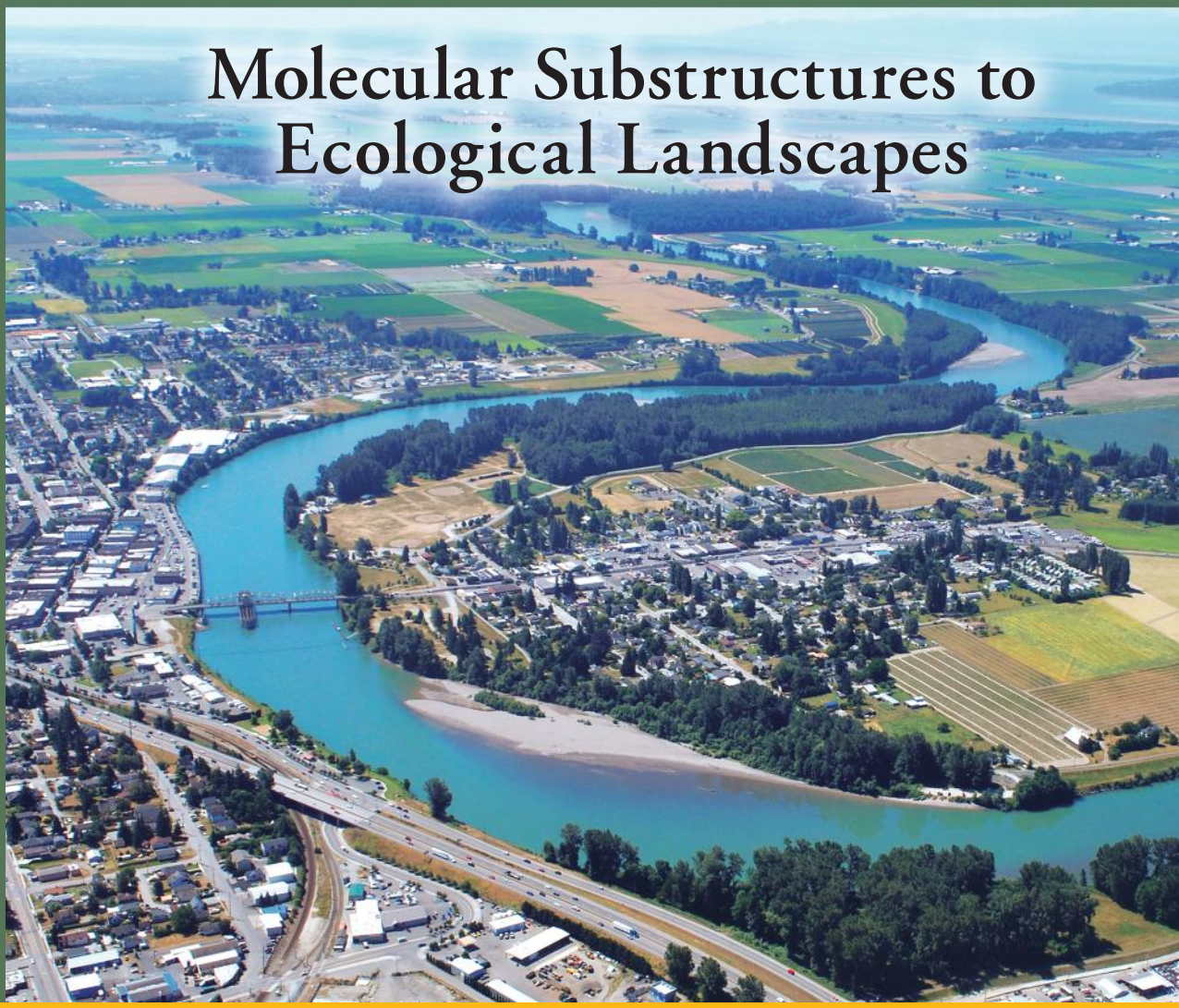
Fourth Edition

Introduction to ENVIRONMENTAL TOXICOLOGY

Molecular Substructures to Ecological Landscapes



Wayne G. Landis | Ruth M. Sofield | Ming-Ho Yu

CRC Press
Taylor & Francis Group

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Preface to the Fourth Edition

Fifteen years ago we submitted the original text because we had no suitable book for teaching courses introducing environmental toxicology and biochemistry. The current edition still reflects those origins. A good textbook presents not just lists of information but also has a design to teach students how the science is connected and how to delineate the frontiers. These connections and frontiers are the items that will stay with the student long after the "facts" are displaced by better information.

The mid-1990s were long ago and our understanding of environmental toxicology was very basic. Computation was still hard, genes stayed put, and it was only then becoming recognized that xenobiotics could have hormonal effects. Ecological risk assessment was in its very early stages and the consideration of the effects of toxicants on landscapes was nascent. These developments are now taken for granted.

The third edition was noteworthy as the work of D. Moore, P. Caux, and M. Newman demonstrated that curve fitting is superior to hypothesis testing for the modeling of concentration-effects data. Endocrine disruption was a major part of the text and risk assessment became a stand-alone chapter. The unifying construct of the hierarchical patch dynamics paradigm was introduced early and used in following chapters to integrate the spatial and temporal scales in environmental toxicology. The third edition explicitly recognized that ecological structures were complex systems being dynamic, not in equilibrium, and historical.

This fourth edition sees the inclusion of a new author, Dr. Ruth M. Sofield, who prepared the chapter on the fate and transport of contaminants. This chapter is a major addition to the text and emphasizes the relationships between chemical structure and the resultant properties with regard to the fate and transport of the material. The relationship between structure and toxicological properties has been a major theme of this book since its inception. In this edition, this fundamental concept is expanded to fate and transport as well. Our current students have the background to utilize the mathematical approaches necessary to predict fate and transport in many systems. Indeed, modeling has become a major theme of this edition.

One of the major enhancements to the fourth edition has been a new emphasis on the use of all types of models in understanding nature. In the early chapters, the use of models in science is discussed and this theme carries throughout the remainder of the book. Inevitably this emphasis on using models to describe toxicological relationships continues to lead to the fundamental flaws in using hypothesis testing to describe toxicity. It is time to move toward the use of models that describe concentration-response relationships and all the diversity of form that may exist. Because the use of biotic indices is incompatible with our current model of how ecological systems operate, the treatment of them recommends a number of methods that take advantage of the increase in

computing power that is available. I am sure that the various indices will take a long time to exit the literature, but there is now no reason to rely upon them for evaluating effects in the field.

This edition also contains a number of discussions on the toxicity by endocrine disruption of atrazine, one of the most controversial arguments in the field. Flame-retardants are now found throughout ecological structures and the implications of these findings are introduced. The existence of synergism among certain classes of organic pesticides has now been clearly demonstrated and has important consequences for understanding the risks of their use. For many years there has been the discussion of the potential population scale effects of contaminants. The recent work by K. Kidd and colleagues in an experimental pond study has conclusively demonstrated that endocrine-disrupting compounds alter the age structure of a population and can drive a species to a local extinction.

As always, it will be interesting and amusing to see what is included in the next edition of this book.

Wayne G. Landis

Acknowledgments

The students of my environmental toxicology courses during the last 20 years at Western Washington University have suffered through the notes, figures, and various graphics, and I thank them for participating in this undertaking. Traci Litwiller compiled the summaries of the various test methods. April Markiewicz generated the again updated reference for the appendix of methods. The late Kevin Short added his graphic expertise to many of the figures and gave me many hints on how to use Illustrator. Linda Landis prepared the study questions and provided her unrelenting support of the project. Although their Dad working on the various editions of this book has been a standard since they were born, my daughters are now old enough to contribute their material support. Margaret Landis helped with much of the editing of the galleys and the cover photograph. Eva Landis contributed original art to illustrate tissue damage to leaves. Thanks to everyone and to my coauthors.

WGL

The two people who helped me see the science of environmental toxicology through the lens that I do today were Philippe Ross and Bruce Honeyman, both at the Colorado School of Mines. Ross taught me to think like a toxicologist and Honeyman taught me to think like an environmental chemist. I feel fortunate to have had both as mentors. The people who taught me to be a teacher are the talented students who I have taught over the years. By working with them, I discovered how to simplify explanations so that we can then build their understanding of complex topics. I always learn as much from them as they do from me, and I am thankful for that. Finally, I would like to thank my husband, Darrell Sofield, for his patience and support of my work, but also for always encouraging me to take a break and go for a run.

RMS

I acknowledge the skilful editorial work of our coauthor Dr. Wayne Landis at Western Washington University. Thanks are also due to Joseph Clements and his associates at Taylor & Francis/CRC Press for their kind assistance.

MHY

Authors

Wayne G. Landis, Ph.D., has been the Director of the Institute of Environmental Toxicology and Chemistry, part of Huxley College of the Environment at Western Washington University, Washington State, since 1989. A graduate of Wake Forest University with a BA in biology in 1974, he subsequently received an MA and a Ph.D. in zoology from Indiana University in 1978 and 1979, respectively. Prior to Dr. Landis's university experience he was a toxicologist for the Chemical Research Defense and Engineering Center at Aberdeen Proving Ground, Maryland.

Dr. Landis has authored more than 120 publications and 300 scientific presentations. He has served on a number of USEPA (U.S. Environmental Protection Agency) and other committees, and consulted for industry; nongovernmental organizations (NGOs); print and electronic media; and federal (U.S. and Canada), state, provincial, and local governments. In 2007, he was selected as a Fellow of the Society for Risk Analysis. Currently, Dr. Landis serves on the board of editors for *Human and Ecological Risk Assessment*; he is one of the founding editors for the new Society of Environmental Toxicology and Chemistry (SETAC) journal *Integrated Environmental Assessment and Management*; and is the environmental risk assessment editor for *Risk Analysis.*

Dr. Landis has had a varied research program. During the 1980s and early 1990s, he discovered and characterized enzymes that degrade organophosphates and bacteria that metabolize riot control materials. He also conducted an extensive research program using microcosms to investigate the effects of jet fuels and other materials on the dynamics of ecological structures. Using patch dynamics models, he also formulated the theory of how to incorporate landscape scale effects as part of environmental toxicology. He is the codeveloper of the Community Conditioning Hypothesis and the Action at a Distance Hypothesis. Dr. Landis's most recent efforts have been to apply ecological risk assessment at regional and landscape scales using the relative risk model. The use of the relative risk model has now been applied to contaminated sites, invasive species, and forestry and species conservation. The approach has been used to estimate risk to sites across the world.

Ruth M. Sofield, Ph.D., is an Associate Professor at the Huxley College of the Environment at Western Washington University. She has been at the university since 2003, and is one of the faculty specializing in environmental toxicology and chemistry. She routinely teaches environmental toxicology, and fate and transport courses at the university, as well as workshops in aquatic toxicology throughout the United States.

Dr. Sofield has a BA in biology from West Virginia University (1993), an MS in environmental science from McNeese State University (Louisiana) (1995), and an MS and Ph.D. in environmental science and engineering from the Colorado School of Mines (1999, 2002). She completed her Ph.D. work in collaboration with the Center for Coastal Environmental Health and Biomolecular Research, Marine Ecotoxicology Branch, National Ocean Service, National Oceanic and Atmospheric Administration (NOAA), Charleston, South Carolina. This work focused on the genetically based tolerance to chemical exposures in *Palaemonetes pugio*. In 2003, Dr. Sofield completed her postdoctoral work on the binding of environmental ligands to uranium and plutonium.

Her current research program is focused on the effects of altered water chemistry and other environmental parameters on metal fate, transport, and aquatic toxicity. These studies range from laboratory bench scale to field scale investigations and include organism exposures combined with chemical modeling.

Ming-Ho Yu, Ph.D., is Professor Emeritus at the Department of Environmental Sciences, Western Washington University. He received his BS degree from National Taiwan University, and MS and Ph.D. from Utah State University. He did his postdoctoral research at Utah State University and the University of Alberta, Canada. Dr. Yu was a visiting professor and conducted research for a year at the Department of Public Health and Hygiene, Iwate Medical University, Morioka, Japan; and also at the Institute of Whole Body Metabolism in Chiba, Japan, for three months. He serves as an associate editor of *Fluoride*, the official journal of the International Society for Fluoride Research. Dr. Yu is coeditor of *Environment Fluoride 1985* (Elsevier, 1986); and the author of *Environmental Toxicology—Impacts of Environmental Toxicants on Living Systems*, Second Edition (CRC Press, 2005), and coauthor of *Introduction to Environmental Toxicology*, Third Edition (CRC Press, 2005).

Chapter 1

Introduction to Environmental Toxicology

As all textbooks do, this volume reflects the points of view of each of the authors, developed from being active researchers, teachers, and participants in various professional societies and governmental panels. Since the early 1990s at Huxley College of the Environment there has been a two-course fundamental introduction to the science of environmental toxicology for which this text was originally developed. That series is supplemented by courses in aquatic toxicology, risk assessment, fate and transport, air pollution, and risk assessment. Since its first edition this book was designed to provide a keystone for the program in environmental toxicology.

The approach is to blend the classic aspects of the field with new developments as they prove fundamental to the understanding of environmental toxicology. Our approach is quantitative, recognizes the connection between molecular interactions and alterations of ecological functions, and understands that the findings of the field can have major implications for the making of environmental policy. We begin by defining the field of environmental toxicology.

1.1 Environmental Toxicology as an Interdisciplinary Science

Environmental toxicology is the study of the impacts of pollutants upon the structure and function of ecological systems. For the purposes of this text, the emphasis will be upon ecological structures, from the molecular to the individual organism to the community and to the ecosystem. The broad scope of environmental toxicology requires a multidisciplinary approach of a variety of specialists. These specialists interact with a variety of other persons, decision and policy makers, the public, educators, and other key individuals, in making decisions about the management

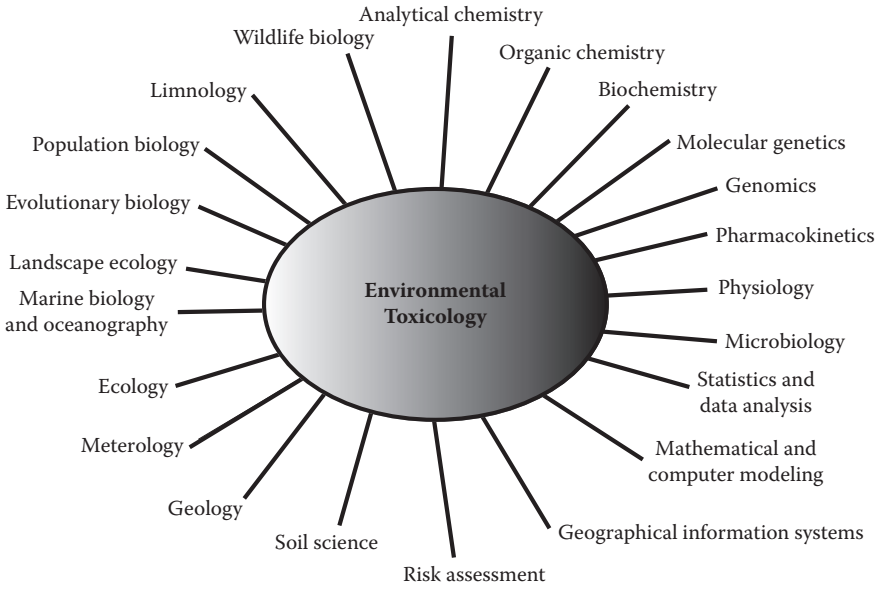

Figure 1.1 The components of environmental toxicology. Environmental toxicology borrows heavily from a variety of scientific disciplines.

of ecological systems. This breadth of scope of environmental toxicology and its application as a management tool make the field both a basic and an applied field of study.

Environmental toxicology takes and assimilates from a variety of disciplines. Terrestrial and aquatic ecologists, chemists, molecular biologists, geneticists, and mathematicians all are important in the evaluation of the impacts of chemicals on biological systems (Figure 1.1.) Ecology provides the bases of our ability to interpret the interactions of species in ecosystems and the impacts that toxicants may have upon the function and structure of a particular ecosystem. Molecular biology and pharmacokinetics operate at the opposite end of the biological hierarchy, describing the interactions of an organism with a toxicant at the molecular level. Analytical chemistry provides data on the environmental concentration of a compound and can also be used to estimate dose to an organism when tissues are analyzed. Organic chemistry provides the basic language and the foundation of both the abiotic and biotic interactions within an ecosystem. Biometrics, the application of statistics to biological problems, provides the tools for data analysis and hypothesis testing. Mathematical and computer modeling enables the researcher to predict effects and to increase the rigor of a hypothesis. Evolutionary biology provides the data for establishing comparisons from species to species and describes the adaptation of species to environmental change. Microbiology and molecular genetics may not only help the environmental toxicologist understand the fate and transformation of environmental pollutants, but also provide the science and the efficient tools to clean up and restore an ecosystem. The science of risk assessment as applied to environmental toxicology may form the framework to guide research and develop specific testable hypotheses.

Of increasing importance to the field are data analysis and the discovery of patterns of data that are of varied types and structures. The fundamental interaction of environmental toxicology is at the molecular level, yet the effects are far ranging and across many biological and physical scales. New tools will lead to new insights to the interaction of chemicals with ecological structures.

1.2 A Brief History and Organizations in Environmental Toxicology

As a discipline, environmental toxicology is relatively new. As of 2009, the 30th annual meeting sponsored by the Society of Environmental Toxicology and Chemistry (SETAC) on environmental toxicology was held. In a rapidly evolving field, this text is only a snapshot of the directions and research of the late 1980s to the early 2000s. The science evolved from the efficacy testing of pesticides in the 1940s to the cleanup of burning rivers, polluted lakes, and wildlife kills in the 1960s. The passage of the National Environmental Policy Act and the establishment of the U.S. Environmental Protection Agency forced the rapid development of the field. The Clean Air and Clean Water standards were required by law to be protective of human health and the environment. The Pellston workshops of the early 1970s provided a focal point for the discussion and consolidation of environmental toxicology. As standards development became important, a relationship with the American Society for Testing and Materials evolved, which has resulted in Committee E-47—Environmental Fate and Effects. This committee is responsible for the writing of many of the important methods used by environmental toxicologists across the world. The Organization for Economic Cooperation and Development serves a similar role in Europe. In 1979, SETAC was founded as a scientific society to support the growing needs of the field. In 1980, 85 persons attended the first SETAC Annual Meeting in Washington, D.C. In 1991, 2,230 scientists and policy makers attended in Seattle, and 3,000 now attend yearly.

As the field of environmental toxicology has grown, so has its sophistication and excitement. Environmental contamination is a fact of life, and scientists are continually called upon to give expert advice, often with little data or time to develop the necessary information. Public outcry can lead to short-term funding and yet a myopic view. Often the concentration of the funding and research is upon the immediate care of dying and sick animals, usually warm-blooded vertebrates, without an appreciation of the damage done to the normal development of the structure and function of an ecosystem. Solutions are required, yet the development of the scientific knowledge and management expertise does not always occur. Once the dying animals are buried and the smell goes away, the long-term and irreversible changes within the ecosystem are often ignored. Likewise, overreaction and the implementation of treatment techniques that are extraordinarily expensive, and that do not provide a reasonable return, can drain funds and other resources from important societal needs.

1.3 Interactions and Connections of Environmental Toxicology to the Management of Ecological Systems

There are many types of interactions that make up the field of environmental toxicology (Figure 1.2). Some are typical to fields of basic research, but because of the use of the information in decision making, there is a broad regulatory interest. Each type of interaction is described below.

1.3.1 Research Programs

This is the most fundamental part of the field of environmental toxicology. This segment includes the identification of toxicity and the causal basis. The effects range from changes at the molecular level to changes in function and structure of ecological systems. Particularly important are the

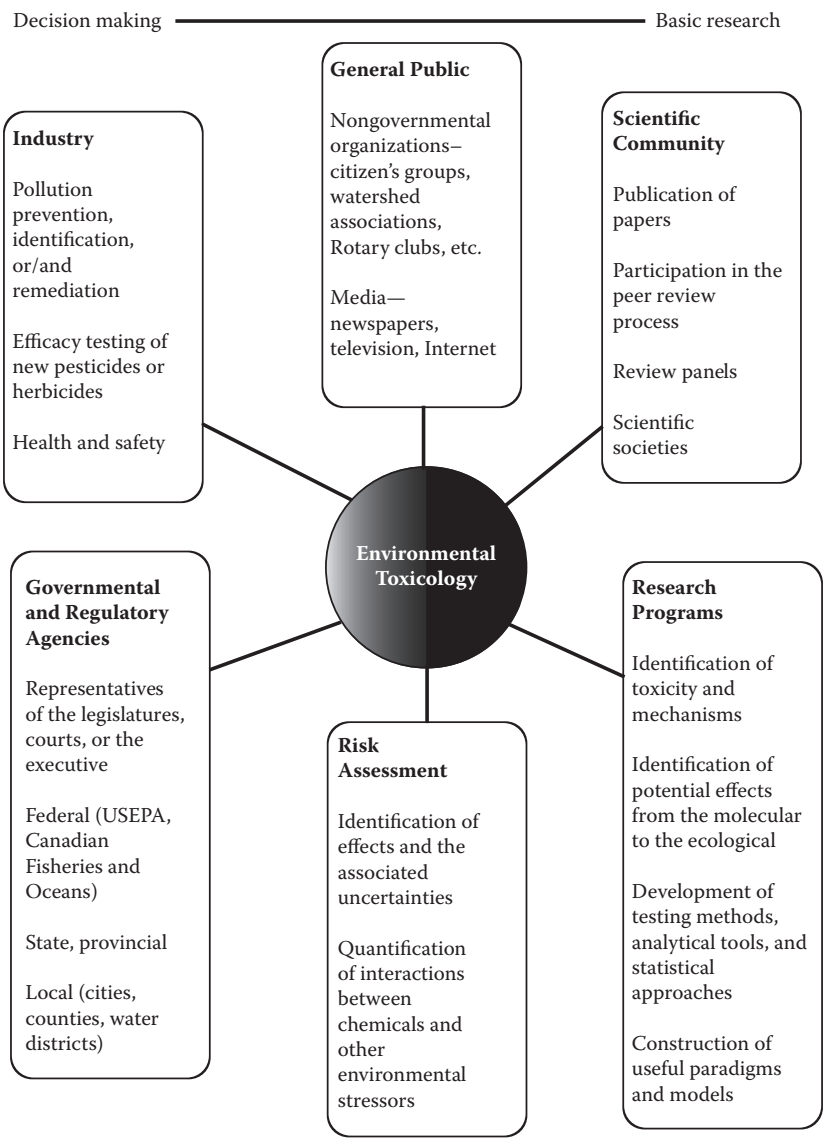

Figure 1.2 Interactions and connections of environmental toxicology to the management of ecological systems. Environmental toxicology borrows heavily from a variety of scientific disciplines. The very nature of the field is multidisciplinary, making knowledge of the basics of biology, chemistry, mathematics, and physics essential.

development of testing methods, analytical tools, and statistical techniques that allow the acquisition of data from such a diverse set of subjects. Underlying all of this is the formation of useful paradigms and models that connect the observations into an integrated structure. The integrated structure can then be useful in formulating predictions about how ecological impacts are caused by chemicals being introduced into the environment.

In order to accomplish these diverse functions it takes a social network of collaboration and expertise, an interactive scientific community.

1.3.2 The Scientific Community

The scientific community is the intellectual and industrial force behind the conduct of the research. Part of the function of the scientific community is the publication of papers in peer-reviewed journals, books, and other publications that report the information generated by the research programs. Participation in the scientific community includes participation in the peer review process, which is a vital but not perfect means of ensuring the quality of the research presented in the literature. Often members of the scientific community participate on review panels examining research priorities, plans, and results for government agencies, industry, and nongovernmental organizations.

An exciting component of participating in the scientific community is attending the variety of scientific symposia and conferences held across the world. These meetings sponsored by scientific societies such as SETAC, the Society of Toxicology (SOT), the Society for Risk Analysis (SRA), and the American Society for Testing and Materials (ASTM) are places to present research results, discuss papers and the implications, meet other researchers, and establish career-long collaborations and friendships. After a postgraduate education these meetings are vital means of keeping up with new developments, including new techniques and the overthrow of paradigms that are a part of a vital science.

Much of the consolidation of new developments within the field of environmental toxicology into frameworks and paradigms occurs at workshops sponsored by a variety of organizations. Among these workshops are the various Pellston workshops coordinated by SETAC, the symposia sponsored by ASTM, and meetings organized and sponsored by many other associations. These workshops are generally smaller than the annual meetings and are of a much narrower scope. However, most of the participants are specialists in the narrow scope of these types of meetings. Typically a special report, summary publication, or even a special journal issue summarizes the papers presented and the major findings or conclusions of the workshop. These publications often serve as landmarks in the development of the field of environmental toxicology and serve as departure points for future research.

1.3.3 Risk Assessment

Increasingly the tool for translating the research and findings of the field of environmental toxicology into predictions of environmental affects and public policy has become risk assessment.

Risk assessment is a broad field of study that incorporates risks due to transportation, disease, social decisions, and even terrorism. In the context of environmental toxicology risk assessment provides predictions of effects as probabilities and reports the uncertainties associated with the prediction. The use of a probabilistic framework allows the quantification of the interactions between chemicals, other environmental stressors, and the target biological or ecological system. A vital part of the risk assessment process is the interaction with decision and policy makers, whether they are located in industry, government, or the general public.

The subarea of risk assessment that deals with the effects of chemicals upon the environment is known as environmental risk assessment or ecological risk assessment. This subarea deals with the effects to nonhuman species of entire ecological systems at landscape and regional scales. Risk assessment as applied to environmental toxicology is discussed extensively in Chapter 14.

As noted above, risk assessment provides a linkage from the science of environmental risk assessment to the making of environmental policy. Policy is made by a variety of groups, including the general public, a variety of governmental entities, and industry.

1.3.4 Governmental and Regulatory Agencies

Governmental agencies at the federal, state and provincial, and local levels have been a major driver for the development of environmental toxicology. These agencies act as the representatives of the legislatures, courts, or the executive in setting environmental policy and rules. These agencies often set standards for chemical concentrations in air, water, soil, sediment, and tissue that are judged to safeguard human health and the valued functions of ecological systems.

In the United States, the U.S. Environmental Protection Agency (U.S. EPA) is often seen as setting important regulations. But many states may have even stricter standards for a variety of chemicals. States may even differ in their approach to setting toxicity limits or in the process of conducting risk assessment. Many other agencies are also involved in setting standards for the protection of wildlife and ecological function. Along with the U.S. EPA, the Department of Fish and Wildlife, the U.S. Army Corps of Engineers, the National Marine Fishery Service, and the U.S. Coast Guard all have some jurisdiction over the release and cleanup of chemicals found in the environment. In the State of Washington, the Department of Ecology, Department of Fish and Wildlife, and Department of Natural Resources are all charged with various aspects of environmental protection.

In Canada, the Federal Department of Fisheries and Oceans has broad powers to protect fish in both marine and freshwater environments. However, provinces also have regulatory ministries, such as the British Columbia Ministry of Water, Land and Air Protection, with broad responsibilities and powers to regulate chemicals in the environment.

Each of these regulatory groups typically has a cadre of environmental toxicologists, risk assessors, and consultants that provide input to the setting of regulatory concentrations of chemicals. Likewise, the industry regulated by these agencies also utilizes similar expertise.

1.3.5 Industry

Industry in this sense includes groups that mine, manufacture, transport, or use chemicals. As discussed in the next section, there are a number of regulations that govern the use and disposal of chemicals. In order to comply with these regulations and prevent toxic materials from adversely impacting the environment, industry applies the science of environmental toxicology in a number of ways. Chemicals under development are subjected to a variety of toxicity tests to ensure that unwarranted toxicity is not a property of the material. Effluents from waste discharges are tested using a variety of bioassays to ensure that the released material does not have an associated toxicity that exceeds regulatory limits. The ability of different effluent treatment regimes to reduce the toxicity can also be evaluated using these same bioassays.

Pesticides, herbicides, fungicides, and rodenticides are materials produced to be toxic to specific groups of pest organisms. These materials must be evaluated in order to test the ability of the chemical to control the pest and also to examine the toxicity of these materials to organisms that are not intended for control. A variety of toxicity tests are performed in order to evaluate the range of toxicity of candidate materials. From these tests decisions are made about how the pesticide can be used, how often, and at what concentrations.

Mining, smelting, and oil production are essential parts of an industrial society, but these processes concentrate heavy metals and other materials in the environment. Environmental toxicity assists in the decision-making process concerning the design of the control mechanisms for mining or smelting waste. Waste materials from the production and refining of oil need to be evaluated for environmental impacts.

Health and safety issues are important features of the testing process as well. Labels and material safety data sheets are developed that discuss both human health and environmental considerations based upon toxicity data.

Industry typically employs its own in-house toxicologists and risk assessors, both as managers of the testing regime and as scientists. Industry widely uses internal and external consultants, laboratories, and academic institutions to perform specialized toxicity testing and risk assessments.

1.3.6 The General Public

In this discussion the general public is considered to be nongovernmental organizations (NGOs), including citizens groups, watershed associations, Rotary and Kiwanis clubs, unions, and specialized environmental groups such as the Sierra Club or World Wildlife Fund. These groups form an important aspect of the decision-making process, since these groups represent the individuals that have a direct stake in the environment.

In some instances, the larger or more well-funded groups may employ specialists in environmental toxicology or hire appropriate consultants. In other instances these groups may have members that can volunteer the necessary expertise.

One of the critical roles that these groups play in the environmental decision-making process is in the articulation of the value that each group derives from the environment. These values can include economic, safety, cultural, or esthetic components, and each is important. Economic values include resource extraction, jobs, shipping, and so forth, that provide a direct financial return. Safety includes providing food, air, and water that do not harm the health of the persons, animals, or plants that occupy the environment. Cultural aspects include preserving those features of the environment that are required or define a group of persons. For example, preserving salmon and shellfish harvesting are important aspects of the culture of the Northwest tribes of Native Americans. Similarly, access to rangeland is an important aspect to ranching in the western United States.

The general public is a critical segment in the support of environmental toxicology and its decision-making process. It has been the demand by the public for clean air, water, and land that has driven the legislative process that has driven the development and use of environmental toxicology. Because the public is fundamental in the decision-making process, it is also important to inform them through the media, presentations at club meetings, open houses, and the Internet. The public is the ultimate customer for our research.

1.4 Legislation

Unlike much of basic research, environmental toxicology has been often defined by and instigated by public policy as written in legislation. Many of these laws in the United States, Canada, and Europe mandate toxicity testing or require an assessment of toxicity. In the United States, federal law can often be supplemented by but not weakened by the states. For example, in the State of Washington there are state and federal responsibilities for the assessment of damage due to a spill of oil or other hazardous substance. The State of Washington also has its own regulations for the control of toxic materials, and administers the National Pollution Discharge Elimination System (NPDES) permits. There are several pieces of legislation that are particularly relevant to the development of environmental toxicology.

The Federal Water Pollution Control Act of 1972, amended in 1976 (33 USC Sections 1251–1376), is commonly known as the Clean Water Act. The stated purpose is to restore and maintain the integrity of the nation's waters. The regulations set by this legislation set maximum allowable concentrations of toxicants allowed in discharges and receiving waters. The results of toxicity testing are commonly used to set these limits. In addition, NPDES permits are now commonly requiring the use of toxicity tests performed on effluents from a variety of manufacturing sites to establish criteria for compliance.

The legislation that controls the registration of pesticides in the United States is the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Originally passed in 1947, the act has been amended by the Federal Environmental Pesticide Control Act of 1972, amendments to FIFRA in 1975, and the Federal Pesticide Act of 1978 (7 USC Section 135 et seq.). Pesticides by definition are toxic materials that are intentionally released to the environment. Many of these compounds provide a measurable economic benefit that is weighed against impact. Essential to the registration of pesticides has been a tiered testing scheme. In a tiered approach, there are specific tests to be performed at each level of the tier. If a compound exhibits particular characteristics, it has the option of passing to the next level of testing. Typically, these tiers range from basic mechanistic data to field tests. In the approach commonly used before the fall of 1992, the top tier included field studies using large man-made ponds or investigations of terrestrial systems dosed with known quantities of pesticide. The field and other ecosystem level approaches are not currently routinely included. A great deal of toxicological data at every level of biological organization has been acquired as part of the registration process.

The Toxic Substance Control Act (TSCA) (1976, 42 USC Sections 2601–2629) is an extremely ambitious program. TSCA attempts to characterize both human health and environmental impacts of every chemical manufactured in the United States. During the Premanufacturing Review Program, the EPA has about 90 days to assess the potential risk of a material to human health and the environment. Given the limited period of notification and the volume of compounds submitted, many of the evaluations use models that relate the structure of a compound to its potential toxicity. Structure activity models have proven useful in screening compounds for toxicity to aquatic and terrestrial organisms as well as mutagenicity and other endpoints. In addition to the toxicity estimation methods, there is a recommended but not binding series of measurements and toxicity tests that may be performed by the manufacturers. The toxicity tests typically involve a single-species approach.

Perhaps the largest toxicity testing program in the world is the European Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH). With many of the same goals as TSCA, this program was initially assumed to cover 30,000 chemicals. However, recent estimates are now up to 143,000 chemicals submitted by 65,000 companies (http://pharmtech.findpharma.com/pharmtech/Online+Only/REACH-Program-May-Carry-Six-Times-the-Expected-Cos/ArticleStandard/Article/detail/623599). REACH includes testing for human health and also environmental toxicity/, accessed December 4, 2009).

Toxicity testing or the utilization of such data is routinely performed in support of the Comprehensive Environmental Response, Compensation and Liability Act of 1980 (42 USC Section 9601 et seq.), abbreviated as CERCLA but more commonly referred to as Superfund. This legislation requires that some assessment of the damage to ecological systems be considered. Research has been conducted that attempts to use a variety of toxicity tests to evaluate the potential damage of chemical contaminants within a site to the environment. This need has given rise to interesting *in situ* methods of detecting toxicity. In the past, this program has generally been driven by human health considerations, but ecological impacts are now becoming important at several sites.

Part of the CERCLA legislation was the creation of the Natural Resource Damage Assessment (NRDA) process. NRDA attempts to quantify the costs of the impact to systems damaged by spills, industrial processes, mining activities, and other areas covered by CERCLA. There have been many discussions about how to assess damage to endangered species, populations of harvested fish, birds, and other wildlife, as well as how to calculate the cost of restoration.

Although the legislation discussed above has provided the principal regulatory force in environmental toxicology, other mandates at the federal and state levels apply. These requirements will likely persist, providing a continuing need for data acquisition in environmental toxicology.

1.5 Introduction to This Textbook

The purpose of this volume is to provide background knowledge so that the short- and long-term effects of chemical pollution can be evaluated and the risks understood. There are 13 more chapters, each with a specific building block toward the understanding of the status of the field of environmental toxicology.

Chapter 2, "Frameworks and Paradigms for Environmental Toxicology," provides an overview of the field of environmental toxicology and introduces the progression from the initial introduction of the toxicant to the environment, to its effect upon the site of action, and finally to the impacts upon an ecosystem. Many of the terms used throughout this text are introduced in this section. After an introduction to toxicity testing, the remainder of the book is organized from the molecular chemistry of receptors to the ecological effects seen at the system level.

Chapter 3 is an introduction to toxicity testing. In this chapter the basics of designing a toxicity test and some of the basics of analysis are presented. The ability to understand and critique toxicity tests and bioassays is critical. Much of our understanding of the impacts of toxicants and the regulations governing acceptable levels is based on toxicity tests. Comparability and accuracy of toxicity tests are also crucial since these data are routinely used to derive structure-activity relationships. Structure-activity relationships are derived that relate the chemical structure of a material to its biological property, be it toxicity or biodegradation. These relationships are particularly useful when decisions are required with limited toxicological data.

After a chapter introducing the design parameters for toxicity tests, Chapter 4, "Survey and Review of Typical Toxicity Test Methods," presents a variety of methods that are used in environmental toxicology to assess the potential hazard of a material. A variety of tests are presented, from single species to ponds, and involving a wide variety of organisms. Tables are included that act as quick summaries of each of the tests described in the chapter. Perhaps not as exciting as contemplating the impacts of toxicants on ecosystems, the tests are the basis of our knowledge of toxicity. The setting of safe levels of chemicals in regulations, the measurement of impacts due to industry and residential outflows, and the estimate of risks are all based on the data derived from these tests. Included in this chapter are brief descriptions of many of the test organisms: freshwater, marine, and terrestrial.

Chapter 5, "Fate and Transport of Contaminants," deals with contaminants once they are introduced to the environment until they enter the organism. This chapter covers how chemicals can move long distances, bioaccumulate and biomagnify, and move into the different physical components of the environment. One of the key components of this chapter is the relationship between structure and the eventual fate of the chemical. Included in this chapter is a detailed table linking structure to physical properties. Extensive modeling is used to illustrate the linkage between physical properties and fate to provide a deeper understanding of the processes for the reader.

Chapter 6, "Uptake and Modes of Action," is an analysis of the routes of exposure allowing a toxicant to enter an organism and the modes of action at the molecular level that cause effects to reverberate throughout an ecological system. The crucial nature of understanding the routes of exposure and their importance in the course of action of the toxicant is brought to light. As the compound reaches the cell, a number of interferences with the normal functioning of the organism take place, from acetylcholinesterase inhibition to the binding of common cellular receptors with disastrous outcomes.

A developing area of research has been that of endocrine disruption. Apparently a wide variety of materials can interfere with or mimic endocrine function. Estrogen mimics and the possible modes of action of these materials are discussed with particular emphasis on dioxins and the polychlorinated biphenyls (PCBs). Other classes of compounds and modes of action are also summarized in this section.

In addition to the biochemistry introduced in this chapter, a great deal of emphasis is placed on the determination of the activity of a compound by an analysis of its structure. Quantitative structure-activity relationships (QSARs), used judiciously, have the ability to help set testing priorities and identify potentially toxic materials in mixtures. Heavily reliant upon the quality of the toxicity data discussed in Chapter 4, these methods use sophisticated statistical techniques or analysis of interaction of a toxicant with the receptor to estimate toxicity. A method that uses structure-activity relationships coupled with availability and an assumed additive model for toxicity is presented to estimate the risk due to polyaromatic hydrocarbons (PAHs).

Even as the route of exposure and the molecular interactions that cause the toxic effects are delineated, that is not the entire story. Chapter 7, "Factors Modifying the Activity of Toxicants," describes the myriad physiological and environmental factors that can alter the exposure of the organism to the toxicant and also the response to the compound. Nutritional status, complexing elements in the environment as well as the organism, and reproductive status can all drastically affect the response of an organism to an environmental exposure.

How to deal with mixtures of toxicants has long been an issue in environmental toxicology. Chapter 7 presents several methods of approaching this issue. Several are empirical, and one method uses QSAR to estimate the toxicity of several PAHs. Finally, there is a demonstration of how toxicity tests can be used to examine the interactive effects of different pesticides.

Many of the examples used in Chapters 2 to 7 focus on organic pollutants; however, inorganic materials comprise an important class of contaminants. Chapter 8, "Inorganic Gaseous Pollutants," describes the mode of action and the creation of a variety of inorganic gaseous pollutants, an increasingly important aspect of environmental toxicology. A major emphasis is placed on the atmospheric chemistry of each pollutant and the effects on a variety of organisms. The chemistry and toxicology of sulfur oxides, ozone, nitrogen oxides, carbon monoxide, and fluoride are reviewed in this chapter.

Chapter 9, "Heavy Metals," is an introduction to a worldwide pollutant, fluoride. Fluoride is a by-product of a variety of industrial processes, notably aluminum smelting. Although controls are common in the developed world, fluoride is a common pollutant in the areas with developing economies.

Chapter 10 is a discussion of the toxicity of metals. Metals are the classical environmental pollutant, and their persistence is the cause of long-lasting concern. Mining, industrial runoff, and the presence of metal contamination in soils and sediments are still major environmental concerns. This chapter covers the fate, speciation, and toxicity of the heavy metals.

As a material enters an ecosystem, a variety of physical and biological transformations can take place, dramatically altering the property of the compound to cause toxicity. Chapter 11,

"Biotransformation, Detoxification, and Biodegradation," reviews the mechanisms that alter the toxicity of a compound. This section is important in understanding and determining the exposure of the environment to a chemical toxicant. In addition, a knowledge of biodegradation and microbial ecology may also yield strategies for the reduction or elimination of xenobiotics.

The next section of the book presents the measurement and prediction of the effects of chemicals upon ecological systems. Because of the breadth of this subject, there are two chapters devoted to it.

Chapter 12, "Ecological Effects from Biomarkers to Populations," deals with broad categories of responses to toxicants as well as specific examples. Biomonitoring and biomonitoring strategies are discussed. One of the key items of this chapter is the issue of using biological indices to measure the response of ecological systems to contaminants, regardless of the type of calculation made. The effects of toxicants upon populations have become a current topic, and this chapter introduces population modeling, nonlinear systems, and patch dynamics to the reader.

Chapter 13, "Ecological Effects: Community to Landscape Scales of Toxicological Impacts," describes several new, exciting, and controversial ideas about the nature of complex systems, chaos, and the interactions with communities that may drastically change our view of ecological systems and their management. It is now fairly clear that ecological structures do not recover structure, although the overall nutrient cycling and energetics may be more robust. Finally, the hierarchical patch dynamic paradigm is introduced as a framework for understanding the effects of chemicals and other stressors upon landscapes and regions.

The discipline that ties together environmental toxicology and links it to environmental management is environmental risk assessment. Chapter 14, "Ecological Risk Assessment," provides a framework for the integration of classical toxicology at the molecular and organismal levels and the prediction of events at the community and ecosystem levels. Exciting research is currently underway to examine the importance of indirect effects, landscape and global changes, and management of these risks. In Chapter 14 we review the current paradigm of the U.S. Environmental Protection Agency. One of the approaches for estimating risks at large spatial scales, the relative risk model, is introduced and a Cherry Point, Washington, case study presented. One of the major themes of this chapter is the role of risk assessment in the management of ecological systems at very large scales. Chemicals are just one of many factors altering the ecological resources that our economy and lives rely on. Risk assessment can be applied to watersheds or even larger regions, invasive species, disease, changes in land use, and climate change, among other stressors.

We hope that the reader finds the journey as exciting as we have.

Study Questions

1. Define *environmental toxicology*.
2. Why must environmental toxicology be considered a broad, multidisciplinary field of study?
3. List seven disciplines that are combined in environmental toxicology.
4. List three important historical events in the history of the discipline of environmental toxicology. For each, give a date (if provided) and the reason the event is so important.
5. What is "the most fundamental part of the field of environmental toxicology"?
6. Why is an integrated structure important to develop in a research program?
7. What part does the scientific community have in the field of environmental toxicology?
8. Define *risk assessment* in the context of environmental toxicology.
9. Name the subarea of risk assessment that deals with effects of chemicals on the environment.

10. What purpose do regulatory agencies serve for the field of environmental toxicology?
11. How does industry apply the field of environmental toxicology? Give three general concerns of industry.
12. Discuss the roles the general public plays in environmental decision making.
13. What is the Federal Water Pollution Control Act of 1972?
14. What legislation controls the registration of pesticides? What is a pesticide?
15. Describe the tiered method of pesticide testing.
16. What is the goal of TSCA?
17. What is the purpose of REACH?
18. Describe CERCLA (Superfund) and the NRDA.

Chapter 2

Frameworks and Paradigms for Environmental Toxicology

2.1 The Fundamentals

As presented in Chapter 1, environmental toxicology draws on a number of scientific fields in its examination of the effects of chemicals upon ecological structures. However, I have found that many undergraduate and graduate students are unfamiliar with the fundamentals of what is meant by *science*.

If you were to refer to *Merriam-Webster's* online dictionary (September 5, 2009), there is one definition that fits the current context of this discussion:

> 3 a: knowledge or a system of knowledge covering general truths or the operation of general laws especially as obtained and tested through scientific method; b: such knowledge or such a system of knowledge concerned with the physical world and its phenomena: natural science.

While we do agree that science is a system of knowledge about the operation of the world, we do not agree that it covers just general truths or is limited to the "natural." In science we strive to know both the specific observations and the general patterns that allow the categorization of knowledge. In science there is nothing but the natural, physical, biological world; there are no supernatural explanations or observations not amenable to the scientific process.

Learning the specific observations or general knowledge about the world is not operational science. To "do" science is much more than memorization, it is exploration. Science is driven by the exploration of key questions, and to know those, it is critical to know what the limits of knowledge are and what questions are important to expanding a general understanding of the field. We agree with Freeman Dyson (2008, p. 3) that scientists innately are rebels, often refuting the authority of the establishment in order to garner a deeper understanding of phenomena.

The process of science does not hold in regard authority due to position or tradition. It regards as important and real only that knowledge or model than can produce predictions that can be tested by observation and experiment and be falsified. In other words, nature is the arbitrator of reality, not social status or convention.

The reliance upon experiment and observation was codified by Popper (first published in 1935, current edition 2002). A distinguishing characteristic of science is that a hypothesis is subject to falsification. If a prediction made by a hypothesis is found to be false, then the hypothesis is false. The converse is not true. If a hypothesis cannot be tested and found to be false, then by definition it is not science but perhaps another form of inquiry. If a hypothesis is tested and the prediction is found to be true, this does not mean that truth has been found. The finding may be due to chance, or because the hypothesis works only under special circumstances. Excellent and classic examples of hypotheses that are only partially true are Newton's laws of motion and gravity: At the scale of the very small or the very large and fast Newton's predictions fail. Quantum mechanics and special and general relativity were created to provide better descriptions of physical phenomena. *Better* in this case means that predictions different from those of classical Newtonian mechanics were made and confirmed by both classes of theories.

A set of theories or models that are generally accepted and become the accepted mode of thought regarding a subject is a paradigm, as formulated by Kuhn (1958, current edition 1996). Paradigms are useful constructs that allow a common frame of reference for formulating additional hypotheses and for organizing the knowledge base.

The critical and creative aspects of a paradigm is that they are malleable and can even be destroyed when the hypotheses that construct them are tested and fail. The classic example of a paradigm shift was the realization that the then current framework of a static earth was replaced by Wegner's old idea of continental drift. Although a mechanism for the drift was not understood, only the hypothesis that the various continents were at one time all one could account for the distribution of fossils and geological features. In addition, the mechanisms for mountain building, earthquakes, and the ring of volcanoes surrounding the Pacific Rim became apparent. A similar paradigm shift appears to be under way in ecology as expressed by Wu and Loucks (1995) and the hierarchical patch dynamics paradigm (HPDP). This paradigm shift will be described further later in this chapter. In the current formulations of science models are a central aspect. It is time to talk about the use of models in science and in environmental toxicology in particular.

2.2 Models

Scientists use a variety of models in order to describe the cause–effect relationships that are observed by observation and experiment. All models have uses and limitations, and it is critical to understand them.

A model is any abstraction of reality that has, demonstrates, or describes the properties of interest to the individual. If that individual is doing science, then that model has to be testable against experiment or experience. Models have a variety of uses in environmental toxicology, but the first example is far afield from that topic.

Figure 2.1 depicts a plastic 1/48 scale model of a North American F-86F aircraft that flew during the Korean War for the U.S. Air Force. The markings of this model accurately represent the appearance of the prototype at a specific time and place. The finish of the model represents the aluminum and other metals that were part of the construction. In the cockpit there are accurate depictions of the instruments, controls, and other equipment of the period. In other words, this

Figure 2.1 Plastic model designed to provide a three-dimensional representation of the historical characteristics of the prototype.

model is a way of representing the historical appearance of the prototype in 1952. In this regard, the model portrays color and dimensional information in a way that a photograph or written description simply cannot. This model is useful as a historical representation of the prototype.

However, this model will tell you very little about how an airplane flies. That is a very different question to be depicted. Although an aircraft can be reproduced in scale form, the air in which it flies and the physics that govern it cannot be scaled. Although the plastic is made as accurate as possible, the shape of the aircraft is not accurate and smooth enough to use in a wind tunnel. The outlines of the control surfaces give only a hint of their use in controlling an aircraft. The internal combustion engine does not scale down appropriately to provide a simulation of the energy involved. In order to model how an aircraft flies, a number of other models need to be built, at much larger scales, or computers used to calculate the dynamics of the air, gravity, thrust, and lift that are involved. As accurate as current models regarding flying are, the first flight of an aircraft is still conducted by specially trained individuals called test pilots, and they board with parachutes just in case. On high-performance military aircraft the test pilot also has an ejection seat just in case the models and reality are really far apart.

Models are a fundamental part of environmental toxicology. At the most fundamental level *in vitro* studies are used to model the interactions of chemicals with specific macromolecules within a cell. Similarly, *Daphnia magna* are used as a model organism to simulate the response of wide ranges of aquatic invertebrates to toxicants. There are also physical models of ecological systems.

Figure 2.2 depicts part of a microcosm experiment designed to model the effects of the water-soluble fraction of jet fuel to an aquatic community. The model was originally developed by Dr. Frieda Taub at the University of Washington and contains a variety of primary producers, detritovores, and herbivores commonly found in freshwater systems. The model is a physical one designed to replicate only certain features of ecological structures.

A Taub microcosm has a trophic structure: Energy comes from light, nutrient cycling occurs, and a variety of population and community dynamics occur within each jar. So each jar replicates in some features the characteristics of freshwater ponds. There are many features that are not simulated on purpose.

In the process of constructing a microcosm experiment the environment is purposefully kept as homogenous as possible to reduce variability to ensure adequate statistical power for analysis. So the variations in physical structure, temperature, nutrients, and other environmental factors are purposefully reduced. The number of species in a microcosm is also controlled. In the Taub

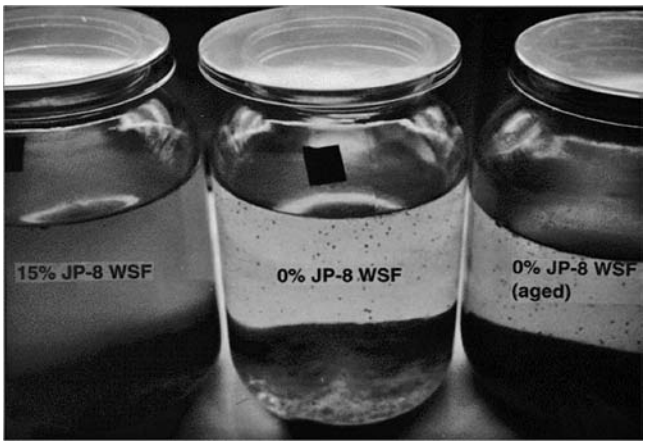

Figure 2.2 The Taub standardized aquatic microcosm: An experimental model of a freshwater ecological structure.

microcosm representatives of detritovores, algae, bacteria, invertebrate herbivores, and protozoa are inoculated from known cultures. Even the simple Taub microcosm exhibits complex dynamics such as predator–prey dynamics, changes in nutrient dynamics, and competitive interactions that are properties of ecological systems outside of the laboratory.

Common classes of models are those constructed as a computer model or simulation of the process being studied. An example of such a model is illustrated in Figure 2.3. The process being modeled is the potential impacts of a toxicant on a population comprised of three patches, with the toxicant isolated in only one of the patches. The entire process is detailed in Chapter 12. The original diagram was a simple three-circle drawing with one of the patches shaded to represent the contaminated patch (Figure 2.3a). This simple diagram was turned into a computer model that includes rates of increase, the carrying capacity for each patch, a concentration-response curve for the toxicant, rates of migration between patches, a stochastic feature designed to represent the exposure of the organisms to the toxicant, and other factors. Stella, a systems modeling program, was used to construct the model. The program graphically represents the model, and this can be seen in Figure 2.3b. The three-patch structure is still apparent. The final model was not designed to show any particular real system, but is just a means of investigating how toxicants may affect patchy populations.

So now we have a simple mathematical representation of many of the features of populations and the interactions of organisms and chemicals. The basic model was modified to represent different concentrations of chemicals, distances between patches, and other features. Hundreds of simulations were performed to examine the dynamics of these three-patch (and later four- and five-patch) models. The outcome of this research, performed by Julann Spromberg, Betina Johns, and myself, gave rise to the action at a distance hypothesis. Action at a distance simply states that a toxicant may affect organisms in uncontaminated patches by affecting the population dynamics. The basic premise of this hypothesis has been demonstrated experimentally but still awaits field confirmation.

One of the great advantages of models is that they allow the investigator to organize the relationships among phenomena and derive undiscovered features of the system. If the hypothesis is sufficient, robust testable hypotheses can be generated to confirm the predictions.

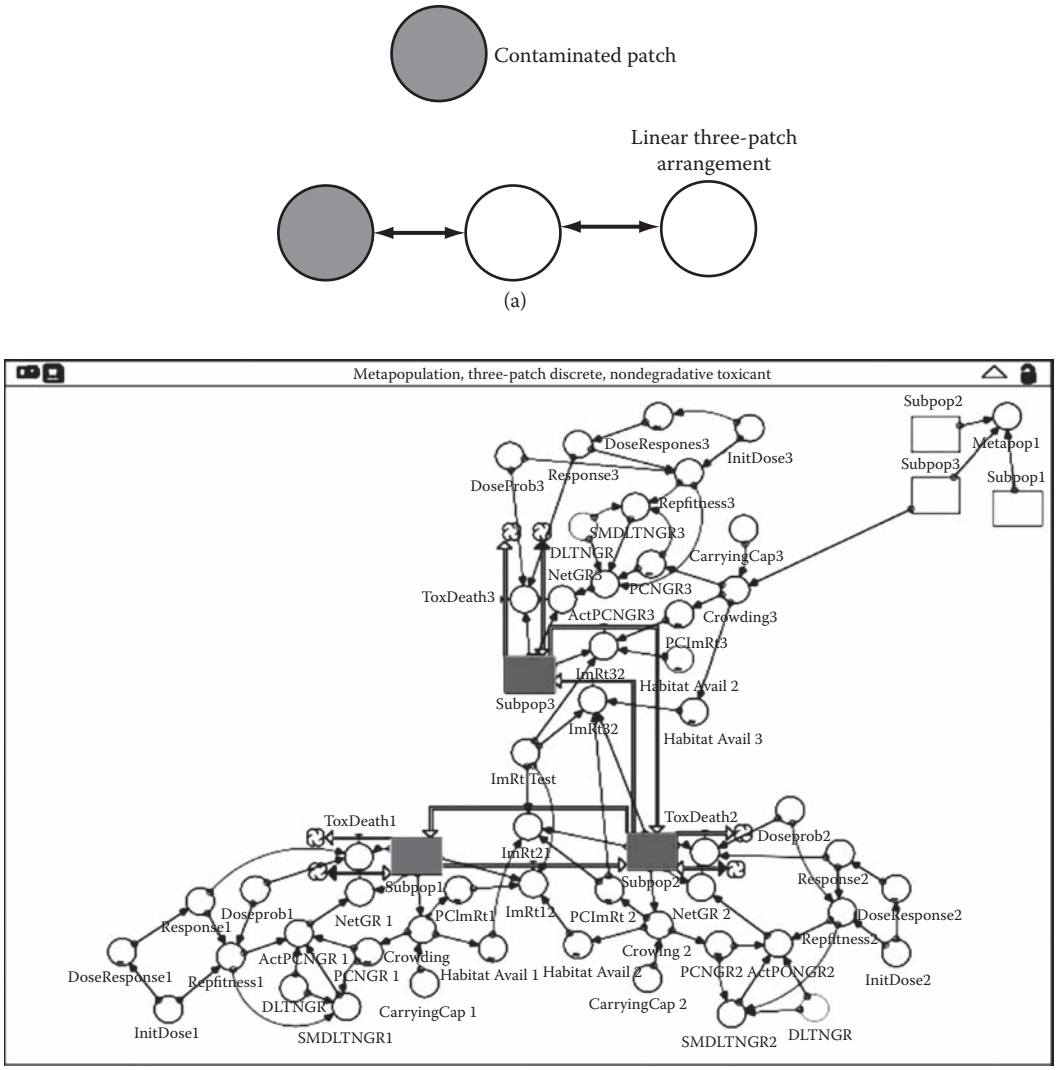

Figure 2.3 The patch dynamics model as a diagram and as a program in Stella. The original diagram used to formulate ideas about the effects of toxicants in a patchy landscape is presented in (a). As described in Chapter 12, the ideas in the original model were written into a Stella program, as presented in (b).

The use of models to organize information, generate hypotheses, and calculate tests to confirm the hypothesis has become recognized as a critical part of the scientific process. Naomi Oreskes and her colleagues (1994) have presented a clear argument as to the limitations of models.

Oreskes et al. (1994) was written in response to the growing number of numerical simulation models being used in the earth sciences and other fields. These models and those in other fields have been claimed by their writers to have been verified and validated. In other words, it was claimed that the models were a true representation of reality. Oreskes et al. pointed out

that this was not possible for open environmental systems. The following argument is derived from this paper.

