control fish (KEH = 13.5 μm); (b) 100 ng/l 17α-MT (KEH = 18 μm); (c) 1 μg/l 17α-MT (KEH = 24.2 μm); (d) 10 μg/l 17α-MT (KEH = 29.7 μm); (e) 500 μg/l 17α-MT (KEH = 36.2 μm); (f) Breeding male (KEH = 34.5 μm).

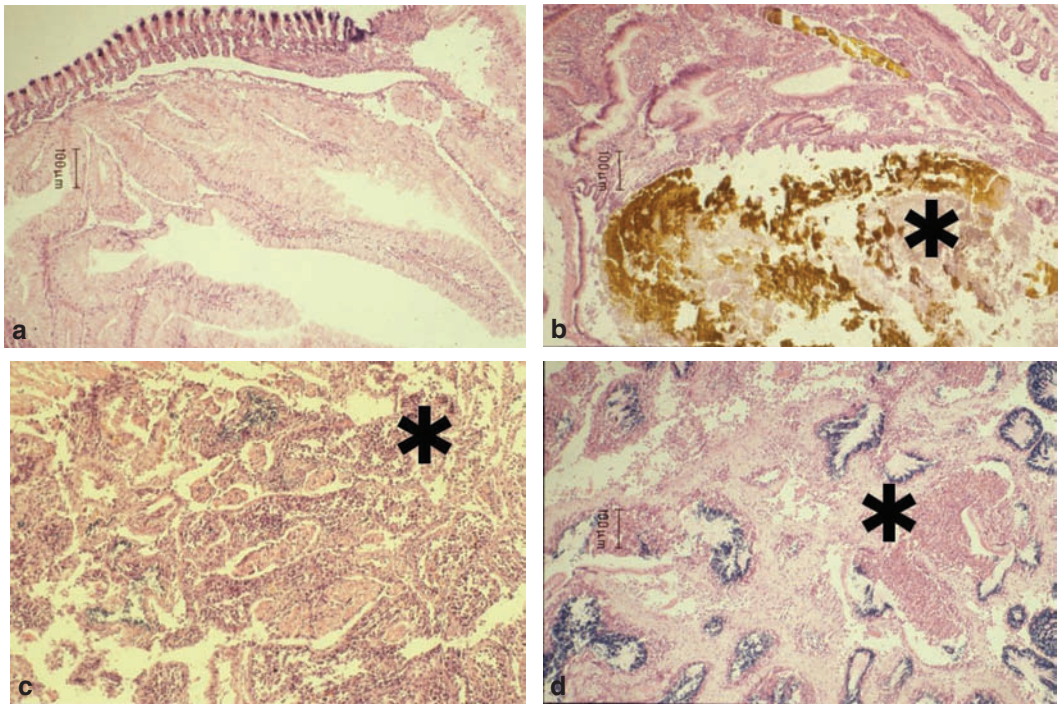

Figure 36.8 Example lesions affecting the health of individual clams and population status in the Gulf of Riga: (a) normal kidney, gill at top; (b) kidney concretion at ''*''; (c) germinoma, undifferentiated cells filling gonadal follicles to left of ''*''; (d) abnormal thickening of connective tissue among gonadal follicles in male clam to left of ''*,'' infiltration of hemocytes in follicles to right of ''*.'' (Photomicrographs courtesy of E.C. Peters.)

Aquatic Toxicology

Techniques in AQUATIC TOXICOLOGY

Volume 2

Edited by

Gary K. Ostrander

With myriad variables involved in the assessment of aquatic ecosystems, it is important for scientists to have a large array of analytical tools at their disposal. However, choosing the appropriate one can become as much an analytical exercise as the research itself. Continuing in the tradition of the first volume of ***Techniques in Aquatic Toxicology***, Gary K. Ostrander again presents an abundance of techniques, reducing them to workable protocols in order to facilitate their selection. This second volume includes both established and recently developed techniques available to aquatic researchers.

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FEATURES

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