To state that a model is valid and verified is simply another way of stating that it is true. The truth of the model is impossible to prove unless the model is a closed system, so that no other variables outside those being modeled can affect the results. Of course, environmental systems such as those in the earth sciences or in environmental toxicology are open. A toxicant is released to an environment with a multitude of different drivers, and not all of them immediately measurable. So at a fundamental level, models cannot be true representations of the systems they intend to reflect.

There are other issues in the use of simulation models. Measurements are inferred from other data. Diversity is not a direct measurement but a construct of species counts, and is dependent on the analysis tool used, the type of sampling gear used, the typology of the environment that was sampled, and the ability of the investigator to differentiate species.

Information about dynamics is inferred from sampling data that may not represent the true dynamics of the system. A sampling interval larger than the true frequency of the dynamic will flatten or obscure the true dynamic of the system.

There is always the scaling problem. How to transfer the information gathered at one scale to another remains an issue. How does a model of simple population dynamics transfer to a species that lives in a spatially and temporally variable environment over relevant timeframes of years to decades?

In the construction of a simulation model there are additional assumptions about cause–effect pathways and input parameters required to make a model work. Many of these assumptions are themselves models or hypotheses that may or may not have been adequately confirmed. These components act as auxiliary hypotheses, hidden in the structure of the model.

Models may also have errors that cancel out or are simply not important in determining the output given the sets of input parameters being used. These errors may not be apparent until other input parameters are used or the test fails its confirmation.

Oreskes and colleagues also make a differentiation between validation and verification. Validation can be defined as meaning that there are no errors in logic or reasoning, and that the code performs as intended. In models with many different interacting pieces it can be difficult to test that each part of the model is performing as expected. With the patch dynamic model described above we were fortunate in its being derived from the work of Jingauro Wu. We were able to set the toxicant concentration at zero and then observe if the model output was that of the Wu models that did not include the toxicant segments. We may share the same errors of logic and reasoning, but the codes provided similar output.

Unfortunately, *validation* is also used to mean that the model reflects the dynamics of reality. This step is better called verification, and it is not clear that this is possible for environmental systems. An open system is affected by a number of external drivers that will not be described by a closed model. Here is a simple example of confounding factors with a model that turns out not to be true:

> If it is sunny tomorrow, I will take my plane and go flying.
>
> If I am found flying, the consequence is that it is sunny.
>
> Nope, I really wanted to go flying.

At best a model can only be called confirmed, not verified. If the predicted outcome occurs, it is not known if it was due to the realism of the model or good fortune. Models making long-term predictions may not be directly testable because of the difficulty in sampling, changes in the surrounding landscapes, or other factors beyond the control of the investigator. However, predictions made by other aspects of the model can be tested to confirm incorporated assumptions or processes.

However, the failure to predict the observed outcome falsifies the model. It may be that there is a programming error or a false assumption. Data used to construct the model may not have been properly calibrated, or assumptions given as "facts" may be wrong. An overlooked feature of a model is its ability to be tested and found incorrect. Models that make too general a prediction are not particularly useful if specific predictions are not testable.

So given the caveats with models, why use them? There are several reasons.

First, models can be used to corroborate a hypothesis. As discussed in Chapter 12, Alan Johnson used an individual-based model to arrive at results that confirmed the action at a distance hypothesis generated by other modeling techniques.

Models can point out discrepancies in other models. In the modeling of the structure-activity relationships, a number of properties, such as molecular weight and the solubility of the organic in lipid, were used to predict toxicity. Although these models predicted the toxicity of a number of organics very well, organophosphates were poorly predicted. When a model was developed that incorporated the presence of an organophosphate into the equation, the prediction of toxicity improved. The interplay between modeling, data, and further modeling identified and confirmed a discrepancy in the original formulation. Structure-activity models are discussed further in Chapter 5.

Models allow *what if* types of questions that can be used in future planning. Models can calculate the future effects of a chemical upon a population, or the cleanup levels necessary to allow a species to increase in number. In risk assessment models, the impact of chemicals can be assessed as land use or pollution levels change. The use of risk assessment is covered extensively in Chapter 13.

In my experience, the second best use of models is in the organizing of thinking about the natural world and in the generation of additional hypotheses. Models generate frameworks into which cause–effect pathways can be generated. The variables that are necessary to generate a useful prediction are clearly stated in the model and can be used to guide experimental or field research.

As stated by Oreskes et al. (1994), perhaps the best use of models is when they are used to challenge existing ideas of how reality works. As discussed later in this chapter, HPDP, as described by Wu and Loucks (1995), is a model challenging the old ideas of equilibrium-based ecological models or even those based upon multiple equilibria. HPDP is also consistent with other novel models of the effects of toxicants on ecological structures. These models, community conditioning, and pollution-induced community tolerance are extensively discussed in Chapter 12.

There are many more discussions of models to follow in this textbook. Please keep these characteristics of models in mind as you proceed.

2.3 Fundamental Models for Environmental Toxicology

Environmental toxicology can be simplified to the understanding of only three functions. These functions are presented in Figure 2.4. First, there is the interaction of the introduced chemical,

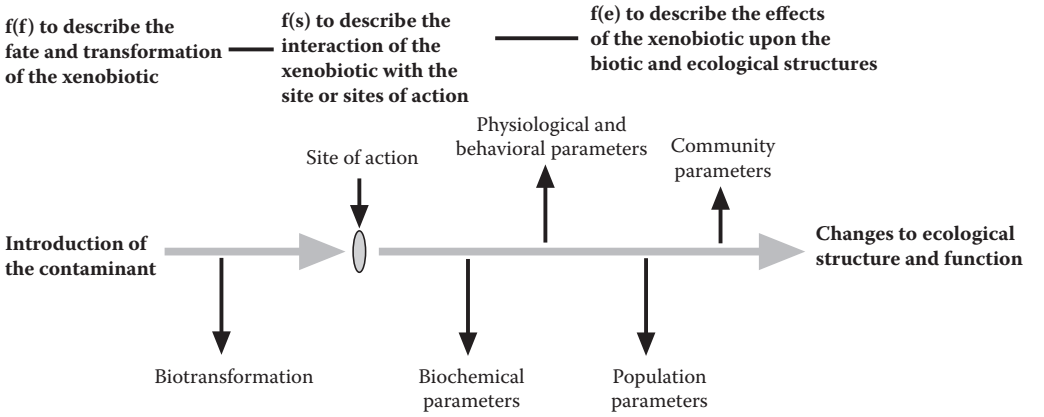

Figure 2.4 The three functions of environmental toxicology and the causal pathway. Only three basic functions need to be described after the introduction of a xenobiotic into the environment. The first describes the fate and distribution of the material in the biosphere and the organism after the initial release to the environment—f(f). The second function describes the interaction of the material with the site of action—f(s). The last function describes the impact of this molecular interaction upon the function of an ecosystem—f(e).

a xenobiotic, with the environment. This interaction controls the amount of toxicant or the dose available to the biota. Second, the xenobiotic interacts with its site of action. The site of action is the particular protein or other biological molecule that interacts with the toxicant. Third, the interaction of the xenobiotic with a site of action at the molecular level produces effects at higher levels of biological organization. If environmental toxicologists could write appropriate functions that would describe the transfer of an effect from its interaction with a specific receptor molecule to the effects seen at the community level, it would be possible to predict accurately the effects of pollutants in the environment. We are far from having a suitable understanding of these functions. The middle of the chapter introduces the critical factors for each of these functions. After the introduction, the three functions of ecological systems are introduced as complex structures that have both spatial and temporal scales. Finally, the hierarchical patch dynamics paradigm is introduced as a framework that may prove useful in combining complexity and scale. Unfortunately, at this time we do not clearly understand how the impacts seen at the population and community levels are propagated from molecular interactions.

2.4 The Classical Viewpoint for Classifying Toxicological Effects

Techniques have been derived to evaluate effects at each step, from the introduction of a xenobiotic to the biosphere to the final series of effects. These techniques are not uniform for each class of toxicant, and mixtures are even more difficult to evaluate. Given this background, however, it is possible to outline the current levels of biological interaction with a xenobiotic:

- Chemical physical–chemical characteristics
- Bioaccumulation/biotransformation/biodegradation
- Site of action
- Biochemical monitoring

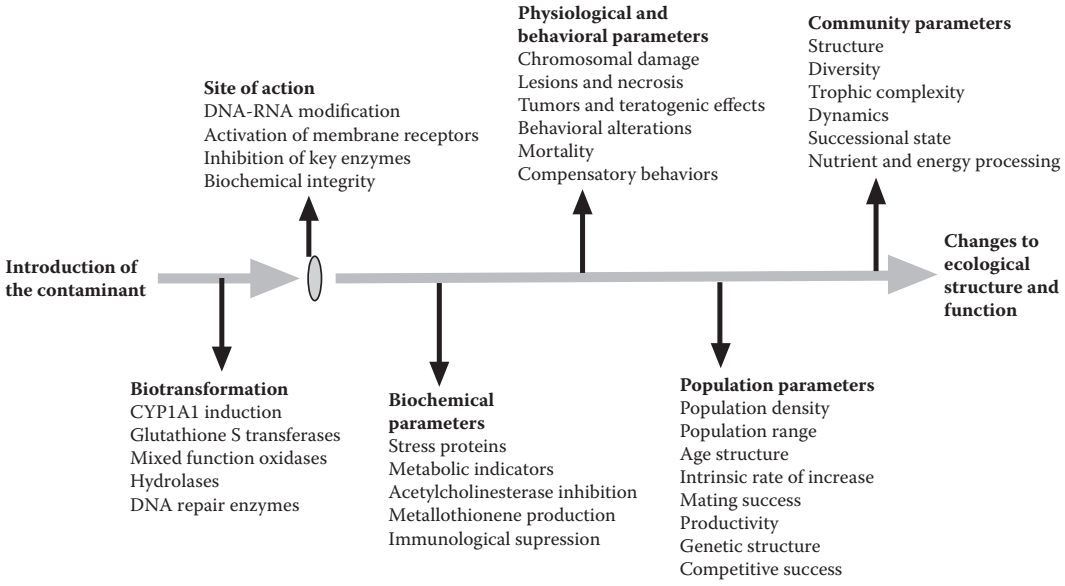

Figure 2.5 Parameters and indications of the interaction of a xenobiotic with the ecosystem. The examples listed are only a selection of the parameters that need to be understood for the explanation of the effects of a xenobiotic upon an ecosystem. However, biological systems appear to be organized within a hierarchy, and that is how environmental toxicology must frame its outlook upon environmental problems.

- Physiological and behavioral
- Population parameters
- Community parameters
- Ecosystem effects

Each level of organization can be observed and examined at various degrees of resolution. The factors falling under each level are illustrated in Figure 2.5. Examples of these factors at each level of biological organization (scale) are given below.

2.5 Chemical Physical–Chemical Characteristics

The interaction of the atoms and electrons within a specific molecule determines the impact of the compound at the molecular level. The contribution of the physical–chemical characteristics of a compound to the observed toxicity is called a quantitative structure-activity relationship (QSAR). QSAR has the potential to enable environmental toxicologists to predict the environmental consequences of toxicants using only structure as a guide. The response of a chemical to ultraviolet radiation and its reactivity with the abiotic constituents of the environment determines a fate of a compound.

It must be remembered that in most cases, the interaction at a molecular level with a xenobiotic is happenstance. Often this interaction is a by-product of the usual physiological function of the particular biological site with some other low molecular weight compound that occurs in the

normal metabolism of the organism. Xenobiotics often mimic these naturally occurring organisms, causing degradation and detoxification in some cases, and toxicity in others.

2.6 Bioaccumulation/Biotransformation/Biodegradation

A great deal can occur to a xenobiotic from its introduction to the environment to its interaction at the site of action. Many materials are altered in specific ways, depending upon the particular chemical characteristics of the environment. Bioaccumulation, the increase in concentration of a chemical in tissue compared to the environment, often occurs with materials that are more soluble in lipid and organics (lipophilic) than in water (hydrophilic). Compounds are often transformed into other materials by the various metabolic systems that reduce or alter the toxicity of materials introduced to the body. This process is biotransformation. Biodegradation is the process that breaks down a xenobiotic into a simpler form. Ultimately, the biodegradation of organics results in the release of CO_2 and H_2O to the environment.

2.7 Receptors and the Mode of Action

The site at which the xenobiotic interacts with the organism at the molecular level is particularly important. This receptor molecule or site of action may be the nucleic acids, specific proteins within nerve synapses or present within the cellular membrane, or it can be very nonspecific. Narcosis may affect the organism not by interaction with a particular key molecule, but by changing the characteristics of the cell membrane. The particular kind of interaction determines whether the effect is broad or more specific within the organism and phylogenetically.

2.8 Biochemical and Molecular Effects

There are broad ranges of effects at this level. We will use as an example, at the most basic and fundamental of changes, alterations to DNA.

DNA adducts and strand breakages are indicators of genotoxic materials, compounds that affect or alter the transmission of genetic material. One advantage to these methods is that the active site can be examined for a variety of organisms. The methodologies are proven and can be used virtually regardless of species. However, damage to the DNA only provides a broad classification as to the type of toxicant. The study of the normal variation and damage to DNA in unpolluted environments has just begun.

Cytogenetic examination of meiotic and mitotic cells can reveal damage to genetic components of the organism. Chromosomal breakage, micronuclei, and various trisomies can be detected microscopically. Few organisms, however, have the requisite chromosomal maps to score accurately more subtle types of damage. Properly developed, cytogenetic examinations may prove to be powerful and sensitive indicators of environmental contamination for certain classes of material.

A more complicated and ultimately complex system directly affected by damage to certain regions of DNA and to cellular proteins is the inhibition of the immunological system of an organism, immunological suppression. Immunological suppression by xenobiotics could have subtle but important impacts on natural populations. Invertebrates and other organisms have a variety of immunological responses that can be examined in the laboratory setting from field collections.

The immunological responses of bivalves in some ways are similar to those of vertebrate systems and can be suppressed or activated by various toxicants. Mammals and birds have well-documented immunological responses, although the impacts of pollutants are not well understood. Considering the importance to the organism, immunological responses could be very valuable at assessing the health of an ecosystem at the population level.

2.9 Physiological and Behavioral Effects

Physiological and behavioral indicators of impact within a population are the classical means by which the health of populations is assessed. The major drawback has been the extrapolation of these factors based upon the health of an individual organism, attributing the damage to a particular pollutant, and extrapolating this to the population level.

Lesions and necrosis in tissues have been the cornerstone of much environmental pathology. Gills are sensitive tissues and often reflect the presence of irritant materials. In addition, damage to the gills has an obvious and direct impact upon the health of the organism. Related to the detection of lesions is the detection of tumors. Tumors in fish, especially flatfish, have been extensively studied as indicators of oncogenic materials in marine sediments. Oncogenesis has also been extensively studied in medaka and trout as a means of determining the pathways responsible for tumor development. Development of tumors in fish more commonly found in natural communities should follow similar mechanisms. As with many indicators of toxicant impact, relating the effect of tumor development to the health and reproduction of a wild population has not been as closely examined as the endpoint.

Reproductive success is certainly another measure of the health of an organism and is the principal indicator of the Darwinian fitness of an organism. In a laboratory situation it certainly is possible to measure fecundity and the success of offspring in their maturation. In nature these parameters may be very difficult to measure accurately. Many factors other than pollution can lead to poor reproductive success. Secondary effects, such as the impact of habitat loss on zooplankton populations essential for fry feeding, will be seen in the depression or elimination of the young age classes.

Mortality is certainly easy to assay on the individual organism. Macroinvertebrates, such as bivalves and cnideria, can be examined, and since they are relatively sessile, the mortality can be attributed to a factor in the immediate environment. Fish, being mobile, can die due to exposure kilometers away or because of multiple intoxications during their migrations. By the time the fish are dying, the other levels of the ecosystem are in a sad state.

The use of the cough response and ventilatory rate of fish has been a promising system for the determination and prevention of environmental contamination. Pioneered at the Virginia Polytechnic Institute and State University, the measurement of the ventilatory rate of fish using electrodes to pick up the muscular contraction of the operculum has been brought to a very high stage of refinement. It is now possible to continually monitor the water quality as perceived by the test organisms with a desktop computer analysis system at a relatively low cost.

2.10 Population Parameters

A variety of endpoints have been used, including number and structure of a population, to indicate stress. Population numbers or density has been widely used for plant, animal, and microbial

populations in spite of the problems in mark recapture and other sampling strategies. Since younger life stages are considered to be more sensitive to a variety of pollutants, shifts in age structure to an older population may indicate stress. In addition, cycles in age structure and population size occur due to the inherent properties of the age structure of the population and predator–prey interactions. Crashes in populations such as those of the striped bass in the Chesapeake Bay do occur and certainly are observed. A crash often does not lend itself to an easy cause–effect relationship, making mitigation strategies difficult to create.

The determination of alterations in genetic structure, that is, the frequency of certain marker alleles, has become increasingly popular. The technology of gel electrophoresis has made this a seemingly easy procedure. Population geneticists have long used this method to observe alterations in gene frequencies in populations of bacteria, protozoans, plants, various vertebrates, and the famous *Drosophila*. The largest drawback in this method is ascribing differential sensitivities to the genotypes in question. Usually, a marker is used that demonstrates heterogeneity within a particular species. Toxicity tests can be performed to provide relative sensitivities. However, the genes that have been looked at to date are not genes controlling xenobiotic metabolism. These genes have some other physiological function and act as a marker for the remainder of the genes within a particular linkage group. In spite of these issues, the method has some promise to provide both populational and biochemical data that may prove useful in certain circumstances, although there are some problems.

Alterations in the competitive abilities of organisms can indicate pollution. Obviously, bacteria that can use a xenobiotic as a carbon or other nutrient source, or that can detoxify a material, have a competitive advantage, with all other factors being equal. Xenobiotics may also enhance species diversity if a particularly competitive species is more sensitive to a particular toxicant. These effects may lead to an increase in plant or algal diversity after the application of a toxicant.

2.11 Community Effects

The structure of biological communities has always been a commonly used indicator of stress in them. Early studies on cultural eutrophication emphasized the impacts of pollution as they altered the species composition and energy flow of aquatic ecosystems. Various biological indices have been developed to judge the health of ecosystems by measuring aspects of the invertebrate, fish, or plant populations. Perhaps the largest drawback is the effort necessary to determine the structure of ecosystems and to understand pollution-induced effects from normal successional changes. There is also the temptation to reduce the data to a single index or other parameter that eliminates the dynamics and stochastic properties of the community.

One of the most widely used indices of community structure has been species diversity. Many measures for diversity are used, from such elementary forms as species number to measures based on information theory. A decrease in species diversity is usually taken as an indication of stress or impact upon a particular ecosystem. Diversity indices, however, hide the dynamic nature of the system and the effects of island biogeography and seasonal state. As demonstrated in microcosm experiments, diversity is often insensitive to toxicant impacts.

Related to diversity is the notion of static and dynamic stability in ecosystems. Traditional dogma stated that diverse ecosystems were more stable and therefore healthier than less rich ecosystems. The work in the early 1970s of May did much to question these almost unquestionable assumptions about properties of ecosystems. We certainly do not doubt the importance of biological diversity, but diversity itself may indicate the longevity and size of the habitat rather than

the inherent properties of the ecosystem. Rarely are basic principals such as island biogeography incorporated into comparisons of species diversity when assessments of community health are made. Diversity should be examined closely as to its worth in determining xenobiotic impacts upon biological communities.

Currently, it is difficult to pick a parameter that describes the health of a biological community and have that form a basis of prediction. A single variable or magic number may not even be possible. In addition, what are often termed biological communities is based upon human constructs. The members of the marine benthic invertebrate community interact with many other types of organisms, microorganisms, vertebrates, and protists, which in many ways determines the diversity and persistence of an organism. Communities can also be defined as functional groups, such as the intertidal community or alpine forest community, which may more accurately describe functional groupings of organisms.

2.12 Ecosystem Effects

Alterations in the species composition and metabolism of an ecosystem are the most dramatic impacts that can be observed. Acid precipitation has been documented to cause dramatic alterations in both aquatic and terrestrial ecosystems. Introduction of nutrients certainly increases the rate of eutrophication.

Effects can occur that alter the landscape pattern of the ecosystem. Changes in global temperatures have had dramatic effects upon species distributions. Combinations of nutrient inputs, utilization, and toxicants have dramatically altered the Chesapeake Bay system.

2.13 An Alternative Framework Incorporating Complexity Theory

The above framework is a classical approach to presenting the impacts of chemicals upon various aspects of biological and ecological systems. It is possible that an alternative exists that more accurately portrays the fundamental properties of each aspect of these systems.

Such a framework is in the initial stages of development and has been published in outline form (Landis et al. 1995, 1996). The basic format of this framework is straightforward. There are two distinctly different types of structures that concern environmental toxicology (Figure 2.6).

Organisms have a central core of information, subject to natural selection, that can impose homeostasis (body temperature) or diversity (immune system) upon the constituents of that system. The genome of an organism is highly redundant, a complete copy existing in virtually every cell, and directed communication and coordination between different segments of the organism is a common occurrence. Unless there are changes in the genetic structure of the germ line, impacts to the somatic cells and structure of the organism are erased upon the establishment of a new generation.

Nonorganismal or ecological structures have fundamentally different properties. There is no central and inheritable repository of information, analogous to the genome, that serves as the blueprint for an ecological system. Furthermore, natural selection is selfish, working upon the phenotype characteristic of a genome and its close relatives, and not upon a structure that exists beyond the confines of a genome.

The lack of a blueprint and the many interactions and nonlinear relationships within an ecosystem mean that the history of past events is written into the structure and dynamics. The

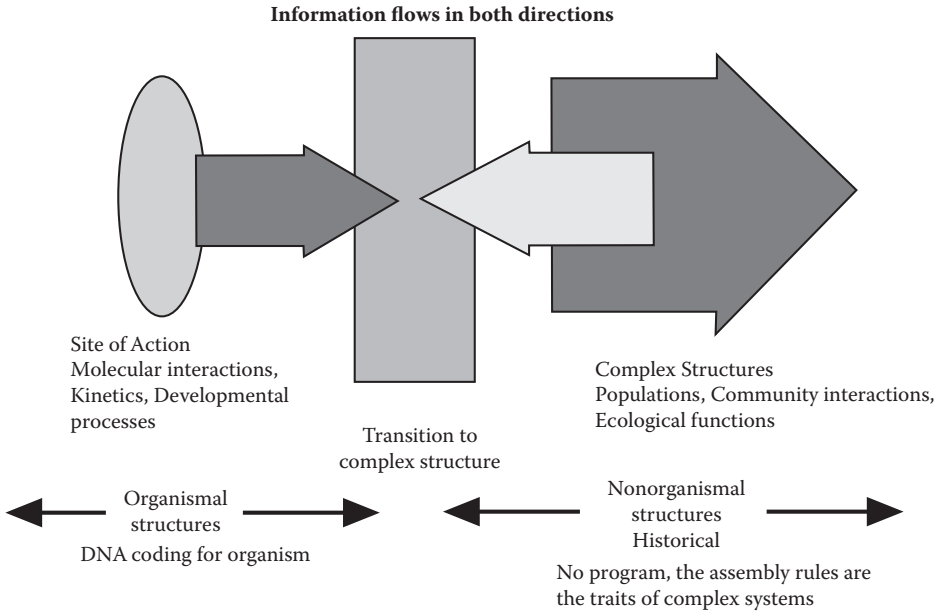

Figure 2.6 Organismal and nonorganismal framework. As the information is passed on to the complex structure it becomes part of the history of the ecosystem.

many nonlinear dynamics and historical nature of ecosystems confer upon the system the property of complexity.

Complex, nonlinear structures have specific properties, listed by Çambel (1993). A few particularly critical to how ecosystems react to contaminants are:

1. Complex structures are neither completely deterministic nor stochastic, and exhibit both characteristics.
2. The causes and effects of the events the system experiences are not proportional.
3. The different parts of complex systems are linked and affect one another in a synergistic manner.
4. Complex systems undergo irreversible processes.
5. Complex systems are dynamic and not in equilibrium; they are constantly moving targets.

These properties are especially important in the design, data analysis, and interpretation of multispecies toxicity tests, field studies, and environmental risk assessment and will be discussed in the appropriate sections. This alternate approach rejects the smooth transition of effects and recognizes that ecosystems have fundamentally different properties and are expected to react unexpectedly to contaminants.

2.14 Spatial and Temporal Scales

Not only are there scales in organization, but also scales over space and time exist. It is crucial to note that all of the functions described in previous sections act at a variety of spatial and temporal

scales (Suter and Barnthouse 1993). Although in many instances these scales appear disconnected, they are in fact intimately intertwined. Effects at the molecular level have ecosystem level effects. Conversely, impacts on a broad scale affect the very sequence of the genetic material as evolution occurs in response to the changes in toxicant concentrations or interspecific interactions.

The range of scales important in environmental toxicology is from a few angstroms of molecular interactions to hundreds of thousands of square kilometers affected by large-scale events. Figure 2.7 presents some of the organizational aspects of ecological systems with their corresponding temporal and spatial scales. The diagram is only a general guide. Molecular activities and degradation may exist over short periods and volumes, but their ultimate impact may be global.

Perhaps the most important example of a new biochemical pathway generating a global impact was the development of photosynthesis. Originally, the atmosphere of earth was reducing. Photosynthesis produces oxygen as a by-product. Oxygen, which is quite toxic, became a major constituent of the atmosphere. This change produced a mass extinction event, yet also provided for the evolution of much more efficient metabolisms.

Conversely, effects at the community and ecosystem level have effects upon lower levels of organization. The structure of the ecological system may allow some individuals of populations to migrate to areas where the species are below a sustainable level or are at extinction. If the pathways to the depleted areas are not too long, the source population may rescue the population that is below a sustainable level. Instead of extinction, a population may be sustainable or

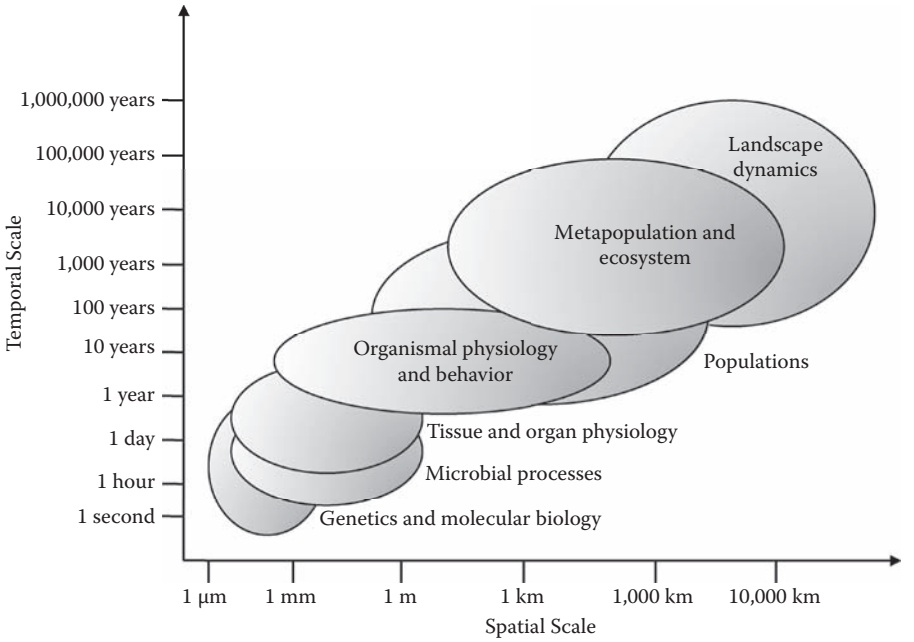

Figure 2.7 The overlap of spatial and temporal scales in environmental toxicology. Not only are there scales in organization, but also scales over space and time exist. Many molecular activities exist over short periods and volumes. Populations can exist over relatively small areas, even a few square meters for microorganisms, and thousands of square kilometers for many bird and mammal populations. Although often diagrammed as discrete, each of these levels is intimately connected and passes one into another along both the space and time scales.

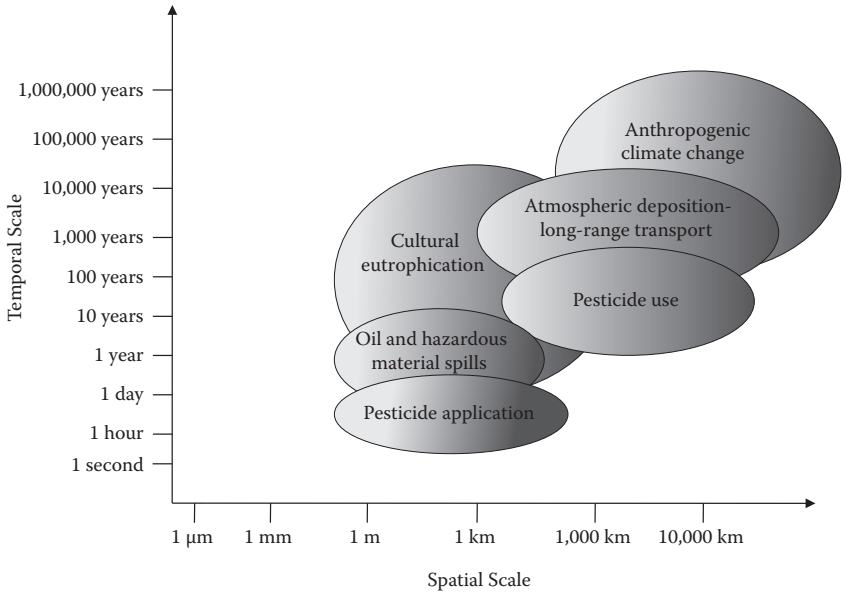

Figure 2.8 The overlap of spatial and temporal scales in chemical contamination. Just as there are scales of ecological processes, contamination events also range in scale. Pesticide applications can range from small-scale household use to large-scale agricultural applications. The addition of surplus nutrients and other materials due to agriculture or human habitation is generally large-scale and long-lived. Acid precipitation generated by the tall stacks of the midwestern United States is a fairly recent phenomenon, but the effects will likely be long-term. However, each of these events has molecular scale interactions.

even increase due to its rescue from a neighboring population. If the structure of the ecological landscape provides few opportunities for rescue, localized extinctions are more likely.

Just as the effects of a toxicant can range over a variety of temporal scales, so can the nature of the input of the toxicant to the system (Figure 2.8). Household or garden use of a pesticide may be an event with a scale of a few minutes and a square meter. The addition of nutrients to ecological systems due to industrialization and agriculture may cover thousands of square kilometers and persist for hundreds or thousands of years. The duration and scale of anthropogenic inputs does vary a great deal; however, it is crucial to realize that the interactions of the toxicant with the organism are still at the molecular level. Small effects can have global implications.

2.15 Combining Scale and Ecological Dynamics: The Hierarchical Patch Dynamic Paradigm

The previous sections have set the requirements for an overall construction for estimating toxicant impacts. An accurate framework for estimating the impacts of toxicants upon ecological systems incorporates a variety of spatial and temporal scales, handles heterogeneity in time and space, and incorporates the wide range of observed ecological dynamics.

HPDP meets the above requirements (Wu and Loucks 1995; Wu and David 2002). The HPDP is a model for describing at a fundamental level the interactions and dynamics of ecological

Table 2.1 The Central Assumptions of the HPDP

1. Ecological systems are spatially structured patch hierarchies with larger patches constructed from smaller patches.
2. Dynamics of an ecological system can be studied as the composite dynamics of individual patches and the interactions of those patches with others at the same and adjacent hierarchical levels.
3. Pattern and process, cause and effect are scale dependent. Interaction occurs when both are at the same domain of scale in space and time.
4. Nonequilibrium and stochastic processes are common and essential for the apparent spatial and temporal patterns and processes found in ecological systems.
5. Perceived stability in ecological systems frequently takes the form of metastability achieved through structural and functional redundancy incorporated in space and time. Patterns that appear stable at one scale may be due to nonequilibrium and stochastic processes occurring at adjacent hierarchies of scale.

Source: Modified after Wu, J., and David, J. L., *Ecol. Model.*, 153, 7–26, 2002.

systems at landscape and regional scales. The HPDP inherently incorporates and predicts a variety of temporal and spatial scales, heterogeneity, and a wide range of dynamics. The basic tenets are listed in Table 2.1 and diagrammed in Figure 2.9. This framework is an alternative to models of ecological systems that incorporate a balance of nature, inherent stability, or multiple equilibria.

The hierarchical portion of HPDP refers to the different levels of scale that are operational in ecological systems. Hierarchy does not imply that the controlling factors are operating in a top-down or bottom-up fashion, but that the level of scale is important in understanding the factors controlling ecological functions. In order to make predictions about one level of the hierarchy, it is critical to understand the contributions from factors at the levels of scale just above and below.

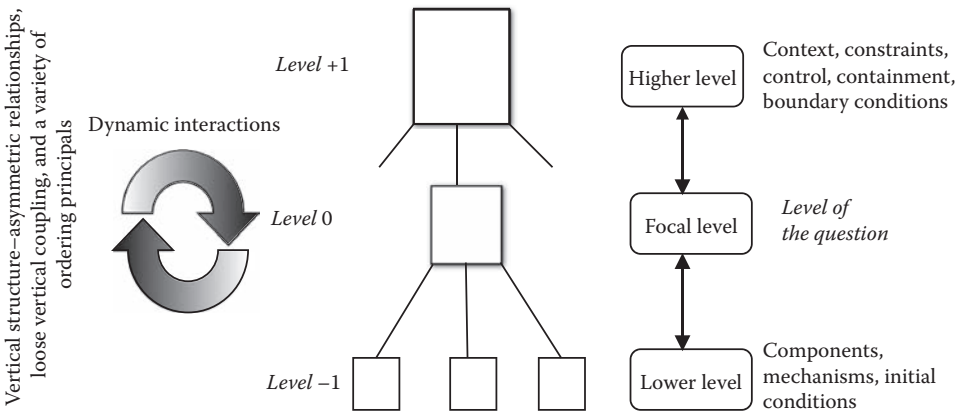

Figure 2.9 Hierarchical patch dynamics paradigm.

The patch aspect of HPDP refers to the location, distribution, and dynamics of patches within an environment. The characteristics of the patches within an environment have a major impact upon the distribution of species, interactions between stressors and receptors, and environmental change. Patches are also assumed to be dynamic in nature, changing location, inherent variability, and composition.

The dynamics of the Pacific herring (*Clupea pallasi*) run at Cherry Point, Washington, are an example of the importance of scale and grain size. The run at Cherry Point spawns in the late spring and early summer along the extreme northwest coast of Washington State. During the rest of the year the members of the Cherry Point run apparently roam the Strait of Georgia and migrate to the western side of Vancouver Island. During the relatively short spawning period at Cherry Point the population is exposed to a variety of factors at the scale of a few kilometers with a fine grain size. These fine-grained factors, compared to the habitat used by the Pacific herring, include spawning habitat, effluents and runoff from the industrial and agricultural areas, salinity changes from freshwater inputs, local predators, and shading due to the piers for the refineries and deposition from an aluminum smelter. In addition, there has been local harvesting of both the adults and eggs as the herring spawn along the nearshore environment. As the population disperses postrun and migrates throughout the area, larger-scale, coarser-grained factors become influential. The northeastern Pacific decadal oscillation (PDO) changes water temperature over a 30-year cycle. The influence of the PDO on water temperature impacts a variety of ecological processes and changes the distribution of predators and prey items within the region. There are also predators that have large-scale home ranges, such as the orcas (killer whales) and salmon. Pacific herring also have a large-scale population structure, with those runs along the British Columbia coast being part of a metapopulation. Finally, there can be exposure to contaminants that exist at broad spatial scales, such as halogenated organics.

HPDP explicitly incorporates, in the case of the Cherry Point herring, these levels of scale and grain size, which are critical to consider (Figure 2.10). A framework that applies the components

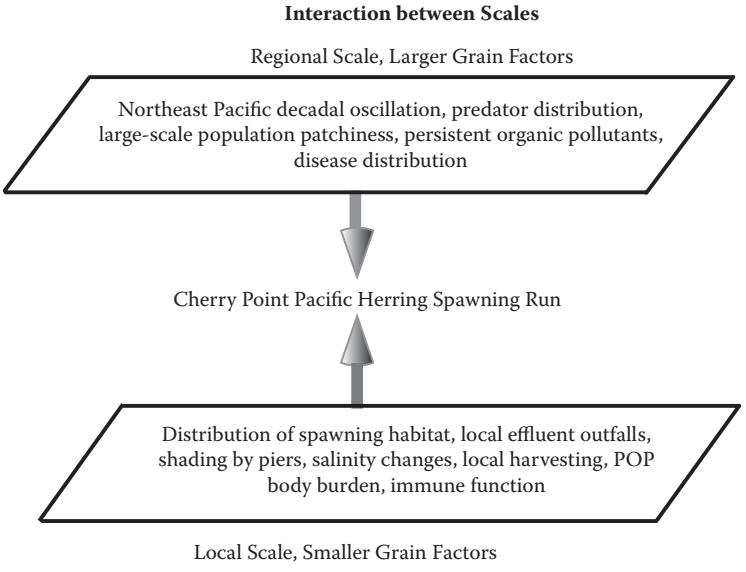

Figure 2.10 The hierarchy of scale illustrated by the Cherry Point run of Pacific herring.

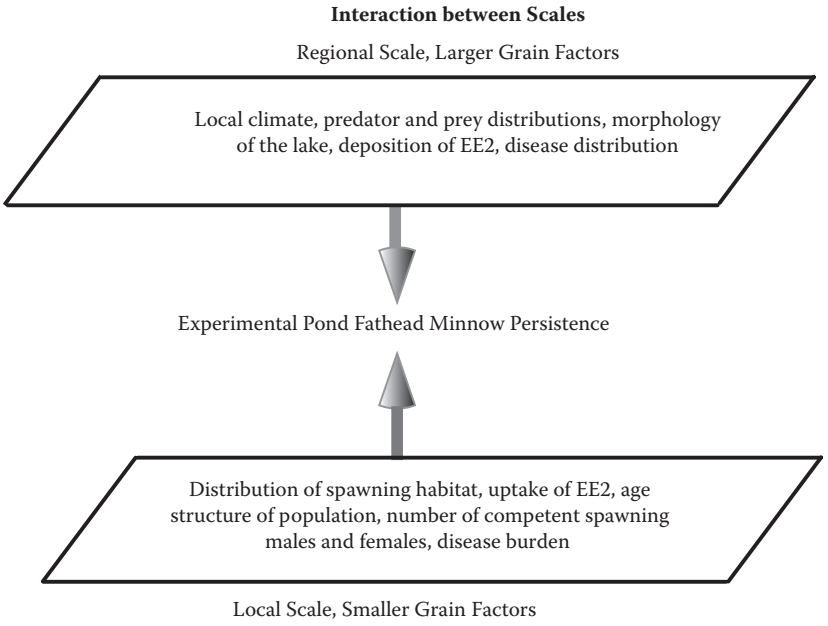

Figure 2.11 The hierarchy of scale for the fathead minnow EE2. (Experiments by Kidd, K. A. et al., *Proc. Natl. Acad. Sci. USA,* 104, 8897–8901, 2007.)

of HPDP immediately places an endpoint or assessment endpoint into an ecological-relevant contextual framework including spatial scale, grain size, and temporal relationships. The framework of HPDP can also be used in experimental pond or smaller-scale scenarios.

Kidd et al. (2007) dosed an experimental pond with 17α-ethynylestradiol (EE2) and observed the population dynamics of fathead minnows (*Pimephales promelas*). The structure of the fathead minnows in the dosed lake progressively aged because of the loss of the addition of newly hatched fish. The physiological states of the male and female fish were observed and marked changes in reproductive ability were noted. In this experiment information at the scales of the population, individual reproductive status, and the fish community structure was available. An HPDP diagram similar to that for the Cherry Point Pacific herring can be constructed for this situation (Figure 2.11). Similar factors to the Cherry Point case can be found at each level of the fathead minnow case.

Wu and David (2002) have also demonstrated that the HPDP framework can incorporate anthropogenic features such as land use boundaries, roads, and urbanization. The HPDP can be used as a framework for incorporating various spatial and temporal scales for a variety of systems.

2.16 Strategy and Tactics in the Use of Models in Environmental Toxicology

Models of every type are used in environmental toxicology. There are three broad classifications of models in ecology (Nisbet and Gurney 1982): tactical, simulation, and strategic.

Tactical models are designed to make specific and short-term predictions or forecasts of specific populations or communities.

Simulation models fall into this category. Detailed information about the species, interactions, and physical characteristics of the system are necessary. Simulation models are generally detailed, requiring complex computations, and not mathematically tractable for simplification.

Strategic models are usually simple and mathematically tractable. These models are designed to explore basic principals of ecology, toxicology, chemistry, geology, and other fields, and are not designed to mimic a particular population or environment. Such models include the logistic equation for population growth, competition equations, and most of the models presented in Chapter 11. Although the models may be simple, complex dynamics yielding intense discussion can result.

Study Questions

1. Define *science* and outline features of the scientific process.
2. Define *paradigm* and how it fits into the process of discovery.
3. Falsification is important in science. Explain why.
4. Why are models important to the field of science and environmental toxicology?
5. List the characteristics of a useful model.
6. Define the three functions to be understood to simplify environmental toxicology.
7. Define *QSAR*.
8. Define *bioaccumulation*, *biotransformation*, and *biodegradation*.
9. What is *site of action*?
10. Describe limits to the use of DNA alteration as an indicator of genotoxic materials.
11. Describe immunological suppression.
12. Name three major physiological indicators of impact by a xenobiotic on a population.
13. Describe a problem with using population parameters to indicate xenobiotic challenge.
14. Name two means by which a xenobiotic can alter competitive abilities of organisms.
15. What are the most dramatic impacts observable on ecosystems by xenobiotics?
16. Is the arrow describing the interactions of the ecological system with a chemical pollutant unidirectional?
17. In what ways are organisms simple structures?
18. What are the characteristics of complex structures?
19. If ecosystems are complex structures, can they be in equilibrium?
20. What are the disadvantages and advantages to the organismal-nonorganismal model compared to the conventional model?
21. Characterize ecological functions and processes by temporal and spatial scales.
22. What are the interactions between the scale of a chemical contamination and that of the affected ecological system?
23. What are the key characteristics of the hierarchical patch dynamics paradigm (HPDP)?
24. Explain how the HPDP is a useful paradigm for framing our understanding of environmental toxicology.

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Chapter 3

An Introduction to Toxicity Testing

3.1 Introduction

Toxicity is the property or properties of a material that produce a harmful effect upon a biological system. A toxicant is the material that produces this biological effect. The majority of the chemicals discussed in this text are of man-made or anthropogenic origin. This is not to deny that extremely toxic materials are produced by biological systems; venom, botulinum endotoxin, and some of the fungal aflatoxins are extremely potent materials. However, compounds that are derived from natural sources are produced in low amounts. Industrial compounds can be produced in the millions of kilograms per year.

Materials introduced into the environment come from two basic types of sources. Point discharges are derived from such sources as sewage discharges, waste streams from industrial sources, hazardous waste disposal sites, and accidental spills. Point discharges are generally easy to characterize as to the types of materials released, rates of release, and total amounts. In contrast, nonpoint discharges are those materials released from agricultural runoffs, contaminated soils and aquatic sediments, atmospheric deposition, and urban runoff from such sources as parking lots and residential areas. Nonpoint discharges are much more difficult to characterize. In most situations, discharges from nonpoint sources are complex mixtures, amounts of toxicants are difficult to characterize, and the rates and timing of discharges are as difficult to predict as the rain. One of the most difficult aspects of nonpoint discharges is that the components can vary in their toxicological characteristics.

Many classes of compounds can exhibit environmental toxicity. One of the most commonly discussed and researched is the pesticides. Pesticide can refer to any compound that exhibits toxicity to an undesirable organism. Since the stochastic processes of evolution link the biochemistry and physiology of all organisms, a compound toxic to a Norway rat is likely to be toxic to other small mammals. Industrial chemicals also are a major concern because of the large amounts

transported and used. Metals from mining operations, manufacturing, and as contaminants in lubricants are also released to the environment. Crude oil and the petroleum products derived from the oil are a significant source of environmental toxicity because of their persistence and common usage in an industrialized society. Many of these compounds, especially metal salts and petroleum, can be found in uncontaminated environments. In many cases, metals such as copper and zinc are essential nutrients. However, it is not just the presence of a compound that poses a toxicological threat, but the relationships between its dose to an organism and its biological effects that determine what environmental concentrations are harmful.

Any chemical material can exhibit harmful effects when the amount introduced to an organism is high enough. Simple exposure to a chemical also does not mean that a harmful effect will result. Of critical importance is the dose, or actual amount of material, that enters an organism, that determines the biological ramifications. At low doses no apparent harmful effects occur. In fact, many toxicity evaluations result in increased growth of the organisms at low doses. Higher doses may result in mortality. The relationship between the dose and biological effect is the dose-response relationship. In some instances, no effects can be observed until a certain threshold concentration is reached. In environmental toxicology, environmental concentration is often used as a substitute for knowing the actual amount or dose of a chemical entering an organism. Care must be taken to realize that dose may be only indirectly related to environmental concentration. The surface-to-volume ratio, shape, external covering, and respiratory systems can all dramatically affect the rates of a chemical's absorption from the environment. Since it is common usage, concentration will be the variable from which mortality will be derived, but with the understanding that concentration and dose are not always directly proportional or comparable from species to species.

3.2 The Dose-Responsive Curve

The graph describing the response of an enzyme, organism, population, or biological community to a range of concentrations of a xenobiotic is the dose-response curve. Enzyme inhibition, DNA damage, death, behavioral changes, and other responses can be described using this relationship.

Table 3.1 presents the data for a typical response over concentration or dose for a particular xenobiotic. At each concentration the percentage or actual number of organisms responding or the magnitude of effects is plotted (Figure 3.1). The distribution that results resembles a sigmoid curve. The origin of this distribution is straightforward. If only the additional mortalities seen at each concentration are plotted, the distribution that results is a normal one, or a bell-shaped curve (Figure 3.2). This distribution is not surprising. Responses or traits from organisms that are

Table 3.1 Toxicity Data for Compound 1

	Dose								
	0.5	*1.0*	*2.0*	*3.0*	*4.0*	*5.0*	*6.0*	*7.0*	*8.0*
Cumulative toxicity	0.0	2.0	7.0	23.0	78.0	92.0	97.0	100.0	100.0
Percent of additional deaths at each concentration	0.0	2.0	5.0	15.0	55.0	15.0	5.0	3.0	0.0

Note: All of the toxicity data are given as a percentage of the total organisms at a particular treatment group. For example, if 7 out of 100 organisms died or expressed other end-points at a concentration of 2 mg/kg, then the responding percentage would be 7%.

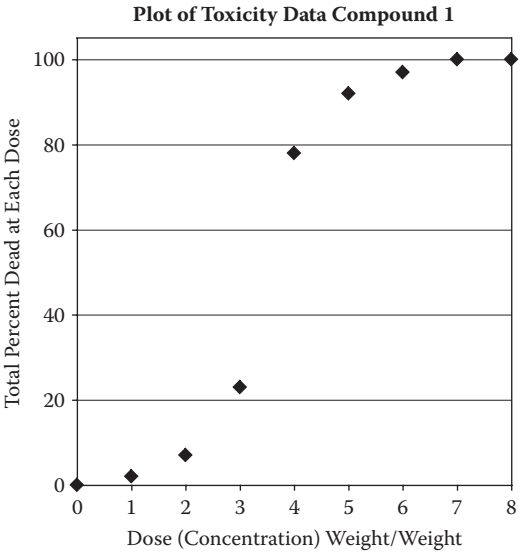

Figure 3.1 Plot of cumulative mortality versus environmental concentration or dose. The data are plotted as the cumulative number of dead by each dose using the data presented in the figure. The X axis is in units of weight to volume (concentration) or weight of toxicant per unit weight of animal (dose).

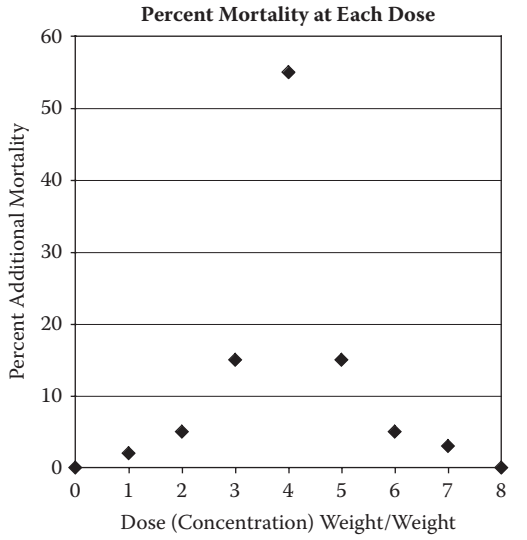

Figure 3.2 Plot of mortality versus environmental concentration or dose. Not surprisingly, the distribution that results is a normal one, or a bell-shaped curve. Responses or traits from organisms that are controlled by numerous sets of genes follow bell-shaped curves. Length, coat color, and fecundity are examples of multigenic traits whose distribution results in a bell-shaped curve. The X axis is in units of weight to volume (concentration) or weight of toxicant per unit weight of animal (dose).

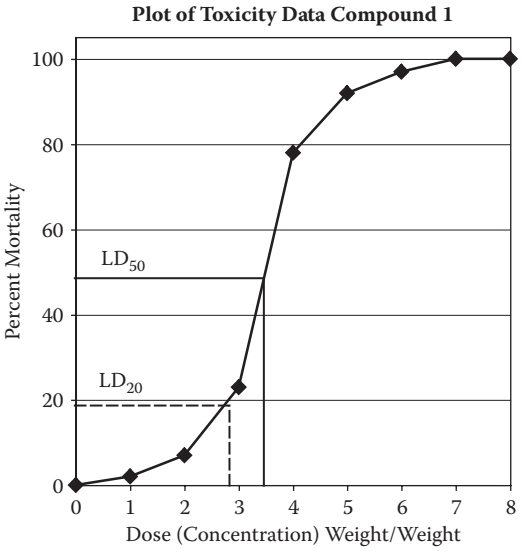

Figure 3.3 The sigmoid dose-response curve. Converted from the discontinuous bar graph of Figure 3.2 to a line graph if mortality is a continuous function of the toxicant, the result is the typical sigmoid dose-response curve. The X axis is in units of weight to volume (concentration) or weight of toxicant per unit weight of animal (dose).

controlled by numerous sets of genes follow bell-shaped curves. Length, coat color, and fecundity are examples of multigenic traits whose distribution results in a normal one.

The distribution of mortality versus concentration or dose is drawn so that the cumulative mortality is plotted at each concentration. At each concentration the total numbers of organisms that have died by that concentration are plotted. The presentation in Figure 3.1 is usually referred to as a dose-response curve. Data are plotted as continuous, and a sigmoid curve usually results (Figure 3.3). Two parameters of this curve are used to describe it: (1) the concentration or dose that results in 50% of the measured effect and (2) the slope of the linear part of the curve that passes through the midpoint. Both parameters are necessary to describe accurately the relationship between chemical concentration and effect. The midpoint is commonly referred to as a LD_{50}, LC_{50}, EC_{50}, or IC_{50}. The definitions are relatively straightforward:

LD_{50}—The dose that causes mortality in 50% of the organisms tested estimated by graphical or computational means.

LC_{50}—The concentration that causes mortality in 50% of the organisms tested estimated by graphical or computational means.

EC_{50}—The concentration that has an effect on 50% of the organisms tested estimated by graphical or computational means. Often this parameter is used for effects that are not death.

IC_{50}—The inhibitory concentration that reduces the normal response of an organism by 50%, as estimated by graphical or computational means. Growth rates of algae, bacteria, and other organisms are often measured as an IC_{50}.

One of the primary reasons for conducting any type of toxicity test is to rank chemicals as to their toxicity. Table 3.2 provides data on toxicity for two different compounds. It is readily

Table 3.2 Toxicity Data for Compounds 2 and 3

	Dose								
	0.5	*1.0*	*2.0*	*3.0*	*4.0*	*5.0*	*6.0*	*7.0*	*8.0*
Compound 2									
Cumulative toxicity	1.0	3.0	6.0	11.0	21.0	36.0	86.0	96.0	100.0
Percent of additional deaths at each concentration	1.0	2.0	3.0	5.0	10.0	15.0	50.0	10.0	4.0
Compound 3									
Cumulative toxicity	0.0	5.0	15.0	30.0	70.0	85.0	95.0	100.0	100.0
Percent of additional deaths at each concentration	0.0	5.0	10.0	15.0	40.0	15.0	10.0	5.0	0.0

apparent that the midpoint for compound 2 will likely be higher than for compound 1. A plot of the cumulative toxicity (Figure 3.4) confirms that the concentration that causes mortality to half of the population for compound 2 is higher than that for compound 1. Linear plots of the data points are superimposed upon the curve (Figure 3.5), confirming that the midpoints are different. Notice, however, that the slopes of the lines are similar.

In most cases the toxicity of a compound is usually reported using only the midpoint, given in a mass per unit mass (mg/kg) or volume (mg/L). This practice is misleading and can lead to a misunderstanding of the true hazard of a compound to a particular xenobiotic. Figure 3.6 provides an example of two compounds with the same LC_{50}s. Plotting the cumulative toxicity and

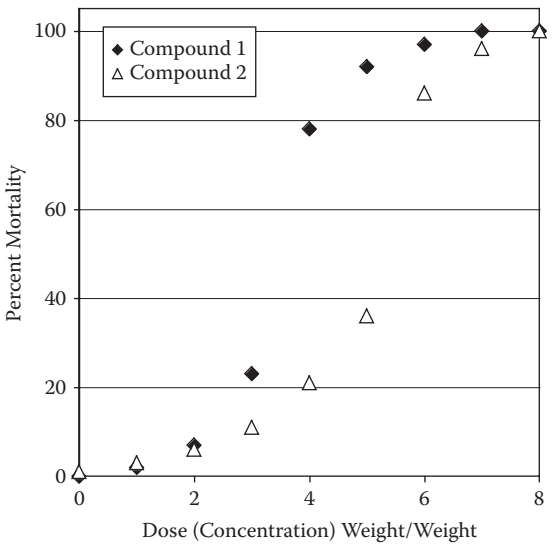

Figure 3.4 Comparison of dose-response curves 1. One of the primary goals of toxicity testing is the comparison or ranking of toxicity. The cumulative plots comparing compounds 1 and 2 demonstrate the distinct nature of the two different toxicity curves.

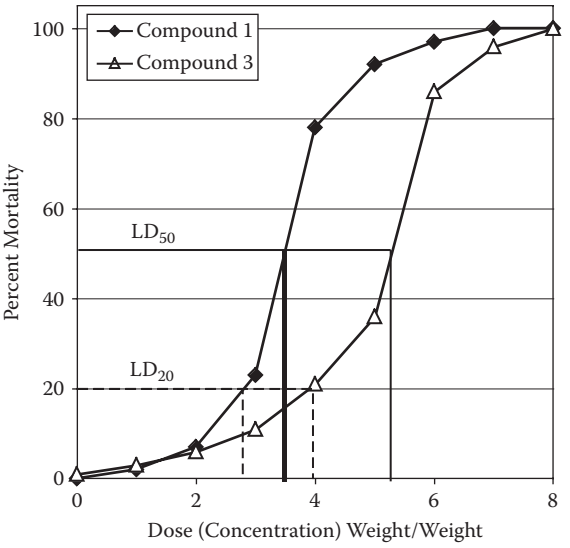

Figure 3.5 Comparison of dose-response curves 2. Plotting the dose-response curve demonstrates that the concentrations that cause mortality to 50% of the population are distinctly different. However, the slopes of the two curves appear to be the same. In many cases this may indicate that the compounds interact similarly at the molecular level.

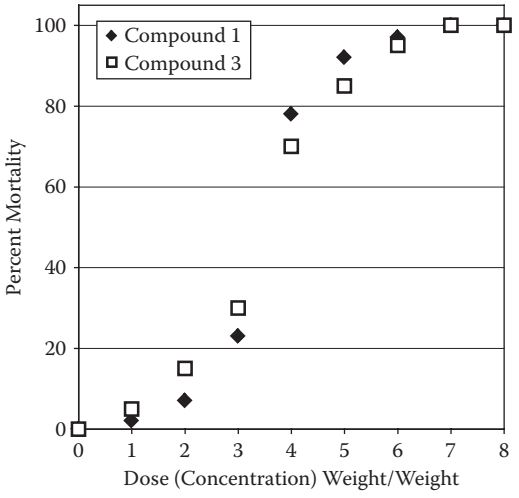

Figure 3.6 Comparison of dose-response curves 3. Cumulative toxicity plots for compounds 1 and 3. Notice that the plots intersect at roughly 50% mortality.

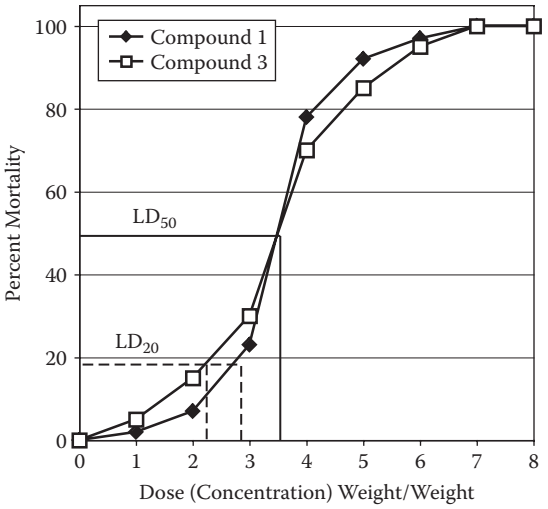

Figure 3.7 Comparison of dose-response curves 4. Although the midpoints of the curves for compounds 1 and 3 are the same, at low concentrations more typical of exposure in the environment compound 3 is more toxic.

superimposing the linear graph, the concurrence of the points is confirmed (Figure 3.7). However, the slopes of the lines are different, with compound 3 having twice the toxicity of compound 1 at a concentration of 2. At low concentrations, those that are often found in the environment, compound 3 has the greater effect.

Conversely, compounds may have different LC_{50}s, but the slopes may be the same. Similar slopes may imply a similar mode of action. In addition, toxicity is not generated by the unit mass of xenobiotic but by the molecule. Molar concentrations or dosages provide a more accurate assessment of the toxicity of a particular compound. This relationship will be explored further in our discussion of quantitative structure-activity relationships. Another weakness of the LC_{50}, EC_{50}, and IC_{50} is that they reflect the environmental concentration of the toxicant over the specified time of the test. Compounds that move into tissues slowly may have a lower toxicity in a 96-hour test simply because the concentration in the tissue has not reached toxic levels within the specified testing time. McCarty (1991, 1992, 1993) has written extensively on this topic and suggests that a lethal body burden or some other measurement be used to reflect tissue concentrations.

3.3 Thresholds and Hormesis

An implicit assumption of the endpoints discussed in the previous section is that there is a threshold concentration or dose. There are actually three competing models for the activity of toxicants at low doses (Figure 3.8). The simplest model is the no threshold assumption: The toxicological effect continues at some degree until the concentration of the toxicant is zero. This model assumes that no threshold concentration exists. The threshold model assumes that the organism, through compensatory mechanisms or the inherent mode of the toxicity of the chemical, can buffer the effects of the toxicant at certain levels of intoxication. Below this concentration there is no effect.

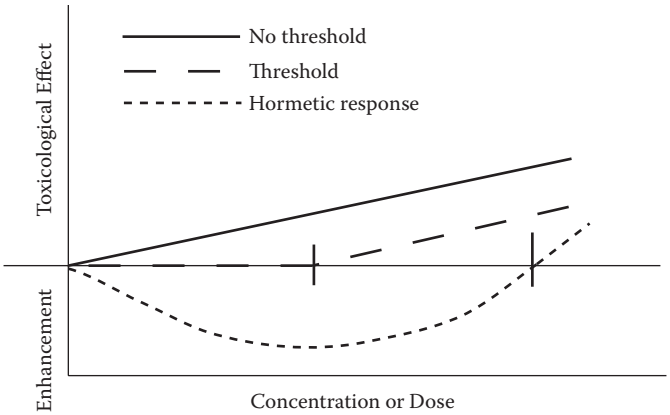

Figure 3.8 Threshold concentration. There are three models on the toxicity of compounds at low concentrations. A compound may have a toxic effect as long as any amount of the compound is available to the organism; there is *no threshold*. Only at zero concentration will the effect disappear. Another model is that a *threshold* dose exists below which the compound exists but no effects can be discerned. A third model, hormesis, states that below a certain concentration a compound enhances the survivorship or other variable being observed. The hormetic response can often be seen in algae growth tests where at low concentrations of a toxicant a larger biomass is produced.

An alternative model, the hormetic response, assumes that at low concentrations survivorship or another parameter can be enhanced by addition of the toxicant (Calabrese and Baldwin 2003). This type of response can often be observed in algal growth tests. All three models are matters of debate at the current time.

3.4 Terminology Based upon Hypothesis Testing

Often other terminology is used to describe the concentrations that have a minimal or nonexistent effect. Those that are currently common are NOEC, NOEL, NOAEC, NOAEL, LOEC, LOEL, MTC, and MATC:

NOEC—No observed effects concentration determined by hypothesis testing.
NOEL—No observed effects level determined by hypothesis testing methods. This parameter is reported as a dose.
NOAEC—No observed adverse effects concentration determined by statistical hypothesis testing methods. The effect is usually chosen for its impact upon the species tested.
NOAEL—No observed adverse effects level determined by statistical hypothesis testing methods. This value is reported as a dose.
LOEC—Lowest observed effects concentration determined by hypothesis testing methods.
LOEL—Lowest observed effects level determined by statistical hypothesis testing methods.
MTC—Minimum threshold concentration determined by statistical hypothesis testing methods.
MATC—Maximum allowable toxicant concentration determined by graphical or statistical methods.

For the NOECs and similar values these concentrations and doses usually refer to the concentration or dose that does not produce a statistically significant effect. The ability to determine accurately a threshold level or no effect level is dependent upon a number of criteria, including:

- Sample size and replication
- Number of endpoints observed
- Number of dosages or concentration
- The ability to measure the endpoints
- Intrinsic variability of the endpoints within the experimental population
- Statistical methodology
- Minimum significant difference (MSD)—The smallest value that would signify a statistical significance between two values

These are the factors used to determine the statistical power of the experiment and the data set. The MSD is rarely reported. Although the NOECs are often reported without any indication of the factors that may add to the uncertainty of the estimate, they are treated as actual measurements or estimates of the shape of the concentration-response curve. At best, these measurements are poor models of the concentration-response. An in-depth discussion of the use of curve fitting and hypothesis testing is covered in Section 3.6.4 and the following text.

3.5 Classification of Toxicity Tests

There are a large number of toxicity tests that have been developed in environmental toxicology because of the large variety of species and ecosystems that have been investigated. However, it is possible to classify the tests using the length of the experiments relative to the life span of the organism and the complexity of the biological community. Figure 3.9 provides a summary of this classification.

Acute toxicity tests cover a relatively short period of an organism's life span. In the case of fish, daphnids, rats, and birds, periods of 24 to 48 hours have been used. Even in the case of the short-lived *Daphnia magna*, a 48-hour period is just barely long enough for it to undergo its first molting. Vertebrates with generally longer life spans undergo an even smaller portion of their life during these toxicity tests. A common misconception is that those toxicity tests of similar periods of time, using bacteria, protests, and algae, also constitute acute toxicity tests. Many bacteria can divide in less than 1 hour under optimal conditions. Most protists and algae are capable of undergoing binary fission in less than a 24-hour period. A 24-hour period to an algal cell may be an entire generation. The tests with unicellular organisms are probably better classified as chronic or growth toxicity tests.

Generally, chronic and sublethal toxicity tests last for a significant portion of an organism's life expectancy. There are many types of toxicity tests that do this. Reproductive tests often examine the reproductive capabilities of an organism. By their nature, these tests must include (1) the gestational period for females and (2) a significant portion of the time for spermatogenesis for males. Growth assays may include an accounting of biomass produced by protists and algae or the development of newly hatched chicks. Chronic tests are not usually multigenerational.

Multispecies toxicity tests, as their name implies, involve the inclusion of two or more organisms and are usually designed so that the organisms interact. The effects of a toxicant upon various

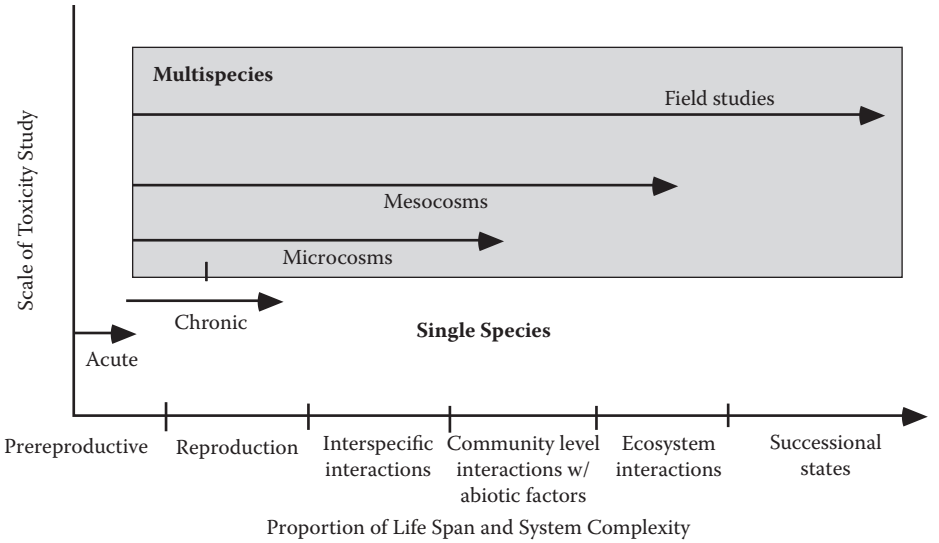

Figure 3.9 Classification of toxicity tests in environmental toxicology. Generally, the two parameters involved are the length of the test relative to the test organism and the species composition of the test system.

aspects of population dynamics, such as predator–prey interactions and competition, are a goal of these tests. Usually these tests are called microcosms (small cosmos). There is no clear definition of what volume, acreage, or other measures of size constitute a microcosm. A larger microcosm is a mesocosm. Mesocosms usually, but not always, have more trophic levels and, in general, a greater complexity than a microcosm toxicity test. Often mesocosms are outside and subject to the natural variations in rainfall, solar intensity, and atmospheric deposition. Microcosms are commonly thought of as creatures of the laboratory. Mesocosms are generally large enough to be able to look at structural and functional dynamics that are usually thought of as an ecosystem level. Unfortunately, one person's mesocosm is another person's microcosm, making classification difficult. The types of multispecies tests are detailed in their own section.

The most difficult, costly, and controversial level of toxicity testing is the field study. Field studies can be observational or experimental. Field studies can include all levels of biological organization and are also affected by the temporal, spatial, and evolutionary heterogeneity that exist in natural systems. One of the major challenges in environmental toxicology is the ability to translate the toxicity tests performed under controlled conditions in the laboratory or test site to the structure and function of real ecosystems. This inability to translate the generally reproducible and repeatable laboratory data to effects upon the systems that environmental toxicology tries to protect is often called the lab-to-field dilemma. Comparisons of laboratory data to field results are an ongoing and important part of research in environmental toxicology.

3.6 Design Parameters for Single-Species Toxicity Tests

Besides the complexity of the biological system and the length of the test, there are more practical aspects to toxicity tests. In aquatic test systems the tests may be classified as static, static renewal, recirculating, or flow-through.

In a static test the test solution is not replaced during the test. This has the advantage of being simpler and more cost-effective. The amount of chemical solution required is small, and so is the toxic waste generation. No special equipment is required. However, oxygen content and toxicant concentration generally decrease through time, while metabolic waste products increase. This method of toxicant application is generally used for short-term tests using small organisms or, surprisingly, the large multispecies microcosm- and mesocosm-type tests.

The next step in complexity is the static renewal. In this exposure scheme a toxicant solution is replaced after a specified time period by a new test solution. This method has the advantage of replacing the toxicant solution so that metabolic waste can be removed and toxicant and oxygen concentrations can be returned to the target concentrations. Still, a relatively small amount of material is required to prepare test solutions, and only small amounts of toxic waste are generated. More handling of the test vessels and the test organisms is required, increasing the chances of accidents or stress to the test organisms. This method of toxicant application is generally used for longer-term tests, such as daphnid chronic and fish early life history tests.

A recirculating methodology is an attempt to maintain the water quality of the test solution without altering the toxicant concentration. A filter may be used to remove waste products, or some form of aeration may be used to maintain dissolved oxygen concentration at a specified level. The advantages to this system are the maintenance of the water quality of the test solution. Disadvantages include an increase in complexity, an uncertainty that the methods of water treatment do not alter the toxicant concentration, and the increased likelihood of mechanical failure.

Technically, the best method for ensuring precise exposure and water quality is the use of a flow-through test methodology. A continuous flow methodology usually involves the application of peristaltic pumps, flow meters, and mixing chambers to ensure an accurate concentration. Continuous flow methods are rarely used. The usual method is an intermittent flow using a proportional diluter (Figure 3.10) to mix the stock solution with diluent to obtain the desired test solutions.

There are two basic types of proportional dilators used to ensure accurate delivery of various toxicant concentrations to the test chambers, the venturi and solenoid systems. The venturi system has the advantage of few moving parts, and these systems can be fashioned at minimal cost. Unfortunately, some height is required to produce enough vacuums to ensure accurate flow and mixing of stock solution of toxicant and the dilution water. A solenoid system consists of a series of valves controlled by sensors in the tanks that open the solenoid valves at the appropriate times to ensure proper mixing. The solenoid system has the advantages of being easy to set up and transport, and often it is extremely durable. Often the tubing can be stainless steel or polypropylene instead of glass. The disadvantages to the solenoid system are an increase in moving parts, expense, and when the electricity stops, so does the diluter. Both of these systems use gravity to move the solutions through the diluter.

3.6.1 Exposure Scenarios

In aquatic test systems exposure is usually a whole body one. That means that the toxicant can enter the organism through the skin, cell wall, respiratory system (gills, stomata), and ingestion. Occasionally a toxicant is injected into an aquatic organism, but that is not usually the case in toxicity tests to screen for effects. Whole body exposures are less common when dealing with terrestrial species. Often an amount of xenobiotic is injected into the musculature (intramuscular), peritoneum (intraperitoneal), or a vein (intravenous) on a weight of toxicant per unit weight of the animal basis. Other toxicity tests place a specified amount into the stomach by a

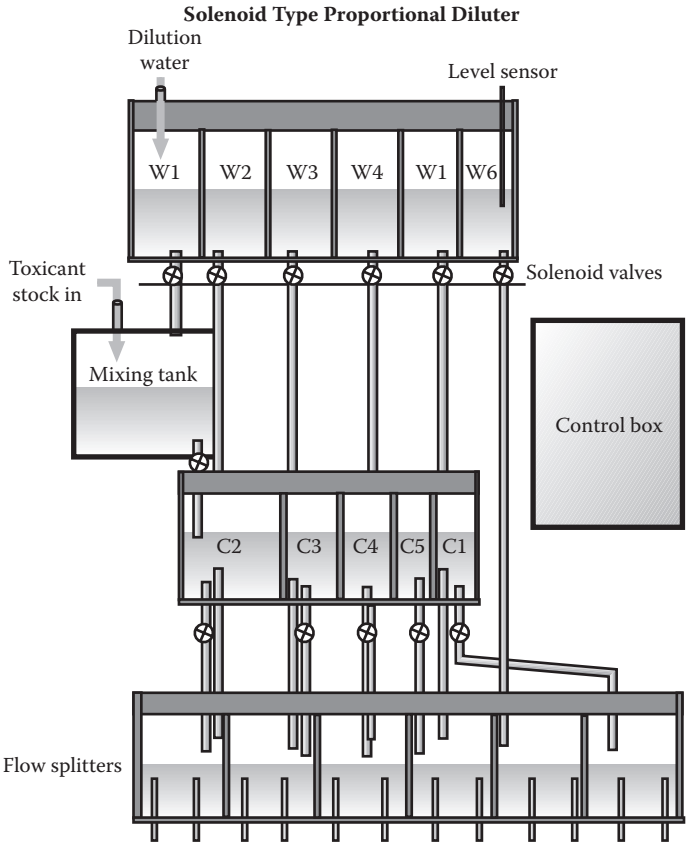

Figure 3.10 Schematic of a proportional diluter. This mechanism ensures that an accurate concentration of the test material is reliably introduced to the test organisms at a specified rate.

tube (gavage) so that the amount of material entering the organism can be carefully quantified. However, feeding studies are conducted so that a specific concentration of toxicant is mixed with food or water to ensure toxicant delivery. Unfortunately, many compounds are not palatable and the test organisms quickly cease to eat.

Other routes of exposure include inhalation for atmosphere-borne pollutants. In many cases of an originally atmospheric exposure, dermal exposure may occur. An alternative method of ensuring an inhalation exposure is to provide an air- or watertight seal limiting exposure to the respiratory apparatus. In the case of rodents, nose-only exposures can be used to limit coat and feet contamination. Dermal exposures are important in the uptake of substances from contaminated soils or from atmospheric deposition.

Plants, soil, and sediment-dwelling organisms have other potential routes of exposure that may be used in toxicity testing. Plants are often exposed through the soil or an atmospheric deposition. Soil invertebrates are often placed in a standardized soil laced with a particular concentration of the test substance. Sediment tests are usually with contaminated sediments or with a material added to standardized sediment.

Often overlooked in toxicity testing can be the multiple routes of exposure that may be inadvertently available during the toxicity test. An inhalation study that exposes the animal to a

toxicant in the atmosphere must also take into account deposition of the material on the feathers or fur and the subsequent self-cleaning, causing an oral exposure. Likewise, exposure is available dermally through the feet, face, or eyes of the animal. In field pesticide experiments, where the exposure might be assumed to be through the ingestion of dead pests, contaminated foliage, soil, and airborne particulate can increase the available routes of exposure, thereby increasing the actual dose to the organism. Soil organisms often consume the soil for nutrition, adding ingestion to a dermal route of exposure.

3.6.2 Test Organisms

One of the most crucial aspects of a toxicity test is the suitability and health of the test organisms or, in the case of multispecies toxicity tests, the introduced community. It is also important to define clearly the goals of the toxicity test. If the protection of a particular economic resource such as a salmon fishery is of overriding importance, it may be important to use a salmonid and its food sources as test species. Toxicity tests are performed to gain an overall picture of the toxicity of a compound to a variety of species. Therefore, the laboratory test species is taken only as representative of a particular class or, in many cases, phylum.

Some of the criteria for choosing a test species for use in a toxicity test are listed and discussed below.

1. *The test organism should be widely available through laboratory culture, procurement from a hatchery or other culture facility, or collection from the field*—In many cases marine organisms are difficult to culture successfully in the laboratory environment, requiring field collection.
2. *The organism should be successfully maintained in the laboratory environment and available in sufficient quantities*—Many species do not fare well in the laboratory. Our lack of knowledge of the exact nutritional requirements, overcrowding, and stress induced by the mere presence of laboratory personnel often make certain species unsuitable for toxicity testing.
3. *The genetics, genetic composition, and history of the culture should be known*—Perhaps the best-documented organisms in laboratory culture are *Escherichia coli* and the laboratory strains of the Norway rat. *E. coli* has been widely used in molecular genetics and biology as the organism of choice. Laboratory rats have long been used as test organisms for the evaluation of human health effects and research, and are usually identified by a series of numbers. Often, each strain has a defined genealogy. Often algae and protozoans are identified by strain and information is available as to their collection site. The American Type Culture Collection is a large repository of numerous procaryotic and eucaryotic organisms. The Star Culture Collection at the University of Texas is a repository for many unicellular algae. However, the majority of toxicity tests in environmental toxicology are conducted with organisms of unknown origin or field collection. Indeed, often the cultures originated from collections and the genetic relationships to the organisms used by other laboratories are poorly known.
4. *The relative sensitivities to various classes of toxicants of the test species should be known relative to the endpoints to be measured*—This criterion is not often realized in environmental toxicology. The invertebrate *Daphnia magna* is one of the most commonly used organisms in aquatic toxicology, yet only the results for approximately 500 compounds are listed in the published literature. The fathead minnow has been the subject of a concerted test program at the U.S. EPA Environmental Research Laboratory–Duluth, conducted by G. Vieth, yet

fewer than 1,000 compounds have been examined. In contrast, the acute toxicity of over 2,000 compounds has been examined using the Norway rat as the test species.

5. *The sensitivity of the test species should be representative of the particular class or phyla that the species represents*—Again, this is an ideal criterion, not often met in the case of most test species. The limiting factor here is often the lack of information on the sensitivity of the organisms not routinely used for toxicity testing. In the case of teleost fish, a fish is a fish, as demonstrated by G. Suter (1993). What this means is that most of the time the toxicity of a compound to a fathead minnow is comparable to the toxicity of the compound to a salmonid. This fact is not surprising given the relative evolutionary distance of the vertebrates compared to the invertebrate classes.

There is the myth of the most sensitive species, and that is the organism that should be tested. J. Cairns (1986) has discussed the impossibility of such an organism, yet it is still held as a criterion to the selection of a test organism. In most cases it is not known what organisms and what endpoints are the most sensitive to a particular toxicant. The effects of toxicants to fungi, nonvascular plants, and mosses are poorly understood, yet these are major components of terrestrial ecosystems. Also, our knowledge of what species exist in a particular type of ecosystem over time and space is still limited. Often the dilemma has to be faced where it is a goal to protect an endangered species from extinction, yet no toxicological data are or can be made available.

3.6.3 Comparison of Test Species

Often the question of the best test species for screening for environmental toxicity has been debated. A wide variety is currently available, representing a number of phyla and families, although a wide swath of biological categories is not represented by any test species. In the aquatic arena, an interesting paper by Doherty (1983) compared four test species for sensitivity to a variety of compounds. The test species were rainbow trout, bluegill sunfish (*Lepomis macrochirus*), fathead minnow, and *D. magna*. A particular strength of the study was the reliance upon data from Betz Laboratories in addition to literature values. Having data from one laboratory reduces the interlaboratory error that is often a part of toxicity testing.

The results were very interesting. There was a high level of correlation ($r > 88\%$) among the four species in all combinations. Of course, three of the species were teleost fish. However, the *Daphnia* also fit the pattern. The exceptions in the correlations were compounds that contained chromium. *D. magna* was much more sensitive than the fish species.

Many other comparisons such as these have been made and are discussed in more detail in Chapter 12. However, in the selection of a test species for screening purposes, there seem to be high correlations between species for a broad number of toxicants. In addition, due to evolutionary events and happenstance, some organisms may be much more sensitive to a particular class of compound. So far, there is no *a priori* means of detecting such sensitivities without substantial biochemical data.

3.6.4 Overview of the Tools for the Analysis of Concentration (Dose)-Effect Relationships

In the design of a toxicity test there is often a compromise between the statistical power of the toxicity test and the practical considerations of personnel and logistics. In order to make these choices in an efficient and informed manner several parameters are considered:

- What is the specific question or questions to be answered by this toxicity test?
- What are the available statistical tools?
- What power, in a statistical sense, is necessary to answer the specific questions?
- What are the logistical constraints of a particular toxicity test?

The most important parameter is a clear identification of the specific question that the toxicity test is supposed to answer. The determination of the LC_{50} within a tight confidence interval will often require many fewer organisms than the determination of an effect at the low end of the dose-response curve. In multispecies toxicity tests and field studies the inherent variability or noise of these systems requires massive data collection and reduction efforts. It is also important to determine ahead of time whether a hypothesis testing or regression approach to data analysis should be attempted.

Over the last several years a variety of statistical tests and other tools have become widely available as computer programs. This increase in statistical tools available can increase the sophistication of the data analysis and in some cases reduce the required workload. Unfortunately, the proliferation of these packages has led to *post hoc* analysis and the misapplication of the methods.

The power of the statistical test is a quantitative measure of the ability to accurately differentiate in populations. The usual case in toxicity testing is the comparison of a treatment group to a control group. Depending on the expected variability of the data and the confidence level chosen, an enormous sample size or number of replicates may be required to achieve the necessary discrimination. If the sample size or replication is too large, then the experimental design may have to be altered.

The logistical aspects of an experimental design should intimately interact with the statistical design. In some cases the toxicity evaluation may be untenable because of the numbers of test vessels or field samples required. Upon full consideration, it may be necessary to rephrase the question or use another test methodology.

3.7 Limitations and Alternatives to Hypothesis Testing

In toxicology we need to test hypotheses regarding cause–effect pathways, changes in population dynamics, patterns in community structures, and distributions of organisms. A conventional means of testing these hypotheses has been classical statistical hypothesis testing. As discussed above, these results are presented as NOECs, LOECs, and so forth.

Newman (2008) has examined in detail the use of hypothesis testing in environmental science and provided clear recommendations. These recommendations are summarized and explained below.

1. *Define and justify the type I and type II error rates based upon the specific risk analysis or injury determination question being addressed by the statistical tool*—The rates should be in part defined by the consequences of each type of error. Conventional practice has been to a cutoff of being 95% sure that any effect is different from the control in the laboratory experiment. For the purposes of academic publication, this criteria has been very useful. The costs of being misled by a significant result are high in misdirected research, wrong hypotheses, and funds being committed because of spurious results.

   However, such criteria may not meet the goal of protection of human health and the environment. A cutoff of being 90% sure that a material is toxic may ensure that even if the result

is spurious, it is likely to err on the side of protection. In other words, the value of damage to human health and the environment is considered to be greater than the costs of being misled by the spurious result. In many instances a 90% criteria (or in conventional parlance, $\alpha = 0.10$) may be more suitable depending on the decision resulting from the study.

2. *Define and justify the effect size (ES) for the statistical test*—The effect size depends upon the decision-making process and the legal and conventional criteria for establishing risk or injury. For example, a 10% reduction in reproductive rate by a toxicant is considered unacceptable. Then the experimental design and the statistical tests used to evaluate the data should be able to, at minimum, detect a 10% reduction. If the minimum significant difference is 20% given the variability in the toxicity test and the analysis method, then that will not meet the regulatory criteria.

   Items 1 and 2 are closely related and should be tied to the decision-making process of risk assessment and injury determination. Reports on the linkage of the experimental design, statistical analysis, and the type of decision to be made are rare in the literature.
3. *Estimate power a priori*—Such an estimate can ensure that the goals expressed in items 1 and 2 can be met, facilitating the decision-making process. In experimental studies, the number of replicates can be determined and control systems implemented to define variability and increase power.
4. *Consider the use of confidence limits on the effect size*—Confidence intervals on an effect size or concentration-response curve provide a great deal of information on both the estimated value and the associated uncertainty. The values can be exhibited as a table or graphically. A detailed discussion of the use of curve fitting regression modeling, the use of confidence intervals, and a comparison of the use and interpretation is presented in Section 3.11.
5. *Do not confuse $p(E/H_o)$ with $p(H_o/E)$*—These terms have very different implications. The expression $p(E/H_o)$ is the probability of the evidence (E) given that the hypothesis (H_o) is true. The expression $p(H_o/E)$ is the probability of the hypothesis given the evidence. The difference in approach is given in the following example.

   A sampling team goes to a site and samples for their favorite indicator species. They do not find that species at that location. The team then concludes that the contamination at that site is the cause. Given the evidence (E, or no indicator species found) it is assumed that the contaminant was the cause keeping those organisms away (H_o). However, the team forgot to take all of the other items of the site into consideration, such as lack of suitable habitat, the dam downstream preventing migration, water temperature, and so forth. Many factors may contribute to the lack of an indicator at a specific location.

   A better approach may be determining the likelihood of a hypothesis being true given the evidence, or $p(H_o/E)$. It may be that the lack of a particular species cannot be tied to a single cause without additional forms of evidence.
6. *Allow an estimation of positive predictive value*—The method of calculation is given in Newman (2008). Simply put, given the α and β error rates for a particular experiment for field design, how likely is a hypothesis true given a significant test result? Given studies with higher β values (field studies, mesocosms), a statistically significant result may have only a 50-50 chance of identifying a true effect.
7. *Publish and note negative results*—The information about negative results and their frequency is important for constructing additional hypotheses, synthesizing patterns for additional inference, and setting further research agendas. In making regulatory and policy decisions, negative results may be as important as results where effects were seen.

8. *Use null, not nil, hypothesis*—The given evidence that a contaminant is of a concentration that would affect the selected receptors or resources, the null hypothesis of an effect (change in community structure, reproductive failure, or individual birds), may be more appropriate than an assumption of no effect, or the nil hypothesis. A no-effect hypothesis is not very useful; it may just mean that the husbandry was poor, the effect was not measured very well, and the hypothesis implies the assumption of a threshold effect.
9. *Avoid definitive inferences from isolated tests*—The goal is to create a self-supporting pattern of evidence that increases the certainty of the analysis. Lines or weights of evidence, inference from mechanisms noted in *in vitro* tests, and comparison of toxicity to related species are all examples of information that can be used to establish a pattern of evidence.

There are alternatives to the conventional frequentist-based tools. Bayesian statistical inference tools can be calculated using modeling frameworks such as WINBUGS. Two of the principal advantages of Bayesian approaches are the ability to be updated by the addition of new information and the innate incorporation of uncertainty. The computational issues that once were an impediment to application are now overcome. Bayesian networks are also modeling networks that are updateable and are an alternative approach to dealing with uncertainty and illuminating sensitive model parameters.

In multispecies toxicity tests multivariate tools have long been used to characterize community structure and the association of variables within a study site or experiment. Confidence intervals can be calculated for output such as principal components analysis. Multivariate methods can provide information about patterns while eliminating errors associated with multiple comparisons tests.

In conclusion, the statistical approaches to either risk analysis or injury determination are similar and depend upon the specific questions to be answered, rather than the study being part of a risk assessment or injury determination.

A number of programs exist for the calculation of the chemical concentration that produces an effect in a certain percentage of the test population. The next few paragraphs review some of the advantages and disadvantages of the various techniques. The goal is to provide an overview, not a statistical text.

3.8 Commonly Used Methods for the Calculation of Endpoints

As reviewed by C. E. Stephan (1977) and Bartell et al. (1992), there are several methods available for the estimation of toxic endpoints. The next few paragraphs discuss some of the advantages and disadvantages of the popular methods.

Graphical interpolation is essentially the plotting of the dose-response curve and reading the concentration that corresponds to the LC_{50} or LC_{10}. This technique does not require concentrations that give a partial kill, say 7 out of 20 test organisms. In addition, data that provide atypical dose-response curves can be analyzed since no previous assumptions are necessary. Another feature that is important is that the raw data must be observed by the researcher, eliminating any outliers or other features that would classify the dose-response curve as atypical. The disadvantage to using a graphical technique is that confidence intervals cannot be calculated and the interpretation is left to human interpolation. Graphing and graphical interpolation would generally be recommended as an exploratory analysis no matter which computational method is finally used. Graphing the

data allows a determination of the properties of the data and often highlights points of interest or violations of the assumptions involved in the other methods of endpoint calculation.

Curve fitting using a variety of regression models is an alternative method to graphing. Each model has its own set of data specifications in order to be successful.

The probit method is perhaps the most widely used for calculating toxicity versus concentration or dose. As its name implies, the method uses a probit transformation of the data. A probit is a unit of divergence from the mean of a normal distribution equal to one standard deviation. The central value of a probit is 5.0, representing the median effect of the toxicity test. A disadvantage of the method is that it requires two sets of partial kills. However, a confidence interval is easily calculated and can then be used to compare toxicity results. There are several programs available for the calculation, and as discussed below, provision of comparable results.

If only one or no partial kills are observed in the data, the Litchfield and Wilcoxin method can be employed. This method can provide confidence intervals, but is partially graphical in nature and employs judgment by the investigator. The probit method is generally preferred, but the Litchfield and Wilcoxin can be used when the partial kill criteria for the probit are not met.

Another transformation of the data is used in the logit method. A logit is calculated by taking the logarithm of the proportion of organisms affected (p) at a concentration divided by 1 – p. A logit transformation of the data can be used, and the curve fitted by a maximum likelihood method. As with some of the other methods, a dearth of partial kill concentrations requires assumptions by the investigator to calculate an EC or LC value.

The Spearman–Karber method must have toxicant concentrations that cover 0 to 100% mortality. Derived values are often comparable to the probit.

Perhaps the most widely applicable method, other than the graphical interpolation, is the moving average. The method can be used only to calculate the LC_{50}, and there is the assumption that the dose-response curve has been correctly linearized. As with the other methods, a partial kill is required to establish a confidence interval.

3.9 Comparison of Calculations of Several Programs for Calculating Probit Analysis

Each of the methods for the estimation of an LC_{50} or other toxicological endpoint is available as a computer program. Examples of commonly available programs are TOXSTAT, SAS-PROBIT, SPSS-PROBIT, DULUTH-TOX, and a program written by C. Stephan, ASTM-PROBIT. Bromaghin and Engeman (1989) and in a separate paper Roberts (1989) compared several of these programs using model data sets.

Bromaghin and Engeman considered the proposed ASTM-PROBIT to be a subset of the SAS Institute program, the SAS log 10 option. Two different data sets were used. The first data set was constructed using a normal distribution with a mean (LD_{50}) of 4.0 and a standard deviation of 1.25. Eleven dosage levels, quite a few compared to a typical aquatic toxicity test, ranging from 1.5 to 6.5 in increments of 0.5, were selected. The second set of test data was normally distributed with a mean of 8 and a standard deviation of 10. Five dosage levels, more typical of a toxicity test, ranging from 2 to 32 by multiples of 2, were used. In other words, the concentrations were 2, 4, 8, 16, and 32. One hundred organisms were assumed to have been used at each test concentration in each data set. The response curves were generated based on two different criteria: (1) The response

Table 3.3 Estimates of LD_{50} Using Probit Analysis and SAS PROBIT and ASTM PROBIT

Data Set (True LD_{50})	*Normality with Respect to:*	*Calculation Method with Estimate (95% Fiducial Limits)*		
		SAS Default	*SAS Log 10*	*ASTM*
1 (4.0)	Dose	4.00 (3.88–4.12)	3.80 (3.59–4.02)	3.80 (3.58–4.02)
	Log 10 dose	4.11 (4.01–4.21)	3.99 (3.90–4.10)	3.99 (3.90–4.10)
2 (8.0)	Dose	8.02 (5.35–10.36)	5.37 (1.46–10.91)	5.37 (1.46–10.91)
	Log 10 dose	12.28 (8.04–16.57)	8.00 (5.61–11.42)	8.00 (5.61–11.42)

is normal with regard to the dosage, and (2) the response is assumed to be normal with respect to either the base 10 or natural logarithm.

As shown in Table 3.3, the resulting estimated value was dependent on the method and the underlying assumptions used to calculate the LC_{50}. SAS log 10 and ASTM PROBIT were consistently identical in the calculated values of the LD_{50}s and the accompanying fiducial limits. Interestingly, the assumption of the normality being based on dose or the log 10 was important. In the first data set, when the normality of the data was based on the log 10 of the dose, the SAS default overestimated the LD_{50} in such a manner that the value was outside the limits given by the SAS log 10 and the ASTM method. In the second data set, the use of the appropriate calculation option was even more crucial. The inappropriate computational method missed the mark in each case and was accompanied by large fiducial limits. Bromaghin and Engeman (1989) conclude that these methods are not robust to departures from the underlying assumptions about the response distributions.

Roberts (1989) made a comparison between several commonly available programs used to calculate probit estimates of LD_{50}s. These programs were:

DULUTH-TOX—Written by C. Stephan of the Duluth Environmental Protection Agency's Environmental Research Laboratory. Used to calculate toxicity endpoints.

ASTM-PROBIT—Also written by C. Stephan, as part of an ASTM Committee E-47 effort to produce a standard method of calculating toxicity estimates.

UG-PROBIT—Developed by the Department of Mathematics and Statistics and the University of Guelph, Canada.

SPSSx-PROBIT—A part of the SPSSx statistical program available commercially and on many mainframes of universities and industries.

SAS-PROBIT—Analogous to the SPSS-PROBIT in that it is a part of the widely available SAS statistical package.

After an extensive analysis, Roberts concluded that most of the programs provided useful and comparable LC_{50} estimates. The exception to this was the UG-PROBIT. The commercially available packages in SAS and SPSSx had the advantages of graphical output and a method for dealing with control mortality. DULUTH-TOX and ASTM-TOX incorporated statistical tests to examine the data to ensure that the assumptions of the probit calculations were met.

The graphic and regression methods are a means of estimating the concentration-response curve. Hypothesis testing is an alternative to the analysis of the concentration-response data.

3.10 Hypothesis Testing

Analysis of variance (ANOVA) is the standard means of evaluating toxicity data to determine the concentrations that are significantly different in effects from the control or not dosed treatment. The usual procedure is (Gelber et al. 1985):

1. Transform the data.
2. Test for equivalence of the control or not dosed treatment with the carrier control.
3. Perform analysis of variance on the treatment groups.
4. Make multiple comparisons between treatment groups to determine which groups are different from the control or not dosed treatment.

Now we will examine each step.

In chronic studies, the data often are expressed as a percentage of control, although this is certainly not necessary. Hatchability, percentage weight gain, survival, and deformities are often expressed as percentage of the control series. The arc-sine square root transformation is commonly used for this type of data before any analysis takes place. Many other types of transformations can be used depending upon the circumstances and types of data. The overall goal is to present the data in a normal distribution so that the parametric ANOVA procedure can be used.

Data such as weight and length and other growth parameters should not be included in the analysis if mortality occurred. Smaller organisms, because they are likely to absorb more of the toxicant on a per mass basis, are generally more sensitive, biasing the results.

If a carrier solvent has been used, it is critical to compare the solvent control to the control treatment to ensure comparability. The common student's t-test can be used to compare the two groups. If any differences exist, then the solvent control must be used as the basis of comparison. Unfortunately, a t-test is not particularly powerful with typical data sets. In addition, multiple endpoints are usually assessed in a chronic toxicity test. The change of a type II error, stating that a difference exists when it does not, is a real possibility with multiple endpoints under consideration.

ANOVA has been the standby for detecting differences between groups in environmental toxicology. Essentially, the ANOVA uses variance within and between the groups to examine the distance of one group or treatment to another. An F score is calculated on the transformed data with the null hypothesis since the effects upon all of the groups are the same. The test is powerful with the assumption met. If the F score is not statistically significant, the treatments all have the same effect and the tested material has no effect. With a nonsignificant F score (generally $p > 0.05$) the analysis stops. If the F score is significant ($p < 0.05$), then the data are examined to determine which groups are different from the controls.

Multiple comparison tests are designed to select the groups that are significantly different from the control or each other. The most commonly used test is Dunnett's procedure. This test is designed to make multiple comparisons simultaneously. However, given the number of comparisons made in a typical chronic test, there is a significant chance that a statistically significant result will be found even if there are no treatment differences. The usual probability level is set at 0.05. Another way of looking at this is that 5 times out of 100 comparisons a

statistically significant result will appear even if no treatment differences exist. Beware of spurious statistical significance.

The overall purpose of the multiple comparisons is a determination of the MATC. The lowest concentration at which an effect is detected is the statistically determined lowest observed effect concentration. The concentration that demonstrates no difference from the control is the no observed effects concentration (NOEL). The maximum allowable toxicant concentration is generally reported as LOEC > MATC > NOEC. The most sensitive endpoint is generally used for this estimation. Perhaps the greatest difficulty in estimating endpoints such as the NOEC and LOEC is their dependence upon the statistical power of the test. Often treatment numbers are determined by parameters other than statistical power, cost, safety, and other logistical factors. A greater statistical power would likely improve the ability to detect significant differences at subsequently lower concentrations. Along with statistical power, the placement of the test concentrations relative to the generally unknown dose-response curve can also alter the interpretation of the NOEC, LOEC, and derived MATC. The closer the spacing and the more concentrations used, the more accurate are these derived parameters.

Gelber et al. (1985) suggest that a major improvement can be made in the analysis of chronic toxicity tests. They suggest that Williams's test (Williams 1971, 1972) is more powerful than Dunnett's since it is designed to detect increasing concentration (dose)-response relationships. A removal of the preliminary ANOVA is also recommended, since performing both the ANOVA and the multiple comparison tests has a 5% error rate. They suggest performing multiple Williams' tests to arrive at the concentration that is not significantly different from the control set.

3.11 Curve Fitting and Regression Modeling versus Hypothesis Testing

There has been a question about which method is more appropriate for the analysis of toxicity data. In order to make a selection, it is important to understand that toxicity data are used for hazard or risk assessment. Curve fitting and regression modeling have clear advantages.

The above methods are generally used to calculate a midpoint in the dose-response curve that results in 50% mortality, or to test the null hypothesis that there is no effect. In ranking compounds by their acute or chronic toxicity this may be an appropriate approach. However, in the estimation of mortality at low concentrations, concentrations that are probably more realistic in a field situation, LC_{10}s or even LC_5s may be more appropriate. As proposed by C. E. Stephan, a regression or curve fitting approach to the evaluation of laboratory toxicity data may be more appropriate for estimating environmental effects. In this instance, a regression is used to calculate the best-fit line through the data. Linear regression after a log transformation can be used along with other regression models. Confidence intervals of the LC_{10} or LC_1 estimation derived from a regression technique can be quite large; however, an estimate of effects at low concentrations can be derived.

Figure 3.11 plots the data in example 3 with the data transformed to a base 10 logarithm. The relationship for this data set is rather linear, and the toxicity at low concentrations can easily be estimated. In this instance, 100% mortality has a log of 2.0, the LC_{50} is 1.7, and the LC_{10} is equal to 1.0.

Hypothesis testing in the determination of NOELs and LOELs also has drawbacks, largely related to the assumptions necessary for the computations. These characteristics have been

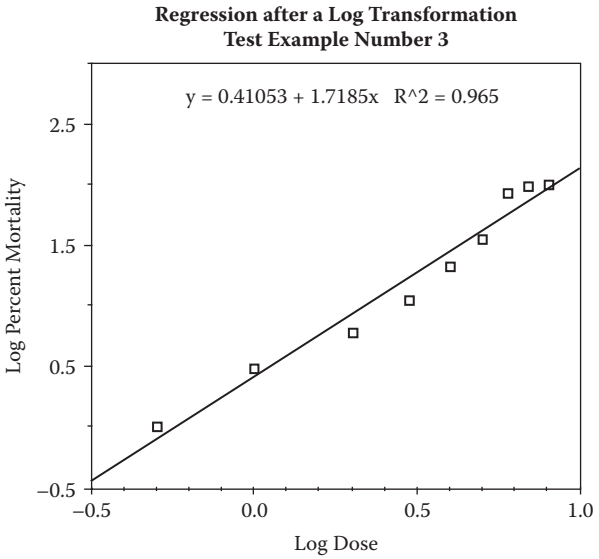

Figure 3.11 Plot of a log-log regression for toxicity data set 3.

listed by Stephan and Rodgers (1985) and compared to curve fitting models for the estimation of endpoints.

First, use of typical hypothesis testing procedures that clearly state the α value (typically 0.05) leave the β value unconstrained, and this skews the importance of reporting the toxic result. In other words, the typical test will be conservative on the side of saying there is not toxicity even when toxicity is present.

Second, the threshold for statistical significance does not innately correspond to a biological response. In other words, hypothesis testing may produce a NOEL that is largely a statistical and experimental design artifact and not biological reality. As discussed earlier in the chapter, there is debate about the existence of a response threshold.

Third, a large variance in the response due to poor experimental design or innate organismal variability in the response will reduce the apparent toxicity of the compound using hypothesis testing.

Fourth, the results are sensitive to the factors of experimental design that determine the statistical power and resolution of the analysis methods. These design parameters are typically the number of replicates for each test concentration, and the number and spacing of the test concentrations.

Fifth, no dose-response relationship is derived using hypothesis testing methods. The lack of dose-response information means that the investigator has no means of evaluating the reasonableness of the test results. Conversely, a specific type of dose-response relationship is not required to conduct the analysis.

There have been studies that directly compared the hypothesis testing approach to regression modeling. These studies are summarized below.

Moore and Caux (1997; Caux and Moore 1997) have investigated methods of regression and have compared this approach to that of the derivation of NOECs, NOELs, LOECs, and so forth, by hypothesis testing. Twenty-four data sets were used that met the criteria of at least one regression method providing an adequate fit and at least two replicates per concentration. Hypothesis testing techniques produced NOELs at levels that corresponded to ECs of between 10 and 30%.

The highest NOEL corresponded to an EC value of 37.4%. LOELs represented EC values of up to 76%. NOELs corresponded to an EC_{30} or higher in 62.4% of the cases. If an EC_{10} is used as the effects cutoff, then 76.9% of the NOELs and 100% of the LOELs exceeded this value.

Crane and Newman (2000) also examined the EC values corresponding to NOEC values. In one instance they examined nine sets of round-robin tests for a fish growth toxicity test. The median NOEC value corresponded to an EC level of 10.5%. However, the ranges were large. When linear alkylbenzene sulfonate (LAS) was tested the EC values corresponding to the NOEC ranged from 3.4 to 38.4%, and for DCA it ranged from 3.3 to 24.1%.

Clearly, hypothesis testing using data from currently used toxicity test protocols cannot effectively detect effects at low concentrations. This is due in part to the lack of statistical power given the number of replicates and the intrinsic laboratory and organismal variability within the experiments. Current assumptions that NOELs are a no effect or safe level are also not warranted. The above studies also indicate that the level of effect that the NOEL represents is highly variable. LOELs are similarly uninformative.

Given these analyses, it is clear that a regression method provides superior information in characterizing toxic responses, especially at concentrations that are protective to populations. However, most toxicological data are reported as summary statistics, an EC_{50} with a NOEC, LOEC, or MATC. It is critical that values such as the EC_{10} or EC_{20} be reported along with the equation for the model generating the estimates or the raw data.

Regression methods do have features that must be considered for a clear understanding of the concentration-response relationship. As regression methods become more common, it will also be necessary to change the decision of toxicity experiments to take advantage of the regression approach.

Moore and Caux (1997), in the same paper examining the relationships between hypothesis testing and effects levels, also characterized some important properties of the regression approach. One of the critical questions is which model to use and how much a difference it makes. Logistic, probit, Weibull, and three-parameter logistic models incorporating a slope parameter were compared in their data sets. The differences in using these models for extrapolation depend upon the structure of the data set.

Figure 3.12 presents two examples of data sets that demonstrate the effect of data structure upon the difference in regression results. These graphs are based upon the figures from Moore and Caux (1997), modified for this comparison. Figure 3.12a presents the observed data along with the line from a logistic model, and the two most divergent models at low effects levels for this data set, the Weibull and positive three-parameter logistic. Note that in this data set the treatments are not replicated and are spaced from high to very low concentrations. Note that the differences in the model predictions at the EC_{50} and the EC_{20} are very low. In this instance the models are interpolating values between data points, with concentrations that correspond to low effects levels.

Figure 3.12b presents a similar analysis. Note that the data all exist above the EC_{50} and each treatment is replicated three times. Again, lines from the logistic model and the two most divergent models at low concentrations (in this experiment the probit and positive three-parameter logistic) are presented. All three models correspond very closely in the region represented by experimental results. However, the models must extrapolate out of this region to estimate the EC_{50} and EC_{20} values. The divergence between the models becomes larger as the distance from the data increases to the point that the EC_{50} for the probit model is essentially identical for the EC_{20} using the positive logistic. As would be expected, a lack of data at the relevant effects levels leads to an increase in variability in estimates.

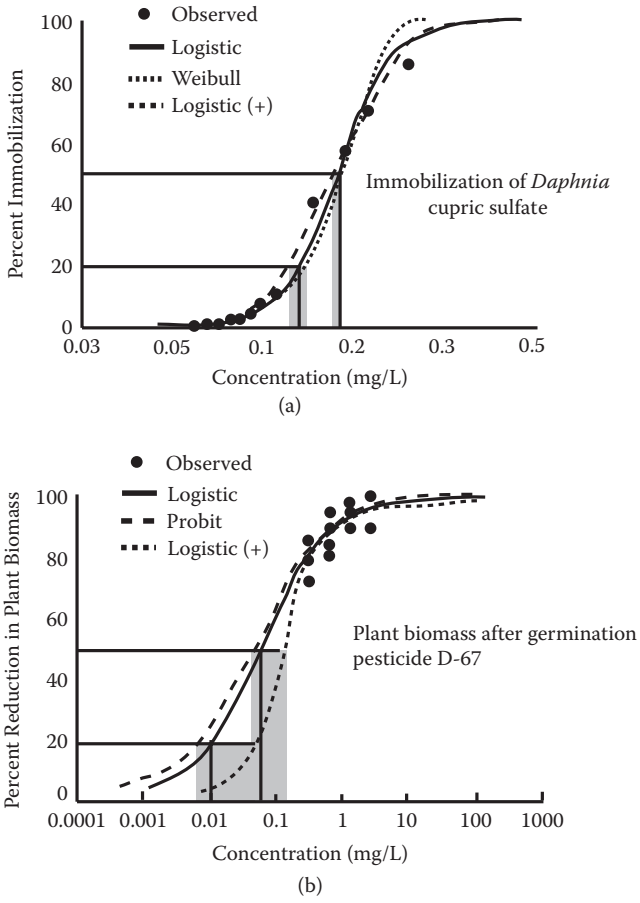

Figure 3.12 Comparison of data range on the variability of curve fitting. (Modified from Moore, D. R. J., and Caux, P.-Y., *Environ. Toxicol. Chem.*, 16, 764–801, 1997.)

In Figure 3.13a the 95% confidence limits are presented curve fitted to a *Daphnia* toxicity test. Test concentrations are from very high to very low with no replication. The confidence intervals at the EC_{50} and EC_{20} are relatively low in each instance. In contrast, Figure 3.13b has fewer test concentrations and four replicates. The test concentrations do not extend to levels corresponding to EC_{20}. Note that the confidence interval is very narrow within the area of the graph represented by data. However, as extrapolation is required at lower concentrations, the confidence interval expands.

This discussion indicates that a greater number of test concentrations is preferable over replicability of a few test concentrations. This is contrary to the design if hypothesis testing is the analysis tool. Stephenson et al. (2000) has performed an in-depth analysis of describing concentration-response relationships for plant species using regression models. They found that the regression approach was very satisfactory when using 11 treatment levels.

In summary, the optimum design strategy for the use of the regression method is to favor a large number of treatment levels, especially at concentrations expected to provide EC_{20} values and lower. Replication of treatment is not as important as providing a broad coverage of toxicant concentrations. For example, if 12 treatment vessels are available, a strategy of examining

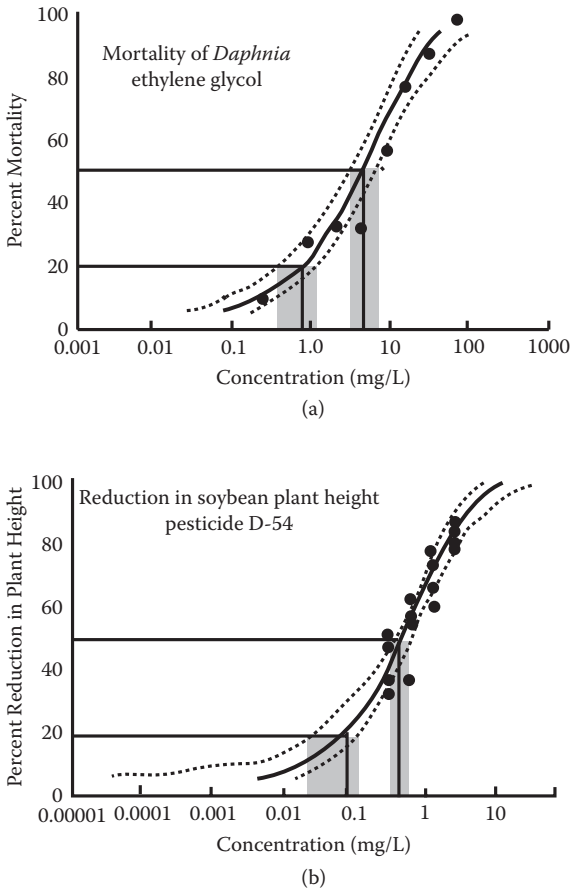

Figure 3.13 Comparison of data range on the confidence intervals of a regression model. (Modified from Moore, D. R. J., and Caux, P.-Y., *Environ. Toxicol. Chem.*, 16, 764–801, 1997.)

12 concentrations, especially those at the lower tail of the expected effects levels, is preferable to having three treatments with four replicates. This is contrary to most current protocols that were originally designed and hypothesis testing.

Adoption of the regression approach is straightforward. Caux and Moore (1997) have published the required program for calculating the regressions presented in Moore and Caux (1997). Stephenson et al. (2000) have published a flow diagram with a step-by-step approach for data analysis using the regression approach.

3.12 The Design of Multispecies Toxicity Tests

Over the last 20 years a variety of multispecies toxicity tests have been developed. These tests, usually referred to as microcosms or mesocosms, range in size from 1 L (the mixed flask culture) to thousands of liters (in the case of the pond mesocosms). A review by Gearing (1989) listed 11 freshwater artificial stream methods, 22 laboratory freshwater microcosms ranging from 0.1 to 8,400 L, and 18 outdoor freshwater microcosms ranging from 8 to 18 million L. In order to evaluate and

design multispecies toxicity tests, it is crucial to understand the fundamental differences compared to single-species tests. A more extensive discussion has been published (Landis et al. 1997) and the major points are summarized below.

3.12.1 The Nature of Multispecies Toxicity Tests

As discussed in Chapter 2, ecological structures including multispecies toxicity tests have a fundamental property of being historical. Brooks et al. (1989), in an extensive literature review and detailed derivation, concluded that ecological systems are time directed—in other words, irreversible with respect to time. Drake (1991) has experimentally demonstrated the historical aspects of ecological structure in a series of microcosm experiments. Design considerations for multispecies toxicity tests must take into account these properties.

Multispecies toxicity tests share the properties of complex systems, as do natural ecological structures, and also have other important characteristics. Multispecies toxicity tests have a trophic structure, although it is simple. The physical aspects of many types of naturally assembled ecological structures can often be mimicked, and there are many successful attempts at incorporating at least some of the nutrient, sunlight, sediment, soil, and other physical features. Multispecies toxicity tests have been successful in modeling a variety of ecological structures.

Evolutionary events also occur within multispecies toxicity tests. Species or strains resistant to xenobiotics do arise. Simple microbial microcosms (chemostats) are often used to force the evolution of new metabolic pathways for pesticide and xenobiotic degradation.

Microcosms do not have some of the characteristics of natural ecological structures. Perhaps primary is that multispecies toxicity tests are by nature smaller in scale, thus reducing the number of species that can survive in these enclosed spaces compared to natural systems. This feature is very important, since after dosing every experimental design must make each replicate an island to prevent cross-contamination and to protect the environment. Therefore, the dynamics of extinction and the coupled stochastic and deterministic features of island biogeography produce effects that must be separated from that of the toxicant. Ensuring that each replicate is as similar as possible over the short-term minimizes the differential effects of the enforced isolation, but eventually divergence occurs.

Coupled with the necessity of making the replicates similar is the elimination of a key ingredient of naturally synthesized ecological structures, the spatial and temporal heterogeneity. Spatial and temporal heterogeneity is one key to species richness, as in the "Paradox of the Plankton" (Hutchinson, 1961). Environmental heterogeneity is key to the establishment of metapopulations, an important factor in the persistence of species.

The design of multispecies toxicity tests runs into a classical dilemma. If the system incorporates all of the heterogeneity of a naturally synthesized ecological structure, then it can become unique, thereby losing the statistical power needed for typical hypothesis testing. If multispecies toxicity tests are complex systems and subject to community conditioning, then the tests are not repeatable in the same sense as a single-species toxicity test or biochemical assay.

Since the information about past events can be kept in a variety of forms, from the dynamics of populations to the genetic sequence of mitochondria, it is necessary to be able to incorporate each of these types of data into the design and analysis of the experiment. Assumptions about recovery are invalid, and tend to cloud the now apparent dynamics of multispecies toxicity tests. The ramifications are critical to the analysis and interpretation of multispecies toxicity tests.

The historical nature of ecological systems, pollution-induced community tolerance, community conditioning, and ecological legacy are discussed in detail in Chapter 13.

3.12.2 Data Analysis and Interpretation of Multispecies Toxicity Tests

A large number of data analysis methods have been used to examine the dynamics of multispecies toxicity tests. The analysis techniques should be able to detect patterns given the properties of multispecies toxicity tests described above. In order to conduct proper statistical analysis, the samples should be true replicates, and in sufficient number to generate sufficient statistical power. The analysis techniques should be multivariate, able to detect a variety of patterns, and to perform hypothesis testing on those patterns.

3.12.2.1 Sample Design

One of the most difficult aspects of designing a multispecies toxicity test is that of having sufficient replication so that the analysis has sufficient power to resolve differences between the reference nondosed replicates and the other treatment groups. This requirement is particularly difficult to meet when examining a broad range of variables with very different distributions and characteristic variances. Logistical considerations are also critical considering the large size and complexity of multispecies tests. However tempting, it is inappropriate to take several samples from the same microcosm and label these samples replicates. This type of sampling is especially tempting in artificial streams where individual sampling trays within a stream are sometimes considered replicates. Such samples are not true replicates since each tray is connected by the water to the tray downstream. Such a sampling may underrepresent the true variance, and is better used to represent the environmental heterogeneity within a single stream. Such pseudoreplication is best avoided since it invalidates the assumptions of statistics used for hypothesis testing.

3.13 Univariate Methods

Univariate ANOVA, just as in single-species testing, has long been a standard of microcosm data analysis. However, because multispecies toxicity tests generally run for weeks or even months, there are problems with using conventional ANOVA. These include the increasing likelihood of introducing a type II error (accepting a false null hypothesis), temporal dependence of the variables, and the difficulty of graphically representing the data set. Conquest and Taub (1989) developed a method to overcome some of the problems by using intervals of nonsignificant difference (IND). This method corrects for the likelihood of type II errors and produces intervals that are easily graphed to ease examination. The method is routinely used to examine data from standardized aquatic microcosm (SAM) toxicity tests, and it is applicable to other multivariate toxicity tests. The major drawback is the examination of a single variable at a time over the course of the experiment. While this addresses the first goal in multispecies toxicity testing, listed above, it ignores the second. In many instances, community level responses are not as straightforward as the classical predator–prey or nutrient limitation dynamics usually picked as examples of single-species responses that represent complex interactions.

However, by definition, these univariate methods of hypothesis testing are inappropriate for multispecies toxicity tests. As such, these methods are an attempt to understand a multivariate system by looking at one univariate projection after another, attempting to find statistically significant differences. Often the power of the statistical tests is quite low due to the few replicates and the high inherent variance of many of the biotic variables.

Perhaps the greatest danger of the use of ANOVA and related univariate tools is the perpetuation of NOELs, LOECs, and related terms based on univariate hypothesis testing. NOECs and LOECs are so dependent upon statistical power and the concentrations chosen by the experimenter that they are artifacts of the experimental design rather than reflecting the intrinsic hazard of the toxicant. Given the historical nature of microcosm systems, such a determination as a NOEC or LOEC is contrary to the properties of complex structures. Instead, measurements such as $NOEC_{community}$ are indications of the resolving power of the experimental design and the parameters chosen to be measured, rather than a measurement of a real characteristic of ecological structures.

3.14 Multivariate Methods

There are a variety of multivariate methods that are available for the exploration of patterns within ecological data sets. Several are extensively discussed in Chapter 11, and this discussion is only a simple introduction. Multivariate statistics have the advantage of examining all of the data, and therefore more accurately reflect the nature of ecological structures. Coupled with association analysis, these techniques can also be used to test the hypothesis that the pattern is related to treatment. Although each method described below is multivariate, not all are equal, and there is no best method for all cases. Each technique makes different assumptions about the relationships among the variables. Some of the techniques attempt to explain variance; others find clusters based on similarity in a distance measure. In some cases the search for patterns is blind to treatment; in others the treatments are known to the algorithm. Each technique provides the opportunity for a different insight into the patterns that exist within the multispecies toxicity test.

Ludwig and Reynolds (1988) provide an excellent introduction to the assumptions, derivations, and use of several multivariate techniques commonly employed for the analysis of ecological communities. Perhaps the most common forms of multivariate analysis are principal components analysis (PCA) and its derivatives. PCA attempts to find orthogonal combinations of variables that account for the variance within a data set. The assumption in PCA is that the relationships are linear; therefore, PCA is best used with a relatively narrow range of variables where a linear response can be assumed. Assuming that ecological structures are complex, nonlinear relationships may be the norm. Another drawback of PCA is the emphasis on the explanation of variance, and the corresponding emphasis upon variables that may be highly variable but only contain noise.

There have been attempts to deal with the issue of nonlinearity in data sets. Detrended principal components (DPCs) use a polynomial expression to remove the nonlinear relationships from the PCA axes. DPC is useful for data sets of moderate nonlinearity. Detrended correspondence analysis uses a more complex algorithm to eliminate the nonlinearity, but requires a more complex computation. Nonmetric multidimensional scaling (NMDS) is a robust method that deals with nonlinearities by using ranks.

A technique derived from a principal components approach is the coupling of PCA with redundancy analysis (RDA) (van der Brink et al. 1996; Van Wijngaarden et al. 1995). The utility of the technique is that it provides a depiction of the treatment trajectories in an ecological space, and the statistical significance can be examined using a permutation test. One of the proposed benefits of the technique is that it can determine recovery, a dubious distinction in light of the groundwork laid in Chapter 2. In common with other PCA techniques, the technique does assume a linear response.

One of the noteworthy characteristics of the previously described techniques is that all are based on knowing the treatment groups, introducing a strong bias into the search for patterns and explanations. Such a bias also makes it difficult to discern new patterns that may be due to other environmental gradients that may be present in the testing facility or part of an outdoor setting. Most of the models assume a linear response. And in common with that assumption is that the variables with the greatest variance are by definition the most important.

Clustering has the advantage of attempting an unbiased search through a data array for patterns. The data are searched for natural groupings or arrays of similar objects. The algorithm has no knowledge of treatment groups, and is attempting to detect patterns and conduct a sorting based on a predetermined set of rules. There are a variety of available techniques. The groupings can then be compared to treatment groups to see if a relationship exists.

Multivariate descriptive methods have proved promising as a method of interpreting dimensions of an ecosystem. One of the first methods used in toxicity testing was the calculation of ecosystem strain developed by Kersting (1988) for a relatively simple (three-species) microcosm. This method has the advantage of using all the measured parameters of an ecosystem to look for treatment-related differences. At about the same time, Johnson (1988a, 1988b) developed a multivariate algorithm using the *n*-dimensional coordinates of a multivariate data set and the distances between these coordinates as a measure of divergence between treatment groups. Both of these methods have the advantage of examining the ecosystem as a whole rather than by single variables, and can track such processes as succession, recovery, and the deviation of a system due to an anthropogenic input.

Developed for the analysis of ecological data (Matthews and Hearne, 1991), nonmetric clustering and association analysis (NCAA) is a multivariate derivative of artificial intelligence research. NCAA has a fundamentally different approach to discovering patterns in data sets.

In NCAA, an accurate description of the data is only part of the goal of the statistical analysis technique. Equally important is the intuitive clarity of the resulting statistics. For example, a linear discriminant function to distinguish between groups might be a complex function of dozens of variables, combined with delicately balanced factors. While the accuracy of the discriminant may be quite good, use of the discriminant for evaluation purposes is limited because humans cannot perceive hyperplanes in highly dimensional space. By contrast, conceptual clustering attempts to distinguish groups using as few variables as possible, and by making simple use of each one. Rather than combining variables in a linear function, for example, conjunctions of elementary yes/no questions could be combined: species A greater than 5, species B less than 2, and species C between 10 and 20. Numerous examples throughout the artificial intelligence literature have proved that this type of *conceptual* statistical analysis of the data provides much more useful insight into the patterns in the data, and is often more accurate and robust. Conceptual statistical analysis attempts to fit the data, but not at the expense of a simple, intuitive result. The uses of nonmetric clustering and other methods have been compared in a number of field and laboratory tests (Matthews and Matthews 1991; Matthews, Matthews, and Ehinger 1991; Landis et al. 1993a, 1993b).

NCAA has proven to be a powerful technique in the analysis of data sets with high dimensionality but with the replication typical of multispecies toxicity tests. Perhaps the biggest asset of NCAA is that it is nondimensional, nonmetric, and it selects the variables that are important in determining the clusters and rejects those that do not contribute. NCAA does not assume a linear relationship among attributes; in fact, it assumes no particular model at all. The principal drawback of NCAA is computationally intensive, and there is no assurance that a global maximum of clustering has been obtained. Furthermore, NCAA is not available as part of packaged statistical programs.

3.15 Visualization

Methods of visualization that are useful in interpreting the dynamics of ecological structures are also available. In the past, numerous graphs of each variable over the course of the experiment were plotted and a pattern searched for by the investigator. Again, there is a danger that important relationships could be missed because of the bias of the investigator or the simple intractability of the patterns. Other methods are available.

An ordination diagram has been used by van der Brink et al. (1996) to plot the path of the various treatment groups using the axes generated by the redundancy analysis. This method has the advantage of seeing a number of variables at once and the trajectory of each treatment over the course of the experiment. The plots are still two-dimensional representations and variability is not pictured.

Landis et al. (1996) have used space-time worms as a method of visualizing the trajectories of the treatment groups. Two variables that NCAA ranks as important in the clustering are plotted along with time. The variability among replicates is represented by the thickness of the cylinder. This technique is particularly useful in depicting the changing nature of the ecological structures and in portraying variability as a characteristic of the experiment. Space-time worms are described in more detail in Chapter 13.

3.16 Summary of Design Guidelines for Multispecies Toxicity Tests

Multispecies toxicity tests come in a wide variety of types (artificial streams, generic freshwater, simulated farm ponds, ditches, experimental plots and forests) and they share basic properties. Experimental designs should take into account and take advantage of these properties to ensure an interpretable experiment result. We propose the following design parameters for experimental design, analysis, and interpretation.

Basic principles:

1. Multispecies toxicity tests are complex structures. Complex structures are nonequilibrium, historical, and nonlinear. To measure the recovery of such a structure is to measure a property that does not exist for a complex structure.
2. Multispecies toxicity tests are not repeatable in the strict sense since each is sensitive to initial conditions. However, common patterns do appear and these should be the focus of the investigation.
3. All impacts can leave lasting effects. Therefore, determination of a NOEC or LOEC is not warranted.

Experimental design:

1. In multispecies toxicity tests the interactions among the component species should be understood.
2. Environmental gradients do exist in a laboratory or a field situation. A random block design to take into account such gradients should be used.
3. Since the systems are all sensitive to initial conditions, equal numbers of replicates for each treatment group should be used to give every treatment an equal chance for deviation.
4. Samples taken from the same experimental unit must not be considered as experimental replicates.

Data analysis:

1. Univariate statistical techniques are not appropriate for multivariate structures. Repeated ANOVAs are not warranted and can even be misleading.
2. Multivariate methods are more suitable for the data analysis of multispecies toxicity tests. No one multivariate technique is always best. Given that many responses of multispecies toxicity tests are nonlinear, techniques that do not assume linear relationships may allow a more accurate interpretation of the test system.
3. Multivariate techniques that account for variability may be misled by the noisy variables and miss the important relationships.
4. Techniques such as PCA may prevent the discovery of novel patterns. Clustering and other exploratory techniques can lead to the discovery of novel patterns and relationships.
5. Do not assume that the combination of variables that is best for determining clusters or treatments on one sampling day will be the most appropriate for every sampling day. As the structure and function of the multispecies toxicity test change over time, so will the important variables.

Multivariate visualization techniques do exist and should be used. These techniques can lead to a much better understanding of the dynamic nature of these structures.

3.17 Standard Methods

Over the years a variety of test methods have been standardized. These protocols are available from the American Society for Testing and Materials (ASTM), the Organization for Economic Cooperation (OECD), and the National Toxicology Program (NTP), and are available as U.S. Environmental Protection Agency publications, from the *Federal Register*, and often from the researchers who developed the standard methodology.

3.17.1 Advantages of Standard Methods

There are distinct advantages to the use of a standard method or guideline in the evaluation of the toxicity of chemicals or mixtures:

1. Uniformity and comparability of test results.
2. Allows replication of the result by other laboratories.
3. Provides criteria as to the suitability of the test data for decision making.
4. Logistics are simplified, little or no developmental work.
5. Data can be compiled with those of other laboratories for use when large data sets are required. Examples are quantitative structure-activity research and risk assessment.
6. The method establishes a defined baseline from which modifications can be made to answer specific research questions.
7. Over the years numerous protocols have been published. Usually, a standard method or guide has the following format for the conduct of a toxicity test using the ASTM methods and guides as an example:
   a. The scope of the method or guide is identified.
   b. References documents, terminology specific to the standards organization, a summary, and the utility of the methodology are listed and discussed.

c. Hazards and recommended safeguards are now routinely listed.
d. Apparatuses to be used are listed and specified. In aquatic toxicity tests the specifications of the dilution water are given a separate listing, reflecting its importance.
e. Specifications for the material undergoing testing are provided.
f. Test organisms are listed along with criteria for health, size, and sources.
g. Experimental procedure is detailed. This listing includes overall design, physical and chemical conditions of the test chambers or other containers, range of concentrations, and measurements to be made.
h. Analytical methodologies for making the measurements during the experiment are often given a separate listing.
i. Acceptability criteria are listed by which to judge the reliability of the toxicity test.
j. Methods for the calculation of results are listed. Often several methods of determining or calculating the EC_{50}, LD_{50}, or NOEL are referenced.
k. Specifications are listed for the documentation of the results.
l. Appendixes are often added to provide specifics for particular species of strains of animals and the alterations to the basic protocol to accommodate these organisms.

3.17.2 Disadvantages of Standard Methods

Standard methods do have a disadvantage: The methods are generally designed to answer very specific questions that are commonly presented. As in the case of acute and chronic toxicity tests, the question is the ranking of the toxicity of a chemical in comparison to other compounds. When the questions are more detailed or the compound has unusual properties, deviations from the standard method should be undertaken. The trap of standard methods is that they may be used blindly: First ask the question, then find or invent the most appropriate method.

Study Questions

1. Anthropogenic toxicants introduced into the environment come from what types of sources?
2. What is a pesticide?
3. What determines a toxicant compound's environmentally harmful concentrations?
4. Define *dose-response relationship*. What is a dose-response curve?
5. Describe the two parameters that determine a dose-response curve.
6. Similar slopes of dose-response curves may imply what about the xenobiotics being compared?
7. Discuss the two prevailing concepts for studying toxicity of compounds at low concentrations.
8. What are the advantages to the use of a standard method in the evaluation of the toxicity of chemicals or mixtures?
9. What are the two general parameters involved in the classification of toxicity tests in environmental toxicology?
10. Describe a microcosm and a mesocosm test.
11. Describe the lab-to-field dilemma.
12. What differences are there between a static and a static-renewal toxicity test?
13. What are the advantages and disadvantages of the recirculating methodology of toxicity testing?

14. Name and describe the best technical method for toxicity testing.
15. What is whole body aquatic test systems exposure?
16. Discuss the criteria for choosing a test species for use in a toxicity test.
17. Discuss the natural source versus laboratory-derived composition of species in multispecies toxicity tests.
18. What are the most important parameters when choosing statistical design parameters for a toxicity test?
19. Compare the various methods for calculating endpoints from an acute or chronic toxicity test.
20. What evaluation method for laboratory toxicity data is more appropriate for estimating environmental effects than the midpoint in a dose-response curve? Why is it more appropriate?
21. List the five drawbacks of hypothesis testing (in determining the NOEL and LOEL), compared to curve fitting models, as per Stephan and Rodgers?
22. Why does a regression method provide superior information for characterizing toxic responses?
23. What is the optimum design strategy of toxicity tests for the use of the regression method?
24. What are the critical design considerations for multispecies toxicity tests?
25. Why are univariate toxicity tests not always appropriate for microcosm studies?
26. List the characteristics that microcosms have with naturally occurring ecosystems.
27. Why are spatial and temporal heterogeneity reduced in microcosm test systems?

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Chapter 4

Survey and Review of Typical Toxicity Test Methods

4.1 Introduction

The importance of understanding the test procedures that are crucial to environmental toxicology cannot be underestimated. The requirements of the tests dictate the design of the laboratory, logistics, and the required personnel. In every interpretation of a concentration (dose)-response relation and the derived point estimates (EC_{20}, EC_{50}, etc.) there should be a clear understanding of the test method used to obtain that estimate. The understanding should include the strengths and weaknesses of the test method and the vagaries of the test organism or organisms. Quite often it is the standard method that is modified by a researcher to answer more specific questions about the effects of xenobiotics. These standard tests form the basis of much of what we know about relative chemical toxicity in a laboratory setting.

Table 4.1 lists a number of toxicity tests currently available from a variety of standard sources. This table is not inclusive since there are more specialized tests for specific location or situations. Many more methods exist, some of which are derivatives of basic toxicity tests. More important than memorization of each test procedure is a good understanding of the general thrust of the various toxicity tests, methods of data analysis, and experimental design.

The following overview starts a consideration of the use of animals in research and especially toxicity testing. In institutions following U.S. regulations the care and use of animals in research is overseen by an independent local committee subject to oversight by federal agencies. The sections following this regulatory overview begin with single-species toxicity tests and conclude with field studies. These summaries are based on the standard methods published by the American Society for Testing and Materials, the U.S. Environmental Protection Agency, and other published sources. Many of these methods are listed in the reference section for this chapter. The survey is broken up into single-species and multispecies tests. Although Chapter 3 discussed at

Table 4.1 Partial List of ASTM Standard Methods for Toxicity Evaluation or Testing

Biodegradation by a shake-flask die-away method
Conducting a 90-day oral toxicity study in rats
Conducting a subchronic inhalation toxicity study in rats
Conducting aqueous direct photolysis tests
Determining the anaerobic biodegradation potential of organic chemicals
Determining a sorption constant (Koc) for an organic chemical in soil and sediments
Inhibition of respiration in microbial cultures in the activated sludge process
Algal growth potential testing with *Selenastrum capricornutum* (*Pseudokirchneriella subcapitata*)
Conducting bioconcentration tests with fishes and saltwater bivalve mollusks
Conducting reproductive studies with avian species
Conducting subacute dietary toxicity tests with avian species
Evaluating environmental fate models of chemicals
Measurement of chlorophyll content of algae in surface waters
Standardized aquatic microcosm: Freshwater
Using brine shrimp nauplii as food for test animals in aquatic toxicology
Using octanol-water partition coefficient to estimate median lethal concentrations for fish due to narcosis
Conduct of micronucleus assays in mammalian bone marrow erythrocytes
Conducting acute toxicity tests on aqueous effluents with fishes, macroinvertebrates, and amphibians
Conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians
Conducting early life stage toxicity tests with fishes
Conducting life cycle toxicity tests with saltwater mysids
Conducting renewal life cycle toxicity tests with *Daphnia magna*
Conducting sediment toxicity tests with freshwater invertebrates
Conducting 10-day static sediment toxicity tests with marine and estuarine amphipods
Conducting static 96-hour toxicity tests with microalgae
Conducting static acute aquatic toxicity screening tests with the mosquito *Wyeomyia smithii* (Coquillett)
Conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve mollusks

Table 4.1 (*Continued*) Partial List of ASTM Standard Methods for Toxicity Evaluation or Testing

Conducting static toxicity tests with the *Lemna gibba* G3
Conducting a terrestrial soil core microcosm test
Conducting three-brood, renewal toxicity tests with *Ceriodaphnia dubia*
Hazard of a material to aquatic organisms and their uses
Assessing the performance of the Chinese hamster ovary cell/hypoxanthine guanine phosphoribosyl transferase gene mutation assay

Note: New methods are continuously being developed. See Appendix 4.1 for those methods current at the time of this writing.

some length the various types of toxicity: acute, chronic, partial life cycle, and so forth, it is in many ways logical to categorize them into organismal and ecosystem type tests. That organizational scheme is what is done here. Since it is difficult to include every toxicity test in a volume of this size, representative tests have been chosen for summary. Inclusion here does not imply an endorsement by the authors, but these tests serve as examples of the kinds of toxicity tests used to evaluate environmental hazards.

4.2 Animal Care and Use Considerations

Since the care and well-being of terrestrial vertebrates have been of great public concern, strict guidelines as to husbandry and the humane treatment of these organisms have been produced by various government agencies, notably the National Institutes of Health. Many toxicologists did not welcome these guidelines during their implementation. The net effect, however, has been in the improvement of research.

Facilities and animal husbandry are major considerations with the avian or any other test using a terrestrial vertebrate. Guidelines exist and are promulgated by the U.S. Department of Agriculture and the National Institutes of Health to ensure that test animals are maintained to an acceptable standard. The organization that ensures compliance with these laws and guidelines is the Institutional Animal Care and Use Committee (IACUC).

4.2.1 Institutional Animal Care and Use Committee

An important consideration in conducting toxicity tests with animals is compliance with the rules as set by the Public Health Service on the humane care and use of laboratory animals. The regulations are set by the Office of Laboratory Animal Welfare (http://grants.nih.gov/grants/olaw/olaw.htm) of the National Institutes of Health. Key sources of information regarding the regulations and compliance with these laws and regulations are found in Box 4.1.

The foundation of these regulations is that the use of animals for research must be governed by the IACUC. This committee ensures compliance with all applicable federal regulations and the policies of that particular institution. The IACUC oversees the use of animals, reviews protocols for using animals for either research or education, and inspects the husbandry and research facilities of the organization.

BOX 4.1 KEY SOURCES OF INFORMATION FOR COMPLIANCE WITH U.S. GOVERNMENT PRINCIPLES FOR THE UTILIZATION AND CARE OF ANIMALS USED IN TESTING, RESEARCH, AND TRAINING

Applied Research Ethics National Association (ARENA)/NIH Office of Laboratory Animal Welfare (OLAW). 2002. *Institutional Animal Care and Use Committee Guidebook*. 2nd ed., National Institutes of Health, Bethesda, MD.

National Research Council, Commission on Life Sciences, Institute of Laboratory Animal Resources. 1996. *Guide for the Care and Use of Laboratory Animals*. National Academy Press, Washington, DC.

NIH Office of Laboratory Animal Welfare (OLAW). August 2002. *Public Health Service Policy on Humane Care and Use of Laboratory Animals*, National Institutes of Health, Bethesda, MD.

The committees are generally composed of the chair, members from the institution, and a member outside the institution to provide community input. At large institutions or companies the IACUC positions may be full-time, with a full-time staff. Smaller organizations often have faculty or staff who serve on the IACUC as part of other duties. An important aspect of the IACUC is that its decisions are final in regards to animal care and welfare and not subject to overturn by the host organization. The regulations specify the independent nature of the committee so that the welfare of the animals is the priority.

The work of the IACUC is overseen by federal inspectors, typically from the Department of Agriculture or the Public Health Service, depending on the kinds of animals used in the research program. The federal inspections can be unannounced and involve an inspection of the facilities, the protocols, and the decision-making process of the IACUC. Failure to comply with the regulations can result in the barring of the institution or company from federal funding, and other penalties can result.

Although the original regulations were originally formulated to ensure the proper care of warm-blooded vertebrates, many institutions now include reptiles, amphibians, and fish in the review process. Protocols for these animals are also reviewed by the IACUC at these institutions using the same guidance as for those organisms specifically described in the federal regulations. There is also discussion to include invertebrates such as octopus and squid, which exhibit complex behaviors and problem solving, in the review process.

The passage of laws and regulations and the creation of IACUCs have had several beneficial impacts on animal research. First, the husbandry and care of animals has been standardized, helping to ensure the health of the organisms and the quality of the data derived from the experiments. Second, research protocols are routinely reviewed by other experts, and the experimental design, statistical analysis, and appropriateness of the surgery or exposure to toxic materials have to be explicitly justified. Experiments that do not meet the standards of the IACUC are not permitted. Third, the presence of the IACUC helps to ensure the continued existence of the social contract between the institution and its local community regarding the use of animals in research. The use of animals in research has long been an issue of public concern. Having community representatives in the IACUC ensures that the local stakeholders are represented.

4.2.2 Replace, Reduce, Refine

An additional consideration when using any animal is the desire to balance the acquisition of data with the pain and suffering of the test organisms. It is crucial to use the fewest numbers of organisms possible and to acquire the maximum amount of data from each toxicity test.

The first consideration should be a careful examination of the requirement that a certain toxicity test or other research program be undertaken. In environmental toxicology it is often necessary to use the organism in the laboratory as a test organism in order to protect the wild populations. If the research or test methodology is required, then there are three other considerations.

Often it is possible to *replace* a toxicity test with an alternative methodology, especially when cellular or mechanistic studies are undertaken. Tissue in laboratory culture, microorganisms, or lower invertebrates can also be used in place of whole animal studies. In the case of screening tests, there now exists a broad variety of quantitative structure-activity models that can predict and actually overestimate acute and chronic toxicity. Compounds that are likely to demonstrate high toxicity can be eliminated from consideration as a product or focused upon for toxicity reduction.

It is also often possible to *reduce* the number of animals used in the evaluation of a chemical or toxic waste site by carefully designing the experiment to maximize the data acquired or by accepting a compromise in the statistical significance and power. Often a slight decrease in the statistical power can result in a large reduction in the number of animals required in a toxicity test.

Finally, it is often possible to *refine* the methodology to require fewer animals. Biochemical and physiological indicators of toxicant stress or indications of mechanisms can help to reduce the number of animals or even the need for such testing.

4.3 Single-Species Toxicity Tests

4.3.1 Daphnia *48-Hour Acute Toxicity Test*

This test, along with the fish 96-hour acute toxicity test, is one of the standbys in aquatic toxicology. *Daphnia magna* and *D. pulex* are the common test species. *D. magna* requires relatively hard water for its culture. *D. magna* are large, commonly available, and easy to culture. *D. pulex* is not quite as large as *D. magna* and tolerates softer water. It is recommended that the test organisms be derived from adults, three generations after introduction into the specific laboratory media.

Water quality is a major factor in the performance of any laboratory aquatic toxicity test. Care must be taken to eliminate other sources of mortality, such as chlorine of chlorinated organics, heavy metal contamination, and contamination by organics in the groundwater or reservoir supply. In some labs with access to high-grade tap or well water, only a minor purification system is required. However, in many cases a further filtration and distillation step may be required. Soft dilution water (40 to 48 mg/l as $CaCO_3$) is recommended for tests with *D. pulex*, and moderately hard water (80 to 100 mg/l as $CaCO_3$) is recommended for tests with *D. magna*. A dilution of water is considered acceptable if *Daphnia* spp. show adequate survival and reproduction when cultured in the water.

Sodium pentachlorophenate (NaPCP) is the reference toxicant that has been suggested for toxicity tests using daphnids. The use of a reference toxicant is important in confirming the health of the daphnids and the quality of the water and test methodology.

In general, 10 neonates that are less than 24 hours old are placed in 125-ml beakers containing 100 ml of test solution with five concentrations and a negative control. The tests are usually run in

triplicate. Death is difficult to observe, so immobility of the daphnids is used as the endpoint. An organism in considered immobile (nonmotile) if it does not resume swimming after prodding with a pipet or glass rod. Measurements are made at 24-hour intervals. No feeding occurs during the course of this toxicity test.

The *Daphnia* 48-hour toxicity test is a useful screen for the toxicity of single compounds, mixtures, or effluents. In some cases the daphnid toxicity test has been used to evaluate the potential pathology or other potential problems with genetically engineered organisms. The advantages of the daphnid toxicity test are its short timeframe, small amounts of hazardous waste are generated, and the test is inexpensive. Often daphnids are more sensitive than vertebrates to a variety of toxicants. The disadvantages include the time-consuming maintenance of test stocks and the sensitivity of the organisms to water quality.

The chronic or partial life cycle toxicity test with *D. magna* is an attempt to look at growth and reproductive success of the test organisms. This test is contrasted to its acute counterpart in Table 4.2. The test follows a set of daphnids through the production of three broods with a measurement of growth (length or mass) of the original organisms along with the numbers of offspring derived from each animal.

One of the most controversial aspects of this test has been the food source during the study. A number of mixtures have been tried with interesting results. A mixture of trout chow and algae has been demonstrated to provided excellent growth, but there are concerns about the consistency of the ingredients. Many laboratories use a combination of algae, *Ankistrodesmus convolutus*, *A. falcatus*, *Chlamydomonas reinhardii*, and *Pseudokirchneriella subcapitata* as the food source.

This toxicity test is usually run as a static renewal, but some researchers have used a continuous flow setup with a proportional diluter. Handling the organisms during the transfer to new media is a potential problem for inexperienced technicians.

Occasionally it is difficult to set up concentrations for the test if the median values for the chronic endpoints are close to the values for a toxicant that induce mortality over the duration of the experiment. Loss of replicates can occur if the mortality rates are high enough. Use of the dose-response curve of the acute data should help in identifying useful boundary conditions for the higher concentrations of xenobiotic.

Closely related to the *D. magna* partial life cycle toxicity test is the three-brood renewal toxicity test with *Ceriodaphnia dubia* (Table 4.3). The test was developed in an attempt to shorten the amount of time, amount of toxicant, and the cost of performing chronic type toxicity tests. This methodology has proven useful in a variety of roles, especially in the testing of effluents. One of the drawbacks and advantages of the method is the small size of the test organism. Adult *C. dubia* are about the same size as first-instar *D. magna*. Handling the first instars and even the adults often takes a dissecting microscope and a steady hand. Conversely, the small size enables the researcher to conduct the test in a minimum of space, and the rapid reproduction rate makes the method one of the shortest life cycle type tests.

As with the *D. magna* tests, one of the problems has been in the successful formulation of a food to ensure the health and reproduction of the *C. dubia* during the course of the toxicity tests. A combination of trout chow, yeast, rye grass powder, and algae has been used. Nonetheless, the *C. dubia* three-brood toxicity test has been proven to be useful and replicable.

4.3.2 Algal 96-Hour Growth Toxicity Test

The purpose of this toxicity test is to examine the toxicity of materials to a variety of freshwater and marine algae, and it is summarized in Table 4.4. In aquatic systems algae are generally responsible

Table 4.2 Comparison of the *D. magna* 48-Hour Acute Toxicity Test with the Common *D. magna* Chronic Toxicity or Partial Life Cycle Test

	Test Type	
	Chronic (Partial Life Cycle)	*Acute 48 Hour*
Organisms	***D. magna***	***D. magna***
Age of test organisms	≤24 hours old	≤24 hours old
Number of organisms per chamber	10	10 (minimum)
Experimental Design		
Test vessel type and size	100 ml beakers	250 ml
Test solution volume	80 ml	200 ml
Number of replicates per sample	2 (minimum)	3 (minimum)
Feeding regime	Various combinations of trout	Do not feed chow, yeast, alfalfa, green algae, and diatoms given in excess
Test duration	21 days	48 hours
Physical and Chemical Parameters		
Water temperature (°C)	20°C	20 ± 2°C
Light quality	Ambient laboratory levels	Ambient laboratory levels
Light intensity	Up to 600 lux	540–1,080 lux
Photoperiod	16 hours light/8 hours dark	16 hours light/8 hours dark (with 15- to 30-minute transition)
pH range	7.0–8.6	7.0–8.6
DO concentration	40–100%	60–100%
Aeration	Not necessary	None
Endpoints		
	Survival, growth, and reproduction	Immobilization

Table 4.3 Summary for Conducting Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*

Test type	Static renewal/chronic
Organisms	
Ceriodaphnia dubia	
Age of test organisms	<12 hours old
Experimental Design	
Test vessel type and size	Test has been conducted with 30 ml beaker with 15 ml of test solution; can use any container made of glass, type 316 stainless steel, or fluorocarbon plastic if (a) each *C. dubia* is in a separate chamber or compartment and (b) each chamber can maintain adequate DO levels for the organism; chambers should be covered with glass, stainless steel, nylon, or fluorocarbon plastic covers or Shimatsu closures
Number of replicates	10
Total number of organisms	At least 10
Number of organisms per chamber	1
Feeding regime	Various combinations of trout chow, yeast, rye grass powder, and algae have been used; types of algae include *Ankistrodesmus convolutus*, *A. falcatus*, *Chlamydomonas reinhardii*, and *Pseudokirchneriella subcapitata*
Test duration	7 days
Physical and Chemical Parameters	
Temperature	25 ± 1°C
Test solution pH	Not specified
DO concentration	40–100%
Endpoint	
Reproduction	

Table 4.4 Summary of Test Conditions for Conducting Static 96-Hour Toxicity Tests with Microalgae

Test type	Static
Organisms	
Freshwater species	*Selenastrum capricornutum (Pseudokirchneriella subcapitata), Scenedesmus subspicatus, Chlorella vulgaris, Microcystis aeruginosa, Anabaena flos-aquae, Navicula pelliculosa*
Saltwater species	*Skeletonema costatum, Thalassiosira pseudonana,* and *Dunaliella tertiolecta*
Number of organisms per chamber (±10%)	*Pseudokirchneriella subcapitata* and other freshwater green algae, 2×10^4 cells/ml
	Navicula pelliculosa, 2×10^4cells/ml
	Microcystis aeruginosa, 5×10^4 cells/ml
	Anabaena flos-aquae, 2×10^4 cells/ml
	Saltwater species, 2×10^4cells/ml
Experimental Design	
Test vessel type and size	Sterile Erlenmeyer flasks of borosilicate glass, any size
Test solution volume	Not to exceed 50% of the flask volume for tests conducted on a shaker, and not more than 20% of the flask volume for tests not conducted on a shaker
Number of replicate chambers per sample	2 or more
Test duration	96 hours
Physical and Chemical Parameters	
Water temperature	24 ± 2°C for freshwater green and blue-green alga
	20 ± 2°C for *Navicula pelliculosa* and other saltwater alga
Light quality	Continuous "cool-white" fluorescent
Light intensity	Should not vary by more than ±15%:
	60 µEinsteins m^2/s (4,300 lm/m^2) for freshwater diatoms and green algae
	30 µEinsteins m^2/s (2,150 lm/m^2) for freshwater blue-green algae

(Continued)

Table 4.4 (*Continued*) Summary of Test Conditions for Conducting Static 96-Hour Toxicity Tests with Microalgae

Test type	Static
	82–90 μEinsteins m^2/s (5,900–6,500 lm/m^2) for *Thalassiosira*
	60 μEinsteins m^2/s (4,300 lm/m^2) for *Skeletonema*
Photoperiod	14 hours light/10 hours dark for *Skeletonema*
Test solution pH	7.5 ± 0.1 for freshwater
	8.0 ± 0.1 for saltwater
Endpoint	
Biomass, cell number, area underneath the growth curve, chlorophyll content	

for a large percentage of the primary production. Impacts upon the unicellular photosynthetic organisms could have long-lasting impacts to the community.

Numerous test organisms have been used in this toxicity test, but those currently recommended by the ASTM guidelines are as follows:

Freshwater:
- Green algae: *Pseudokirchneriella subcapitata*, *Scenedesmus subspicatus*, *Chlorella vulgaris*
- Blue-green algae (bacteria): *Microcystus aeruginosa*, *Anabena flos-aquae*
- Diatom: *Navicula pelliculosa*

Saltwater:
- Diatom: *Skeltonema costatum*, *Thalassiosira pseudonana*
- Flagellate: *Dunaliella tertiolecta*

Other test organisms can be used, if necessary, for a particular toxicity assessment or research. The methodology is very adaptable.

Depending upon the test organism, between 2×10^4 and 5×10^4 cells are used to inoculate the test vessel and the concentration of cells is determined daily. Cell counts are made daily by using a hemocytometer or an electronic particle counter such as the Coulter counter. Chlorophyll can be measured spectrophotometrically or fluorometrically. The fluorometric determinations are more accurate at low concentrations of test organism. Other measurements that have been used include DNA content, ATP charge, and ^{14}C assimilation.

If only standing biomass is the endpoint to be measured, then only cell concentration at the end of the exposure period has to be determined. However, measurements such as area under the curve and growth rate are important variables in determining the ecological impacts of a toxicant. These valuable endpoints require measurements of cell density each day for the duration of the toxicity test. Other measurements to ensure the replicability of the data include pH, temperature, and light intensity.

Whenever possible, toxicant concentration should also be taken at the beginning and end of the test. Errors in measurement, degradation, or volitization can produce a concentration different from that of the expected or nominal concentration.

Good microbiological sterile technique is required to ensure a minimum of cross-contamination with other algae and to prevent the introduction of bacteria. The degradation of the toxicant

by introduced bacteria can alter the apparent toxicity, even to the point of eliminating the test compound from the media.

Another interesting aspect of this test is the enhancement of algal growth often found at low concentrations of toxicant. The spontaneous hydrolysis or other breakdown of the test compound may provide nutrients in addition to the nutrients contained in effluents. It is crucial that the data be appropriately plotted and analyzed.

4.3.3 Acute Toxicity Tests with Aquatic Vertebrates and Macroinvertebrates

As with the daphnid toxicity tests, toxicity tests using a variety of fish species, amphibians, and macroinvertebrates have long been the standbys of aquatic toxicity evaluations. Table 4.5 summarizes the species and methods used in these tests.

One of the major problems in conducting these toxicity tests is the reliable supply of healthy test organisms. Many of the fish species used to stock ponds and lakes are available through hatcheries. Specialist suppliers also exist for the species that are routinely used for toxicity evaluations. In some cases it is required that wild organisms are collected and acclimated to the laboratory environment before conducting the toxicity test. Wild collected animals have some advantages and drawbacks. The major advantage is that if the organism is collected locally, the sensitivity demonstrated in the toxicity test is representative of that particular native population. Care must be taken, however, to not unduly stress the collected organisms or the resultant stress may cause an overestimate of the toxicity of the compound being examined. The major difficulty of using organisms collected from wild populations is the variation among populations in sensitivity to the toxicant or to the laboratory culture collections. With mobile organisms it may be difficult to consistently collect organisms from the same breeding population. Also, the act of collecting the organisms may seriously deplete their numbers, especially in areas near the testing facility. Care should be taken not to deplete local populations. Another solution is to maintain a habitat adjacent to the facility as a source of the test organisms under the control and regulation of the testing laboratory.

Another difficulty in conducting a broad series of toxicity tests is the assurance of adequate water quality and volume for a variety of species. For testing freshwater species the solution is often the investment in a well system with the water filtered and sterilized. Occasionally the testing facility may be adjacent to a body of water that can supply a consistent and uncontaminated source of water for the culture of the test organisms and also act as a source of dilution water. Laboratories on the Great Lakes or marine laboratories often have access to large volumes of relatively clean water. The least desirable but often the only option available is the use of distilled tap water for culture and diluent. At the least, the tap water should be doubly distilled and filtered before being used to make culture media. Systems that use distilled water supplied by a central system, filtered though an ion exchange system and then glass distilled, have proven reliable. Unfortunately, the necessity of using distilled water cuts down on the volumes available for large-scale flow-through test systems. Finally, it is important to constantly monitor the quality of the water source. The choice of deionizing or filtering units is also important. Apparently some resins do leach out small amounts of materials toxic to fish and invertebrates. A positive control using a toxicant with well-known LC_{50} values should give an indication of the suitability of the test solutions. Measurement of variables such as hardness, pH, alkalinity, and in the case of marine systems, salinity, can prevent disasters or unreliable test results.

Table 4.5 Summary for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians

Test Type	*Static Renewal, Flow-Through*
Organisms	
Freshwater vertebrates	Frog (*Rana* sp.), toad (*Bufo* sp.), coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Salmo gairdneri*), brook trout (*Salvelinus fontinalis*), goldfish (*Carassius auratus*), fathead minnow (*Pimephales promelas*), channel catfish (*Ictalarus punctatus*), bluegill (*Lepomis macrochirus*), green sunfish (*Lepomis cyanellus*)
Freshwater invertebrates	Daphnids (*Daphnia magna, D. pulex, D. pulicaria*), amphipods (*Gammarus lacustris, G. fasciatus, G. pseudolimnaeus*), crayfish (*Orconectes* sp., *Combarus* sp., *Procambarus* sp., *Pacifastacus leniusculus*), stoneflies (*Pteronarcys* sp.), mayflies (*Baetis* sp., *Ephemerella* sp.), mayflies (*Hexagenia limbata, H. bilineata*), midges (*Chironomus* sp.), snails (*Physa integra, P. heterostropha, Amnicola limosa*), planaria (*Dugesia tigrina*)
Saltwater vertebrates	Sheepshead minnow (*Cyprinodon variegatus*), mummichog (*Fundulus heteroclitus*), longnose killifish (*Fundulus similis*), silverside (*Menidia* sp.), threespine stickleback (*Gasterosteus aculeatus*), pinfish (*Lagodon rhomboides*), spot (*Leiostomus xanthurus*), shiner perch (*Cymatogaster aggregata*), tidepool sculpin (*Oligocottus maculosus*), sanddab (*Citharichthys stigmaeus*), flounder (*Paralichthys dentatus, P. lethostigma*), starry flounder (*Platichthys stellatus*), English sole (*Parophrys vetulus*), herring (*Clupea harengus*)
Saltwater invertebrates	Copepods (*Acartia clausi, Acartia tonsa*), shrimp (*Penaeus setiferus, P. duorarum, P. aztecus*), grass shrimp (*Palaemonetes pugio, P. intermedius, P. vulgaris*), sand shrimp (*Crangon septemspinosa*), shrimp (*Pandalus jordani, P. danae*), bay shrimp (*Crangon nigricauda*), mysid (*Mysidopsis bahia, M. bigelowi, M. almyra*), blue crab (*Callinectes sapidus*), shore crab (*Hemigrapsus* sp., *Pachygrapsus* sp.), green crab (*Carcinus maenas*), fiddler crab (*Uca* sp.), oyster (*Crassostrea virginica, C. gigas*), polychaete (*Capitella capitata*)
Age and size of test organisms	All organisms should be as uniform as possible in age and size

Table 4.5 (*Continued*) Summary for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians

Test Type	*Static Renewal, Flow-Through*
Fish	Juvenile
	Weight between 0.1 and 5.0 g
	Total length of longest fish should be no more than twice that of the shortest fish
Invertebrates	Except for deposition tests with bivalve mollusks and tests with copepods, immature organisms should be used whenever possible
Daphnids	Less than 24 hours old
	Amphipods, mayflies, and stoneflies: Early instar
	Midges: Second or third instar
	Saltwater mysids: Less than 24 hours postrelease from the brood sac
	Do not use ovigerous decapod crustaceans or polychaetes with visible developing eggs in coelom
Amphibians	Use young larvae whenever possible
Experimental Design	
Test vessel type and size	Smallest horizontal dimension should be three times the largest horizontal dimension of the largest organism
	Depth should be at least three times the height of the largest organism
Solution volume	At least 150 mm deep for organisms over 0.5 g each and at least 50 mm deep for smaller organisms
Feeding regime	Feed at least once a day a food that will support normal function
Test duration	Daphnids and midge larvae: 48 hours
	All other species: 96 hours in static tests, at least 96 hours in renewal and flow-through tests
Physical and Chemical Parameters	
Freshwater Vertebrates	*Water Temperature (°C)*
Frog, *Rana* sp.	22
Toad, *Bufo* sp.	22

(Continued)

Table 4.5 (*Continued*) Summary for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians

Freshwater Vertebrates	*Water Temperature (°C)*
Coho salmon, *Oncorhynchus kisutch*	12
Rainbow trout, *Salmo gairdneri*	12
Brook trout, *Salvelinus fontinalis*	12
Goldfish, *Carassius auratus*	17, 22
Fathead minnow, *Pimephales promelas*	25
Channel catfish, *Ictalurus punctatus*	17, 22
Bluegill, *Lepomis macrochirus*	17,22
Green sunfish, *Lepomis cyanellus*	17, 22
Freshwater Invertebrates	*Water Temperature (°C)*
Daphnids, *Daphnia magna*, *D. pulex*, *D. pulicaria*	20
Amphipods, *Gammarus lacustris*, *G. fasciatus*, *G. pseudolimnaeus*	17
Crayfish, *Orconectes* sp., *Combarus* sp., *Procambarus* sp.	17, 22
Pacifastacus leniusculus	17
Stoneflies, *Pteronarcys* sp.	12
Mayflies, *Baetis* sp., *Ephemerella* sp.	17
Mayflies, *Hexagenia limbata*, *H. bilineata*	22
Midges, *Chironomus* sp.	22
Snails, *Physa integra*, *P. heterostropha*, *Amnicola limosa*	22
Planaria, *Dugesia tigrina*	22
Saltwater Vertebrates	*Water Temperature (°C)*
Sheepshead minnow, *Cyprinodon variegatus*	22
Mummichog, *Fundulus heteroclitus*	22
Saltwater Vertebrates	*Water Temperature (°C)*
Longnose killifish, *Fundulus similis*	22
Silverside, *Menidia* sp.	22

Table 4.5 (*Continued*) Summary for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians

Saltwater Vertebrates	*Water Temperature (°C)*
Threespine stickleback, *Gasterosteus aculeatus*	17
Pinfish, *Lagodon rhomboides*	22
Spot, *Leiostomus xanthurus*	22
Shiner perch, *Cymatogaster aggregata*	12
Tidepool sculpin, *Oligocottus maculosus*	12
Sanddab, *Citharichthys stigmaeus*	12
Flounder, *Paralichthys dentatus, lethostigma*	22
Starry flounder, *Platichthys stellatus*	12
English sole, *Parophrys vetulus*	12
Herring, *Clupea harengus*	12
Saltwater Invertebrates	*Water Temperature (°C)*
Copepods, *Acartia clausi*	12
Acartia tonsa	22
Shrimp, *Penaeus setiferus, P. duorarum, P. aztecus*	22
Grass shrimp, *Palaemonetes pugio, P. intermedius, P. vulgaris*	22
Sand shrimp, *Crangon septemspinosa*	17
Shrimp, *Pandalus jordani, P. danae*	12
Bay shrimp, *Crangon*	17
Mysid, *Mysidopsis bahia, M. bigelowi, M. almyra*	27
Blue crab, *Callinectes sapidus*	22
Shore crab, *Hemigrapsus* sp., *Pachygrapsus* sp.	12
Green crab, *Carcinus maenas*	22
Fiddler crab, *Uca* sp.	22
Oyster, *Crassostrea virginica, C. gigas*	22

(Continued)

Table 4.5 (*Continued*) Summary for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians

Saltwater Invertebrates	*Water Temperature (°C)*
Polychaete, *Capitella capitata*	22
Light quality	Not specified
Light intensity	Not specified
Photoperiod	16 hours light/8 hours dark with a 15- to 30-minute transition period
Test solution pH	Very soft: 6.4–6.9
	Soft: 7.2–7.6
	Hard: 7.6–8.0
	Very hard: 8.0–8.4
DO concentration	60–100% for static test during first 48 hours
	40–100% for static test after 48 hours
	60–100% for renewal and flow-through tests (all times)
Endpoint	
Death, immobilization	

The fish species used in these tests can be far ranging, although the most popular are the fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), channel catfish (*Ictalarus punctatus*), and rainbow trout (*Oncorhynchus mykiss*). Andromonas fish are usually represented by the Coho salmon (*O. kisutch*). Marine species used are often the sheepshead minnow (*Cyprinodon variegatus*), mummichog (*Fundulus heteroclitus*), and silversides (*Menidia* sp.).

A variety of invertebrates are also used in these series of tests. Freshwater invertebrates are represented by daphnids, insect larvae, crayfish, and mollusks. Various mysid, shrimp, and crab species are used to represent marine invertebrates.

4.3.4 Terrestrial Vertebrate Toxicity Tests

In parallel to the short-term toxicity tests with aquatic species are the standard mammal and bird toxicity tests. The methodologies are typically classed as to period and mode of exposure. Two examples of mammalian tests are summarized in Tables 4.6 and 4.7. The small mammal toxicity tests were originally and are still used primarily for the extrapolation of toxicity and hazard to humans. The advantage to this developmental process is that a great deal of toxicity data occurs for a variety of compounds, in both their structure and their mode of action. Often, the only toxicity data available for a compound are a rat or mouse toxicity endpoint. An enormous amount of physiological and behavioral data is available due to the extensive testing, and much of what forms the foundation of traditional toxicology was formed using these methods. The strains of rodents used are often well characterized genetically, with some having extensive pedigrees available. The drawback to environmental toxicology, however, is that the focus has traditionally been

Table 4.6 Summary of Test Conditions for Conducting a Subchronic Inhalation Toxicity Study in Rats

Test Type	*Subchronic*		
Organisms			
Variety of rodent species may be used; rat is preferred			
Age and size of organisms	Ideally before 6 weeks old, not more than 8 weeks old; weight variation not to exceed ±20% for each sex		
Experimental Design			
Test Chamber Size			
	Weight of Rat (g)	*Floor Area/Rat (cm^2)*	
	<100	109.68 (17.0 $in.^2$)	
	100–200	148.40 (23.0 $in.^2$)	
	200–300	187.11 (29.0 $in.^2$)	
	300–400	258.08 (40.0 $in.^2$)	
	400–500	387.15 (60.0 $in.^2$)	
	>500	451.64 (70.0 $in.^2$)	
	Height should be at least 17.8 cm (7 in.)		
Exposure to test substance	Ideally for 6 hours/day on a 7 day/week basis; if necessary, exposure on a 5 day/week is considered acceptable; test substance is introduced into the chamber air supply; a suitable analytical control system should be used		
Number of test groups	3		
Number of organisms per group	20 rats (10 male, 10 female)		
Number of organisms per chamber	1 individual		
Feeding regime	Withhold food and water during exposure period		
Test duration	90 days		
Clinical examinations	Urinalysis, hematology, blood chemistry, and necropsy		
Physical and Chemical Parameters			
Temperature	22 ± 2°C		
Humidity	Ideally 40–60%		
Oxygen content	19%		
Dynamic airflow	12–15 air changes/hour		
Endpoint			
Death			

Table 4.7 Summary of Test Conditions for Conducting a 90-Day Oral Toxicity Study in Rats

Test Type	*Subchronic*		
Organisms			
Rats; other rodents may be used with appropriate modifications and justifications			
Age and size of organism	Ideally before rats are 6 weeks old and not more than 8 weeks old; weight variation should not exceed ±20% of the mean weight for each sex		
Feeding regime	Any unmedicated commercial diet that meets the minimum nutritional standards of the test species		
Experimental Design			
Test Chamber Size			
	Weight of Rat (g)	*Floor Area/Rat (cm^2)*	
	<100	109.69 (17.0 $in.^2$)	
	100–200	148.40 (23.0 $in.^2$)	
	200–300	187.11 (29.0 $in.^2$)	
	300–400	258.08 (40.0 $in.^2$)	
	400–500	387.15 (60.0 $in.^2$)	
	>500	451.64 (70.0 $in.^2$)	
	Height should be at least 17.8 cm (7 in.)		
Test chamber type	All metal cages with wire-mesh bottoms, suspended in racks		
Number of test groups	At least 4		
Number of test organisms per group	20 (10 male, 10 female)		
Number of test organisms per chamber	1 individual		
Dosage	Administer through the diet, the drinking water, by capsule or gavage; if by gavage, a 5 day/week dosing regiment is acceptable		
Test duration	90 days		
Clinical examinations	Urinalysis, hematology, blood chemistry, and necropsy		
Physical and Chemical Parameters			
Temperature	22 ± 2°C		
Endpoint			
Death			

the extrapolation of the toxicity data to primates and not toward other classes of mammals. It is difficult to accurately extrapolate rodent oral toxicity data to cattle since cattle have drastically different digestive systems. It is possible to use other species of rodents and other small mammals with strains having originated from wild-caught organisms, and these tests may prove useful in assessing the interspecific variability of a toxic response.

In contrast, the avian toxicity tests have been developed over the last two decades in order to assess the effects of environmental contaminants, especially the effects of pesticides to nontarget species. The methods are similar, in general, to other short-term toxicity tests. A variety of species from different families of birds have been used, although standardization as to strain of each species has not been as extensive as with the mammalian toxicity tests. Examples of an acute feeding study and a reproductive test are presented in Tables 4.8 and 4.9.

It should not be assumed that one method exists for each of these tests. In many cases subtle differences exist between protocols that are acceptable. Table 4.10 compares two methods: the ASTM consensus method and the U.S. Environmental Protection Agency (EPA) method. The ASTM method is broader and includes species that the U.S. EPA method does not. This allows the U.S. EPA method to be more specific since fewer species are involved. Both tests are for a maximum of 14 days. Other differences are in the experimental chambers. The ASTM standard includes a general description of the test chamber; the U.S. EPA standard includes the size and specifications for the materials. Although both standards are used, differences do exist, and it is important to understand the specifications and potential differences when comparing toxicity results.

4.3.5 Frog Embryo Teratogenesis Assay: FETAX

This toxicity test is one of the few amphibian-based toxicity tests and is summarized in Table 4.11. *Xenopus laevis*, the South African clawed frog, is the amphibian species used in this toxicity test. J. Bantle and colleagues (1992) have developed and perfected this methodology over the last 10 years. The methodology has been performed in a number of laboratories with repeatable results. This toxicity test has a number of uses. FETAX (Frog Embryo Teratogenesis Assay: Xenopus) has been touted as an alternative to performing the mammalian teratogenicity test, and its correlation with known mammalian teratogens is very good. Teratogenicity of runoff, water collected from lakes and streams and even elutriates from soil samples has been evaluated using the same basic methodology.

One of the major advantages is the database that has been obtained on the test organism, *Xenopus*. *Xenopus* is a research organism widely used in developmental research and in the genetics of development. The animals are also easy to mate and large numbers of eggs are produced, ensuring large sample sizes. Compared to mammals, birds, and reptiles, it is easy to observe malformations or other teratogenic effects since the developing embryos are in the open.

FETAX is a rapid test for identifying developmental toxicants. Data may be extrapolated to other species, including mammals. FETAX might be used to prioritize hazardous waste samples for further tests that use mammals. Validation studies using compounds with known mammalian or human developmental toxicity suggest the predictive accuracy rate compares favorably with other currently available in vitro teratogenesis screening assays (Bantle et al. 1992). It is important to measure developmental toxicity because embryo mortality, malformation, and growth inhibition can often occur at concentrations far less than those required to affect adult organisms. Because of the sensitivity of embryonic and early life stages, FETAX provides information that might be useful in estimating the chronic toxicity of a test material to aquatic organisms.

The criticism often presented about the FETAX is that it is a poor representation of native species of amphibians or other vertebrates. *Xenopus* is, of course, not native to the Americas, but

Table 4.8 Summary for Conducting Subacute Dietary Toxicity Tests with Avian Species

Test Type	*Avian Subacute Dietary*
Organisms	
Test to be done primarily with northern bobwhite (*Colinus virginianus*), Japanese quail (*Coturnix japonica*), mallard (*Anas platyrhynchos*), and ring-necked pheasant (*Phasianus colchicus*)	
Age of organism	14 days, 14 days, 5 days, and 10 days, respectively
Experimental Design	
Test chamber	Construction materials in contact with birds should not be toxic, or capable of adsorbing or absorbing test substances
	Materials that can be dissolved by water or loosened by pecking should not be used; stainless or galvanized steel, or materials coated with plastics are acceptable; any material or pen shape is acceptable provided the birds are able to move about freely and that pens can be kept clean
Test substance	One concentration should kill more than 0% but less than 50%, and one concentration should kill more than 50% but less than 100%; these results can be obtained with four to six treatment levels
Number of organisms per group	Minimum of 10 birds for each test concentration
Number of organisms per replicate	Minimum of 5
Feeding	Test substance is mixed with feed; birds shall be fed *ad libitum*
Test duration	Treated diets are available for 5 days, then replaced with untreated feed; birds are held for a minimum of 3 days following treatment
Clinical examinations	Body weight (record at beginning and end) and feed consumption
Physical and Chemical Parameters	
Temperature	A temperature gradient from approximately 38°C to approximately 22°C should be established in brooders
Photoperiod	Minimum of 14 hours of light
Humidity	45–70% (higher relative humidities may be appropriate for waterfowl)
Ventilation	Sufficient to supply 10–15 air changes per hour
Endpoint	
Mortality	

Table 4.9 Summary for Conducting Reproductive Studies with Avian Species

Test Type	*Avian Reproduction*
Organisms	
Ring-necked pheasant (*Phasianus colchicus*), bobwhite (*Colinus virginianus*), Japanese quail (*Coturnix japonica*), chicken (*Tympanuchus cupido*), mallard (*Anas platyrhynchos*), black duck (*Anas rubripes*), screech owl (*Otus asio*), American kestrel, ring dove (*Streptopelia risoria*), gray partridge, crowned guinea-fowl	
Age of organism	Should be within ±10% of the mean age of the group
Feeding	Feed and water should be available *ad libitum*; feed consumption should be measured for 7-day periods throughout the study
Experimental Design	
Test chamber type and size	Materials that can be dissolved by water or loosened by pecking should not be used; stainless steel, galvanized steel, or materials coated with perfluorocarbon plastics are acceptable; any design is acceptable such that the birds are able to move about freely and the pens kept clean
Test concentration	(1) At least one concentration must produce an effect
	(2) The highest test concentration must contain at least 0.1% (1,000 ppm)
	(3) The highest test concentration must be 100 times the highest measured or expected field concentration
Number of test groups	A minimum of 16 pens per test concentration and control group should be used
Number of organisms per chamber	Pairs or groups containing no more than one male
Exposure to test substance	Mix test substance directly into feed
Clinical examinations	Eggs laid; normal eggs; fertile eggs; hatchability; normal young; survival; weight of young; eggshell thickness; residue analysis
Physical and Chemical Parameters	
Temperature	About 21°C for adults
	For hatchlings, the amount and duration of heat is species specified; a temperature gradient should be established from an appropriate heat source and range down to about 21°C
Humidity	45–70% (higher relative humidities may be appropriate for waterfowl)

(Continued)

Table 4.9 (*Continued*) Summary for Conducting Reproductive Studies with Avian Species

Light quality	Should emit a spectrum simulating daylight
Light intensity	65 lux (6 ft candle)
Photoperiod	For adults: 8 hours light/16 hours dark prior to photostimulation
	17 hours light/7 hours dark from onset of photostimulation
	For hatchlings: At least 14 hours of light for precocial species
Endpoint	
Reproduction	

Table 4.10 Comparison of ASTM and U.S. EPA Standards for Conducting Subacute Dietary Toxicity Tests with Avian Species

	Test Type	
	ASTM Avian Subacute Dietary	*EPA Avian Subacute Dietary*
Organisms		
	Northern bobwhite (*Colinus virginianus*)	Northern bobwhite (*Colinus virginianus*)
	Japanese quail (*Coturnix japonica*)	Mallard (*Anas platyrhynchos*)
	Mallard *(Anas platyrhynchos*)	
	Ring-necked pheasant (*Phasianus colchicus*)	
Age of organism	14 days, 14 days, 5 days, and 10 days, respectively	10–14 days and 5–10 days, respectively
Experimental Design		
Test chamber	Construction materials in contact with birds should not be toxic, or capable of adsorbing or absorbing test substances; materials that can be dissolved by water or loosened by pecking should not be used; stainless or galvanized steel, or materials coated with plastics are acceptable; any material or pen shape is acceptable provided the birds are able to move about freely and that pens can be kept clean	Bobwhite: 35 × 100 × 24

Table 4.10 (*Continued*) Comparison of ASTM and U.S. EPA Standards for Conducting Subacute Dietary Toxicity Tests with Avian Species

	Test Type	
	ASTM Avian Subacute Dietary	*EPA Avian Subacute Dietary*
	Floors and external walls of wire mesh; ceilings and walls of galvanized sheeting	
Test substance	One concentration should kill more than 0% but less than 50%, and one concentration should kill more than 50% but less than 100%; these results can be obtained with four to six treatment levels	Dose levels should attempt to produce mortality ranging from 10 to 90%
Number of concentrations	4 concentrations minimum	5 or 6 strongly recommended plus additional groups for control
Number of organisms per group	Minimum of 10 birds for each test concentration	10 per level
Number of organisms per replicate	Minimum of 5	About 10
Feeding	Test substance is mixed with feed; birds shall be fed *ad libitum*	Standard commercial game bird or water fowl diet (mash); test substance should be added directly to the diet without a vehicle, if possible
Test duration	Treated diets are available for 5 days then replaced with untreated feed; birds are held for a minimum of 3 days following treatment	8 days, two phases: Phase 1: 5 days treated diet for experimental "clean" diet for control Phase 2: 3-day observation, clean diet for both groups
Clinical examinations	Body weight (record at beginning and end) and feed consumption	Body weight and feed consumption
Physical and Chemical Parameters		
Temperature	A temperature gradient from approximately 38°C to approximately 22°C should be established in brooders	22–27°C outside, about 35°C inside brooder

(Continued)

Table 4.10 (*Continued*) Comparison of ASTM and U.S. EPA Standards for Conducting Subacute Dietary Toxicity Tests with Avian Species

	Test Type	
	ASTM Avian Subacute Dietary	*EPA Avian Subacute Dietary*
Photoperiod	Minimum of 14 hours of light	Diurnal recommended, 24-hour lighting acceptable
Humidity	45–70% (higher relative humidities may be appropriate for waterfowl)	30–80%
Ventilation	Sufficient to supply 10–15 air changes per hour	Adequate supply should be maintained
Endpoint		
	Mortality	Mortality

it is a typical amphibian and its comparability in teratogenic response to mammalian species has already been documented. *Xenopus* are also widely available, and the basic methodology can also be transferable to other frogs and toads.

In addition to the American Society for Testing and Materials method, several useful documents are produced by Oklahoma State University in support of the test method. Particularly useful is an atlas of malformations, making it easier to score the results of the toxicity test. Given the relative ease of performing the toxicity test and the supporting documentation, FETAX has found rapid acceptance as a teratogenicity screen in environmental toxicology.

Although useful in forming the backbone of most toxicological research, the single-species toxicity test is not without shortcomings. In the role of providing toxicity data for environmental scenarios, these relatively simple toxicity tests have provided a great deal of information and controversy. The ability to examine the relationships between chemical structure and function is based on a large database produced by comparable toxicity determinations. In addition, the large number of chemicals tested with these methods and organisms provide a relative ranking as to acute toxicity. As will be discussed in detail in following chapters, the usefulness of these tests in predicting environmental effects is questionable. The situations the organisms are in are decidedly not natural, and typically are chosen for the cost-effective production of reliable and repeatable toxicity data. Effects at low doses over long periods of time are not generally considered, as well as the species-to-species interactions.

4.4 Multispecies Toxicity Tests

Toxicity tests using artificially contained communities have long been a resource in environmental toxicology. The nature and design criteria for these types of tests are discussed in Chapter 3. Many different methodologies have been developed (Table 4.12). Each has particular advantages and disadvantages, and none have been demonstrated to faithfully reproduce an entire ecosystem. However, as a research tool to look at secondary effects, bioaccumulation, and fate, the various multispecies toxicity tests have been demonstrated to be useful.

Table 4.11 The Frog Embryo Teratogenesis Assay: Xenopus (FETAX)

Test Type	*96-Hour Static Renewal*
Organism	
Xenopus laevis	
Age of parent organism	Adult male: At least 2 years of age
	Adult female: At least 3 years of age
Size of parent organism	Adult male: 7.5–10 cm in crown-rump length
	Adult female: 10–12.5 cm in length
Feeding	Adult: 3 feedings per week of ground beef liver; liquid multiple vitamins should be added to the liver in concentrations from 0.05 to 0.075 cc/5 g liver
Experimental Design	
Test vessel type and size	Adults: Large aquarium or fiber glass or stainless steel raceways; side of tank should be opaque and at least 30 cm high
	Breeding adults: 5- or 10-gallon aquarium fitted with a 1 cm mesh suspended approximately 3 cm from the bottom of the tank; nylon or plastic mesh is recommended; aquarium should be fitted with a bubbler to oxygenate the water; the top of aquarium should be covered with an opaque porous material such as a fiber glass furnace filter
	Embryos: 60 mm glass or 55 mm disposable polystyrene Petri dishes
Test solution volume	Adults: Water depth should be 7–14 cm
	Embryos: 10 ml per dish
Exposure to test substance	Continuous throughout test
Replacement of test material	Every 24 hours
Number of concentrations	5
Number of replicates per sample	2
Number of organisms per chamber	Adults: 4–6 per 1,800 cm^2 of water surface area
	Breeding adults: 2
	Embryos: 25

(*Continued*)

Table 4.11 (*Continued*) The Frog Embryo Teratogenesis Assay: Xenopus (FETAX)

Test duration	96 hours
Physical and Chemical Parameters	
Temperature	Adult: 23 ± 3°C
	Embryos: 24 ± 2°C
Photoperiod	12 hours light/12 hours dark
pH range	6.5–9
TOC	10 mg/L
Alkalinity and hardness	Between 16 and 400 mg/L as $CaCO^3$
Endpoint	
Acute (mortality) and subacute (teratogenesis)	

Table 4.12 Listing of Current Multispecies Toxicity Tests

Aquatic Microcosms
Benthic-pelagic microcosm
Compartmentalized lake
Mixed-flask culture microcosm
Pond microcosm
Sediment core microcosm
Ecocore microcosm
Ecocore II microcosm
Standard aquatic microcosm
Stream microcosm
Waste treatment microcosm
Terrestrial Microcosms
Root microcosm system
Soil core microcosm
Soil in a jar
Terrestrial microcosm chamber
Terrestrial microcosm system
Versacore

The overriding characteristic of multispecies toxicity tests is that they consist of at least two or more interacting species. Which two or more species and their derivation, along with the volume and complexity of substrate and heterogeneity of the environment, are matters of debate. Much current theory on the coexistence of species and their interactions emphasizes the role of environmental heterogeneity upon the formation and continuance of a community. Yet in the conduct of a multispecies toxicity test the goal is often to minimize the heterogeneity to allow the performance of traditional hypothesis testing statistics. On the other hand, including the heterogeneity of nature would require a system so large and complex that it would in essence be a field study with all of the problems assigning cause and effect inherent to those types of studies. It is perhaps more important to use good scientific methodology and emphasize the question being asked, as opposed to which multispecies toxicity test is the best mimic for the natural ecosystem. An emphasis upon the specific question will likely select for itself one of the current methods, with slight modification as best for that particular situation.

Multispecies toxicity tests range widely in size and complexity. This is the case for both aquatic and terrestrial systems.

In the aquatic arena some of the biodegradation tests are done with volumes of less than a liter. Tests to evaluate community interaction conducted in a laboratory have test vessels ranging in size from 1 L to 55 gallons. Larger test systems can also be used outside the laboratory. A proposed outdoor aquatic microcosm uses large tanks of approximately 800 L capacities. Larger still are the pond mesocosms used for pesticide evaluations. These systems are designed to mimic farm ponds in size and morphology.

Terrestrial microcosms also see a comparable range in size and complexity. A microbial community living within the soil in a test tube can be used to examine biodegradation. A soil core is comparable in size and utility to the laboratory microcosms described above. In some cases, terrestrial microcosms can be established with a variety of plant cover and include small mammals and insects. Field plots are the terrestrial equivalent of the larger outdoor aquatic microcosms. These field plots can vary in size but usually contain a cover crop or simulated ecosystem, and are fenced to prevent escape of the test vertebrates or the migration of other organisms into the test plot. Ecosystems ranging from agroecosystems to wetlands have been examined in this manner. Compared to the aquatic multispecies toxicity tests, the terrestrial systems have not undergone the same level of standardization. This is due to the length of time most of these tests require and the specialized nature of most of the test systems, rather than any lack of completeness of the method. The development of outdoor multispecies tests for the evaluation of terrestrial effects of pesticides and hazardous waste is a current topic of intense research.

One of the ongoing debates in environmental toxicology has been the suitability of the extrapolation and realism of the various multispecies toxicity tests that have been developed over the last 15 years. One of the major criticisms of small-scale systems is that the low diversity of the system is not representative of natural systems in dynamic complexity (Sugiura 1992). Given the above discussion and the conclusions derived from it, much of this debate may have been misdirected. The small-scale systems used in our study have been demonstrated to express complex dynamics. Kersting and van Wijngaarden (1992) found that even the three-compartment microecosystem, as developed by Kersting (1984, 1985, 1988), expresses indirect effects as measured by pH changes after dosing with chloropyrifos. Since even full-scale systems cannot serve as reliable predictors of the dynamics of other full-scale systems, it is impossible to suggest that any artificially created system can provide a generic representation of any full-scale system. Debate should probably revert to more productive areas, such as improvements in culture, sampling, and measurement techniques, or other characteristics of these systems. A more worthwhile goal is

probably the understanding of the scaling factors, in a full *n*-dimensional representation, which should enable the accurate representation of specific ecosystem characteristics. Certain aspects of a community may be included in one system to answer specific questions that in another system would be entirely inappropriate. If questions as to detritus quality are important, then the system should include that particular component. In other words, the system should attempt to answer the particular scientific question.

4.4.1 Standardized Aquatic Microcosm

The standardized aquatic microcosm (SAM) was developed by Frieda Taub and colleagues to examine the effects of toxicants on multispecies systems in the laboratory. Figure 4.1 illustrates the course of events over the 64 days of the experiment, and Table 4.13 provides a tabular overview. The microcosms are prepared by the introduction of 10 algal, 4 invertebrate, and 1 bacterial species into 3 L of sterile defined medium. Test containers are 4 L glass jars. A sediment consisting of 200 g silica sand and 0.5 g of ground chitin is autoclaved separately and then added to the already autoclaved jar and media. A photograph of a typical setup for the SAM system can be found in Figure 4.2a.

Numbers of organisms, dissolved oxygen (DO), and pH are determined twice weekly. Nutrients (nitrate, nitrite, ammonia, and phosphate) are sampled and measured twice weekly for the first four weeks, then only once weekly thereafter. Room temperature is set at 20 ± 2°C. Illumination is set at 79.2 $\mu Em^{-2}\ s^{-1}$ PhAR with a range of 78.6 to 80.4 and a 16/8 day/night cycle.

The test is conducted in a temperature-controlled facility on a worktable of approximately 0.85 × 2.6 m, with a light hung 0.56 m from the top of the table. Originally 30 jars are placed under the lights, but at day 4 the microcosms are culled to the 24 test systems. Three treatment groups and a control are used.

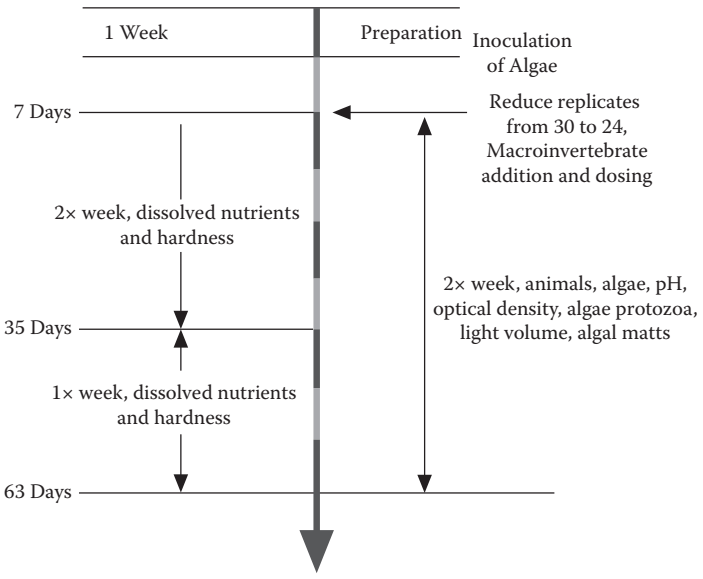


Figure 4.1 Timeline for the standardized aquatic microcosm. The 63-day toxicity test is specific in its sampling requirements, acclimation times, and dosing.

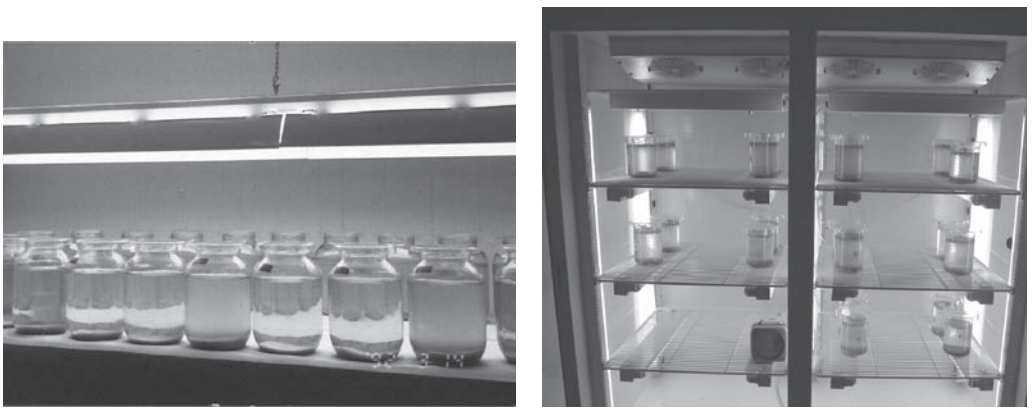
Table 4.13 Summary of Test Conditions for Standardized Aquatic Microcosms: Freshwater

Test Type	*Multispecies*
Organisms	
Type and number of test organisms per chamber	Algae (added on day 0 at initial concentration of 10^3 cells for each algae species): *Anabaena cylindrica*, *Ankistrodesmus* sp., *Chlamydomonas reinhardi* 90, *Chlorella vulgaris*, *Lyngbya* sp., *Nitzschia kutzigiana* (diatom 216), *Scenedesmus obliquus*, *Pseudokirchneriella subcapitata*, *Stigeoclonium* sp., and *Ulothrix* sp.
	Animals (added on day 4 at the initial numbers indicated in parentheses): *Daphnia magna* (16/microcosm), *Hyalella azteca* (12/microcosm), *Cypridopsis* sp. or *Cyprinotus* sp. (ostracod) (6/microcosm), hypotrichs (protozoa) (0.1/ml) (optional), and *Philodina* sp. (rotifer) (0.03/ml)
Experimental Design	
Test vessel type and size	1-gallon (3.8 L) glass jars are recommended; soft glass is satisfactory if new containers are used; measurements should be 16.0 cm wide at the shoulder, 25 cm tall, with 10.6 cm openings
Medium volume	500 ml added to each container
Number of replicates	6
Number of concentrations	4
Reinoculation	Once per week add one drop (circa 0.05 ml) to each microcosm from a mix of the 10 species = 5×10^2 cells of each alga added per microcosm
Addition of test materials	Add material on day 7; test material may be added biweekly or weekly after sampling
Sampling frequency	2 times each week until end of test
Test duration	63 days
Physical and Chemical Parameters	
Temperature	Incubator or temperature-controlled room is required providing an environment at 20–25°C with minimal dimensions of 2.6 × 0.85 × 0.8 m high
Work surface	Table at least 2.6 × 0.85 m and having a white or light-colored top or covering
Light quality	Warm white light

(Continued)

Table 4.13 (*Continued*) Summary of Test Conditions for Standardized Aquatic Microcosms: Freshwater

Light intensity	80 μE m^{-2} photosynthetically active radiation s^{-1} (850–1,000 fc)
Photoperiod	12 hours light/12 hours dark
Microcosm medium	Medium T82MV
Sediment	Composed of silica sand (200 g), ground, crude chitin (0.5 g), and cellulose powder (0.5 g) added to each container
pH level	Adjust to pH 7
Endpoint	
Cell counts for algae, population estimates for invertebrates, pH, DO, nutrient levels	



(a) (b)

Figure 4.2 Experimental setup for laboratory microcosm systems. (a) The larger standard aquatic microcosm is shown in the dedicated experimental room. Note the differences in the color of the SAM replicated due to differences in algal growth in each treatment. (b) The smaller 1 L mixed-flask culture microcosm system is shown in the incubator. The MFC takes much less laboratory space than the larger SAM. (See color insert following page 268.)

All data are recorded onto standard computer entry forms, checked for accuracy, and input to the Macintosh-compatible data analysis system (SAMS) developed by the University of Washington under contract with the Chemical Research, Development and Engineering Center. Parameters calculated included the DO, DO gain and loss, nutrient concentrations, net photosynthesis/respiration ratio (P/R), pH, algal species diversity, daphnid fecundity, algal biovolume, and biovolume of available algae. The statistical significance of each of these parameters compared to the controls is also computed for each sampling day.

4.4.2 Mixed-Flask Culture Microcosms

The mixed-flask culture (MFC) microcosms are smaller systems of approximately 1 L and are inoculated with 50 ml of a stock culture originally derived from a natural system (Figure 4.2b). Over a 6-month period repeated inocula are made into a stock tank so that a number of interactions can be established. At the end of the 6-month period the material from this stock tank is ready for inoculation into the test vessels. Six weeks is allowed for the establishment of the freshwater community followed by an experimental duration of 12 to 14 weeks. In contrast to the SAM, the MFC method relies upon the initial inoculum to provide the prerequisite components of the microcosm community. The protocol requires two species of single-celled green algae or diatoms, one species of filamentous green alga, one species of nitrogen-fixing blue-green alga, one grazing macroinvertebrate, one benthic, detrital feeding macroinvertebrate, and bacteria and protozoan species. Four treatment groups are recommended with five replicates for each group. The MFC has been used for the evaluation of procaryotic organisms introduced into the environment. A summary of this method is found in Table 4.14.

An implicit assumption of the MFC is that the acclimation time is sufficient for coevolution to occur and that coevolution is important to assess the impacts of xenobiotics upon communities. The use of a "natural" inoculum should increase species diversity and complexity over a protocol such as the SAM, but the smaller size of the test vessel would tend to decrease species number. Debate also exists as to the applicability of coevolution in the evaluation of test chemicals. If algal populations and others are primarily regulated by density-independent factors, then population-specific interspecific interactions may not be particularly important. If ecosystems are loosely connected in an ecological sense, then coevolved assemblages may be rare. On the other hand, in enclosed systems that are islands, these relationships may have had an opportunity to occur and coevolved interactions may be important in the assessment of toxicological impacts.

4.4.3 FIFRA Microcosm

Aquatic microcosms too large to be contained in the average laboratory have been routinely manufactured and used to attempt to obtain enough volume to contain fish as grazers or invertebrate predators. Proposed in late 1991 was a microcosm-mesocosm blend that is substantially larger than the MFC or the SAM experimental units. The experimental protocol is termed the Outdoor Aquatic Microcosm Tests to Support Pesticide Registrations (Table 4.15), but it is also called the FIFRA microcosm to reflect its origin as a pesticide testing methodology. The FIFRA microcosm is a system of approximately 6 m^3 in volume for each experimental unit with an inherent flexibility in design. Macrophytes can be included or not, along with a variety of fish species, invertebrates, and emergent invertebrates. A diagrammatic representation of one system for the examination of the effects of a model herbicide is presented in Figure 4.3.

Table 4.14 Summary of Test Conditions for Adaptation of Mixed-Flask Culture Microcosms for Testing the Survival and Effects of Introduced Microorganisms

Test Type	*Multispecies*
Organisms	
Number and type of organism	1. Two species of single-celled green algae or diatoms
	2. One species of filamentous green alga
	3. One species of nitrogen-fixing blue-green alga (bacteria)
	4. One grazing macroinvertebrate
	5. One benthic, detrital feeding macroinvertebrate
	6. Bacteria and protozoa species
Experimental Design	
Test vessel type and size	1 L beakers covered with a large petri dish
Volume/mass	50 ml of acid-washed sand sediment and 900 ml of Taub no. 82 medium, into which 50 ml of inoculum was introduced
Number of groups	4
Number of replicate chambers per group	5
Reinoculation	10 ml of stock community each week
Test duration	12–18 weeks
	Allow to mature 6 weeks prior to treatment; follow 6–12 weeks after exposure
Physical and Chemical Parameters	
Temperature	20°C
Photoperiod	12 hours light/12 hours dark
Endpoint	
Oxygen content, algal densities, microbial activity, respiratory activity, biomass, protozoan population	

Table 4.15 Summary of Test Conditions for Conducting Outdoor Aquatic Microcosm Tests to Support Pesticide Registrations

Test Type	*Multispecies Toxicity Test*
Organisms	
Add: Bluegill sunfish (*Lepomis macrochirus*), fathead minnow (*Promephales promelas*), channel catfish (*Ictalurus punctatus*), or others may be present: phytoplankton, periphyton, zooplankton, emergent insects, and benthic macroinvertebrates	
Size of organism	Biomass of fish added to the microcosms should not exceed 2 g/m^3 of water
Experimental Design	
Test vessel size and type	Tanks with a surface area of at least 5 m^2, a depth of at least 1.25 m, and a volume of at least 6 m^3 made of fiberglass or some other inert material; smaller tanks could be used for special purposes in studies without fish
Addition of test material	Allow microcosms to age for approximately 6–8 weeks before adding test material; apply by spraying across water surface, apply the test material in a soil-water slurry, or apply test material in a water-based stock solution
Sampling	Begins approximately 2 weeks after the microcosms are constructed and continues for 2 or 3 months after the last treatment with test material; frequency depends upon the characteristics of test substance and on treatment regime
Dosage levels, frequency of test material addition, and number of replicates per dosage level are determined based on the objectives of the study	
Physical and Chemical Parameters	
Temperature	Maintained by partially burying tanks in the ground or immersing in a flat-bottomed pond
Sediment	Obtained from existing pond, containing a natural benthic community, added to each microcosm directly on the bottom, in trays or other containers; sediment should be 5 cm thick
Water	Obtained from healthy, ecologically active pond; water level should be set in the beginning and not allowed to vary more than ±10% throughout study; if water level falls more than 10%, add pond water, fresh well water, or rainwater; if water level rises more than 10%, surplus should be released and retained
Weather	Should be recorded at the study site or records obtained from a nearby weather station; data should include air temperature, solar radiation, precipitation, wind speed and direction, and relative humidity or evaporation

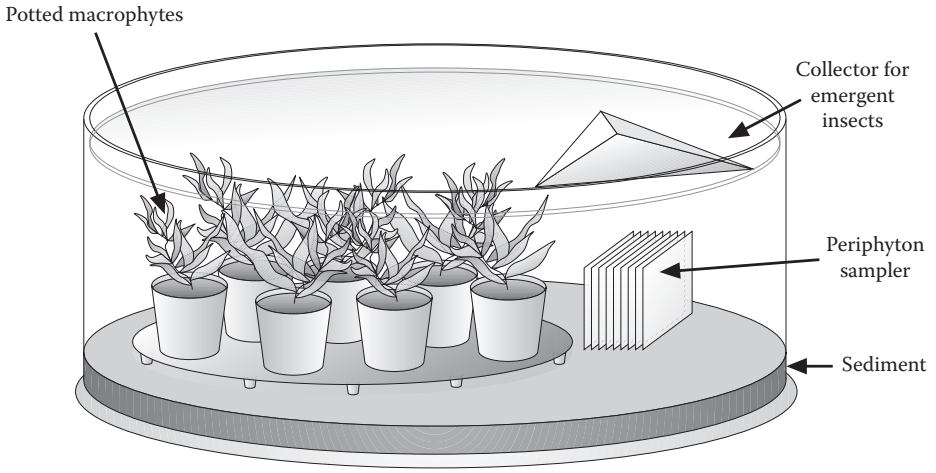


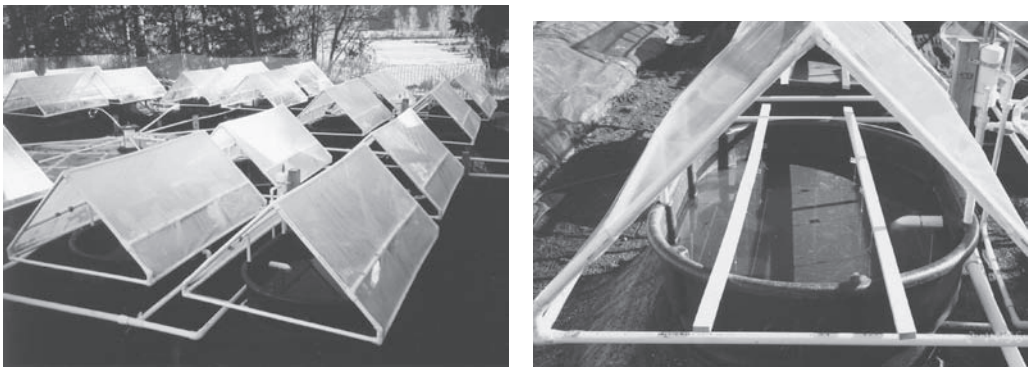
Figure 4.3 FIFRA microcosm experimental unit. An example of a microcosm experimental unit designed to test the effects of an herbicide on an aquatic environment. This particular setup does not include fish since the predatory effects would tend to hide lower trophic level effects upon the invertebrate populations. Typically, a FIFRA microcosm experiment includes fish species, especially when acetylcholinesterase inhibitors or other toxicants particularly effective against animal species are tested.

The flexibility in design is a recognition that this protocol originated to replace larger pond mesocosms mandated by the Office of Pesticide Programs to examine the potential impacts of pesticides to nontarget aquatic organisms. The larger systems were designed to simulate farm ponds and tended to be unwieldy and difficult to sample with a concurrent problem with the data analysis. The FIFRA microcosm was an attempt to design a flexible system able to answer specific questions concerning the fate and effects of a material in a more tightly controlled outdoor system.

One of the interesting aspects of the FIFRA microcosm system is the variety of methods used to ensure a uniform temperature among the experimental replicates during the course of the experiment. Basically, two methods have been used. The first method is to bury the test system in the ground and use the ground as an insulator and temperature regulator. This has been used extensively. In certain instances water can be used as the insulator. The experimental units are placed in the pond when the water is removed and then replaced as the plumbing and experimental setups are established. In some locations it may also be important to provide shade and to prevent a deluge from adding sufficient volume to cause an overflow of the test vessels.

Although the FIFRA microcosm has a number of advantages, there are also compromises. The few experiments that have been conducted and the variance in methodologies have not given an accurate representation of the repeatability or replicability of the experiments. In addition, the method is somewhat local specific, since the temperature, diurnal cycle, and to some extent, the experimental organisms are controlled by the local environmental conditions. On the other hand, the sensitivity to local conditions can also act as a more accurate model of local fate and effects of the test material.

As of this writing, no ASTM or comparable consensus method exists for this larger microcosm system; this is due to the relative newness of the methodology. The publication "Aquatic Mesocosm Studies in Ecological Risk Assessment" (Graney et al. 1994) reviews and discusses the system typically used for the purposes of pesticide registration.



(a) (b)

Figure 4.4 Large microcosm/mesocosm experimental unit. The larger multispecies toxicity tests are larger in size and require considerable construction and logistical support. (a) An overall view of the 24 replicate systems. (b) A newly filled mesocosm unit. The tents over each unit are to prevent rainfall from entering each replicate system. (See color insert.)

Although not as large as many of the mesocosms used in pesticide testing, Figure 4.4 depicts two views of an outdoor mesocosm system. In this instance, 24 replicates were fed from a common system until the experimental units were selected at random for treatments. The vessels are drinking troughs for sheep. Note that each is covered with plastic sheeting to keep out the rain typical of the region. These types are systems are very labor intensive to sample and to count the numerous algal and invertebrate species in each of the systems.

4.4.4 Soil Core Microcosm

The soil core microcosm (SCM) is one of the first test vehicles developed for the evaluation of xenobiotics on an agroecosystem, with it accompanying plants, soil invertebrates, and microbial processes. Table 4.16 summarizes the basic protocol.

The SCM is a hybrid methodology with cores derived from an outdoor environment brought into a laboratory setting to more accurately control the environmental variables. In this manner, the intrinsic heterogeneity of the terrestrial ecosystem is preserved, although successional changes can occur due to the small size of the experimental unit. Because of the design of the experimental container, extensive nutrient and chemical fate analyses can be performed. A typical greenhouse area is required with proper ventilation for the reduction of occupational exposure.

Although a useful methodology and an ASTM standard, few examples of SCM experiments exist in the open literature. This may be due to the somewhat specialized facilities required or the performance of proprietary research that is often unreported.

4.5 Summary

This chapter reviewed a wide variety of toxicity tests, yet only a small fraction of the toxicity tests that are currently performed or exist. These tests cover the entire range of biological organization that can be expected to fit into a laboratory or outdoor contained setting. There are a few caveats that must be dealt with when exploring the topic of toxicity testing.

Table 4.16 Summary of Test Conditions for Conducting a Terrestrial Soil Core Microcosm Test

Test Type	*Multispecies Toxicity Test*
Organisms	
Varies; dependent on site being tested	
Experimental Design	
Microcosm size and type	60 cm deep by 17 cm diameter plastic pipe made of ultra-high molecular weight, high density, and nonplasticized polyethylene and contains an intact soil core covered by homogenized topsoil; tube sits on a Buchner funnel covered by a thin layer of glass wool
Soil volume	40 cm intact soil core
	20 cm homogenized topsoil
Number of replicates	Each cart holds 6–8 microcosms; place microcosms paired for analyses in different carts to ensure that all microcosms are housed under similar conditions
Number of concentrations	3
Leaching	At least once before dosing and once every 2 or 3 weeks after dosing
Test duration	12 or more weeks
Physical and Chemical Parameters	
Temperature	Based on season of region being tested; insulated cart is used to prevent drastic temperature changes
Lighting	Based on season of region being tested
Watering	Determined on the basis of site history; use either purified laboratory water or rainwater that has been collected, filtered, and stored in a cooler at 4°C
Endpoint	
Many	

First, there is a tendency to overextrapolate from the results of a few tests that were convenient to perform or mandated by regulation or convention. The danger is extrapolating to situations or to ask questions that the toxicity test was not designed to answer. Examples are numerous. Many single-species tests are extrapolated to establish a safety level to protect a particular habitat or indigenous population. If direct, relatively short-term effects are the points of concern, then these tests are probably sufficient; however, if long-term effects are also a concern, then other multispecies tests or field studies should be conducted.

Second, there is an element of fashion or style attributed to a method because of either overzealous salesmanship, undue conservatism, or lack of knowledge of alternatives that often comes to play in the selection and review of a test method. The test should be able to stand alone as a means of answering specific questions about the effect of a xenobiotic. Tests that lack an adequate statistical or theoretical foundation should be avoided. Acquisition of data should not be an end unto itself. A well-designed toxicity evaluation should be comprised of toxicity tests that address particular questions that are the basis of the environmental concerns.

Third, many times the toxicity tests are selected on the basis of cost, and this is a valid parameter. A FIFRA mesocosm may cost as much as $750,000, compared to as little as $500 for a *D. magna* acute toxicity test. The danger is from both ends of the spectrum. The more expensive multispecies test is not necessarily better unless it answers specific questions left unanswered by the simpler tests. In fact, the large multispecies tests are performed only after a thorough review and evaluation of simpler testing procedures. Likewise, the simpler and less costly toxicity tests may not adequately address the fate and effects of a xenobiotic, leaving a great deal of uncertainty in the prediction of environmental effects.

Study Questions

1. Discuss the major factor in the performance of a laboratory aquatic toxicity test.
2. Why is the use of a reference toxicant important in the *Daphnia* toxicity test?
3. What are the advantages of the daphnid toxicity test?
4. What is the chronic or partial life cycle toxicity test?
5. Why is the three-brood renewal toxicity test with *Ceriodaphnia dubia* used?
6. How could low concentrations of toxicant in an algal 96-hour growth toxicity test lead to a false analysis of toxicity if not properly data analyzed?
7. Discuss two major problems in conducting acute toxicity tests with aquatic vertebrates and macroinvertebrates.
8. How can terrestrial vertebrate toxicity tests be modified to better assess interspecific variability of a toxic response?
9. Discuss the replace, reduce, and refine considerations in a required research or test methodology.
10. What are the advantages of the FETAX test?
11. Why have terrestrial systems not undergone the same level of standardization as the aquatic multispecies systems?
12. Discuss coevolution as a component of the mixed-flask culture microcosm.
13. Discuss the two methods used to ensure a uniform temperature among experimental replicates during a FIFRA microcosm experiment.
14. Discuss the three caveats to be dealt with in the topic of toxicity testing.

Appendix 4.1: The Natural History and Utilization of Selected Test Species

Aquatic Vertebrates

Coho Salmon *(Oncorhynchus kisutch)*

Description: Body fusiform, streamlined, laterally compressed, usually 18 to 24 inches (457 to 610 mm) in length and 8 to 12 pounds in weight as marine adults and 10.8 to 25.8 inches (279 to 656 mm) fork length in Great Lakes freshwater populations; body depth moderate, greater in breeding males.

Color: Adults in ocean or Great Lakes are steel blue to slightly green on dorsal surface, sides brilliant silver, ventral surface white, small black spots on back, sides above lateral line, base of dorsal fin, and upper lobe of caudal fin.

Distribution: This species occurs naturally only in the Pacific Ocean and its tributary drainage. It is known in freshwater in North America from Monterey Bay, California (in the sea infrequently to Baja California), to Point Hope, Alaska. In Asia, it occurs from the Anadyr River, USSR, south to Hokkaido, Japan.

Biology: Adults migrate from the sea or lake late in the season and over a prolonged period. Spawning is from early September to early October. Spawning takes place in swifter water of shallow, gravel areas of river tributaries from October to March, but usually October to November, or November to January in North America.

Toxicity testing: Species can be used as a model salmonid.

Rainbow Trout *(Oncorhynchus mykiss)*

Description: Body trout-like, elongate, average length is 12 to 18 inches (305 to 457 mm); no nuptial tubercles but minor changes to head, mouth, and color, especially in spawning males.

Color: Variable with habitat, size, and sexual condition. Stream residents and spawners are darker, colors more intense, lake residents lighter, brighter, more silvery.

Systematic notes: Populations in different watersheds have long been called by different scientific names, and still by different regional common names in the south.

Distribution: Native range was eastern Pacific Ocean and the freshwater, mainly west of the Rocky Mountains, from northwest Mexico (including extreme northern Baja California) to the Kuskokwim River, Alaska; probably native in the drainages of the Peace and Athabasca rivers east of the Rocky Mountains. Has been widely introduced throughout North America in suitable localities. Also introduced into New Zealand, Australia and Tasmania, South America, Africa, Japan, Southern Asia, Europe, and Hawaii.

Biology: Spring spawners, temperature from 50 to 60°F (10.0 to 15.5°C) (FF of C, 184 to 191).

Brook Trout *(Salvelinus fontinalis)*

Description: Average length is 10 to 12 inches (254 to 305 mm); breeding males may develop a hook (or kype) at the front of the lower jaw.

Color: Back is olive green to dark brown, at times almost black, sides lighter, becoming silvery white below; light-green or cream-colored wavy lines or vermiculations on top of head and on back, broken up into spots on sides.

Distribution: North American endemic species and under natural conditions occurs only in northeastern North America.

- Brook trout spawn in late summer or autumn, varying with latitude and temperature.
- A stable and well-defined species (FF of C, 208+).

Goldfish *(Carassius auratus)*

Description: Body stout, thick set, average total length about 5 to 10 inches (127 to 254 mm).

Color: Overall coloration variable, from olive green through gold (often with black blotches) to creamy white.

Systematic notes: Goldfish hybridize readily with carp.

Distribution: Native to eastern Asia; goldfish originated in China, spread to Japan, parts of Europe, and throughout parts of North America.

Biology: A spring-spawning species and seeks warm, weedy shallows in May or June to deposit its eggs (FF of C, 389 to 390).

Fathead Minnow *(Pimephales promelas)*

Description: Body short, average length about 2 inches (51 mm), thick set, compressed laterally and deep bodied, often with a pronounced belly.

Color: Overall coloration usually dark.

Systematic notes: The fathead minnow varies greatly in many characters throughout its wide geographic range, and some populations have been designated as subspecifically distinct.

Distribution: The fathead minnow ranges through most of central North America, from Louisiana and Chihuahua, Mexico, north to the Great Slave Lake drainage, and from New Brunswick on the east to Alberta on the west (FF of C, 480 to 482).

Channel Catfish *(Ictalurus punctatus)*

Description: Average length is 14 to 21 inches (356 to 533 mm), weight is 2 to 4 pounds.

Color: Individuals less than 12 to 14 inches (305 to 356 mm) are pale blue to pale olive with silvery overcast. Adults with dorsal surface of head and back, and upper side steel blue to gray; lower sides lighter; ventral surface of head, and body to pelvic fins, dirty white to silver white. Barbels are darkly colored.

Systematic notes: There was, for many years, considerable taxonomic and nomenclatural confusion associated with what we now recognize as this species. Differences in shape and color, now known to be associated with sex, size, season, and locality, were once construed to be indicative of several different species or subspecies.

Distribution: Restricted to the freshwaters, and to a limited extent brackish waters, of east and central North America.

Biology: Locally abundant in certain parts of Canada but poorly known; very little published information.

Bluegill *(Lepomis macrochirus)*

Description: Has a very deep, compressed body and individuals are usually 7 to 8 inches (178 to 203 mm) in length.

Color: Dorsal surface green, olive to almost brown, with several vague vertical bands extending down sides; upper sides brown to green, shading into brown, orange, or pink; lower sides and abdomen silver to white.

Distribution: Native range of bluegill is restricted to the freshwaters of eastern and central North America; has been introduced throughout the United States, into Africa and possibly other areas off the North American continent.

Biology: No detailed account of the life history of a Canadian population; spawning takes place in late spring to early and mid-summer (in Canada) with peak activity in early July (FF of C, 719 to 723).

Green Sunfish *(Lepomis cyanellus)*

Distribution: A deep-bodied, laterally compressed fish, usually not over 5 inches (127 mm) in length in Canada.

Color: Body generally brown to olive with an emerald sheen, darker on dorsal surfaces and upper sides, sides light yellow-green, upper sides with 7 to 12 dark but vague vertical bars; ventral surface yellow to white.

Distribution: Restricted to the freshwaters of east-central North America.

Biology: Spawning occurs in late spring and summer; multiple spawnings occur.

Invertebrates: Freshwater

Daphnids *(Daphnia magna, D. pulex, D. pulicaria, Ceriodaphnia dubia)*

Description: Water flea (Cladocera). These are small, laterally flattened forms that usually measure 0.2 to 3 mm. Body is covered by a carapace, but head and antennae are usually apparent. Body does not appear segmented and possesses five or six pairs of legs. Carapace often ends in a spine.

Distribution: Some 135 species of freshwater water fleas are known from North America, where the group is widespread and can be found in most freshwater environments. Most species occur in open waters, where they swim intermittently. The second pair of antennae is used primarily to propel them. Movement is generally vertical, with the head directed upwards. Many of these open-water forms are also known for their vertical migration, which generally consists of upward movement in the dark and downward migration during daylight hours. Some water fleas are primarily benthic. *Daphnia* is commonly maintained in laboratories for assaying toxic substances in water. Water fleas are often of great importance in the diets of fishes, especially young fishes, and predaceous insects, such as many of the Diptera larvae.

Amphipods *(Gammarus lacustris, G. fasciatus, G. pseudolimnaeus, Hyalella azteca)*

Description: Scuds (amphipoda) are laterally flattened, often colorful forms that usually measure 5 to 20 mm when mature. Head and first thoracic segment form a cephalothorax. The

remainder of the thorax possesses seven pairs of legs, the first two pairs being modified for grasping.

Distribution: Three families and approximately 90 species of scuds occur in North America. The family Talitridae contains one widely distributed North American species, *Hyalella azteca*, which is common in springs, streams, lakes, and ponds. The family Haustoriidae also contains only one species in North America, *Pontooporeeia hoyi*. Somewhat atypical of scuds, this species is confined to the bottom and open waters of deep, cold lakes. The family Gammaridae is the most important group and is divided into about eight genera.

Scuds occur primarily in shallow waters of all kinds. They are benthic and often rest among vegetation and debris or occasionally slightly within soft substrate. They also swim, however, and are sometimes known as side swimmers. They are generally omnivore-detritivores but rarely predaceous. Several species are restricted to particular spring or cave habitats, whereas others are more widespread in larger surface water habitats and sometimes occur in very large numbers (McCafferty 1981, p. 389).

- *Gammarus*: Reach densities of thousands of individuals per square meter where detrital food and cover are abundant.
- *Hyalella azteca*: Produce multiple broods during an extended breeding season; warm water species.
- *G. lacustris*: Cold water species; a period of short days and long nights (typical of winter) is needed to induce reproduction.

Crayfish *(Orconectes* sp.*, Combarus* sp.*, Procambarus* sp.*, Pacifastacus leniusculus)*

Description: Decapoda; these are somewhat flattened either dorsoventrally or laterally and range in size from 10 to 150 mm. Head and entire thorax form a large cephalothorax covered by a carapace. Cephalothorax possesses five pairs of legs; first two or three pairs are pincer-like at their ends, and first pair is often very robust.

Distribution: The freshwater Decapoda in North America comprise four species of the family Atyidae, which are restricted to certain caves of the southeastern states and coastal streams of California. The family of Astacidae (crayfish) is widely distributed, except that they are not generally found in the Rocky Mountain region. They occur in a wide variety of shallow freshwater habitats, and some live in swamps and wetlands. They are benthic and, at least in daylight hours, usually remain hidden in burrows or under stones and debris. They retreat rapidly backwards when disturbed. Depending on the species, crayfishes may be herbivores, carnivores, detritivores, or omnivores; their very robust first pair of legs (chelae) is used to cut or crush food. These chelae are also used as defensive weapons. Prawns and river shrimps are generally swimmers (McCafferty 1981, pp. 390–391).

Stoneflies *(Pteronarcys* sp.*)*

Description: They are all freshwater inhabitants as larvae. As a group they are close relatives of the cockroaches and have retained the primitive condition of possessing tails but demonstrate the advanced ability to fold their wings over the back of the body. Their common name undoubtedly is derived from the fact that individuals of many common species are found crawling or hiding among stones in streams or along stream banks.

Distribution: Close to 500 species are represented in North America. Many stoneflies are known as clean-water insects, since they are often restricted to highly oxygenated water. As such, some are excellent biotic indicators of water quality. Adults of stoneflies can be found throughout the year, some being adapted for winter emergence (McCafferty 1981, p. 148).

Mayflies *(Baetis* sp.*, Ephemerella* sp.*, Hexagenia limbata, H. bilineata)*

Over 700 species occurring in North America is possible; as a group, mayflies are one of the most common and important members of the bottom-dwelling freshwater community. Because most species are detritivores or herbivores and are themselves a preferred food of many freshwater carnivores, including other insects and fishes, they form a fundamental link in the freshwater food chain. Many species are highly susceptible to water pollution or occur in very predictable kinds of environments. It is for this reason that mayflies have proven very useful in the analysis or biomonitoring of water quality. Several species emerge in mass numbers, and these mass emergences are among the most spectacular in the insect world. In North America, mayflies may also be known locally by such names as willowflies, shadflies, drakes, duns, spinners, fishflies, and Canadian soldiers.

Midges *(Chironomus* sp.*)*

Larvae are slender, commonly cylindrical, and slightly curved forms that usually measure 2 to 20 mm but are occasionally larger. Body has a pair of prothoracic prolegs and a pair of terminal prolegs. Terminal segment usually has a short dorsal pair of tubercles or projections, each with a variable tuft of hairs (dorsal pranal brushes).

Larvae of this very large, common, and geographically widespread family are distinctive.

Pupae of most species live with cylindrical or conical cocoons. Others are free swimming, and some resemble mosquito larvae. This group is probably the most adapted of all aquatic insects. The larvae of this group are often used as an indicator of environmental quality. Habitats of immatures range from littoral marine waters to mountain torrents, from mangrove swamps to Arctic bogs, and from clear deep lakes to heavily polluted waters. They can be expected in almost all inland waters. Most species are bottom dwelling, and many live within tubes or loosely constructed silk-lined cases in the substrate. A few build distinctive cases. These benthic forms can occur in extremely high densities; their tube cases sometimes cover large areas of the bottom, virtually becoming substrate themselves for other organisms, such as encrusting diatoms (McCafferty 1981, p. 310).

Snails *(Physa integra, P. heterostropha, Amnicola limosa) (Mollusca, Gastropoda)*

Description: These possess a single (univalve), usually drab-colored shell that is either spiraled or coiled or low and cone-like. They generally range in size from 2 to 70 mm. Part of the body protrudes from the aperture of the shell and bears a head with a pair of tentacles.

Distribution: The gastropods are well represented in marine, freshwater, and terrestrial environments. Several hundred species of freshwater snails occur in North America. They are benthic organisms that slowly move about on the substrate of almost all shallow freshwater

habitats. Some are known to burrow into soft substrates or detritus during periods of drying in vernal habitats or when shallow habitats become frozen solid.

Calcium carbonate is used in the production of the shell, and it is for this reason that many freshwater snails are more common in hard-water habitats, although some do well in soft water. Many feed on the encrusted growths of algae over which they creep. Others are detritivores or omnivores. Certain freshwater fishes feed extensively on snails, and most marsh fly larvae are predators and parasites of snails.

Planaria *(Dugesia tigrina, Platyhelminthes, Turbellaria)*

Description: These are soft-bodied, elongate, worm-like forms, usually dorsoventrally flattened or at least flattened ventrally. They are generally less than 1 mm in length, but some range to 30 mm. Most are dark colored, and many are mottled. Head area is commonly arrowhead shaped. A pair of dorsal eyespots is usually present. Mouth and anus are combined into a single ventral opening, usually at about midlength along the body.

The phylum Platyhelminthes includes the so-called flatworms, many of which are parasitic or marine. Most of the free-living, freshwater forms are planarians, and a few of these are large enough to be considered macroorganisms.

Planarians are usually associated with the substrate of shallow waters. They are often found on the underside of rocks and detritus. Most are carnivores and scavengers that feed on a variety of soft invertebrates.

Invertebrates: Saltwater

Copepods *(Acartia clausi, Acartia tonsa)*

Description: These are generally less than 3 mm in length. Body is divided into a cephalothorax, thorax, and abdomen. A carapace covers cephalothorax. Six pairs of legs are usually present, the first of which is modified for feeding and the remaining five pairs for swimming. Body lacks lateral abdominal appendages.

About 180 species of copepods occur in North America. Two groups of copepods (the Caligoida and Lernaeopodoida) are parasitic on fishes and are highly modified for this type of existence. The vast majority of copepods are free living. One genus (*Cyclopoida*) is parasitic on fishes.

Free-living copepods are planktonic or benthic in a wide variety of freshwater environments. Some species of cyclopoid and calanoid copepods occur in extremely high densities. Some of the planktonic copepods have a daily vertical migration in lakes, similar to that of some water fleas. Parasitic copepods can become a serious economic problem in fish hatcheries. Many free-living copepods are important in the food chain of many fishes.

Algae

Chlamydomonas reinhardi

These are unicellular, green alga that possesses one nucleus, one chloroplast, and several mitochondria. It is facultatively photosynthetic, and it can grow in the dark with acetate as carbon and energy source. It has a sexual life cycle controlled by two mating type alleles of a single gene, called *mt*; the mating types, and their allele determinants, are called *mt*+ and *mt*–, respectively.

Ulothrix sp.

These are filamentous members of the Chlorophyta, a multicellular alga that is immobile in the mature state. Reproduction frequently involves the formation and liberation of motile cells, asexual reproductive cells (zoospores), or gametes. The structure of the motile reproductive cells of multicellular algae thus often reveals their relatedness to a particular group of unicellular flagellates.

Microcystis aeruginosa

Phototroph; blue-green bacteria.

Anabaena flos-aquae

Blue-green bacterium that contains gas vacuoles, which account for the phase-bright appearance of the vegetative cells.

Avian Species

Mallard *(Anas platyrhynchos)*

Male: Grayish with green head, narrow white ring around neck, ruddy breast, and white tail. *Female*: A mottled brown duck with whitish tail and conspicuous white borders on each side of metallic violet-blue wing patch. Breeding occurs in western North America east to Great Lakes area; winters from Great Lakes and southern New England south to the Gulf of Mexico.

Species commonly used in acute and chronic toxicity testing as a representative waterfowl.

Northern Bobwhite *(Colinus virginianus)*

These animals are a small, ruddy, chicken-like bird, near the size of a meadowlark. The male shows a conspicuous white throat and stripe over the eye; the female is buffy. The common habitat is in farming country from the Gulf of Mexico north to South Dakota, south Minnesota, south Ontario, and southwest Maine.

This species is extensively used as a model galliform for a variety of acute, chronic, and even field studies. It may be regarded as the white rat of bird toxicity testing.

Ring-Necked Pheasant *(Phasianus colchicus)*

A large chicken-like or gamecock-like bird having a long, sweeping, pointed tail. The male is highly colored with a white neck ring; the female is mottled brown with a moderately long pointed tail. The species was introduced to the Americas and is currently established in farming country mainly in the northeastern quarter of the United States.

Larger than the bobwhite, this is another representative galliform not as commonly used as the northern bobwhite for toxicity testing.

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Chapter 5

The Fate and Transport of Contaminants

5.1 Introduction

The need to consider the fate and transport of contaminants in the environment is a characteristic that distinguishes environmental toxicology from human toxicology. In human toxicology, chemical structure and delivery form are typically controlled. In environmental toxicology, after a chemical has been released into the environment many processes can alter it before an exposure occurs, and therefore make exact knowledge of the exposure profile a challenge to understand. A chemical released purposefully or accidentally, for example, can be degraded into other chemicals, called degradates, through chemical, physical, and biological mechanisms. The parent chemical and its degradates can also be dissolved in solutions such as water, present as gases, sorbed to solid materials, or present as precipitates. A study of the fate and transport of a chemical will describe the ultimate disposition of the chemical (i.e., the fate) and how the chemical got there (i.e., the transport).

There are many questions related to environmental toxicology that knowledge of chemical fate and transport can help answer, for example:

- Which parent chemicals and degradates will be present in the environment?
- What is the concentration of each of those chemicals?
- Are the parent chemicals and degradates dissolved, sorbed, or gaseous?
- Are the parent chemicals and degradates likely to persist, and how long will they be present?
- Are the chemicals bioavailable to higher tropic level organisms?
- Are the parent chemicals and degradates likely to bioconcentrate, biomagnify, or bioaccumulate?

All of these questions are ultimately related to the degree of bioavailability of a chemical and its degradates. In assessing the fate and transport of a chemical, three broad areas should always be considered: (1) the properties of the chemical, (2) the properties of the environment, and when present, (3) the properties of the organism.

The properties of the chemical and the environment may be difficult to distinguish from each other. It is often the case, for example, that a change in an environmental characteristic such as pH or salinity changes the properties of some chemicals. It is important, nonetheless, to consider each of these general properties separately and as they relate to each other. Among the properties of a chemical when assessed separately are whether the chemical is ionized, what it is complexed to, and what the chemical structure is. The degree of chlorination in organochlorine chemicals, for example, dictates how volatile it is. Table 5.1 provides other examples of how chemical properties, such as structure, influence behavior in the environment. All of the chemicals in this table have been identified by different organizations as persistent, bioaccumulative, or toxic. Even the untrained eye will note that many of these chemicals are halogenated and organic; the structure is clearly an important chemical property.

The properties of the organism are not often specifically considered in fate and transport, but organisms can play important roles. For example, organisms can be a transport mechanism and can play a role in the fate of chemicals through storage. Additionally, organisms can metabolize a chemical, which reduces the mass of chemical in the environment. The scale at which the fate and transport are being assessed should also be established since local, regional, and global scales can all be important to consider.

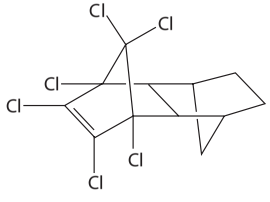
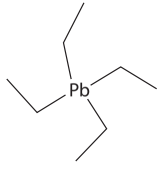
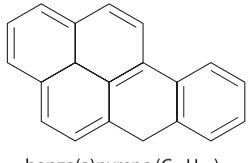
5.2 Transport Mechanisms

Many examples of chemical contaminants measured some distance from the original source exist. Perchlorate from a manufacturing facility in Nevada has been found in Arizona lettuce that had been irrigated with water from the Colorado River (Sanchez et al. 2005). Remediation efforts are under way at the U.S. Department of Energy Hanford site to stop migration of chromium-contaminated groundwater to the Columbia River, Washington (NRC 2001). Organochlorine pesticides have been reported in the glacial ice of the Canadian Rocky Mountains and the Italian Alps (Donald et al. 1999; Villa et al. 2003). Polar bears in the Svalbard archipelago in arctic Norway are contaminated with polybrominated diphenyl ethers (PBDEs) and perfluorinated compounds (PFCs) such as perfluorooctane sulfonate (PFOS) (Smithwick et al. 2005; Muir et al. 2006). In each case, the contaminants were transported from the source by surface water, groundwater, or air. This transport can be described as advection, diffusion, and dispersion. Although these processes occur together in the environment, they are considered individually here.

5.2.1 Advection, Diffusion, and Dispersion

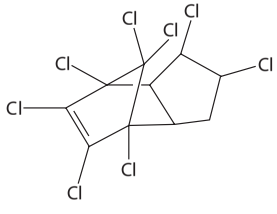
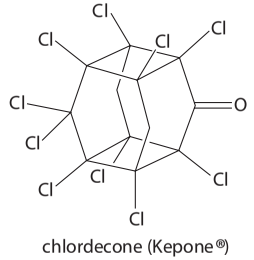
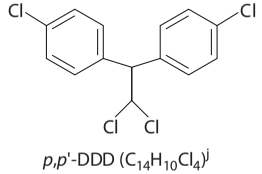
In the case of advection, a chemical moves at the same velocity as the bulk flow of the media. Examples of this are an orange flowing with the water in a stream or a feather carried by the wind. Molecular and turbulent or eddy diffusion are the two types of diffusion that can be described. In both cases, there is random movement. With molecular diffusion, the chemical molecules move randomly from a higher concentration to a lower concentration. If red dye is added to a glass of stationary water, there will initially be both clear and dark red water together. Molecular diffusion will cause the red color to spread until all of the water becomes the same color. With turbulent or

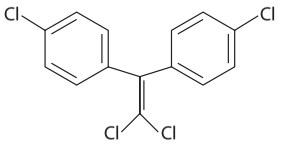
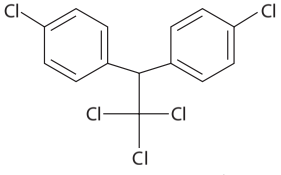
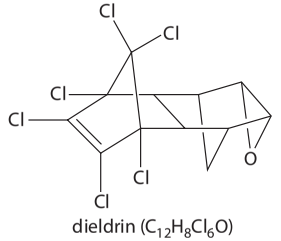
Table 5.1 Chemical Properties of POP and PBT Chemicals

Chemical	*Chemical Group and Use*	*Naturally Occurring*	*Listing*[a]	*Regulatory Status for Use in the United States*	$t_{1/2}$ *(years) (hydrolysis)*[b] $t_{1/2}$ *(days) (atmosphere)*[c]	H_c[d] *(atm-m³/mol)* *VP (mmHg)*[e]	*Log* K_{ow}[f] *Log BCF*[g]
 aldrin ($C_{12}H_8Cl_6$)	Organochlorine insecticide. Aldrin is metabolized to dieldrin *in vivo* and degraded by photolysis and microorganisms to dieldrin in environment.	No	SC, BNS (grouped with dieldrin in BNS)	Department of Agriculture banned in 1970, EPA allowed limited use starting in 1972, final registered use canceled in 1989.[h]	NA 0.166	3.87E-04 1.87E-06	6.75 3.65
Alkyl Lead							
 tetraethyl lead ($C_8H_{20}Pb$)	Organometals that include tetramethyl lead and tetraethyl lead. Used as a gasoline "antiknock" additive.	Yes, as a result of microbial alkylation of Pb or degradate of higher alkyl-Pb	BNS	Use in motor vehicle gasoline, banned in 1996.	NA 0.217	8.26E-01 0.47[i]	4.88 2.505
 benzo(a)pyrene ($C_{20}H_{12}$)	PAH occurs in mixture with other PAHs. Formed petrogenically and pyrogenically during incomplete combustion of organic materials.	Yes, volcanoes, forest fires, crude oil, and shale oil	BNS	Not produced commercially in the United States, no known use. Releases managed under specific regulations, e.g., CAA, CWA, RCRA.	NA 0.214	8.10E-07 9.82E-10	6.11 2.561

(Continued)

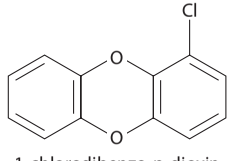
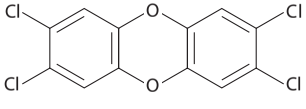
Table 5.1 (*Continued*) Chemical Properties of POP and PBT Chemicals

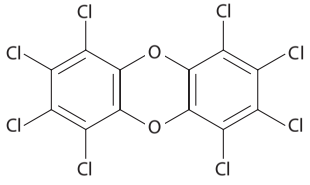
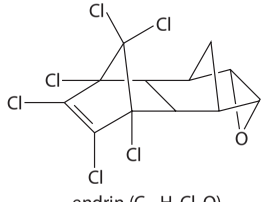
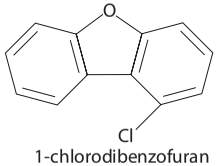
Chemical	*Chemical Group and Use*	*Naturally Occurring*	*Listing*[a]	*Regulatory Status for Use in the United States*	$t_{1/2}$ *(years) (hydrolysis)*[b] $t_{1/2}$ *(days) (atmosphere)*[c]	H_c[d] *(atm-m³/mol)* *VP (mmHg)*[e]	*Log* K_{ow}[f] *Log BCF*[g]
 chlordane ($C_{10}H_6Cl_8$)	Organochlorine insecticide. Technical chlordane contains > 140 related chemicals.	No	SC, BNS	All registered products canceled in1988.[h]	NA 2.123	7.03E-05 2.00E-05	6.26 4.269
 chlordecone (Kepone®) ($C_{10}Cl_{10}O$)	Organochlorine insecticide. Degrades very slowly in environment.	No	COP4	All registered products canceled 1978.[h]	NA NA	1.76E-10 7.91E-09	4.91 3.589
 *p,p'*-DDD ($C_{14}H_{10}Cl_4$)[j]	Organochlorine insecticide (*p,p'*-DDD) and treatment for cancer of adrenal grand (*p,p'*-DDD). Degradate from biodegradation and metabolite of DDT.	No	BNS	All registered pesticide products canceled in 1971.[h] Still used for cancer treatment.	NA 2.462	4.34E-05 1.95E-05	5.87 4.235

 *p,p′*-DDE ($C_{14}H_8Cl_4$)[j]	No commercial use. Degradate from biodegradation and metabolite of DDT.	No	BNS	NA	NA	3.52E-05	6
					1.44	6.85E-05	3.88
 *p,p′*-DDT ($C_{14}H_9Cl_5$)[j]	Organochlorine insecticide. Technical grade includes *p,p′*-DDT, *o,p′*-DDT, and *o,o′*-DDT.	No	SC, BNS	All registered products canceled in 1972 except in the case of public health emergency.[h]	NA	1.53E-05	6.79
					3.114	7.47E-06	3.818
 dieldrin ($C_{12}H_8Cl_6O$)	Organochlorine insecticide. Is a metabolite and degradate of aldrin.	No	SC, BNS (grouped with aldrin in BNS)	Department of Agriculture banned in 1970, EPA allowed limited use starting in 1972, final registered use canceled in 1989.[h]	12.823 (Ka at pH 7)	5.41E-07	5.45
					1.163	2.74E-06	3.802

(Continued)

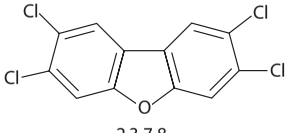
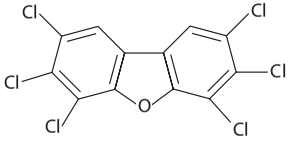
Table 5.1 (*Continued*) Chemical Properties of POP and PBT Chemicals

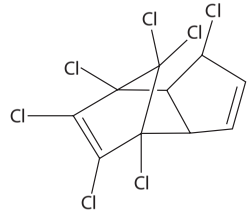
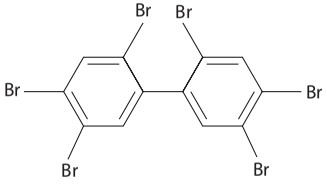
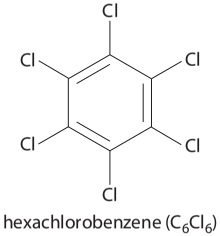
Chemical	*Chemical Group and Use*	*Naturally Occurring*	*Listing*[a]	*Regulatory Status for Use in the United States*	$t_{1/2}$ *(years) (hydrolysis)*[b] $t_{1/2}$ *(days) (atmosphere)*[c]	H_c[d] *(atm-m³/mol)* *VP (mmHg)*[e]	*Log* K_{ow}[f] *Log BCF*[g]
Dioxins (Polychlorinated Dibenzo-*p*-Dioxins (PCDDs)—Congeners with Range of Chlorination Included							
 1-chlorodibenzo-p-dioxin ($C_{12}H_7ClO_2$)	Includes 75 congeners and occurs with PCDFs. Not manufactured commercially, but unintentionally produced as by-product of incomplete combustion of fossil fuels, incineration of waste, and uncontrolled chemical reactions with chlorine.	Yes, volcanoes and forest fires	SC, BNS (grouped with PCDFs in BNS)	Releases managed under specific regulations, e.g., CAA, CWA, RCRA.	NA 1.678	8.67E-06 1.04E-04	4.99 2.867
 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) ($C_{12}H_4Cl_4O_2$)					NA 14.204	3.53E-06 1.95E-08	6.92 3.797

 1,2,3,4,6,7,8,9-octochlorodibenzo-p-dioxin ($C_{12}Cl_8O_2$)					NA	1.06E-06	9.5
					197.438	5.40E-12	2.222
 endrin ($C_{12}H_8Cl_6O$)	Organochlorine insecticide, rodenticide, and avicide.	No	SC, BNS (II)	All registered products canceled in 1991.[h]	12.823 (Ka at pH 7)	5.41E-07	5.45
					1.163	2.74E-06	3.802
Furans (PCDFs)—Congeners with Range of Chlorination Included							
 1-chlorodibenzofuran ($C_{12}H_7ClO$)	Includes 135 congeners and occurs with PCDDs. Not manufactured commercially, but unintentionally produced as by-product of incomplete combustion of fossil fuels, incineration of waste, and uncontrolled chemical reactions with chlorine.	Yes, trace amounts possibly from sources such as forest fires	SC, BNS (grouped with PCDDs)	Releases managed under specific regulations, e.g., CAA, CWA, RCRA.	NA	3.80E-05	4.36
					2.534	1.07E-04	2.565

(Continued)

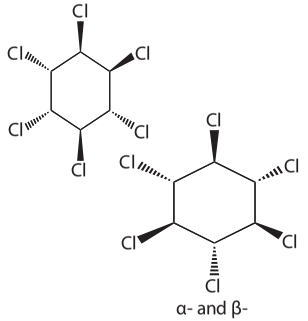
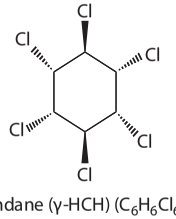
Table 5.1 (*Continued*) Chemical Properties of POP and PBT Chemicals

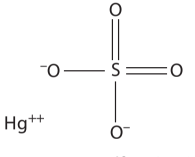
Chemical	*Chemical Group and Use*	*Naturally Occurring*	*Listing*[a]	*Regulatory Status for Use in the United States*	$t_{1/2}$ *(years) (hydrolysis)*[b] $t_{1/2}$ *(days) (atmosphere)*[c]	H_c[d] *(atm-m³/mol)* *VP (mmHg)*[e]	*Log* K_{ow}[f] *Log BCF*[g]
 2,3,7,8-tetrachlorodibenzofuran ($C_{12}H_4Cl_4O$)					NA 42.258	1.54E-05 1.47E-07	6.29 2.934
 2,3,4,6,7,8-hexachlorodibenzofuran ($C_{12}H_2Cl_6O$)					NA 205.745	8.48E-06 7.7E-08	7.58 2.744

 heptachlor ($C_{10}H_5Cl_7$)	Organochlorine insecticide. Degradate and a constituent of technical grade chlordane.	No	SC, BNS (II)	Most registered products banned in 1974, final products banned in 1988 with exception of current use for fire ants in underground power transformers.[h]	NA 0.175	1.76E-04 2.38E-04	5.86 3.813
 hexabromobiphenyl (HBB) $2,2^1,4,4^1,5,5^1$-HBB or BB-153 ($C_{12}H_4Br_6$)	Organobromine flame retardant used in consumer products. HBB is one of three commercial mixtures. Most common congeners in HBB mixture are 2,2′,4,4′,5,5′-HBB (53.9–68.0%) and 2,2',3,4,4',5,5'-HBB (7.0–27.3%).	No	COP4	Manufacture discontinued in United States in 1976.	NA 82.964	1.65E-06 2.49E-09	9.10 2.467
 hexachlorobenzene (C_6Cl_6)	Organochlorine fungicide. Also used to make fireworks, ammunition, and rubber. Can be a byproduct of chemical manufacturing.	No	SC, BNS	Not manufactured as a commercial end product, but still released by industry as a by-product. Registration as pesticide voluntarily canceled in 1984.	NA 633.112	8.92E-04 3.05E-06	5.86 4.201

(Continued)

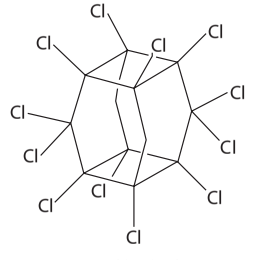
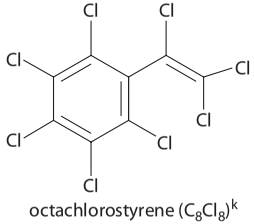
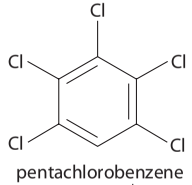
Table 5.1 (*Continued*) Chemical Properties of POP and PBT Chemicals

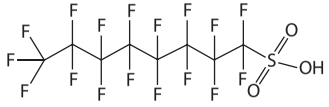
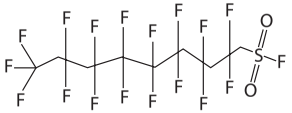
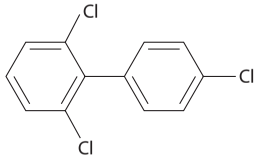
Chemical	*Chemical Group and Use*	*Naturally Occurring*	*Listing*[a]	*Regulatory Status for Use in the United States*	*$t_{1/2}$ (years) (hydrolysis)*[b] / *$t_{1/2}$ (days) (atmosphere)*[c]	*H_c*[d] *(atm-m³/mol)* / *VP (mmHg)*[e]	*Log K_{ow}*[f] / *Log BCF*[g]
 α- and β-hexachlorocyclohexane (HCH) ($C_6Cl_6H_6$)	Organochlorine fungicide (technical grade HCH). Also used in production of other chemicals and produced as a by-product of lindane. Are 8 isomers of HCH. Technical grade HCH includes 60–70% α-HCH and 5–12% β-HCH.	No	COP4 (α and β), BNS (II) lists HCH without isomers defined.	Technical grade HCH banned for production and use in 1976.	3.558 E+10 (Kb at pH 7)	2.56E-04	4.26
					18.659	5.06E-04	3.121
 lindane (γ-HCH) ($C_6H_6Cl_6$)	Organochlorine pesticide. An isomer (gamma) of HCH.	No	COP4	Commercial production reported to end in 1976.[h]	3.558 E+10 (Kb at pH 7)	2.56E-04	4.26
					18.659	5.06E-04	3.121

Mercury and Mercury Compounds							
Hg mercury (Hg)	Exists in 3 general forms: elemental (pure form), inorganic Hg (combined with elements besides carbon), and organic (combined with carbon). Uses of Hg are extensive and include pesticides, pharmaceuticals, paint colorings, catalysts, used in alkaline batteries, lamps, gold mining, and dental amalgams.	Yes, usually found as HgS (cinnabar)	BNS	Releases controlled under specific regulations, e.g., CAA, CWA, and RCRA.	NA	NV	0.62 (OED)
					NA	NV	0.02
$H_3C–Hg–CH_3$ dimethyl mercury (HgC_2H_6)					NA	2.13E-03	0.62
					0.655	50.5[i]	1.369
$H_3C–Hg$ methyl mercury ($HgCH_3$)					NA	7.22E-03	0.08
					1.31	77[i]	–0.027
 mercuric sulfate ($HgSO_4$)					NA	NV	–0.07 (OED)
					NA	NV	–0.041

(Continued)

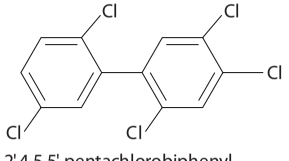
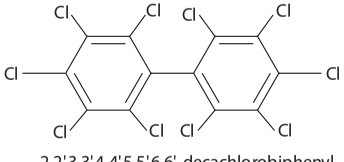
Table 5.1 (*Continued*) Chemical Properties of POP and PBT Chemicals

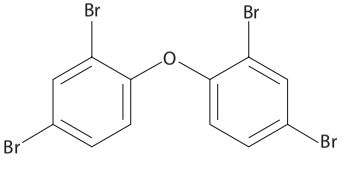
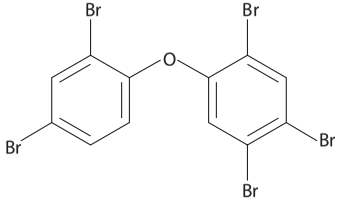
Chemical	*Chemical Group and Use*	*Naturally Occurring*	*Listing*[a]	*Regulatory Status for Use in the United States*	*$t_{1/2}$ (years) (hydrolysis)*[b] *$t_{1/2}$ (days) (atmosphere)*[c]	*H_c*[d] *(atm-m³/mol)* *VP (mmHg)*[e]	*Log K_{ow}*[f] *Log BCF*[g]
 mirex ($C_{10}Cl_{12}$)	Organochlorine insecticide and flame-retardant additive.	No	SC, BNS	All registered products canceled 1977.[h]	NA NA	1.28E-06 2.92E-06	7.01 3.762
 octachlorostyrene (C_8Cl_8)[k]	Organochlorine. Not intentionally manufactured, but is a by-product from eletrolytic production of magnesium.[k]	No[k]	BNS	National Action Plan in draft form.[k]	NA 9.967	2.30E-4 1.32E-05	7.46 3.522
 pentachlorobenzene (C_6HCl_5)[l]	Organochlorine used in the manufacture of other chemicals. Can be a degradation product of other organochlorine chemicals.[l]	No[l]	COP4, BNS (II)	Releases controlled under specific regulations, e.g., CERCLA, CWA, and RCRA.[l]	NA 184.851	1.20E-3 1.71E-03	5.22 3.889

Perfluorooctane Sulfonic Acid (PFOS), Its Salts, and Perfluorooctane Sulfonyl Fluoride (PFOSF)							
 perfluorooctane sulfonic acid (PFOS) ($C_8HF_{17}O_3S$)	Perfluoroalkyls. Manufactured for consumer products such as fire-fighting foam, photo imaging, hydraulic fluids, and textiles. Also a degradation product of related perfluoroalkyl chemicals.	No	COP4	Manufacturer stopped production of PFOS in 2002 EPA requires 90 days' notification prior to manufacture or import of listed perfluoroalkyl sulfonate compounds, for any use in the United States.	NA	1.10E-02	6.28
					76.4	6.40E-03	3.831
 perfluorooctane sulfonyl fluoride ($C_8F_{18}O_2S$)					NA	679E+01	9.62
					NA	5.75E+00	2.087
Polychlorinated Biphenyls (PCBs)—Representative Congeners Included							
 2, 4', 6-trichlorobiphenyl (PCB-32) ($C_{12}H_7Cl_3$)	Organochlorines used as coolants and lubricants in transformers, capacitors, and other electrical equipment. Marketed as Aroclor in the United States. 209 different congeners possible and always present as a mixture in the environment.	No	SC, BNS	Manufacture stopped in 1977.	NA	1.68E-04	5.69
					9.022	4.00E-05	4.205

(Continued)

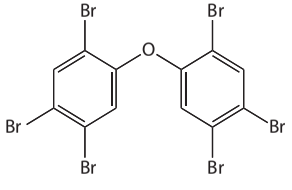
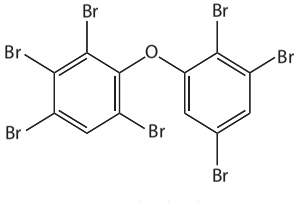
Table 5.1 (*Continued*) Chemical Properties of POP and PBT Chemicals

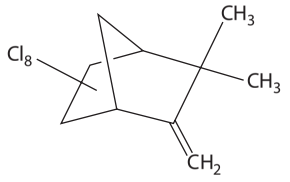
Chemical	*Chemical Group and Use*	*Naturally Occurring*	*Listing*[a]	*Regulatory Status for Use in the United States*	$t_{1/2}$ *(years) (hydrolysis)*[b] $t_{1/2}$ *(days) (atmosphere)*[c]	H_c[d] *(atm-m³/mol)* *VP (mmHg)*[e]	*Log* K_{ow}[f] *Log BCF*[g]
 2,2',4,5,5' pentachlorobiphenyl (PCB-101) ($C_{12}H_5Cl_5$)					NA 31.947	9.24E-05 2.22E-6	6.98 4.06
 2,2',3,3',4,4',5,5',6,6'-decachlorobiphenyl (PCB-209) ($C_{12}Cl_{10}$)					NA 588.56	2.06E-05 1.02E-10	10.2 3.267

Polybrominated Diphenyl Ethers (PBDEs)—Representative Congeners Included							
 2,2',4,4'-tetrabromodiphenyl ether (BDE-47)($C_{12}H_6Br_4O$)	Organobromine flame-retardant chemicals added to consumer products. 209 congeners possible, but only a limited number are present in commercial mixtures. Always present as a mixture in the environment.	No[m]	COP4 (hexaBDE and heptaBDE, and tetraBDE and pentaBDE)[n]	Manufacturer of hexaBDE and octaBDE commercial mixtures voluntarily stopped production in 2004. As of January 1, 2005, EPA requires 90 days' notification prior to manufacture or import, for any use and manufacture of decaBDE commercial mixtures limited at state level.[o,p]	NA	2.97E-06	6.77
					10.661	2.41E-07	3.699
 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) ($C_{12}H_5Br_5O$)					NA	1.18E-06	7.66
					19.439	1.08E-08	3.868

(Continued)

Table 5.1 (*Continued*) Chemical Properties of POP and PBT Chemicals

Chemical	*Chemical Group and Use*	*Naturally Occurring*	*Listing*[a]	*Regulatory Status for Use in the United States*	$t_{1/2}$ *(years) (hydrolysis)*[b] $t_{1/2}$ *(days) (atmosphere)*[c]	H_c[d] *(atm-m³/mol)* *VP (mmHg)*[e]	*Log* K_{ow}[f] *Log BCF*[g]
 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE-153) ($C_{12}H_4Br_6O$)					NA 46.163	4.71E-07 2.87E-09	8.55 2.624
 2,2',3,3',4,5',6-heptabromodiphenyl ether (BDE-175) ($C_{12}H_3Br_7O$)					NA 43.488	1.88E-07 3.29E-10	9.44 1.890

 toxaphene ($C_{10}H_{10}Cl_8$)	Mixture of 670 chlorinated terpenes with high percent of chlorination, used as an insecticide.	No	SC, BNS	All registered products canceled in 1990.[h]	3.34E+9 (Kb at pH 7)	4.35E-05	6.79
					4.749	1.42E-06	4.032

Note: Unless stated otherwise, all information is from the most current Toxicological Profile Information Sheet. (Agency for Toxic Substances and Disease Registry for each chemical. http://www.atsdr.cdc.gov/toxpro2.html.)

[a] SC = Stockholm Convention; BNS = Binational Toxics Strategy Level I; BNS (II) = Binational Toxics Strategy Level II, not all Level II listed here; COP4 = Conference of the Parties 4.

[b] Estimate from HYDROWIN v. 2.00 in EPI Suite (U.S. EPA and SRC 2008). Aqueous hydrolysis rates only for esters, carbamates, epoxides, halomethanes, selected alkyl halides, and phosphorus esters. NA = not available.

[c] Estimate from AOPWIN v. 1.92 in EPI Suite (U.S. EPA and SRC 2008). Hydroxyl radical reaction with 12 hours light and 1.5E6 OH/cm^3. NA = not available.

[d] Estimate from HENRYWIN v. 3.20 in EPI Suite (U.S. EPA and SRC 2008), using bond method. At 25°C.

[e] Estimate from MPBPWIN v. 1.43 in EPI Suite (U.S. EPA and SRC 2008), using modified grain method unless stated otherwise. At 25°C.

[f] Estimate from KOWWIN v. 1.67 in EPI Suite (U.S. EPA and SRC 2008).

[g] Estimate from BCFBAF v. 3.00 in EPI Suite (U.S. EPA and SRC 2008), using Arnot-Gobas method (upper trophic level).

[h] See U.S. EPA (1990) for more information.

[i] Mean of Antoine and grain methods.

[j] Grouped with DDT in BNS (as DDT, DDD, and DDE). Technical grade DDT included up to 14 chemicals, including 65–80% (*p,p'*)DDT.

[k] From U.S. EPA (2000).

[l] From U.S. EPA (1999a).

[m] Methoxylated polybrominated diphenyl ethers have been found to naturally occur in some marine organisms (e.g., Teuten et al. 2005).

[n] Listed as (1) hexa- and heptaBDEs present in commercial octaBDE and (2) tetra- and pentaBDEs present in commercial pentaBDE. A third commercial formula (decaBDE) is not included in this listing.

[o] From U.S. EPA (2006).

[p] From U.S. EPA (2009).

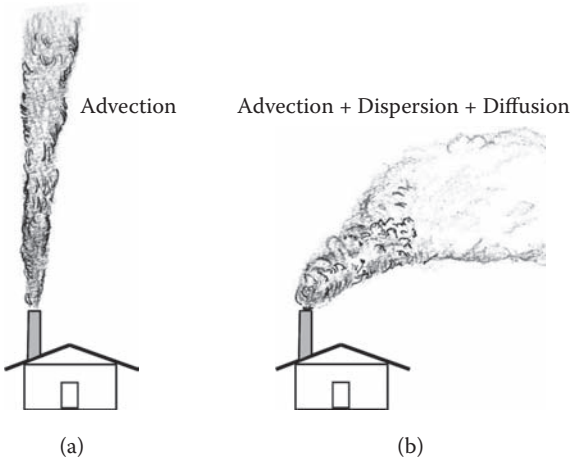


Figure 5.1 Smoke moving by (a) primarily advection and (b) advection, dispersion, and diffusion, resulting in a lower concentration of smoke in a larger volume of air.

eddy diffusion, random motion of the media causes the chemical to mix. If the glass of water is shaken instead of stationary, the turbulence will cause the red color to spread quicker throughout the glass. In the environment, this turbulent diffusion can be caused by wind interacting with buildings or trees or by meandering stream channels and changes in streambed topography. Other environmental mixing events that can be described as turbulent diffusion are vertical mixing across a boundary layer of different water densities, such as in a stratified lake, or the heating of a land surface that causes air to rise adiabatically. A final transport mechanism is dispersion. Dispersion is similar to turbulent diffusion except there is a pattern to the media's motion. This can occur when there are different velocities of the media in the longitudinal direction of transport. For example, in a cross section of stream, the velocity is greatest in the stream center below the water surface and lower near the channel surface. This results in some parcels of water, and the chemicals advected by them, moving faster than other water parcels. Groundwater flow provides another example of dispersion, with water parcels moving along different paths around soil particles.

From a modeling perspective, advection is described by the direction and magnitude of the media's velocity, and diffusion/dispersion can be accounted for with Fickian mixing models. With respect to transport in the environment, advection typically accounts for the greatest movement of contaminants from the source, whereas diffusion and dispersion result in mixing of the chemical throughout the media of interest, which causes the maximum chemical concentration to decrease. A house chimney releasing smoke provides an example of how these processes work together to transport contaminants in the environment. If the smoke were moving by advection only, it would occupy a consistent cross-sectional area as it is advected. Instead, a plume of smoke spreads in three dimensions and takes up a larger cross-sectional area as it is advected, diffused, and dispersed from the source. A consequence of the diffusion and dispersion is that a larger volume of air is contaminated with a lower concentration of the chemical (Figure 5.1).

5.2.2 Long-Range Atmospheric Transport (LRAT)

Transport in air provides an interesting and important example to expand upon since this is a primary mechanism through which global transport of contaminants occurs. Chemicals can be

transported atmospherically as a gas, dissolved in atmospheric water, or sorbed to aerosols and larger particles. The properties of the chemical dictate in which form the contaminants are most likely to exist. Once sorbed or dissolved in atmospheric water, the chemicals can be removed by wet deposition such as rain, snow, and fog. Gravitational settling or sedimentation of the aerosol can result in dry deposition of the sorbed contaminants back to the terrestrial or aquatic environments. Direct condensation of the gas onto the earth's surface is another type of dry deposition that removes the contaminant from the atmosphere.

Semivolatile organic compounds (SVOCs) are chemicals, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), phthalates, and organochlorine pesticides, that can slowly volatilize under environmentally relevant conditions (He and Balasubramanian 2009). Organic chemicals in water can be identified as SVOCs if the Henry's constant (H_c) is 10^{-5} to 10^{-7} atm-m^3/mol (MacKay and Shui 1981; Zhang 2007). Since volatilization is temperature dependent, the SVOCs can volatilize from regions of usage in tropical or temperate regions and be transported atmospherically to regions with lower temperatures, like the high-latitude ecosystems of the Arctic. With a decrease in temperature, cold condensation can occur and the low ambient temperatures can trap the SVOCs, an effect called cold trapping (Rahn and Heidam 1981; Wania and MacKay 1996). The result is a net accumulation of SVOCs in high-latitude regions. The process of contaminant volatilization followed by condensation, resulting in LRAT, is called global distillation and explains the presence of PCBs, DDT, and other organochlorine pesticides in the Arctic and Antarctic, where local sources of these contaminants do not exist. Depending on the properties of the chemical (i.e., vapor pressure and H_c) and the environment (i.e., seasonally driven ambient temperatures, and wind speed and direction), the chemical may volatilize and condense just once or several times, a phenomenon called the grasshopper effect (Figure 5.2a) (Wania and MacKay 1993, 1996; Fernández and Grimalt 2003).

To complicate matters further, different chemicals condense at different temperatures. The result is more volatile contaminants are transported farther than less volatile contaminants, a process known as global fractionation (Wania and MacKay 1993). More chlorinated PCB congeners, for example, will be deposited closer to the source than the less chlorinated congeners, resulting in a spatial redistribution of the PCB congeners. On a more regional scale, distillation and fractionation of semivolatile contaminants can also occur along the temperature gradients associated with high-altitude mountain ecosystems (Figure 5.2b). Although there is evidence to support this altitudinal-driven regional distillation, called mountain cold trapping or orographic cold trapping, the patterns are complex and no single pattern predominates. Daly and Wania (2005) describe the complexities of understanding mountain cold trapping with a need to consider additional environmental properties such as distance from source, climatic season, wind speed, surface type (e.g., snow cover, vegetation type and extent, rocky), and organic content of the soils.

5.3 Persistence

Persistence has been defined as the residence time of a chemical in a specific environmental compartment (Greenhalgh et al. 1980). Chemicals that are identified as persistent will remain in the environment for long periods, meaning there is extended potential for exposure and subsequent toxicity. Transport processes such as diffusion and dispersion decrease the maximum chemical concentration in an environmental compartment, but these processes do not affect the persistence of a chemical since the total mass is not changed. Advection may remove a chemical from one location, and therefore decrease the persistence on a local scale. However, the total mass does not

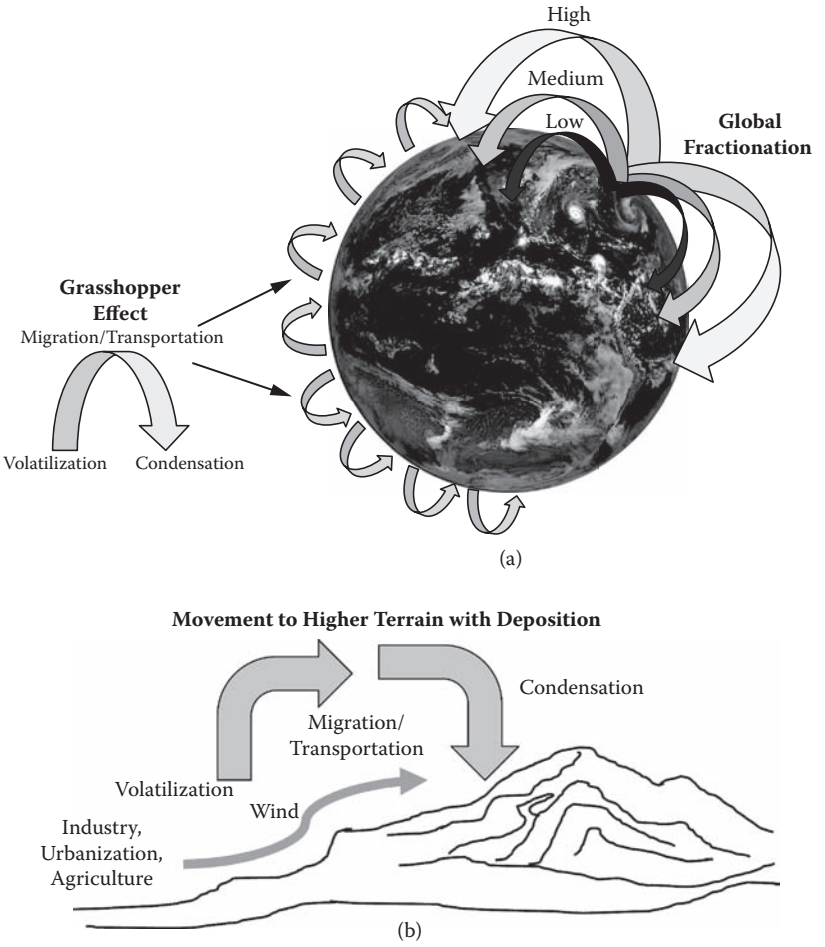


Figure 5.2 Atmospheric movement of SVOCs. (a) Global distillation of SVOCs. Chemicals are transported atmospherically in single or multiple steps of volatilization followed by condensation, where multiple steps are known as the grasshopper effect. Global fractionation occurs because chemicals with greatest volatility are transported the farthest, and those with the lowest volatility are transported the least. (See color insert following page 268.) (b) Mountain cold trapping of SVOCs. The temperature gradients associated with high-altitude mountain systems can cause SVOCs to volatilize and condensate, ultimately resulting in increased deposition in these systems.

change since the chemical is only transported to another location and persistence is not affected at the larger scale. Abiotic and biotic degradation reactions are the major mechanisms through which persistence is affected.

The Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention) was the result of an international effort to identify and manage a class of contaminants that are persistent, bioaccumulative, toxic, and subject to long-range transport. The Stockholm Convention, put forth for signatures in 2001 in Stockholm, Sweden, and effective in 2004, is administered by the United Nations Environment Program. It includes a list of 12 persistent organic pollutants (POPs), known as the dirty dozen (Table 5.1). Parties to the Stockholm Convention agreed to reduce or

eliminate the manufacture, use, and import of these POPs with the intent of limiting environmental releases. In May 2009, the Conference of the Parties (COP) to the Stockholm Convention met for the fourth time in Geneva, Switzerland. At this meeting (COP4), nine new POPs were added to the Stockholm Convention (Table 5.1). A similar classification of persistent chemicals was developed under the 1997 Great Lakes Binational Toxics Strategy (BNS), signed by Canada and the United States, to be administered by the U.S. Environmental Protection Agency (EPA) and Environment Canada (1997). Twelve persistent, bioaccumulative, and toxic (PBTs) chemicals were identified as level I PBTs, to be managed so that environmental release is reduced or eliminated. A level II list includes chemicals for which pollution prevention activities are encouraged. The BNS was followed in 1998 by the draft *Multimedia Strategy for Priority Persistent, Bioaccumulative, and Toxic (PBT) Pollutants* (PBT Strategy) from the U.S. EPA. This document led to a requirement for more stringent reporting under the Toxics Release Inventory (TRI) of the Emergency Planning and Community Right to Know Act (EPCRA), and screening for PBT properties under the New Chemical Program (NCP) of the Toxics Substances Control Act (TSCA) in the United States.

Several approaches have been developed to identify persistent contaminants. For the BNS, the level I chemicals were selected based upon their nomination to other lists related to toxicants in Great Lake Basin ecosystems. The new European chemical legislation, Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH), has taken a more typical approach. Under REACH, criteria for persistent and very persistent substances are set based on the half-life of the chemical in different environmental compartments. If a chemical half-life exceeds the criteria for any of the compartments, the chemical is considered persistent. Some of the adopted criteria are listed in Table 5.2.

5.4 Biotransport

A final transport mechanism for chemicals, including those not volatile enough to be transported atmospherically, is biotransport or the special case of biovector transport. With biotransport, contaminants that have accumulated in or on an organism are transported by the moving organism. Biovector transport is more specific and occurs when an organism transports a contaminant and deposits that contaminant in a new location identified as the receptor site. The direction of transport in this case is unidirectional, and a net accumulation of contaminants would be expected at the receptor site (Blais et al. 2007). Migratory organisms, such as anadromous Pacific salmon, are one of the best examples of biovector transport. Krümmel et al. (2003) found a strong correlation between the density of spawning salmon in Alaskan nursery lakes and PCB concentration in sediments. The salmon, which accumulate the majority of the PCB body burden during the ocean phase, release the lipophilic PCBs upon death and decomposition, and the lipophilic contaminants can reenter the abiotic transport cycle. In another study, Ewald et al. (1998) measured organic contaminant concentrations in whole body lipids of sockeye salmon during spawning migration in the Copper River, Alaska, in arctic graylings resident to the spawning lakes, and in arctic graylings from a reference lake with no spawning salmon. The same contaminants were analyzed in the atmospheric deposition to the lakes. No correlation was found between the concentrations in graylings from either lake and atmospheric deposition. The DDT and PCB congener concentrations in the graylings were significantly greater in the salmon spawning lake than in the salmon-free lake. The pattern of contaminants in the salmon was similar to that in the graylings in the salmon lake, further supporting the salmon as the primary source of organic contaminants in the spawning lake. Other migratory animals, including birds and whales, can also transport

Table 5.2 Criteria Used to Determine Persistence, Bioaccumulative, and Toxic Properties of a Chemical

	Persistence				*Bioaccumulative*		
Program and Regulatory Agency/Organization	*Half-Life in Water (days)*	*Half-Life in Soil (days)*	*Half-Life in Sediment (days)*	*Half-Life in Air (days)*	*BCF*	*Log K_{ow}*	*Toxic*
Stockholm Convention—United Nations Environment Program (UNEP 2001)	>60	>180	>180	>2	>5,000	>5	Toxicity or ecotoxicity data that indicate the potential for damage to human health or to the environment
REACH PBT—European Chemical Agency (European Commission 2006)	Marine > 60, or freshwater/ estuarine > 40	>120	Marine > 180, or freshwater/ estuarine > 120	NA	>2,000	NA	1. NOEC in marine or freshwater < 0.01 mg/L 2. Carcinogenic, mutagenic, or toxic for reproduction 3. Other evidence of chronic toxicity

REACH vPvB[a]—European Chemical Agency (European Commission 2006)	>60	>180	>180	NA	>5,000	NA	NA
Canadian Environmental Protection Act (CEPA)—Environment Canada (Government of Canada 1999, 2000)	≥182	≥182	≥365	≥2	≥5,000	NA	Interpreted under CEPA
TRI of EPCRA—U.S. EPA (1999b)	≥60 (persistent) >180 (very persistent)	≥60 (persistent) >180 (very persistent)	≥60 (persistent) >180 (very persistent)	>2 (persistent)	≥1,000 (bioaccumulative) >5,000 (very bioaccumulative)	NA	Interpreted under EPCRA

Note: NA = Not applicable.

[a] Very persistent and very bioaccumulative.

contaminants. In the case of birds, guano and loss of feathers are the biological processes through which contaminants are deposited at the receptor site (Blais et al. 2007).

5.5 Abiotic Degradation/Transformation

Abiotic transformation, which includes chemical and photolytic reactions, is a process through which molecules are converted to new chemicals called transformation products. If the transformed product is an intermediate to complete degradation or mineralization, the product can more specifically be called a degradate. Since products formed during abiotic reactions are available for biotic reactions that may result in mineralization, abiotic reactions here will be described as degradation reactions and the products as degradates. As with biotic degradation, several degradation steps may occur and several pathways may be present, resulting in the formation of many degradates. The degradation steps and pathways depend on the chemical, the environmental media in which degradation is occurring, and environmental properties of that compartment (Figure 5.3). Table 5.3 compares other characteristics of abiotic and biotic degradation.

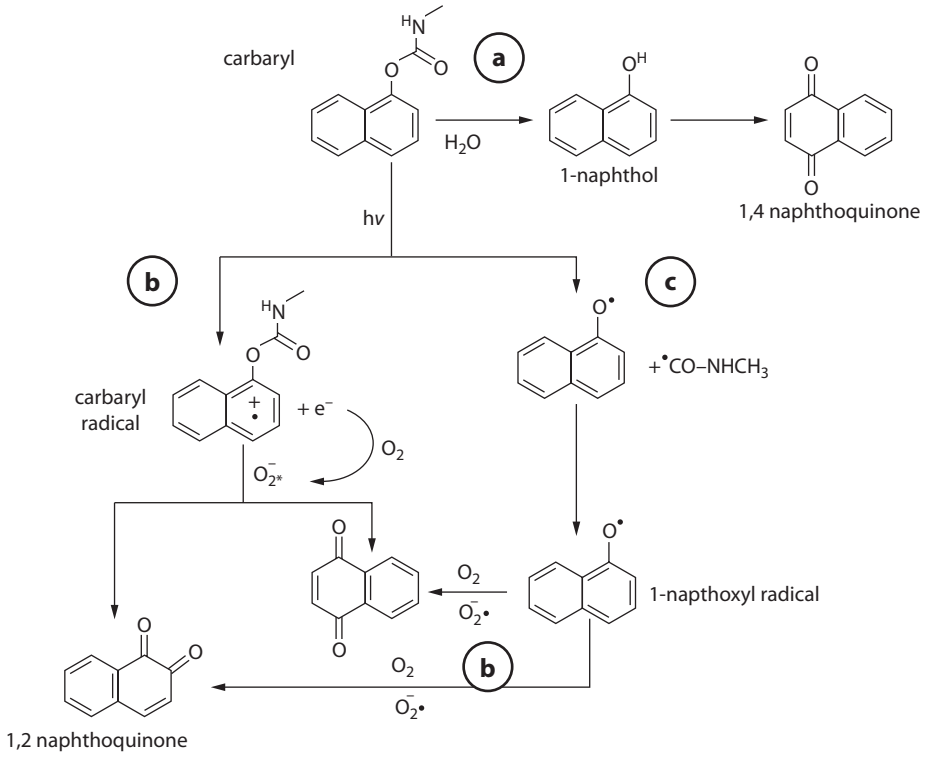


Figure 5.3 Degradation pathways of carbaryl, a carbamate pesticide. (a) Primary degradation pathway by hydrolysis in the absence of ultraviolet radiation (UVR). (From Wang, Q. Q., and Lemley, A. T., *J. Agric. Food Chem.,* 50, 2331–2337, 2002.) Degradation pathways of direct photolysis (hv) in oxygen-rich water (b) and in water (c). (Proposed by Brahmia, O., and Richard, C., *J. Photochem. Photobiol. A,* 156, 9–14, 2003.) Indirect photolysis has also been shown to occur with natural organic matter (NOM) and nitrates as the photosensitizers producing •OH, which interact with the carbaryl.

Table 5.3 A Comparison of Some Characteristics of Abiotic and Biotic Degradation

	Abiotic Degradation	*Biotic Degradation (Biodegradation)*
Mechanism	Chemical and photolytic	Enzymatically catalyzed by microorganisms
Common degradation reactions	Oxidation, reduction, hydrolysis, photolysis, dealkylation, dehalogenation, aromatic ring cleavage	Oxidation, reduction, hydrolysis, dealkylation, dehalogenation, aromatic ring cleavage
Relative rate of degradation	Lower	Higher
Completeness of degradation	Mineralization not typical	Can lead to complete mineralization of organic contaminants
Effect on chemical persistence	Decreases persistence of parent chemical	Decreases persistence of parent chemical

Compared to the parent chemical, the degradates may be as toxic, less toxic (where the process of decreasing toxicity is inactivation or detoxication), or more toxic (where the process of increasing toxicity is activation or toxication). An analysis of the toxicity of pesticides, for example, showed 41% of the parent chemicals were less toxic and 20% were more toxic than their degradates to fish, daphnia, or algae (Boxall et al. 2004). Degradation reactions are typically represented as a half-life ($t_{1/2}$) with units of time (T) or as a rate constant (k) with units of T^{-1}. The two can be related to each other with the first-order decay equation (Equations 5.1 and 5.2). Since $t_{1/2}$ and k are inversely related, a greater $t_{1/2}$ and a smaller k indicate the chemical is more persistent.

$$C_t = C_0 e^{-kt} \tag{5.1}$$

$$k = \frac{0.693}{t_{1/2}} \tag{5.2}$$

Hydrolysis reactions, the cleavage of a molecule with water, results in two new molecules with an H or OH added. The rates of these reactions are typically pH and temperature dependent. Since hydrolysis reactions are considered pseudo-first-order reactions, the rate of hydrolysis depends on the concentration of parent chemical. The chemical classes that can be hydrolyzed include alkyl halides, amides, amines, esters, epoxides, and nitriles (Cronin and Livingston 2004). Organophosphate insecticides, and carbamate insecticides and herbicides are esters of phosphoric and carbamic acids, respectively, and provide a good example of environmentally relevant contaminants that can be degraded hydrolytically (Figure 5.3).

Photolysis or photodegradation is the process of chemical bond breakage by light and can be an important removal or production mechanism in surface waters and the atmosphere. The rates at which photodegradation occur are highly dependent on the properties of the environment, such as the energy and wavelength of light, with high energy and short wavelengths (i.e., ultraviolet and blue wavebands) being important. The double-bonded carbons of organic molecules,

such as aromatic rings and alkenes, are chromophores that can absorb light energy in the form of photons. An electron in the organic molecule is elevated to an excited state with energy to be dissipated. The release of energy can result in chemical reactions (e.g., isomerization, hydrogen atom abstraction, and fragmentation) leading to new products. If the light-absorbing molecule is a contaminant, this process is primary or direct photodegradation. Several PAHs, particularly the three- to five-ring PAHs, are more toxic in the presence of ultraviolet light. This increase in toxicity has been termed phototoxicity. One demonstrated mechanism of phototoxicity is photomodification, a type of primary photodegradation (Lampi et al. 2006). If, instead of direct absorption, the photon is absorbed by a chromophore in close proximity to the contaminant, secondary or indirect photodegradation can occur. In this case, the molecule with the chromophore is defined as a photosensitizer. The energy from the elevated electron in the photosensitizer can be transferred to the contaminant as an e^- or H^+ or can result in short-lived reactive oxygen species or photooxidants, such as $OH^{\cdot}$, $NO_3^{\cdot}$, O_3, and $^{\cdot}O_2^-$, which can interact with the contaminant. Natural organic matter (NOM), nitrate, and nitrite are common photosensitizers in natural waters. Typically knowledge of secondary photodegradation is obtained from controlled laboratory experiments; for example, significant degradation of PCBs in the presence of photosensitizers can occur in bench-scale systems (e.g., Lin et al. 1996; Jones et al. 2003; Poster et al. 2003). A study by Kolpin and Kalkhoff (1993) demonstrates the challenges of understanding secondary photodegradation in the field, although they were able to support that atrazine in a stream was most likely photodegraded indirectly, with nitrates being a possible photosensitizer.

5.6 Multimedia Box Models

One of the key features of the PBT Strategy is a multimedia approach. This recognizes the fact that chemicals, particularly SVOCs, may be found in multiple environmental compartments or phases (e.g., water, sediment, organisms, air, and soil). Box models provide one method for assessing chemical fate and transport in multiple environmental compartments. Key features of a box model are that multiple compartments of interest are established to create the environmental system, the volume of those compartments is known or estimated and does not change, each compartment is assumed to be well mixed so that concentrations are consistent throughout a compartment and diffusion/dispersion can be neglected, and pathways for the chemical to move between the compartments are established. From there, additional assumptions about the environmental system are made:

- Open versus closed—If the environmental system is not open to additional inputs or outputs (e.g., advection does not occur), the system is considered closed. An example is a closed bucket with water, air, and chemical. If additional inputs or losses can occur from the system, it is considered open (Figure 5.4). An open system would typically be the most environmentally realistic.
- Equilibrium versus nonequilibrium—Equilibrium is based on how the chemical partitions between the different compartments in the system. Note that this differs from chemical equilibrium, which occurs when forward and reverse chemical reactions proceed at the same rate and there is no net change in the amounts of products or reactants. If the ratio of the chemical concentrations in different compartments is constant over time, the system is at equilibrium. In this case, individual molecules of the chemical may move between the compartments, but there is no net change in the concentration in each compartment, and so no

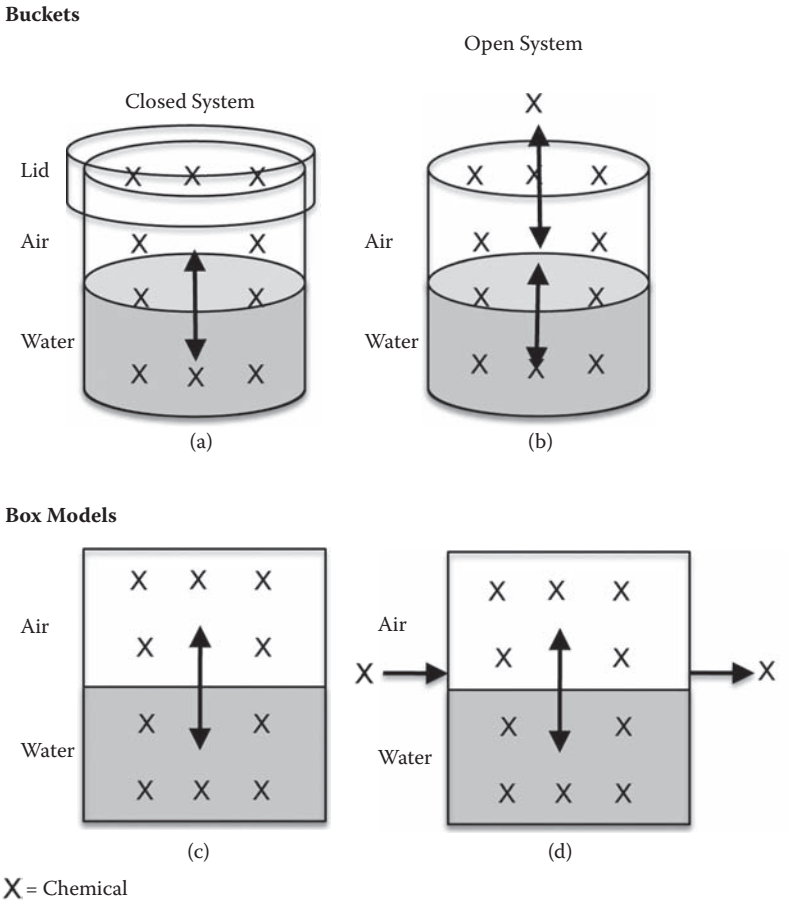


Figure 5.4 Representation of how a real system (buckets in a and b) is simplified into a box model (c and d). Real system (a) and box model (c) are a two-phase closed system, and real system (b) and box model (d) are a two-phase open system. In all cases, the chemical can distribute between the two phases.

change in the ratio. If the ratio of the chemical concentration in the different compartments changes with time, the system is in a nonequilibrium state (Figure 5.5).

- Steady state versus unsteady state—This condition deals with the change in chemical mass in the environmental system over time, where the system is defined as one or multiple compartments. If there is no change, the system is at steady state. If the chemical mass changes with time, the system is at unsteady state (Figure 5.6). The timeframe over which the system is evaluated should be considered in the case of steady state versus unsteady state. For example, the epilimnion of a lake that is stratified may reach steady state during the summer, but may be at unsteady state if spring and fall turnover are included in the timeframe.

Mass balance equations can be used to quantify chemical partitioning in an environmental system because of the law of conservation of mass. In the most basic closed system, the total mass (M_T) in the system is equal to the sum of the masses in each compartment (Equation 5.3). This assumes that there is no advection (implicit in the definition of a closed system) and that

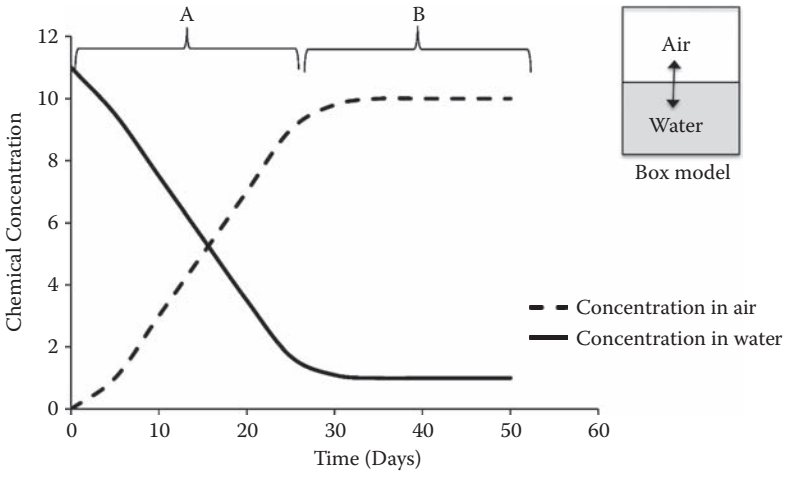


Figure 5.5 A two-compartment closed system of air and water with a chemical that is at equilibrium when the ratio is 10:1 (C_A:C_w). In this case, (A) ratio is not 10:1, nonequilibrium; (B) ratio is 10:1, equilibrium.

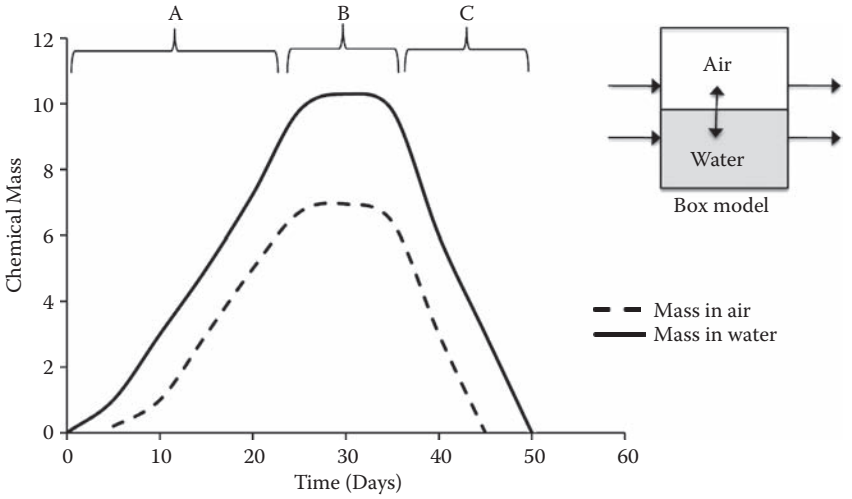


Figure 5.6 A two-compartment open system of air and water. In this case, (A) mass of chemical in system is increasing, system is at unsteady state; (B) mass of chemical in system is not changing, system is at steady state; and (C) mass of chemical in system is decreasing, system is at unsteady state.

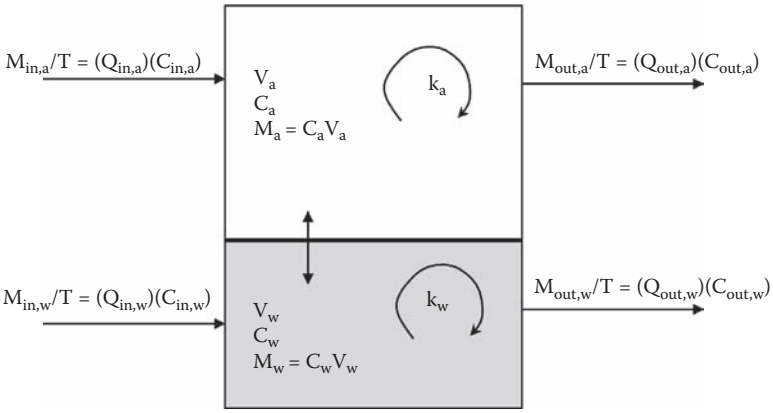


Figure 5.7 Conceptual model of a two-phase system with water and air. Symbols represent those used in the mass balance. In this example, there is one input and one output for air and water. The symbols inside each compartment represent the volume, concentration, mass, and degradation constant in each phase.

the system is at a steady state (e.g., no degradation reactions or internal emissions to increase or decrease the total chemical mass). Since the mass in a compartment (M_i) is equal to the concentration in a compartment (C_i) multiplied by the volume of that compartment (V_i), substitutions can be made (Equation 5.4).

$$M_T = \sum_i M_i \tag{5.3}$$

$$M_T = \sum_i C_i V_i \tag{5.4}$$

The second mass balance equation of importance is used in the open system (Equation 5.5). In this case, the mass inputs to the system can be advection in, emissions from sources in the system (e.g., industrial releases), or production of a chemical through degradation reactions when the degradate is the chemical of concern. The mass outputs from the system can be advection out or loss of a chemical through degradation reactions. The component of advection is calculated as the flow into or out of a compartment (Q_i) multiplied by the concentration of the chemical in the advected media (C_i). Chemical input or output resulting from degradation can be represented by the product of V_i, C_i, and the degradation rate constant (k) in a compartment (Equation 5.6 and Figure 5.7). Recall that the degradation rate constant (k) can be determined from the half-life (Equation 5.2). If a degradate is more toxic than the parent chemical, it may be important to account for its production with a formation rate constant. This mass balance (Equation 5.6) can be simplified if an assumption of steady state is made since there would be no change in the rate of mass stored in the system, $dM/dT = 0$ (Equation 5.7).

$$\frac{dM}{dT} = \sum_i \frac{M_{in,i}}{T} - \sum_i \frac{M_{out,i}}{T} \tag{5.5}$$

$$\frac{dM}{dT} = \sum Q_{in,i}C_i + \sum k_{produced}V_iC_i - \sum Q_{out,i}C_i - \sum k_{degraded}V_iC_i \quad (5.6)$$

$$\sum Q_{in,i}C_i + \sum k_{produced}V_iC_i = \sum Q_{out,i}C_i + \sum k_{degraded}V_iC_i \quad (5.7)$$

5.7 Equilibrium

An assumption often made, at least with initial modeling efforts, is that a system is at equilibrium. In order to account for equilibrium with the mass balance equation, equilibrium partitioning constants are empirically determined, often in controlled laboratory settings. Among the more common equilibrium partitioning constants are K_{ow}, *BCF*, K_{oc}, K_d, and H_c. In all cases, these constants describe the ratio at which a chemical will exist between two environmental phases at equilibrium. Each is discussed separately below.

K_{ow} (dimensionless) is the octanol-water partition coefficient and is essentially a measure of lipophilicity, where K_{ow} is defined as the

$$K_{ow} = \frac{\text{concentration of chemical in octanol}}{\text{concentration of chemical in water}} = \frac{C_o}{C_w} \text{ at equilibrium}$$

Octanol ($C_8H_{18}O$) is an organic liquid that is similar to fatty tissue, and therefore is used as a surrogate for lipids. K_{ow} values are often reported as log K_{ow} values and are widely available for many chemicals. Chemicals with a log K_{ow} between 2 and about 6 have been identified as lipophilic, and those with log $K_{ow} > 6$ as superhydrophobic. Analyses of how K_{ow} relates to BCF have demonstrated that chemicals with a log K_{ow} between approximately 3 and 6 are linearly related. Chemicals with a log $K_{ow} > 6$ to 7 are not directly correlated to the BCF, and less of the chemical bioaccumulates than would be expected by the log K_{ow} (Connell 1990, 1993). The criteria used to define a POP or PBT as bioaccumulative under the Stockholm Convention and under TSCA are based on K_{ow} or *BCF* values (Table 5.1). Log K_{ow} is also represented by log *P*.

BCF ($L_{water}/kg_{organism}$) is the bioconcentration factor, where

$$BCF = \frac{\text{concentration of chemical in an organism (or biota)}}{\text{concentration of chemical in water}} = \frac{C_b}{C_w} \text{ at equilibrium}$$

BCF is similar to the K_{ow} in that it is a measure of how likely a chemical is to bioaccumulate. The difference when *BCF* is empirically measured is that *BCF* uses the concentration of chemical in an actual organism, typically the whole body, as opposed to in octanol, and thus accounts for processes such as metabolism and elimination. *BCF* values will vary depending on the organism for which they were determined. Although *BCF* values are not as widely available as K_{ow} values, *BCF* can be estimated from K_{ow} values for many chemicals. Related partitioning constants include the biota sediment accumulation factor (BSAF), the biomagnification factor (BMF), and the bioaccumulation factor (BAF). As with *BCF*, the numerator

BOX 5.1 A NOTE ON UNITS FOR THE QUANTITATIVELY CHALLENGED AND SIMPLIFICATIONS FOR THE HABITUAL SKEPTIC

For the quantitatively challenged or the beginning modeler: If using mass balance equations is a challenge, the best two pieces of advice are to *never* skip steps when rearranging and substituting into equations and *always* check units. Incorrect units are a clear indication that the answer is wrong! The abbreviations for units used here are:

- L^3 = length cubed or volume (e.g., cubic meters)
- M = mass (e.g., grams, moles)
- P = pressure (e.g., pascal, atmosphere)
- T = time (e.g., seconds, minutes, hours, days, years)

The units for the symbols used in Equation 5.1 through Equation 5.9 are included below. The subscripts describe the relevant environmental phase. For example, L^3_{phase} is the volume of the environmental phase of interest.

- Bioconcentration factor (BCF) = $L^3_{\text{water}}/M_{\text{biota}}$
- Concentration (C) = $M_{\text{chemical}}/L^3_{\text{phase}}$ or $M_{\text{chemical}}/M_{\text{phase}}$
- Degradation constant (k) = T^{-1} or $1/T$
- Flow (Q or G) = $L^3_{\text{water or air}}/T$
- Fugacity (f) = P
- Fugacity capacity (Z) = $M/(P \times L^3)$
- Half-life ($t_{1/2}$) = T
- Henry's constant (H_c) = $(P \times L^3_{\text{water}})/M_{\text{chemical}}$
- Henry's constant (H_c^*) = dimensionless
- Organic carbon partitioning coefficient (K_{oc}) = $L^3_{\text{water}}/M_{\text{organic carbon}}$
- Octanol-water partition coefficient (K_{ow}) = dimensionless
- Sediment/soil distribution coefficient (K_d) = $L^3_{\text{water}}/M_{\text{soil or sediment}}$

For the habitual skeptic: You may think that model simplifications such as closed systems, equilibrium, or steady state are useless since environmental systems rarely, if ever, display these conditions. These simplifications leading to imperfect models can provide a solid foundation for countless applications. To name a few, these imperfect models can:

- Provide relative comparisons of one chemical's persistence, fate, and transport to another chemical or with different environmental conditions.
- Aid in determining what processes control the fate and transport of a chemical or where a chemical is likely to be at the greatest levels. With this information needs for future research or sampling efforts can be identified.
- Aid in decision making when further data collection is not possible.
- Provide a screening tool for predicting fate and transport of new chemicals.

The environment is complex, so no model will ever capture all of the important details needed to describe fate and transport exactly. The reality is that simplifications must be

made. Given this, the challenge is to be a critical thinker instead of a habitual skeptic. A critical thinker carefully evaluates the context in which the models were developed and applied. A critical thinker confirms that the assumptions of the model are explicitly stated. With this knowledge, the limitations of the models can be identified. It can then be confirmed that the results are interpreted within the context of the assumptions, and that implications are not extrapolated beyond the limitations of the model. Finally, an important goal for all critical thinkers is to find ways to contribute new ideas, for this is how advances will be made in the environmental sciences.

is C_b for each of these, but the denominator is the concentration of the chemical in sediment, diet, and from all sources, respectively. These concepts are further developed in Arnot and Gobas (2006).

K_{oc} ($L_{water}/kg_{organic\ carbon}$) is the organic carbon partitioning coefficient. Both K_{oc} and K_d deal with sorption of a chemical (the sorbate) to a solid substance (the sorbent). *Sorption* is a general term that includes both adsorption (the accumulation of the sorbate at the surface of the sorbent) and absorption (the incorporation of the sorbate into the sorbent). By definition,

$$K_{oc} = \frac{\text{concentration of chemical sorbed to organic carbon}}{\text{concentration of chemical in water}} = \frac{C_{oc}}{C_w} \text{ at equilibrium}$$

Soils, sediment, suspended solids in aquatic systems, and atmospheric particulate matter (aerosols) may contain organic carbon as a component. It has been established that organic carbon is the primary sorbent for nonpolar organic chemicals when the fraction of organic carbon (foc) in the soil/sediment is > 0.001. K_{oc} values are widely available and require knowledge of the organic carbon content of any compartments having a sorbent. As with BCF, if the K_{oc} has not been determined for a chemical, it often can be estimated from the K_{ow}. An equilibrium partitioning (EqP) approach using K_{oc} and K_{ow} values can be used to derive equilibrium sediment benchmarks (ESBs) to protect benthic organisms from contaminant exposures. The U.S. EPA has recommended K_{oc}-based procedures for ESB derivations for endrin, dieldrin, and PAH mixtures (Berry et al. 2003a, 2003b; Hansen et al. 2003).

K_d ($L_{water}/kg_{sorbent}$) is the sediment/soil distribution coefficient, where

$$K_d = \frac{\text{concentration of chemical sorbed to soil or sediment}}{\text{concentration of chemical in water}} = \frac{C_s}{C_w} \text{ at equilibrium}$$

This coefficient is also represented as a sediment/soil partition coefficient (K_p). K_d is highly dependent on the sorbent, sorbate, and environmental properties. K_d can be empirically determined for a soil or sediment, but several estimation methods are available. For example, for nonpolar organic chemicals, when the foc is >0.001, $K_d = K_{oc}$(foc). This relationship is also a good first estimate for polar organic chemicals. Other methods used to estimate K_d or K_p (for metals, and polar and ionizable organic chemicals) are included in Boethling and MacKay (2001) and U.S. EPA (2002).

H_c or K_H is Henry's law constant, where H_c can be defined as

$$H_c = \frac{\text{concentration of chemical in air}}{\text{concentration of chemical in water}} = \frac{C_a}{C_w} \text{ at equilibrium}$$

Caution is urged when working with H_c since it can also be defined as the inverse of its definition here. This constant can be dimensionless or have units of PL^3/M. For clarity, a dimensionless Henry's law constant can be represented as H_c^*. A simple calculation can be used to convert between H_c^* and H_c using the temperature (in K) and the gas constant (R = 0.821 L atm/mol K), where

$$H_c^* = \frac{H_c}{RT}$$

if the chemical's solubility in water is less than a few percent (MacKay and Shiu 1981). H_c can often be estimated as

$$\frac{VP_{chemical}}{c_{w,sat}} = \frac{\text{vapor pressure of the chemical}}{\text{maximum solubility of the chemical in water}}$$

H_c is highly dependent on the temperature of the system.

When equilibrium is assumed, the partitioning coefficients can be substituted into the mass balance equations. In a closed or open system at steady state, these substitutions will often allow for algebraic solutions to the mass balance equation.

Example of the Flame-Retardant Contaminated Fish

Assume a closed system at equilibrium and steady state. The compartments that make up the system are water, air, and fish. The temperature is 25°C. It is known that commercial octaBDE containing 10 mg of BDE-153 was added to the system (Figure 5.8). The volume of each compartment is known and the partitioning coefficients (K_{ow} and H_c) can be looked up for BDE-153 (see Table 5.1, the PBDE section). What is the concentration and mass of the BDE-153 in the fish?

Known:

$$V_a = 2.0\text{E+11 m}^3$$

$$V_w = 1.5\text{E+8 m}^3$$

$$V_{\text{fish lipids}} = 0.2 \text{ m}^3$$

$$\text{Log } K_{ow} = 8.55,\ K_{ow} = 10^{\log Kow} = 10^{8.55} = 3.55\text{E+8}$$

$$H_c = 4.71\text{E-7 atm-m}^3\text{/mol},\ H_c^* = \frac{4.71\text{E-7 atm m}^3\text{/mol}}{(8.21\text{E-5 m}^3\text{ atm/mol K})(298\text{ K})} = 1.93\text{E-5}$$

In solving this mass balance equation, **unknown values are identified with bold text**. Using Equation 5.4 as the starting point, the three compartments can be included as

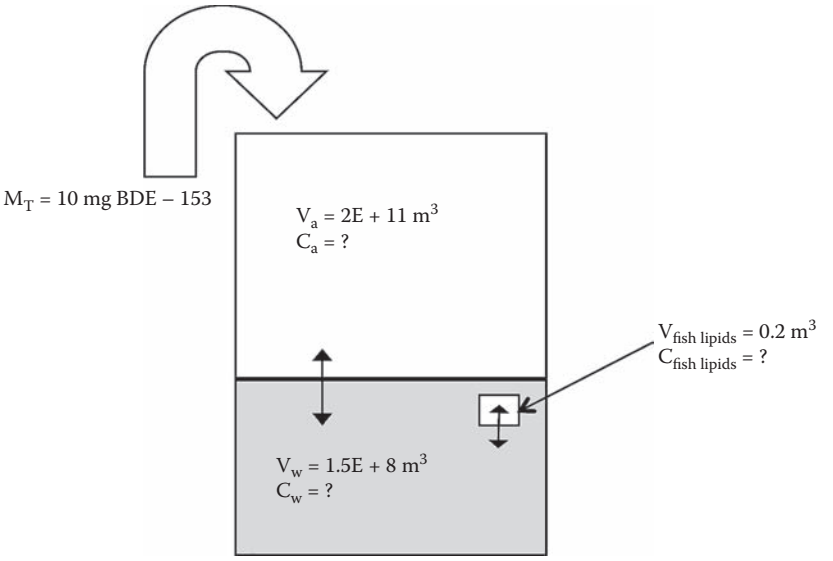


Figure 5.8 Conceptual model of the flame-retardant contaminated fish.

$$M_T = \boldsymbol{C_w} V_w + \boldsymbol{C_a} V_a + \boldsymbol{C_o} V_{fish\ lipids}$$

Rearrange the equilibrium ratio between water and air (H_c), and between water and octanol (K_{ow}):

$$\boldsymbol{C_a} = \boldsymbol{C_w}(H_c^*) \quad \text{and} \quad \boldsymbol{C_o} = \boldsymbol{C_w}(K_{ow})$$

Substitute into the mass balance equation:

$$M_T = \boldsymbol{C_w} V_w + \boldsymbol{C_w} H_c^* V_a + \boldsymbol{C_w} K_{ow} V_{fish\,lipids}$$

Factor out the C_w:

$$M_T = \boldsymbol{C_w}(V_w + H_c^* V_a + K_{ow} V_{fish\,lipids})$$

Rearrange to solve for C_w:

$$\boldsymbol{C_w} = \frac{M_T}{(V_w + H_c^* V_a + K_{ow} V_{fish\,lipids})} =$$

$$\frac{10\ \text{mg}}{1.5\text{E+8}\ \text{m}^3 + (1.93\text{E-5} \times 2.0\text{E+11}\ \text{m}^3) + (3.55\text{E+8} \times 0.2\ \text{m}^3)} = 4.15\text{E-8}\ \text{mg/m}^3$$

Now that C_w is known, solve for C_a and C_o:

$$\boldsymbol{C_a} = C_w(H_c^*) = 4.15\text{E-8}\ \text{mg/m}^3\ (1.93\text{E-5}) = 8.6\text{E-13}\ \text{mg/m}^3$$

$$\boldsymbol{C_o} = C_w(K_{ow}) = 4.15\text{E-8}\ \text{mg/m}^3\ (3.55\text{E+8}) = 1.6\text{E+1}\ \text{mg/m}^3$$

Based on these calculations, the greatest concentration of BDE-153 would be in the fish lipids. Be careful about this answer, though; using the M = CV relationship, the greatest mass of BDE-153 is in the water, then the fish, then the air.

$$\boldsymbol{M_w} = (4.15\text{E-}8 \text{ mg/m}^3) * (1.5\text{E+}8 \text{ m}^3) = 6.67 \text{ mg}$$

$$\boldsymbol{M_o} = (1.6\text{E+}1 \text{ mg/m}^3) * (0.2 \text{ m}^3) = 3.16 \text{ mg}$$

$$\boldsymbol{M_a} = (8.6\text{E-}13 \text{ mg/m}^3) * (2\text{E+}11 \text{ m}^3) = 0.17 \text{ mg}$$

5.8 The Fugacity Approach

A common approach to multimedia fate and transport models is the fugacity approach developed by MacKay (1979, 2001). Fugacity (f) is a thermodynamic concept defined as the escaping tendency of a molecule or substance, and it has units of pressure. At equilibrium, the fugacity in all environmental phases is equal. Fugacity can be related to concentration in a phase by a fugacity capacity constant (Z), where $C_i = f_i Z_i$. The Z values are calculated for a specific chemical with equations derived for each environmental phase and typically have units of mol/atm m^3. For example, the fugacity capacity for air (Z_a) = $1/RT$, whereas the fugacity capacity for water (Z_w) = $1/H_c$. It should be apparent how properties of the environment (e.g., temperature) and properties of the chemical (e.g., the Henry's law constant) will affect chemical fate.

The mass balance equations described earlier (e.g., Equations 5.4 and 5.6) can be used with the fugacity approach, by substituting $f_i Z_i$ for C_i. For example, in a three-compartment system (e.g., air, water, and sediment) that is closed and at steady state, the mass balance equation would be

$$M_T = f_a Z_a V_a + f_w Z_w V_w + f_s Z_s V_s \tag{5.8}$$

If the system is assumed to be at equilibrium, then $f_a = f_w = f_s$ and the fugacity can be solved with

$$f = \frac{M_T}{(Z_a V_a + Z_w V_w + Z_s V_s)} \tag{5.9}$$

Similar substitutions can be made with Equation 5.6, although the symbol for flow is G in the fugacity models, and D is used to represent GZ and kVZ.

Several computer models that use the fugacity approach are available through the Canadian Center for Environmental Modeling (CEMC). The basic models, with their assumptions, include level I (closed system at equilibrium and steady state), level II (open system at equilibrium and steady state), level III (open system at steady state and nonequilibrium), and level IV (open system, unsteady state, and nonequilibrium). The acceptance of the fugacity modeling approach is evidenced by its widespread use. For example, the U.S. EPA PBTprofiler, which is used for the NCP of TSCA and reporting requirements under the TRI of EPCRA, uses a level III fugacity model. CalTox is another level III model used by the California EPA to predict human exposures to contaminants. The European Union System for the Evaluation of Substances (EUSES) is also a level III fugacity model used to assess the general risk of substances.

5.9 Bioconcentration versus Biomagnification

One of the potential fates of contaminants is the accumulation of chemicals in or on an organism. This is often reported as a concentration or body burden. The units for the concentration are typically mass of chemical per mass (or volume) of organism. The concentration may be measured in specific compartments of the organism, such as lipids, in which case the units would be mass of chemical per mass of lipids. Body burden is the amount of chemical in an individual organism and has units of mass of chemical per individual.

Bioconcentration and biomagnification are special cases of bioaccumulation, which is the net accumulation of a chemical in or on an organism from all sources in the environment. Bioconcentration is the accumulation of a chemical in or on an organism when the source of chemical is solely water. This term was created specifically for the field of aquatic toxicology and explains why the BCF relates the chemical concentration in the organism to the concentration in the water. The reality of bioconcentration is that in a field exposure, it would be challenging to identify what portion of a body burden came from water versus the other sources (e.g., food and sediment); thus, bioconcentration is a concept primarily used in controlled laboratory studies, and fate and transport models. Biomagnification, also called biological magnification, is the accumulation of a chemical in an organism when the source of a chemical is primarily food and there is an increase in the organism concentration as trophic levels increase. Lipophilic chemicals, such as PCBs, PBDEs, dioxins, DDT, and other chlorinated organic pesticides that are poorly metabolized, are likely to biomagnify.

It is important to understand that not every chemical that can bioaccumulate will also biomagnify. This is best demonstrated with PAHs. Benzo(a)pyrene (BaP) has an estimated log K_{ow} of 6.11 (Table 5.1), which indicates that it is lipophilic and likely to bioaccumulate and biomagnify. Since K_{ow} is determined based on partitioning into octanol, it does not take into account metabolic processes that occur in the organism. Some aquatic invertebrates, such as marine bivalves, do not readily metabolize PAHs, while benzo(a)pyrene is rapidly metabolized by the mixed-function oxidase (MFO) enzyme system in fish and eliminated (James 1989; Varanasi et al. 1989). In a field situation, elevated benzo(a)pyrene concentrations would be present in mussels, indicating bioavailability and bioaccumulation of the PAH. Trophic transfer would occur since consumers, such as fish, would be exposed to the accumulated PAHs and any metabolites in the mussel. Because the fish rapidly metabolize the PAH, however, the benzo(a)pyrene would not bioaccumulate in the fish or biomagnify in this food web. This example underscores the need to understand properties of the organism (e.g., rate of PAH metabolism).

Other properties of an organism that may be important to understand include the lipid content, sex, age, and trophic guild. For lipophilic chemicals, an organism with a higher lipid content will have a greater body burden. Zebra mussels with a higher lipid content had a greater BCF and uptake kinetics for benzo(a)pyrene and 2,2',4,4',5,'5-hexachlorobiphenyl than did mussels with a lower lipid content (Bruner et al. 1994). Marine mammals provide good examples of the effect of sex on body burdens of liopophilic chemicals. During lactation, accumulated lipophilic chemicals in the adult are redistributed to the milk and adult females pass the chemicals on to their calves. Gestation provides another opportunity to transfer chemicals from the mother to the fetus. These lactational and gestational processes, that are called maternal transfer, result in a decrease in the maternal body burden and an increase in the calf's body burden. A study with stranded pilot whales found the blubber concentrations of ΣPCBs and ΣDDTs were lower in sexually mature females than in young females not of reproductive age, and lower in females than males when length was taken into account (Tilbury et al. 1999). In this same study, the organochlorine concentrations

were lower in maternal blubber than in the fetus, supporting maternal transfer of these chemicals. The importance of age and trophic level is demonstrated by another study. Researchers measured the greatest PCB congener lipid concentrations and the second greatest dioxin/furan concentrations in wild chinook salmon compared to wild chum, coho, sockeye, and pink salmon. The difference was attributed to the greater age at maturity of the chinook salmon, since they would have more time to accumulate the lipophilic chemicals, and to the higher trophic level at which the chinook salmon feed, since these chemicals do biomagnify (Ikonomou et al. 2007). Species differences and other life history traits, such as geographic origin, migration route, reproductive history, and birth order, as they relate to bioaccumulation of lipophilic chemicals, have been discussed in more detail by several authors (e.g., Ross et al. 2000; Missildine et al. 2005; Hickie et al. 2007).

A final organism property of interest is related to the catabolism of fats. During hibernation, migration, starvation, and seasonally caused temperature changes, organisms utilize stored fats. When the fats are catabolized, the chemicals are released into the blood and transported systemically where they may interact with bioactive target sites and cause toxicity, or they may be metabolized and possibly eliminated. The released chemicals and metabolites that remain after systemic distribution will ultimately repartition between the organism's body compartments with a greater concentration in the remaining lipids. Christensen et al. (2007), for example, found that the ΣPCBs and ΣPBDEs increased 2.21 and 1.58 times, respectively, when prehibernation fat concentrations were compared to posthibernation concentrations in grizzly bears. Ewald et al. (1998) found muscle lipid concentrations of ΣPCBs increased as lipid content decreased with migration distance in sockeye salmon. Several researchers have investigated effects of starvation on pesticide remobilization in avian species. For example, lipid content in caged robins decreased following starvation, resulting in relocation of p,p'-DDT and p,p'-DDE to brain tissue (Södergren and Ulfstrand 1972). Finally, Montie et al. (2008) suggested that seasonal temperature increases might result in a decrease in adipocyte lipids of dolphins since there is less need for the insulating properties of the lipids. A redistribution of PCBs from the deep blubber into the circulatory system is a proposed result.

5.10 Bioavailability

Bioavailability in environmental toxicology can be defined as the degree to which a chemical in the environment is available to reach a target site on or in a living organism. The target site may be at the site of exposure (local toxicity), or it may be inside the organism requiring the chemical to first be absorbed across membranes (e.g., across the dermis, gastrointestinal tract, or gills) and transported systemically to the target site. The target site may be a biomolecule that the chemical interacts with to cause an effect, such as ion channels at the gill surface or a gene on DNA. The target site may also be a biomolecule that sequesters or detoxifies the chemical so that no effect occurs in the exposed organism (e.g., lipids or MFO). Chemicals that have accumulated in or on an organism are, by definition, bioavailable to that organism, but it is important to recognize that those accumulated chemicals may also be bioavailable to consumers.

The concept of bioavailability connects environmental fate and transport with environmental toxicology. For example, chemicals bound to organic carbon (determined with K_{oc}) are generally considered less bioavailable to aquatic organisms than unbound chemicals. Physical processes, such as sedimentation, decrease the likelihood of interaction between organisms and a chemical. This can decrease bioavailabilty. Environmental properties, such as pH, temperature, and redox conditions, can also influence bioavailability. For example, van Weerelt et al. (1984) found that

Cr^{+6} was bioavailable in water because it concentrated significantly in soft tissues of barnacles. In its reduced form, however, the Cr^{3+} precipitated in the seawater and was filtered and passed through the gastrointestinal system of the barnacles without accumulating. From these examples and the ones that follow, it should be clear that knowledge of chemical concentration in the environment alone is not enough to assess the toxicity of many contaminants since bioavailability is typically not accounted for by those measurements.

5.10.1 Measures of Bioavailability

Since bioavailability requires the accumulation of a chemical in or on a living organism, the most direct way to assess bioavailability is to expose organisms to the contaminated environment of interest and measure the amount of accumulated chemical in the organism. When conducted with animals, these assessments typically include a period of depuration following the exposure so that chemicals in the gut and intestines are not included in the accumulated concentrations. Several protocols have been developed using earthworms to assess bioavailability of soil contaminants since they directly contact the soil and have low MFO activity, which minimizes metabolism of many organic chemicals (Dean 2007). Bivalve mollusks have been used to assess bioavailability of chemicals in aquatic environments. As with earthworms, MFO activity is low so that metabolism of many organic chemicals is not a confounding factor in determining bioavailability. Additionally, they are sessile and attached, so accumulated chemical levels are representative of the environment from which they were collected (McElroy et al. 1989). The National Oceanic and Atmospheric Association (NOAA) Mussel Watch Program provides an example of the widespread acceptance of bivalves as a tool for monitoring bioavailable contamination. In the program, which began in 1986, over 140 contaminants are analyzed annually or less frequently in resident mussels and oysters collected from nearly 300 sites in the Great Lakes and coastal United States (Kimbrough et al. 2008).

Other techniques have been designed to assess the influence of desorption of chemicals from sediments during digestive solubilization in the gut. In the method developed by Mayer et al. (1996), digestive fluids from benthic invertebrates are collected and incubated with contaminated sediment. The solubilized concentrations of contaminants are then measured. When this method was applied with lugworm and sea cucumbers, significantly more Cu and PAHs were solubilized in their digestive fluids than in seawater alone. Additionally, the lugworm digestive fluids solubilized more copper than the sea cucumber's, an example of an organism-specific property that may influence uptake and toxicity of metals.

In situ measurements of bioavailability have been conducted with semipermeable membrane devices (SPMDs). The SPMD is a passive sampler filled with a high molecular weight lipid that mimics fatty tissue, such as triolein ($C_{57}H_{104}O_6$), encased in a low-density polyethylene membrane tube. Following deployment and acclimation in the field (water or air), the samplers are collected and the nonpolar organic contaminants (e.g., PAHs, PCBs, polychlorinated dibenzo-*p*-dioxins [PCDDs]) are extracted from the triolein into an organic solvent and analyzed. Since deployment can be anywhere from days to months, the accumulated chemical concentrations represent a time-weighted average. In a recent study, SPMDs were simultaneously deployed in a lake with caged crucian carp. After 32 days, PAH concentrations in the SPMD and carp muscle tissue were measured and 7-ethoxyresorufin-o-deethylase (EROD) bioassays were conducted. Although the final concentrations were lower in the carp, the pattern of PAH accumulation was similar between the carp and SPMDs, supporting SPMD use as a measure of bioavailable PAHs (Ke et al. 2007). Another passive sampling device often used to measure bioavailable metal contaminants

in sediment is a peeper. A peeper is a vessel filled with deionized distilled water and closed with a dialysis membrane. The peeper is buried in sediment so that contaminants in the interstitial water can diffuse into the peeper. After equilibration, the peeper is removed and contaminants in the peeper are analyzed, with measured concentrations representing bioavailable contaminants (Hesslein 1976; Serbst et al. 2003).

5.10.2 Metal Speciation and the Biotic Ligand Model

With respect to metal toxicity, the speciation of metals is important to consider since this affects the bioavailability of a metal. In aquatic environments, for example, the exact speciation of a dissolved metal depends on the composition and concentration of other dissolved and suspended components in the water. Inorganic ligands such as Cl^- and SO_4^{2-}, for example, may complex with a metal to form a metal:chloride or metal:sulfate species. Dissolved organic carbon (DOC) may also complex the metal and contribute another species of the metal to solution. It is clear, therefore, that site-specific characteristics, such as salinity, alkalinity, and dissolved organic carbon content, can alter the bioavailability of a metal.

The free ion activity model (FIAM) is based on the concept that the ionized uncomplexed metal is the toxic form of a metal in an aquatic environment (Morel 1983; Campbell 1995). Further work in freshwater systems has elucidated the modes through which the free ion of certain metals (e.g., Cu, Ag, Co, Zn, Cd, Pb) causes acute toxicity. These metals bind with ion channels at the gill surface of fish or a similar site on aquatic invertebrates. These channels, which typically regulate essential elements such as Ca^{2+} and Na^+, do not allow passage of the essential elements if the toxic metals are present. This understanding of the mode of action of acute metal toxicity led to the development of modeling tools that can be used to predict site-specific toxicity, with the characteristics of the site waters taken into account (Paquin et al. 2002). The biotic ligand model (BLM) is the most advanced of these modeling tools available.

A key feature of the BLM is that understandings of the physiological mode of action and chemical thermodynamics have been combined (Figure 5.9). The ion channels in aquatic animals are the biotic ligands. Sorption constants for the different metals to the biotic ligands, which have been experimentally and theoretically determined, are included in the database of the BLM along with the stability constants for the metals with the inorganic and organic ligands dissolved in the water. Cations dissolved in the water, such as Ca^{2+} and Mg^{2+}, can also bind to the biotic ligand and effectively compete with the toxic metal for those binding sites. Since an increase in the concentration of these competing cations results in less of the toxic metal bound to the biotic ligand, these competing cations are protective. This explains why an increase in hardness reduces the toxicity of metals. From a regulatory perspective, the BLM has been accepted by the U.S. EPA for guiding the water quality criteria for copper (HydroQual 2007; U.S. EPA 2007). Visual MINTEQ version 2.53 (Gustafsson, 2007) is another chemical equilibrium computer model that can be used to predict metal toxicity to certain aquatic organisms using the BLM approach. Recently a terrestrial BLM (TBLM) for Ni and Cu bioavailability in soils was developed (Thakali et al. 2006a, 2006b).

5.10.3 Acid Volatile Sulfide (AVS)/Simultaneously Extracted Metals (SEM) in Anoxic Sediment

In sediments, the concentration of contaminants present in the interstitial water is a good approximation of the bioavailable concentration. This is the underlying theory of the ESBs for the nonionic

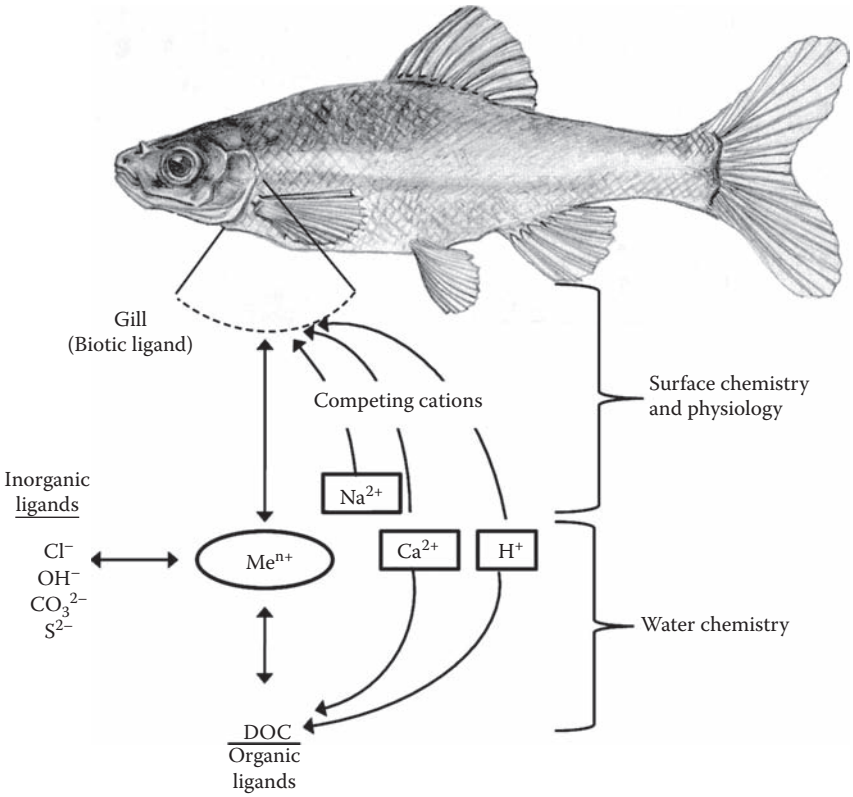


Figure 5.9 Conceptual model of the biotic ligand model. (After Paquin, P. R. et al., *Comp. Biochem. Physiol. C*, 133, 3–35, 2002. Art credit: Rob Harper, 2009.)

organic chemicals endrin, dieldrin, and PAHs, where K_{oc} is used to predict interstitial water concentrations. An ESB for metal mixtures (Cd, Cu, Pb, Ni, Ag, and Zn) using the EqP approach has also been proposed (Hansen et al. 2005), but partitioning is thought to include both organic carbon and acid volatile sulfide (AVS) (Di Toro et al. 1990, 2005).

The bioavailability of divalent metals in anoxic sediments has experimentally been shown to be controlled by AVS (Di Toro et al. 1990, 1992). In these sediments, sulfur is present in a reduced form and iron can exist as amorphous FeS(s). Free metal (Me^{2+}) can displace the Fe to form soluble Fe^{2+} and the precipitate MeS(s) in the interstitial water:

$$Me^{2+} + Fe(s) \leftrightarrow MeS(s) + Fe^{2+} \tag{5.10}$$

Since the free metal is the bioavailable form, the production of MeS(s) reduces the bioavailability of the toxic metal. To measure AVS, sediment is digested in cold 6 N HCl and volatile sulfides are trapped and measured. The Cd, Cu, Pb, Ni, Ag, and Zn present in the HCl digest are the simultaneously extracted metals (SEM). The molar concentrations of SEM and AVS are then compared. If SEM < AVS, toxicity is not predicted to occur since there is enough sulfide to precipitate the metals. If SEM > AVS, toxicity may or may not occur. In this case, there is not enough sulfide to precipitate the toxic metal, so it is predicted to be present in a bioavailable form as Me^{2+} and available for uptake and toxicity. Because other environmental characteristics, such as

the presence of organic carbon, may also reduce toxicity, it is a challenge to predict toxicity with only an understanding of SEM and AVS. Models that also account for organic carbon should be utilized when possible (e.g., Di Toro et al. 2005).

Other limitations related to a focus on only AVS and SEM to predict toxicity are demonstrated by De Jonge et al. (2009). These researchers found that *in situ* bioaccumulation of metals, including Ni and Pb, in chironomids and tubificid worms was not related to AVS, with significant accumulation occurring when SEM << AVS. Instead, total metal concentrations in the sediment, and organic carbon and clay content normalized total metals were the factors that best correlated with accumulation. As the authors and Lee et al. (2000) point out, benthic invertebrates are exposed to metals from both the pore water and ingestion of sediment-bound metals. With ingestion, metal speciation is likely to be modified in the gut lumen because of the different chemical environment, meaning that SEM/AVS in the sediment is not the primary predictor of toxicity. Other limitations of the model include seasonal and spatial variations in AVS concentrations, and insufficient knowledge of how well the model works for environmentally realistic conditions (van den Hoop et al. 1997; Lee et al. 2000; De Jonge et al. 2009).

5.11 Summary

Knowledge of the fate and transport of contaminants is a necessary component of environmental toxicology. The route of exposure, amount of exposure, and chemical form in the environment can be established with this knowledge. This leads to better sampling and experimental designs, which improve the environmental toxicological assessments and predictions. Fate and transport may seem to be an overwhelming topic, but rest assured that this field has involved years of developing and integrating expertise from many scientific disciplines. Contributions to our understanding of fate and transport will continue to come from multiple disciplines, including toxicology. Emerging toxicological issues will help to direct research in the fate and transport of chemicals in the environment, which will promote and enhance improvements to both fields of study. Opportunities to contribute to and advance these disciplines are plentiful, particularly when experts from each understand both the utility and limitations of the other discipline.

Study Questions

1. What are two characteristics of contaminants in environmental toxicology that need to be considered?
2. List the three broad areas to be considered when assessing fate and transport of a chemical.
3. Define *advection*, *diffusion*, and *dispersion* as contaminant transport methods.
4. Contrast the effects of advection versus diffusion and dispersion on contaminant transport.
5. Define and describe *LRAT*.
6. What are SVOCs? What is cold trapping?
7. Describe the terms *global distillation* and *grasshopper effect*.
8. What is global fractionation?
9. Define and describe *persistence*. What are the major mechanisms through which persistence is affected?
10. What are POPs? What are PBTs?
11. What is the REACH criteria for persistence?

12. Define and give an example of *biotransport*.
13. What is a degradate? How does it compare to the parent chemical?
14. Describe the role hydrolysis reactions play in degradation.
15. Describe photolysis. How is it involved in decontamination?
16. Explain the box model method. What are the three additional assumptions?
17. What factors does a critical thinker need to consider when using the box model?
18. Define *fugacity*. Of what importance is it?
19. What is body burden? Define *bioconcentration* and *biomagnification*.
20. Explain an example of maternal transfer of body burden.
21. Define *bioavailability*.
22. Describe two bioavailability assessment techniques.
23. How does speciation of a metal factor in the bioavailabilty of the metal?
24. Explain FIAM.
25. How can AVS and SEM predict toxicity?
26. What major ideas are presented in the summary section of this chapter?

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Chapter 6

Uptake and Modes of Action

Now that the book has covered the fundamentals of environmental toxicology, toxicity testing, data analysis, and the fate and transport of chemicals, it is time to examine in detail the toxicological effects of chemicals. This chapter details damage at several levels. Damage can occur at the system level, but many of the effects are initiated at the molecular scale. This chapter presents several modes of action and the molecular basis for the interaction between the xenobiotic and the affected molecular receptor.

A key point of this chapter is the relationship between the structure of the xenobiotic and the toxicity. There are several ways of doing the analysis, from statistical models to detailed computer analysis and computation.

6.1 The Damage Process

An environmental pollutant at a sufficiently high concentration can critically influence the physiological processes of a living organism. In order for a pollutant to exert its toxicity on an organism, it must first enter the host and reach its target site. Although it is difficult, if not impossible, to generalize the precise mechanism by which each specific pollutant affects living organisms, some features that are shared by different pollutants are presented here.

6.2 Atmospheric Pollutants and Plants

An atmospheric pollutant-induced plant injury may follow a pathway that includes exposure, uptake, transport, storage, metabolism, and excretion (Figure 6.1). To cause injury to any vegetation, an air pollutant must first enter the plant in question. Although the atmospheric concentration of a pollutant is important, the actual amount that gets into the plant is of more concern. The conductance through the stoma, which regulates the passage of ambient air into the cells, is especially critical. Uptake is dependent upon the physical and chemical properties along the gas-to-liquid diffusion pathway. Pollutant flow may be restricted by the physical structures of the leaf or scavenging by competing chemical reactions. The leaf orientation and morphology, including epidermal

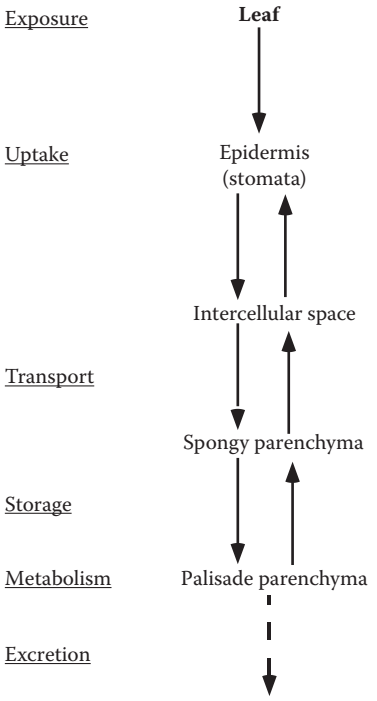


Figure 6.1 Schematic pathway of plant injury induced by atmospheric pollutants.

characteristics, and air movement across the leaf are important determinants affecting the initial flux of gases to the leaf surface. More pollutant enters a leaf when there is some air movement.

Stomatal resistance is vitally important in affecting pollutant uptake. The resistance is determined by stomatal size, number, anatomical characteristics, and the size of the stomatal aperture. Little or no uptake will occur when the stoma is closed. Stomatal opening is regulated by internal CO_2 content, light, humidity, temperature, water availability, and nutrient status, particularly potassium (K) levels. Research shows that K^+ ions in the guard cells regulate the guard cell turgor and opening of the stoma. It should be mentioned that although stomatal resistance is an important factor regulating pollutant uptake, genetic sensitivity of individual species and cultivars is the overriding factor determining plant injury. Of particular importance is the pollutant concentration within the leaf, more so than the ambient concentration itself, which is considered to be most critical to plant health.

6.2.1 Plant Injury

The epidermis is the first target of atmospheric pollution as the pollutant first passes through the stomata of the epidermal tissue. In passing through the intercellular spaces, a pollutant may dissolve in the surface water of the leaf cells, affecting cellular pH. A pollutant may not remain in its original form as it passes into solution. In fact, it may be converted into a different form, which may be more reactive and toxic than the original form. For example, the free radicals that are formed following the initial reaction in the cell may exert more serious damage to cellular materials than they otherwise would. The pollutant, either in its original form or in an altered form, may then react with different cellular components such as cytoplasmic membrane or membranes of the organelles,

and enzymes or their cofactors, coenzymes, and substrates, thus affecting cellular metabolism and causing plant injury. Changes in the ultrastructure of various organelles such as chloroplasts and mitochondria can impair photosynthesis and energy metabolism of the plant cell.

As an environmental pollutant moves in the liquid phase from the substomatal regions to the cellular sites of perturbation, it may encounter many obstacles along the pathway. Scavenger reactions between endogenous components and the pollutant may occur, influencing the toxicity of the pollutant. For example, ascorbate, which occurs widely in plant cells, may absorb or neutralize a pollutant. On the other hand, an oxidant such as ozone may react with membrane material to form other toxic substances, such as aldehydes, ketones, and various free radicals, which in turn adversely affect the cell.

Certain air pollutants can inhibit the activity of some enzymes in the cell. For instance, heavy metals such as Pb and Cd may inhibit the activity of an enzyme by disrupting the function of its active site containing sulfhydryl (–SH) group. Similarly, SO_2 may oxidize and break apart the sulfur bonds on critical enzyme molecules in the membrane, impairing cellular function.

The net result of all this is an unhealthy plant. Even before visible symptoms are discernible, an exposed plant may be weakened and its growth impaired. Ultimately, visible symptoms characterizing the effect of a specific pollutant may appear, and death of the plant may follow.

6.2.2 Vertebrates

A pollutant may get into an animal through a series of pathways. The routes may include exposure, uptake, transport, storage, metabolism, and excretion. Figure 6.2 shows the pathways a pollutant may follow as it enters a vertebrate.

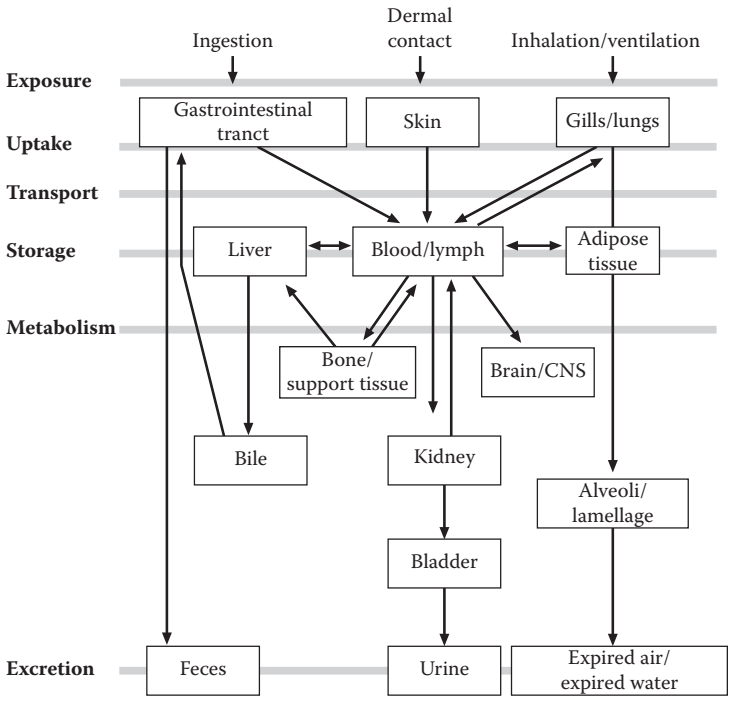


Figure 6.2 Routes of absorption, translocation, and excretion of toxicants in a vertebrate.

6.2.2.1 Exposure

As mentioned earlier, exposure to a pollutant by a host organism constitutes the initial stage in the manifestation of toxicity. In a mammalian organism, exposure of the body occurs through dermal or eye contact, inhalation, or ingestion.

6.2.2.2 Uptake

The immediate and long-term effects of a pollutant are directly related to the mode of entry. The portals of entry for an atmospheric pollutant are the skin, gastrointestinal tract, and lungs. For a toxicant, by far the most common means of entry into the body system is by absorption through the skin. In this case, the points of entry are through the hair follicles, sweat glands, and open wounds.

To be taken up into the body and finally carried to the cell, a pollutant must pass through a number of biological membranes. These include not only the peripheral tissue membranes but also the capillary and cell membranes. Thus, the nature of these membranes and the chemical and physical properties of the toxicant in question are important factors affecting uptake. The mechanisms by which chemical agents pass through the membranes include: (1) filtration through spaces or pores in membranes; (2) passive diffusion through the spaces or pores, or by dissolving in the lipid material of the membrane; (3) facilitated transport, whereby specialized transport systems carry water-soluble substances across the membrane by a lipid-soluble "carrier" molecule, which complexes with the chemical; and (4) active transport, which requires energy and a carrier. Of these mechanisms, active transport is the only one where a toxicant can move against a concentration gradient, i.e., move from a low-concentration compartment to a high-concentration compartment. This is the reason for the need of energy expenditure.

6.2.2.3 Transport

Once absorbed, a rapid transport of the substance throughout the body takes place. A pollutant or chemical agent may be transported via the blood stream or lymphatic system and distributed to various body tissues, such as those of storage depots and sites of metabolism or biotransformation.

6.2.2.4 Storage

The storage depots include the liver, lungs, kidneys, bone, adipose tissue, and others. They may or may not be the sites of the toxic action of the agent. It is possible that a toxicant that is transported to a storage depot may be stored there only temporarily; under certain physiological conditions, the agent may be removed from the depot and translocated again. Similarly, following biotransformation, a toxic agent may be transported to a storage depot, or to a site where it is finally excreted. Translocation of a toxicant among tissues may be carried out through binding to a blood protein—a lipoprotein, for example.

6.2.2.5 Metabolism

The metabolism of toxicants may occur at the portals of entry or in such organs as the liver, lungs, gastrointestinal tract, skin, and kidneys. The liver plays a central role in metabolizing xenobiotics (chemicals foreign to the body). A rich supply of nonspecific enzymes in the liver enables it to

metabolize a broad spectrum of organic materials. The reactions involved in the metabolism of these materials include two phases: phases I and II. Phase I reactions involve the introduction of a reactive polar group into the xenobiotic through oxidation, reduction, or hydrolysis, forming a primary metabolite. Phase II reactions, on the other hand, involve conjugation reactions in which an endogenous substance combines with the metabolite, forming a complex secondary metabolite. An important feature of these reactions is the conversion of a lipophilic compound to a more water soluble, and thus a more excretable, metabolite. While many toxicants are detoxified through these processes, others may be activated as well.

6.2.2.6 Excretion

The final step involved in the action of a pollutant is excretion from the body. Excretion may take place through the lungs, kidneys, or intestinal tract. A pollutant may be excreted in its original form or as its metabolite(s), depending on its chemical properties. Excretion is the most permanent means by which toxic substances are removed from the body.

6.3 Mechanisms of Action

The toxic action of pollutants involves compounds with intrinsic toxicity or activated metabolites. These interact with cellular components at their site of action to initiate toxic effects. The effects may be manifested anywhere in the body. The consequence of such action may be reflected in the inhibition of oxidative metabolism and the central nervous system (CNS), or interaction with nucleic acids resulting in carcinogenesis or injury to the reproductive system. The biological action of a pollutant may be terminated by storage, metabolic transformation, or excretion.

Although the precise mechanism by which each of the many environmental pollutants exerts its toxicity remains to be elucidated, four principal mechanisms are described here. In general, a pollutant may cause an adverse effect on a living organism through (1) disruption or destruction of cellular structure, (2) direct chemical combination with a cell constituent, (3) its influence on enzymes, and (4) initiation of a secondary action. These are examined below.

6.3.1 Disruption or Destruction of Cellular Structure

A pollutant may exert its injurious effect on an organ by causing structural damage to its tissues. For example, airborne pollutants such as SO_2, O_3, NO_2, and fluoride are known to be phytotoxic. Sensitive plants exposed to any of these pollutants at a sufficiently high concentration can exhibit structural damage, followed by cellular destruction. Evidence suggests that low concentrations of SO_2 can injure epidermal and guard cells, leading to enhanced stomatal conductance and greater entry of the pollutant into the plant (Black and Unsworth 1980). Similarly, after entry into the substomatal cavity of plant leaves, O_3, or the free radicals produced from it, may react with protein or lipid membrane components and disrupt the cellular structure of the leaf (Heath 1980; Grimes et al. 1983).

When inhaled by animals or humans, sufficient quantities of O_3 and sulfuric acid mists can cause damage to surface layers of the respiratory system. Exposure to high levels of O_3 leads to pulmonary edema (Mueller and Hitchcock 1969), i.e., a leakage of fluid into the gas exchange parts of the lung. This implies that exposure to O_3 can cause disruption of the lung tissue.

6.3.2 Direct Chemical Combination with a Cellular Constituent

A pollutant may combine with a cell constituent and form a complex. This often leads to impaired function. For example, carbon monoxide (CO) in the blood readily binds to hemoglobin (Hb) to form carboxyhemoglobin (COHb):

$$CO + Hb \rightarrow COHb \tag{6.1}$$

Since hemoglobin in the body is essential in the carbon dioxide–oxygen exchange system between the lungs and the tissues, interference with the functioning of hemoglobin following the formation of COHb can be detrimental.

Another example is cadmium (Cd), a highly toxic heavy metal. Once absorbed, Cd in the body is mainly bound to a protein called metallothionein. This protein is involved in the transport and selective storage of Cd. A rather selective accumulation of Cd occurs in the kidneys, leading to eventual tubular dysfunction with proteinuria (Friberg et al. 1974).

6.3.3 Effect on Enzymes

The most distinguished feature of reactions that occur in a living cell is the participation of protein catalysts called enzymes. As with any catalyst, the basic function of an enzyme is *to increase the rate of a reaction*. All protein enzymes are globular, with each enzyme having a specific function because of its specific globular structure. However, the optimum activity of many enzymes depends on the presence of nonprotein substances called cofactors. The molecular partnership of protein-cofactor is termed a holoenzyme and exhibits maximal catalytic activity. The protein component without its cofactor is termed an apoenzyme and exhibits very low activity, or none at all.

Protein	**+ Cofactor**	**→ Protein-Cofactor complex**
apoenzyme: (inactive or less active)	(inorganic ion or organic substance; inactive as a catalyst)	holoenzyme: (optimally active catalyst)

(6.2)

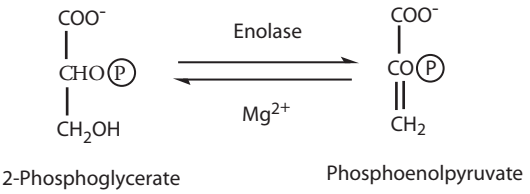
There are two categories of cofactors: the organic and inorganic. The inorganic cofactors include several metallic ions, such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, K^{+}, and Na^{+} ions. The organic cofactors include certain substances of diverse structure, and are usually called coenzymes. Coenzymes are especially important in animal and human nutrition because most of them are vitamins or substances produced from vitamins. For example, following ingestion, niacin (a B vitamin) is converted to either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). Both compounds often act as coenzymes in biological reactions. Environmental pollutants may inhibit the action of enzymes in different ways, as shown below:

1. A pollutant may combine with the active site or sites of an enzyme, thus inactivating it. For example, a heavy metal such as Hg, Pb, or Cd can attach itself to the thiol or sulfhydryl

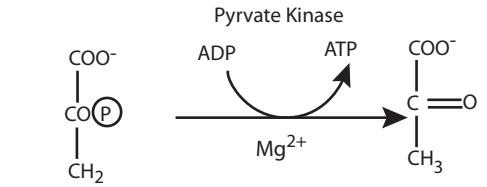
(SH) group on an enzyme molecule, forming a covalent bond with the sulfur atom. This will lead to inactivation of the enzyme if the –SH group is the active site. Transaminases and delta-aminolevulinate dehydratase are susceptible to inhibition by Pb because these enzymes contain the –SH group at their active sites. The interaction involved is shown below:

$$2\ \text{Enz} - \text{SH} + \text{Pb}^{2+} \rightarrow \text{Enz} - \text{S} - \text{Pb} - \text{S} - \text{Enz} + 2\ \text{H}^{+} \tag{6.3}$$

2. Many enzymes require cofactors, often cations, for their activity. These ions provide electrophilic centers in the active site. A pollutant may inhibit an enzyme by inactivating the cofactor involved. For instance, fluoride is known to be a potent inhibitor of enolase, a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Equation 6.4). The resultant phosphoenolpyruvate is then converted to pyruvate, a reaction catalyzed by pyruvate kinase (Equation 6.5). Both enzymes require Mg^{2+} ion for their activity. Fluoride is a potent inhibitor of enolase. Mg^{2+} and inorganic phosphate are believed to form an ionic complex with F^{-} ion, which is responsible for inhibition of the enzyme, apparently by preventing the interaction of the enzyme with its substrate, (a complex of Mg^{2+} and 2-phosphoglycerate).



2-Phosphoglycerate Phosphoenolpyruvate (6.4)



Phosphoenolpyruvate Pyruvate (6.5)

3. A pollutant may exert its toxicity through competition with the cofactor for the active site, thus leading to enzyme inhibition. For example, beryllium (Be) competes with Mg and Mn, while Cd replaces Zn in some enzymes.
4. The activity of an enzyme may be inhibited by the presence of a toxic metabolite. Sodium fluoroacetate, known as rat poison 1080, is extremely toxic to animals. The toxic action, however, is not due to sodium fluoroacetate itself; rather, it is due to a metabolic conversion product called fluorocitrate, which is formed through a reaction commonly known as lethal synthesis. (Figure 6.3). The resulting fluorocitrate is toxic because it inhibits aconitase, the enzyme responsible for the conversion of citrate into cis-aconitate and then into isocitrate in the tricarboxylic acid (TCA) cycle. Inhibition of aconitase results in citrate accumulation. The cycle stops for lack of metabolites, leading to disruption of cellular energy metabolism.

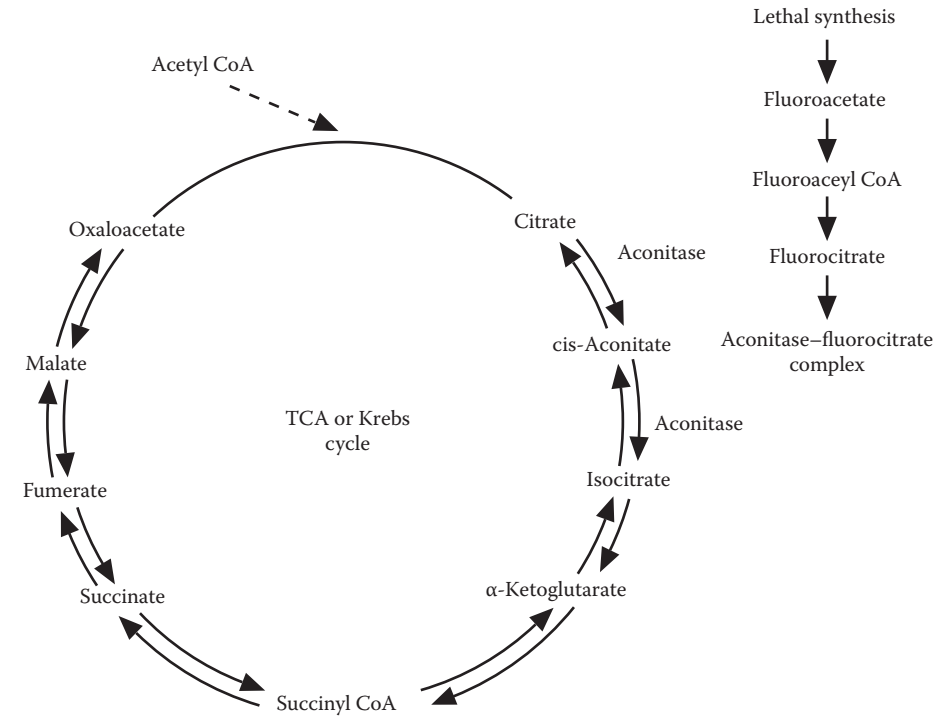


Figure 6.3 Synthesis of fluorocitrate from fluoroacetate through lethal synthesis. Inhibition of aconitase shuts down the TCA cycle.

6.3.4 Secondary Action as a Result of the Presence of a Pollutant

The presence of a pollutant in a living system may cause the release of certain substances that can cause injury to cells. Several examples are given to illustrate this phenomenon.

Subsequent to inhalation of pollen, allergic response occurs in many individuals, leading to a common symptom of hay fever. This is due to the release of *histamine*, a substance formed from the amino acid histidine through decarboxylation (Figure 6.4). Histamine is made and stored in the mast cell and in many other cells of the body. Release of histamine occurs in anaphylaxis, or as a consequence of allergies; it is also triggered by certain drugs and chemicals. Histamine is a powerful vasodilator and causes dilation and increased permeability of blood vessels. It stimulates secretion of pepsin; it can reduce the blood pressure and can induce shock, if severe enough. In

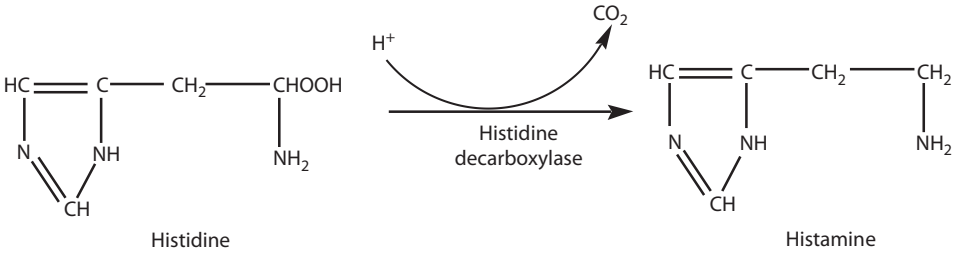


Figure 6.4 Formation of histamine from histidine.

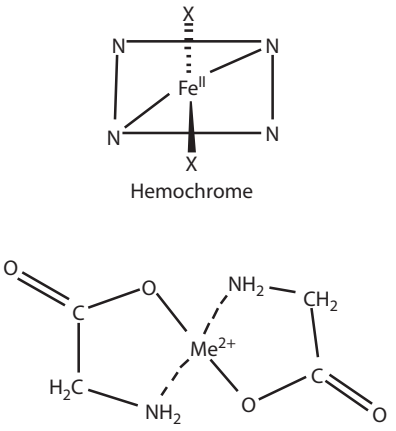


Figure 6.5 Examples of chelation.

excessive concentrations histamine can cause vascular collapse. Antihistamines such as diphenylhydramine and antigen are compounds similar to histamine structurally, and can prevent physiologic changes induced by histamine by inhibiting its function.

Another example is seen with the effect of carbon tetrachloride (CCl_4) on humans. Once taken up into the body, CCl_4 causes a massive discharge of epinephrine from the sympathetic nervous system, leading to liver damage. Epinephrine is a potent hormone and is involved in many critical biological reactions in animals and humans, including such diverse functions as stimulation of glycogenolysis, lipolysis, and glucagon secretion; inhibition of glucose uptake by muscle; and insulin secretion. It also causes the blood pressure to increase. Like other hormones, epinephrine is rapidly broken down as soon as its function is finished. Metabolism of the hormone occurs mainly in the liver.

A third example involves *chelation*. This is a process wherein atoms of a metal in solution are sequestered by ring-shaped molecules, as illustrated in Figure 6.5. The rings of atoms, usually with O, N, or S as electron donator, have the metal as an electron acceptor. Within this ring the metal is more firmly gripped than if it were attached to separate molecules. In forming strain-free stable chelate rings, there must be at least two atoms that can attach to a metal ion. The iron in a hemoglobin molecule and the magnesium in a chlorophyll molecule are two examples of this kind. Through chelation, some biologically active compounds are absorbed and retained in the body, whereas others may be removed from living systems more readily.

The toxicity of certain chemicals may be the result of chelation. For example, experiments have shown that when rabbits were exposed to CS_2 at 250 ppm, there was a rapid outpouring of tissue Zn in urine. The loss of body Zn is primarily due to a chemical reaction of CS_2 with free amino groups of tissue protein, to form thiocarbamate and thiazolidone (Figure 6.6). The resultant thiazolidone may make copper less available for essential enzyme functions. For example, copper is an essential metal component of several tissue oxidases such as cytochrome oxidase and delta-aminolevulinic acid dehydrase. Removal of copper from the enzyme systems leads to inactivation of the enzymes.

It has been suggested that metal chelation may be one of the mechanisms involved in carcinogenesis. Many carcinogens possess structures or can be converted to certain chemical structures capable of metal binding. This in turn will permit the entrance of certain metals into cells. Once inside the cells, interaction between normal metals and abnormal metals can occur, thus altering

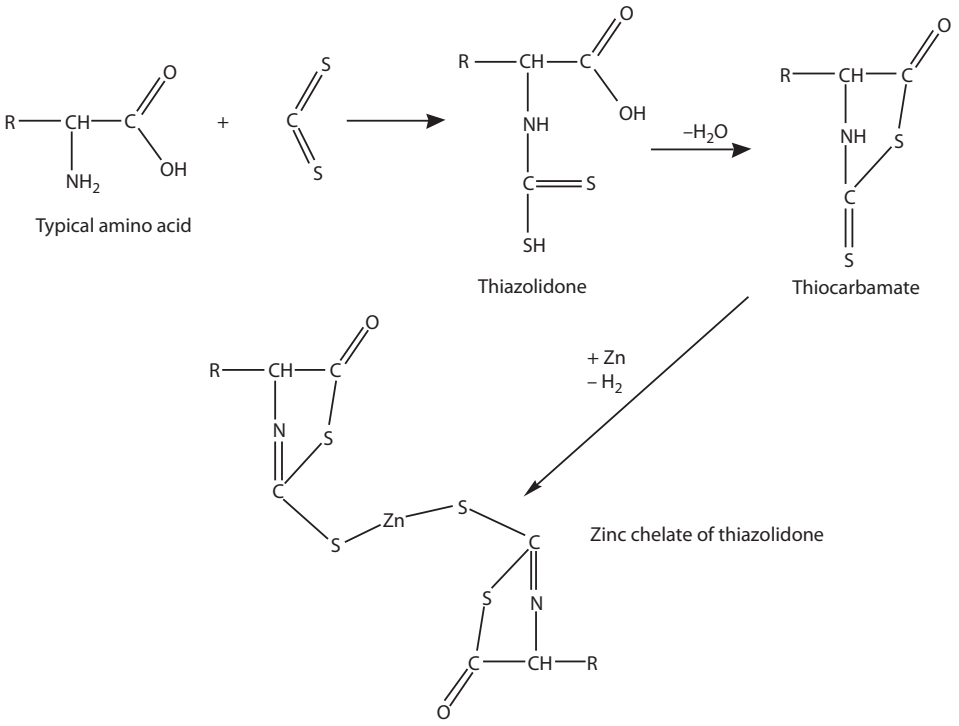


Figure 6.6 Reactions of CS_2 with proteins and amino acids.

cell metabolism. Certain anticancer agents may function through metal binding; i.e., they may inactivate more toxic metals than the useful metals within the cells. There are, moreover, numerous toxic environmental chemicals that become chelating agents through the usual metabolic processes.

6.3.5 Metal Shift

Metal shift refers to the phenomenon in which certain metals shift from one organ to another as a result of the presence of a pollutant. This is among the earliest biological indicators of toxic response. For example, rats fed vanadium (V) at concentrations up to 150 ppm were shown to cause iron (Fe) to move into the liver and spleen. When V concentrations were at 250 ppm or above, however, Fe moved out of the liver and spleen. As a result, the Fe level in the spleen was decreased to one-half to one-third of the normal content, while that in the liver was decreased to one-third of the normal level (Furst 1960). These results indicate that treatment with V will lead to depletion of Fe in these tissues.

Yoshida et al. (1991) have also made a similar observation. Their experiments showed the phenomenon of metal shift in rats exposed to fluoride (F). When rats were exposed to F, the serum Zn levels increased, whereas the levels of Se and Al in the whiskers were decreased.

Rats exposed to O_3 showed a similar phenomenon. When the rats were exposed to this pulmonary irritant for 4 hours, the animals showed increased levels of Cu, Mo, and Zn in the lungs, whereas these metals were decreased in the liver. This would indicate altered hemodynamics and changes in cellular permeability in a secondarily affected organ.

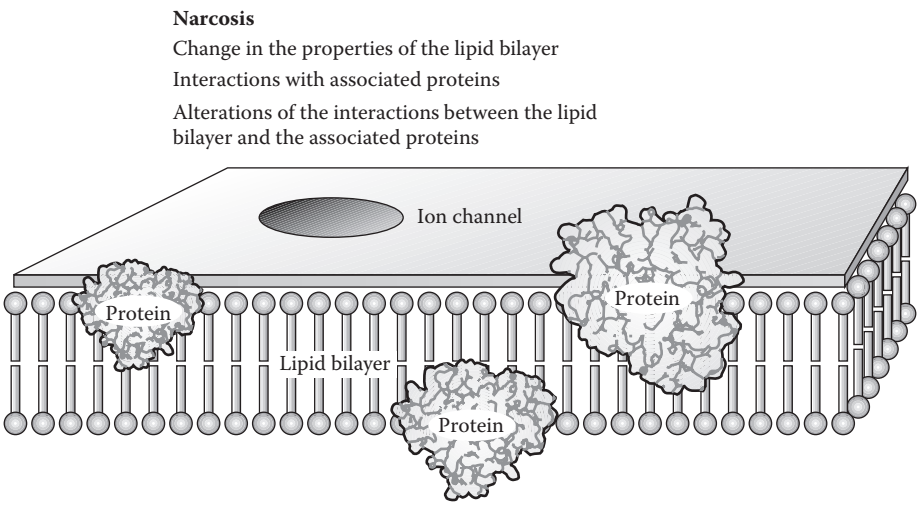


Figure 6.7 Schematic of cell membrane with associated proteins.

6.4 Specific Modes of Action in Detail

6.4.1 Narcosis

Narcosis is perhaps the most common mode of action of common industrial pollutants. A variety of compounds, especially those used as solvents, exhibit this mode of action during the typical toxicity test. Although a common mode of action from the point of view of symptomology, several different molecular mechanisms may be at play.

Figure 6.7 is a diagram of a typical cellular membrane with the lipid bilayer and its associated proteins. Three sites of action within the membrane may actually be the place where a molecule exhibits its effect. First, the actual mode of action may be an alteration of the physical-chemical properties of the lipid bilayer. Changes to the fluidity or other aspects may sharply alter the passage of molecules through the membrane. Second, the molecule may interact directly with the protein associated with the membrane. Many of the proteins are ion pumps, receptors for regulatory molecules, or have some other regulatory function. Finally, the toxicant may alter the interaction of the lipid bilayer with the inserted protein. This change in the bilayer-protein interaction then changes the ability of the protein to perform its function. Each of these modes can be relatively nonspecific and the impact of lipid solubility is obvious. Lipid-soluble materials can readily enter the membrane and then alter its function. In fact, most of the models that portray the relationship between structure of the toxicant and the narcotic effect rely extensively, if not exclusively, on the ratio of the compound's solubility in octanol compared to water.

The fact that not all compounds with narcosis as the mode of action work similarly is depicted in Figure 6.8. Apparently, at higher values of log P, the nonpolar compounds demonstrate a lesser slope. Perhaps two different mechanisms are at play.

6.4.2 Organophosphates

The organophosphates are compounds widely used as insecticides and chemical warfare agents. Although extremely toxic in some cases, these materials are generally short lived in

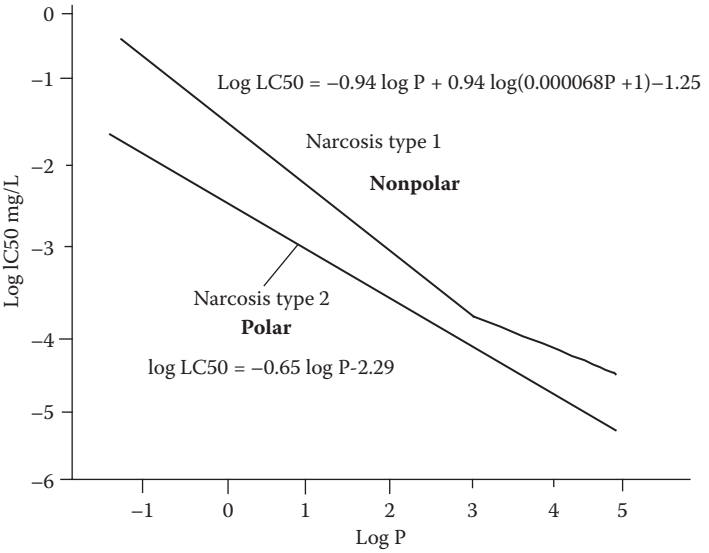


Figure 6.8 Comparison of the relationship between polar and nonpolar compounds, toxicity, and the octanol-water partition coefficient. Note that the slopes are similar for both groups of compounds until higher log P values.

the environment compared to halogenated organics and related compounds. The toxicity of an organophosphate is related to its leaving group, the double-bonded atom, usually O or S, and the phosphorus ligands, the groups surrounding the phosphate in the compound. Several examples of typical organophosphates are shown in Figure 6.9. The more toxic compounds generally have short phosphonate side groups with fluoride or a cyano leaving group. The metabolic replacement of sulfur by oxygen in the liver or other detoxification organ activates the sulfur-containing organophosphate into a much more potent form. The extreme toxicity of these compounds is due to their ability to bind to the amino acid serine, rendering it incapable of participating in a catalytic reaction within an enzyme and the further blocking of the active site by the organophosphate residue. Although many proteins have serine in their active sites and are affected by organophosphates, the acute toxicity of these compounds is usually attributed to their ability to bind to the critical nervous system enzyme acetylcholinesterase.

In normal transmission of a nervous impulse from nerve to nerve, acetylcholine is released into the synapse in order to excite the receiving neuron (Figure 6.10). Unless acetylcholine is rapidly broken down, the receiving nerve is constantly fired, resulting in uncoordinated muscle movement, nausea, dizziness, and eventually seizures and unconsciousness. The serine enzyme acetylcholinesterase is responsible for the expedient breakdown of the neurotransmitter acetylcholinesterase.

Typically, acetylcholine is catalytically degraded by the initial binding of the acetylcholine to the amino acid serine with a proton donated by the amino acid. This process is graphically demonstrated in Figure 6.11. This results in the release of the choline group, with the remainder binding to serine. With the addition of a molecule of water, the serine is reactivated with the release of the acetyl group from the active site.

Organophosphates are able to participate in part of the reaction depicted above. However, as shown in the accompanying figure (Figure 6.12), all does not work as if the organophosphate were acetylcholinesterase. The typical organophosphate is able to enter at the active site and the initial

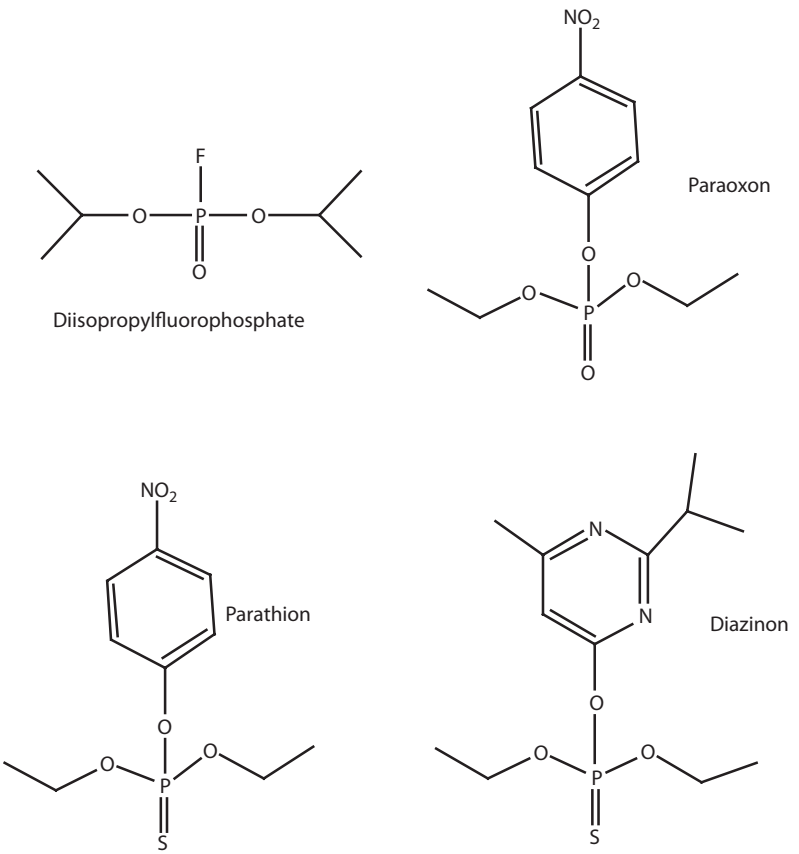


Figure 6.9 Typical organophosphates and related compounds.

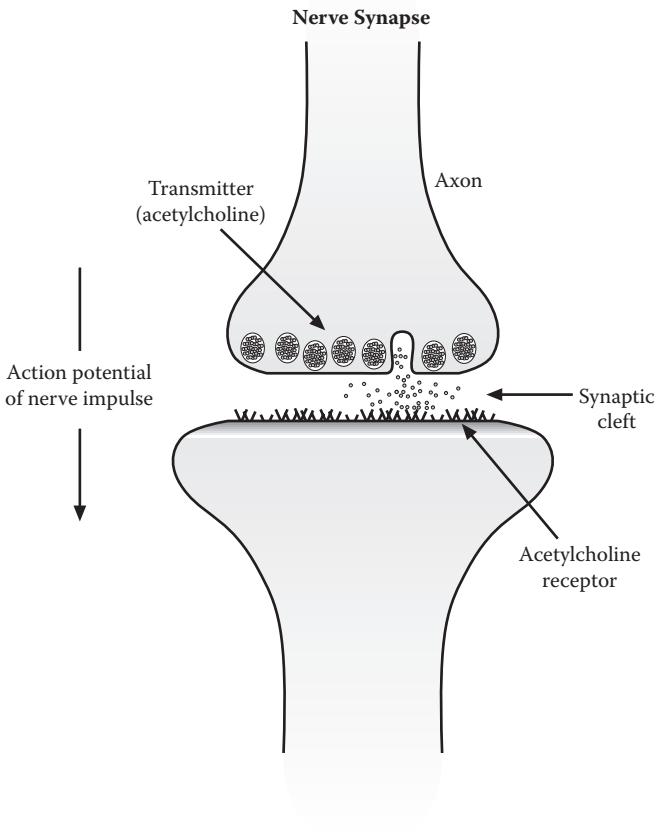


Figure 6.10 Schematic of the synapse. Acetylcholine is an important neurotransmitter, and the intervention of acetylcholinesterase prevents subsequent firing of the adjacent neuron.

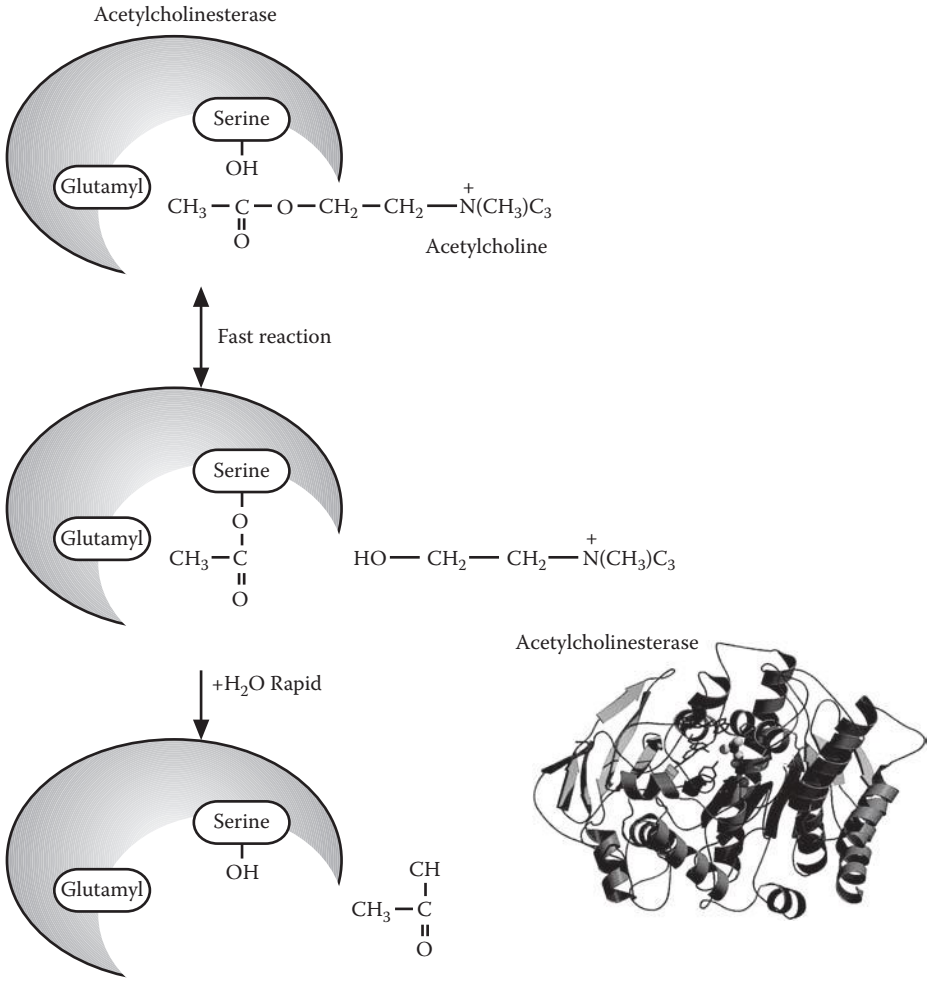


Figure 6.11 Normal hydrolysis of acetylcholinesterase. The amino acid serine is important in the donation of a proton used in the catalytic process. The proton is regenerated during the reaction.

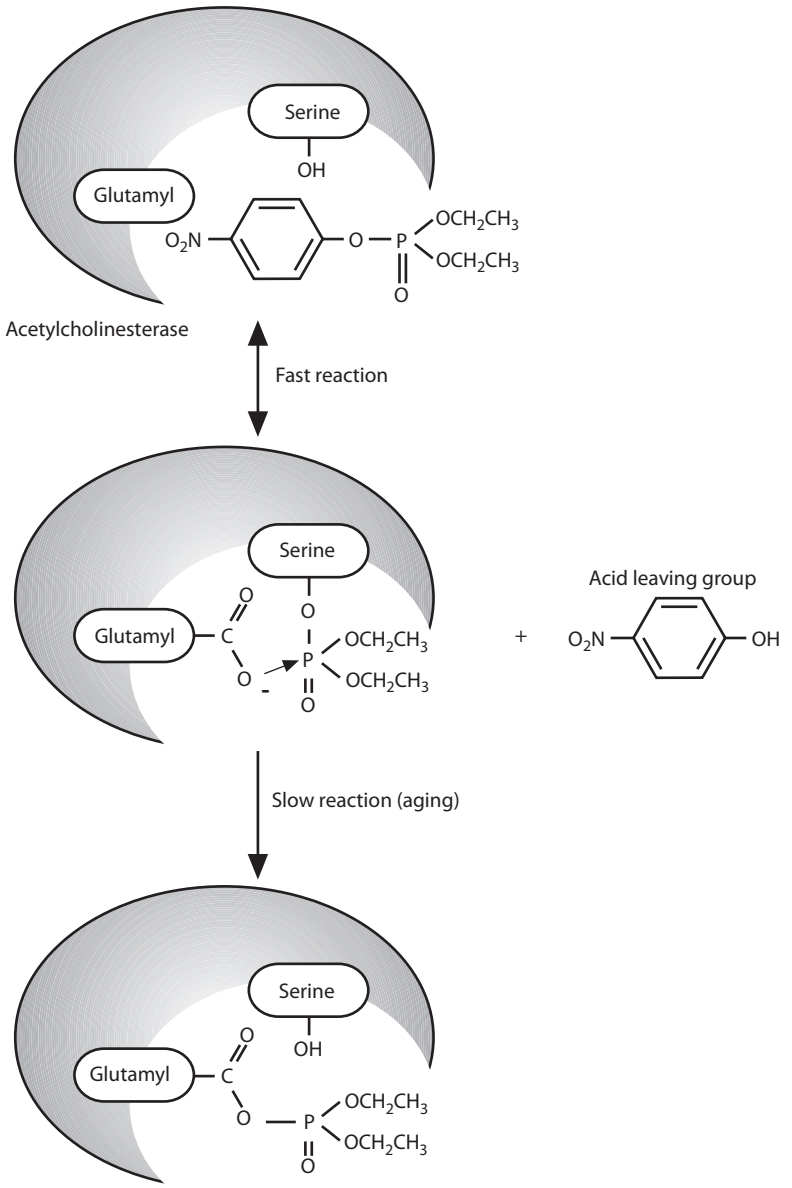


Figure 6.12 Inhibition of acetylcholinesterase by an organophosphate. The initial binding of the organophosphate to the active site prevents the normal substrate from entering the active site. The aging process subsequently binds the organophosphate to the active site permanently, inactivating the enzyme.

proton donation does occur, resulting in the linkage of the serine to the phosphate. This is a two-step process. First, a Michaelis complex is formed among the –OH group and the phosphate, and then the covalent bond between the serine and phosphate is formed, resulting in the loss of a nitrophenol, fluoride, or other leaving group. These reactions are reversible. The next step is an irreversible binding at a glutamyl residue that "ages" the protein. This next step is relatively slower than the initial binding to the organophosphate, but is variable from organophosphate to organophosphate. Compounds typically used as chemical warfare agents have relatively fast aging reactions.

The binding of an organophosphate to acetylcholinesterase can be used to an advantage. Inhibition of acetylcholinesterase and its relative butylcholinesterase is routinely used as an indication of exposure to an organophosphate or other inhibitory compound.

Lastly, organophosphates bind to other proteins and likely affect many other metabolic pathways. It has been shown that organophosphates bind to a variety of liver proteins, and these proteins act, accidentally perhaps, as sinks protecting enzymes of the CNS from exposure. Of course, a second dose of an organophosphate soon after would likely be more toxic, not because of the increased toxicity of the molecule but because of the prior filling of this sink.

6.4.3 Modes of Action of Chemical Warfare Agents

There are numerous materials that have been used as chemical weapons or have been suggested as agent materials. The purpose of this section is to provide a very short introduction to the kinds of materials used as chemical weapons and their modes of action.

The development of chemical warfare agents parallels the development of industrial chemicals. Initially the materials produced had broad-acting mechanisms for reacting with biological materials. Eventually chemicals with more specific modes of action were developed and deployed. During World War I a number of very toxic materials were developed and used (Figure 6.13). These materials generally have a broad mechanism of action, such as the alkylation of a broad range of biological molecules.

Perhaps the most iconic chemical warfare material is mustard agent. Mustard is a highly reactive material that rapidly denatures a variety of biological molecules. Blisters occur on the skin, eyes are damaged, and the lungs fill with fluid. Mustard is also a potent mutagen, being one of the first chemical mutagens discovered. Mustard alkylates nucleotide bases, causing a transition from one base to another in the genetic code. Mustard is also very persistent in the environment, although hydrolysis can occur. Anecdotal evidence suggests that agent spilled or dispersed on the

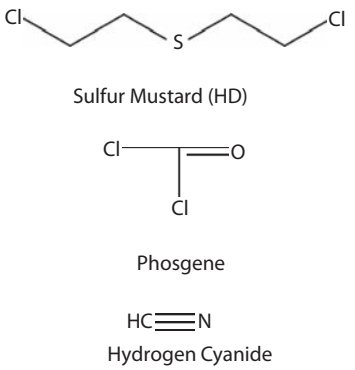


Figure 6.13 Classic chemical warfare agents.

ground persisted from WWI to the 1970s. Mustard agent can be easily synthesized from chemicals commonly found in the chemical industry.

Phosgene is converted in the lungs to HCl and Cl_2, materials that rapidly react with –OH, –SH, and $–NH_2$ groups in proteins, inactivating and denaturing them. These reactions lead to the destruction of the blood-air barrier, resulting in the filling of the lungs with fluid. Phosgene is also very persistent. The manufacture of phosgene is still common because of its use as a chemical feedstock for a variety of industrial processes.

Hydrogen cyanide can cause blistering, but its primary mode of action is the inhibition of cellular respiration by the inhibition of cytochrome c oxidase. Hydrogen cyanide is easily synthesized and is used in the manufacture of synthetic fibers, plastics, and dyes.

These chemicals were directly derived from or are still used in the manufacture of chemicals at industrial scales. Mustard was extensively used in WWI and comprised the chemical arsenal of the United States during WWII. After WWII, mustard was still manufactured and found its way into a number of munitions, including bombs and rockets. These munitions were disposed of by incineration during the 1990s and early 2000s.

In the late 1930s pesticide development moved toward more specific modes of action. So did the development of chemical warfare materials. Acetylcholinesterase inhibitors were discovered at the outbreak of WWII and remained classified until after the war. Figure 6.14 illustrates two acetylcholinesterase inhibiting classes, the G agents and the V agents. The G agents were developed and deployed during WWII by Germany, and subsequently manufactured in large amounts by the United States and the Soviet Union during the Cold War.

Diisopropylfluorophosphate (DFP) can be regarded as a prototypical organophosphate acetylcholinesterase inhibitor and G agent. As discussed previously, the OP binds into the active site of acetylcholinesterase, and after the aging process, the enzyme is irreversibly inactivated. Sarin is essentially half of a DFP molecule and is much more toxic. Soman is similar but with a two-carbon chain after the oxygen, with a branching methyl group at the first carbon. DFP, soman, and sarin all have an F– as the acid leaving group.

During the 1950s, a new group of compounds, the V agents, were developed and manufactured. The compounds are structurally similar to the G agents, except that the leaving group is the branch containing both sulfur and nitrogen. The remaining structure is very similar to that of the G agents.

The G and V agents closely resemble the insecticides paraoxon and parathion, discussed previously. In the pesticides previously presented, the nitrophenol group was the acid leaving entity, compared to the chemical warfare agents. Essentially, the chemical warfare agents are pesticides designed to be particularly toxic to mammals and humans rather than arthropods.

Pesticides and the chemical warfare agents can easily be produced by the same chemical manufacturing industry of any industrialized nation. Creating each is more a matter of introducing the correct feedstocks than inventing a new technology. Small amounts can also be synthesized in the laboratory, but amounts useful as insecticides or chemical warfare agents require a large manufacturing infrastructure. For chemical weapons it is also required that a weapon be constructed that effectively delivers the material to the battlefield. This development and manufacturing process also requires a substantial manufacturing base. These infrastructures tend to be substantial and readily identified. The disputes tend to arise over the fact that the same manufacturing infrastructure can be used for weapons against either insects or humans.

Disposal of chemical warfare agents has been an issue. All of the acetylcholinesterase inhibitors can be destroyed by an excess of base that promotes hydrolysis of the agent. However, this neutralizing process produces large amounts of still hazardous materials. There are enzymes that degrade

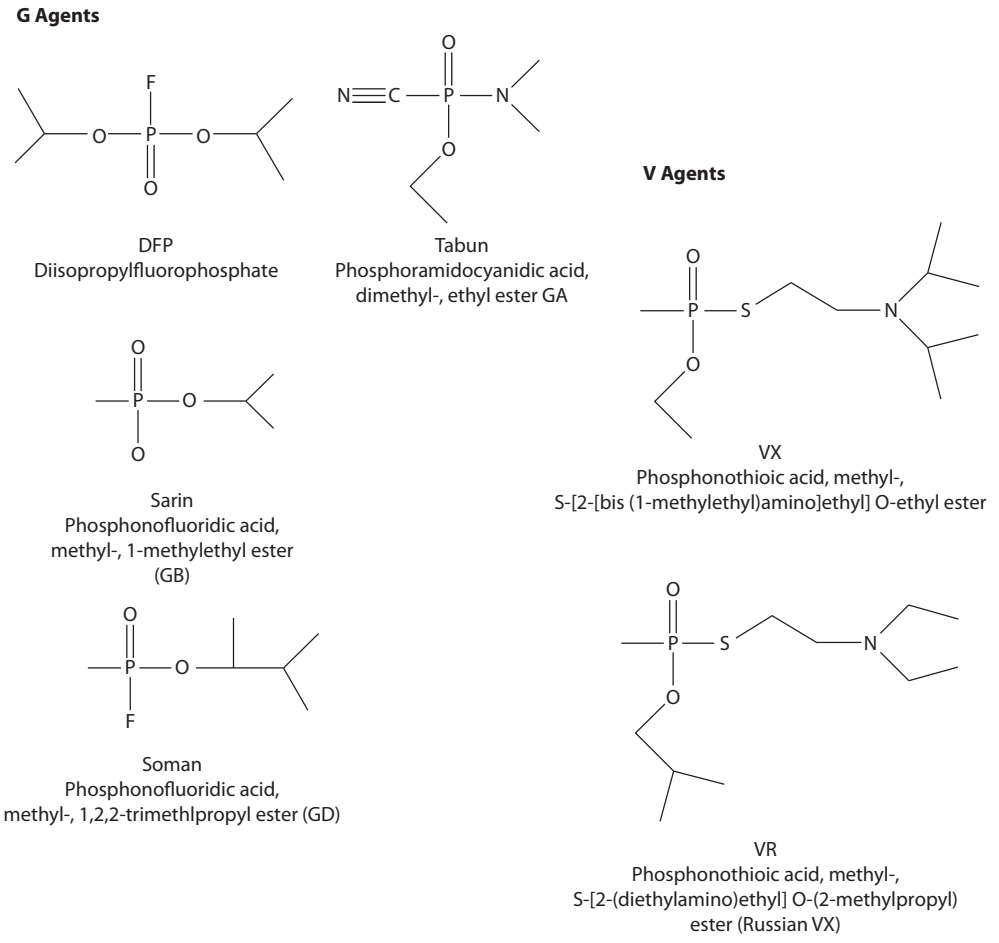


Figure 6.14 Agents developed since WWII that inhibit acetylcholinesterase activity.

the organophosphates, but these have not been used at large scales. Incineration also has been used successfully in the destruction of the American stockpiles of agent and weapons.

Chemical agents such as those above have been used since WWI. However, in WWI the extensive use of chlorine, phosgene, and mustard did not result in a shift in the battlefield in Europe. Agents were not used in the battlefield during WWII, although both the Axis and the Allies had stockpiles of weapons. Iraq did used mustard agent extensively during the Iran–Iraq War of the 1980s. The agent was effective against closely bunched, ill-trained, and ill-equipped shoulders of the Iranian army, but this use did not result in a dramatic change in the outcome of the war. Iraq did use chemical warfare agents against the Kurdish population during the 1980s, with dramatic results against a defenseless population. It is not clear that chemical agents are militarily useful, but they are a tool of terror against unprotected populations.

6.4.4 Monohaloacetic Acids

Monhaloacetic acids are compounds derived from acetic acid with the substitution of a halogen to replace one of the hydrogens. Chloroacetate, fluoroacetate, iodoacetate, and bromoacetate are

compounds that vary in toxicity and mode of action, although they are closely related. Sodium fluoroacetate was a widely used mammalian pesticide known as compound 1080. Chloroacetic acid is used as a feedstock, and that resulted in manufacturing in large amounts.

Hayes compared the toxicity of chloroacetate, fluroacetate, and iodoacetate in rats. The 24-hour LD_{50} values were 108, 5, and 60 mg/kg, respectively. LD_{90} doses were delivered to rats and the time until death (LT) was determined. The LT_{50} values for chloroacetate, fluroacetate, and iodoacetate were 130, 310, and 480 minutes, respectively. Based upon this comparative study, fluroacetate was the most toxic, iodoacetate the intermediate, and chloroacetic acid the least toxic of the three compounds. Bromoacetic acid is not as well studied, although it is a potent enzyme inhibitor.

Although the monohaloacetic acids have similar chemical properties and structure, the unique properties of halogen cause very different physiological effects. Figure 6.15 is a spatial representation of the four monohaloacetic acids compared to acetic acid. As shown in the figure, fluoroacetic acid and acetic acid are very similar in configuration. The small size of the fluorine

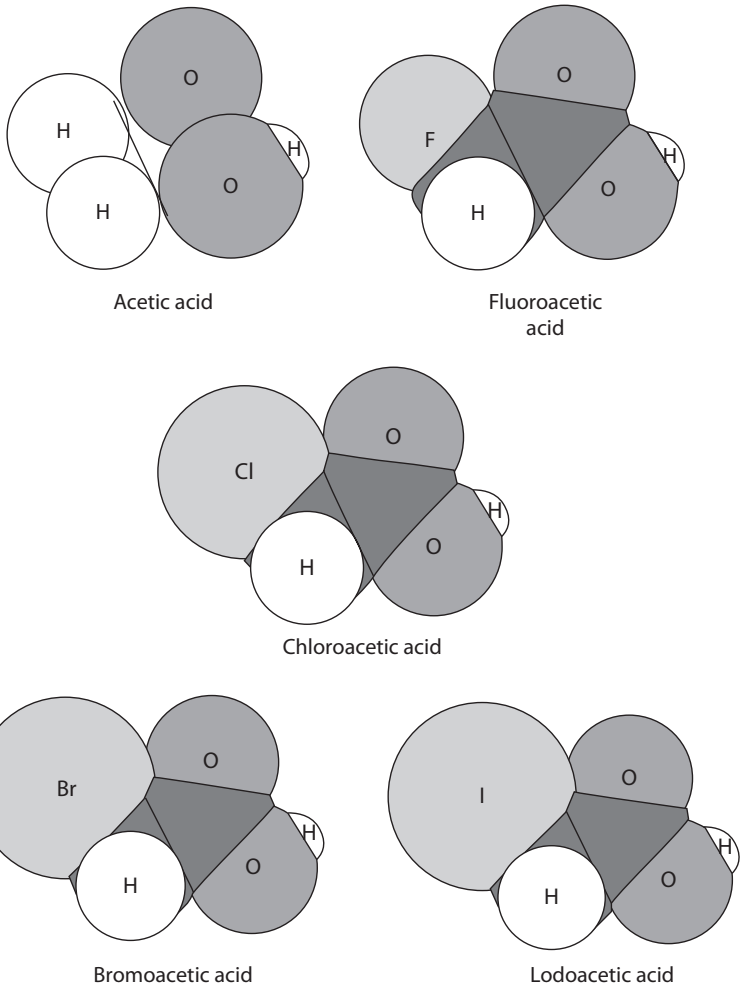


Figure 6.15 Relative configurations of the monohaloacetic acids and acetic acid.

atom allows fluoroacetate to mimic acetate in the TCA cycle, as described previously in this chapter. Briefly, the fluoroacetate is metabolized in the TCA cycle to the point where fluorocitric acid is synthesized in the place of citric acid. Aconitase accepts the molecule into the active site, but the strong electronegativity of the fluorine prevents the enzyme from catalyzing the reaction or dislodging the molecule. Since there is competition for the active site of the enzyme, fluorocitrate is a competitive inhibitor of aconitase, and the inhibition is reversible.

In contrast, iodoacetic and bromoacetic acids inhibit enzymes by alkylating sulfhydryl (–SH) and amino ($–NH_2$) groups. This involves the replacement of the hydrogen atom by the acetic acid group $–CH_2COOH$. This reaction prevents these proton donor groups from participating in the biochemical reactions requiring the addition of the proton. Enzymes containing these proton donor groups are inhibited. Examples of such enzymes are guinea pig monoamine oxidase, GAPD, and various enzymes involved in glycolysis. Iodoacetic and bromoacetic acids do not enter the TCA cycle due to the relatively large size of the halogen. However, since competition for the active site of the affected enzyme does not occur, they are irreversible inhibitors of enzyme function.

Chloroacetate is an intermediate case. Apparently –SH groups and acetate oxidation are affected. The relatively small chlorine atom may allow chloroacetic acid to slowly enter the TCA cycle and inhibit aconitase while at the same time alkylating –SH groups.

6.5 Receptor-Mediated Toxicity, Endocrine Disruption

Of recent concern has been the ability of some xenobiotics to mimic the effects of steroidal hormones. Before the toxic mechanism can be understood, it is necessary to understand the role of steroidal hormones as regulators of cellular processes.

A clear introduction to the mechanisms of hormonal function and disruption has been provided by Eubanks (1997) and is summarized here. Hormones are regulatory molecules produced by the endocrine system that fit precisely to proteins called receptors. This interaction is very precise and constitutes the reception of a chemical message by a particular cell. Upon reception of the message dramatic changes can occur in the cell, although extremely small amounts of the hormone may be present. The reaction to the interaction of the hormone and receptor is specific to the type of cell involved. In this manner, a host of dramatic changes can occur to a variety of cellular and tissue types, all caused by the change in concentration of a specific hormone. The concentration of hormones is regulated by a negative feedback system.

Hormones initiate these changes by altering the transcription of specific genes within the cellular nucleus. Figure 6.16 shows the typical mechanisms of hormonal-receptor interaction. Androgens and estrogens are steroids that are very lipid soluble, facilitating the passage of the hormone past the lipid bilayer and into the cytoplasm. In the cytoplasm is the receptor, often comprised of protein subunits. Upon binding of the receptor and the hormone, a conformational change occurs, perhaps releasing some of these subunits and producing a unique receptor complex. This receptor complex moves into the nucleus. In the nucleus the receptor complex may interact with other proteins to bind to specific promoter regions of DNA. Transcription and subsequent translation of specific gene products may then occur, altering the metabolism of the cell. In some instances the receptor complex may repress transcription.

Androgens and estrogens are two steroidal hormones that regulate a variety of reproductive and other characteristics. Androgens include testosterone and androsterone and initiate male sexual development. Estrogens include estradiol, estrone, and estriol and are important in the development of female sex characteristics and in regulating female receptiveness and reproduction.

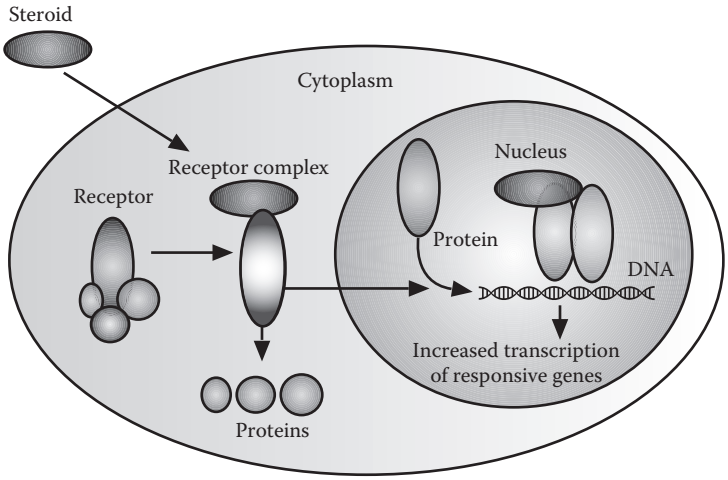


Figure 6.16 Generalized regulatory role of steroidal hormones. The steroid combines with a cytoplasmic receptor and becomes a new receptor-ligand complex. This complex then can enter the nucleus, initiating transcription.

Since only small amounts of hormone are necessary to induce dramatic cellular and physiological effects, an organism should be sensitive to any alteration in the amount of hormone or a blockage of the estrogen or other receptor. Toxicants that are endocrine disrupters work in two basic ways (Figure 6.17). In the first instance, the toxicant mimics the hormone, producing a change in the structure of the receptor, and initiates a response. Toxicity may be due to an inappropriate excess of hormone-producing gene products or inhibiting transcription at inappropriate times. Males may become feminized if an estrogen mimic is present. The second major mechanism is that the xenobiotic is a hormone block. In this instance the xenobiotic binds to the receptor and prevents the hormone from entering the active site. The xenobiotic occupies the active site but does not induce the conformational changes necessary to ensure the correct hormonal response. If sufficient toxicant is available, the viable receptors may no longer be present to mediate the hormonal signals. In the instance of a xenobiotic blocking an estrogen receptor, masculinization of females can occur.

The different affinities for the estrogen receptor have been demonstrated by Vonier et al. (1996), investigating the binding of a variety of xenobiotics to alligator estrogen receptor (ER) (see Table 6.1). Inhibition of a titrated estradiol binding to alligator ER was the basis of the assay. 17B-Estradiol was used as a positive control. A variety of compounds were able to inhibit [^{3}H]17B-estradiol binding at low concentrations. The compounds *o*,*p*'-DDD and *o*,*p*'-DDT were particularly potent, while the related compounds *p*,*p*'-DDD and *p*,*p*'-DDT, differing in only the substitution pattern, did not exhibit inhibition up to the limit of solubility. A variety of results were noted for the compounds tested, and several had no statistically significant inhibition of the estrogen binding to the alligator ER.

6.5.1 Specificity of the Hormone-Receptor Interaction

As noted above, closely related isomers of DDD and DDT had very different abilities to inhibit binding to the alligator ER. Two factors are involved. First is the conformation of the receptor,

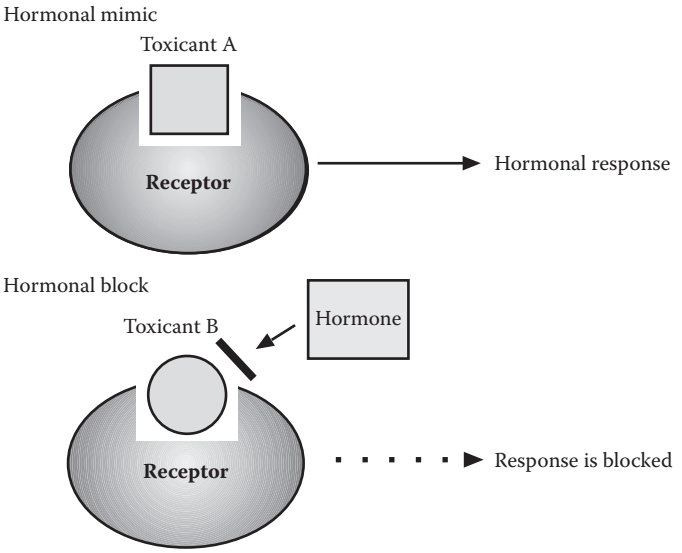


Figure 6.17 Mechanisms for the xenobiotic disruption of hormonal activity. In certain cases the xenobiotic may interact with the hormone receptor in such a manner that a hormonal response is generated. In some instances this hormonal response is inappropriate for the sex or normal breeding state of the organism. In other instances the toxicant may interact with the receptor in such a manner that it binds tightly to the receptor site but does not initiate the conformational changes that confer the normal cellular interactions of the receptor. In this instance the hormone is blocked from interacting with the receptor and the response is blocked.

and second is the three-dimensional structure of the xenobiotic and its resemblance to a natural ligand. As a model system to investigate the structure-activity relationships of molecules that react to specific sites in hormones, we will use the well-studied molecules 1,3,7,8-TCDD (dioxin) and various polychlorinated biphenyls (PCBs).

Dioxin and PCBs were hypothesized to be toxic because of three modes of action (McKinney and Waller 1994). First, these compounds are toxic due to their irreversible chemical reactivity in binding to a variety of macromolecules such as DNA. Second, these compounds are highly lipid soluble and may accumulate in lipid-rich cellular components. Third, these compounds can reversibly react to specific sites in receptors and enzymes. Overall toxicity is certainly due to a combination of these items, although we will concentrate on the third mode of action.

2,3,7,8-TCDD is often regarded as a highly toxic material. However, that toxicity is in one manner very specific. Table 6.2 presents data for the toxicity of TCDD to a variety of plant and invertebrate species. Unlike the common perception, TCDD is not particularly toxic to a wide range of invertebrates. At relatively high concentrations and particularly body burdens, and for a significant duration of exposure, the TCDD has little or no effect. Conversely, Table 6.3 presents data for vertebrates. At concentrations hundreds or even a thousand times less than those for the invertebrate species, the mortality was 100%. Obviously vertebrates have something that invertebrates do not.

Vertebrates apparently have a specific protein, the aryl hydrocarbon (Ah) receptor (see below), which has a great affinity for 2,3,7,8-TCDD. Although a functionally similar receptor no doubt exists in invertebrates, the vertebrate receptor has a great affinity for dioxin.

Table 6.1 The Inhibition of Alligator Estrogen Receptor by a Variety of Xenobiotics

Chemical	*Alligator ER Binding IC_{50} (μM)*
17B-Estradiol	0.0078
o,p′-DDD	2.26
o,p′-DDT	9.1
DDDH	11.1
o,p′-DDE	37.25
Dicofol	45.6
p,p′-DDT	>50[a]
p,p′-DDD	>50[a]
p,p′-DDE	>50[a]
Methoxychlor	NS
Atrazine	20.7
Alachlor	27.5
Kepone	34
Aroclor 1242	37.2
Endosulfan I	>50[a]
Toxaphene	NS
2,4-D	NS

Source: After Vonier, P. M. et al., *Environ. Health Perspect.,* 104, 1318–1322, 1996.

Note: Estradiol, DDD, and related compounds were strong inhibitors of radioactive estradiol to the receptor. NS, not significant; 2,4-D, 2,4-(dichlorphenoxy) acetic acid.

[a] Compounds inhibited binding but were insoluble at concentrations necessary for an IC_{50}.

Table 6.2 Toxicity of TCDD to a Variety of Invertebrates

Test Species	*Water Concentration (ng/L)*[a]	*Organism Concentration (pg/g)*[b]	*Duration of Exposure*	*Effects*
Algae, *Oedogonlum cardiacum*	1,330	2,295,000	33 days	No toxic effect
Vascular plant, duckweed, *Lemna minor*	1,300		33 days	No toxic effect
	7.13	30,700	33 days	No toxic effect
Annelid, worm, *Paranals* sp.	200[c]		55 days	No decrease in reproductive success
Mollusk, snail (adult), *Physa* sp.	1,330	502,000	33 days	No toxic effect
Arthropod, cladoceran (adult), *Daphnia magna*	1,330	1,570,00	33 days	No toxic effect

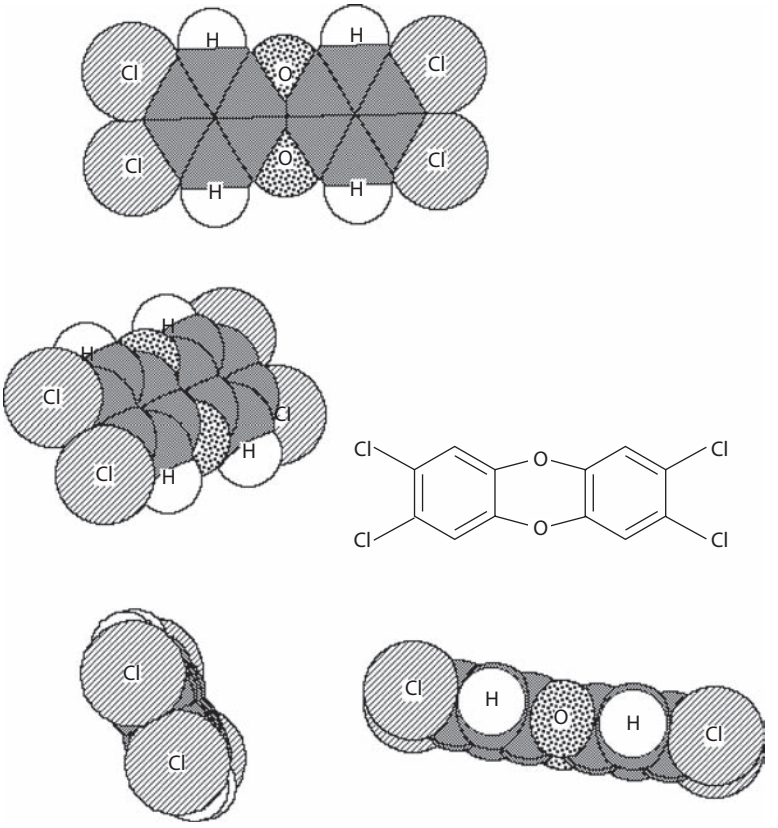
[a] Measured TCDD concentration in water.
[b] Measured TCDD concentration in organism (wet weight).
[c] Unmeasured TCDD concentration in water or organism (wet weight).

Table 6.3 Toxicity of TCDD to a Variety of Vertebrates

Test Species	*Water Concentration (ng/L)*[a]	*Organism Concentration (pg/g)*[b]	*Duration of Exposure*	*Effects*
Fish, Coho salmon, *Oncorhychus kisutch*, Juvenile (3.5 g)	5.60		96 hours	50% mortality
Mink, *Mustela vison*, Newborn		1,000[c]	Daily for 12 days	100% mortality after 14 days

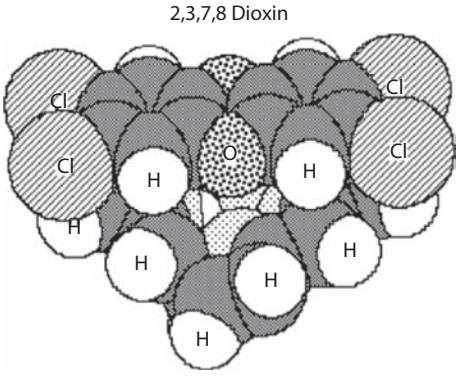
[a] Measured TCDD concentration in water.
[b] Measured TCDD concentration in organism (wet weight).
[c] Unmeasured TCDD concentration in water or organism (wet weight).

McKinney and Waller (1994) published a paper on the relationship of the structure to the modes of action of dioxin and PCBs. The dioxin 2,3,7,8-TCDD has a very specific conformation; it is locked in a planar configuration and has three chlorine atoms on each end of the molecule (Figure 6.18). This specific configuration apparently allows for very specific modes of action. One of the important modes of action of dioxin is its ability to stack when reacting with a variety of ring structures in proteins (Figure 6.19). In this instance the dioxin sticks or Velcros itself to the ring structure of the protein. A second proposed mode of action of dioxin in interacting with receptors is that the three end chlorines are important in reacting to the Ah receptor. The chlorines interact with a C-shaped receptor within a protein that acts as a vice to clamp the xenobiotic within the recognition site (Figure 6.20). The specific interaction of the TCDD with the Ah receptor and its hormone-like cellular activity has been determined.



2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Figure 6.18 The three-dimensional structure of 2,3,7,8-TCDD.



Porphine ring system

Figure 6.19 Stacking model for dioxin toxicity.

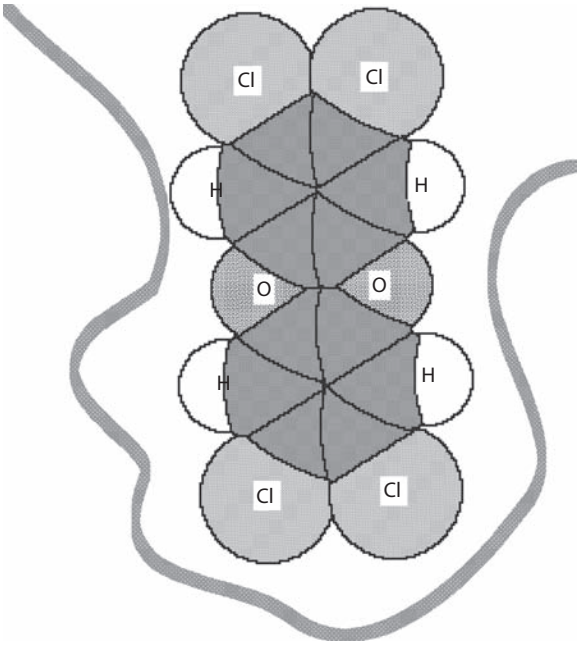


Figure 6.20 Cleft type model for dioxin toxicity.

6.5.2 The Receptor Pathway for TCDD

The endocrine-related pathway for TCDD toxicity is now understood. As in the general diagram for endocrine action in Figure 6.21, the pathway is receptor mediated and is summarized below. TCDD, PCB, or similar compounds operate as the ligand for a specific receptor.

In a vertebrate, once TCDD enters it, the cell binds to a specific Ah receptor complex (Figure 6.21). This complex is comprised by the arylhydrocarbon receptor (AHR) binding protein that is associated with two heat shock proteins of 90 kilodaltons (hsp90). Also in this complex is the X associated protein 2 (XAP2). This complex exists in the cytoplasm of the cell and cannot interact with nuclear DNA until it binds to TCDD or similar molecules. As the Ah receptor complex binds to TCDD it becomes active and may pass into the nucleus by the nuclear pore. Once inside the nucleus the XAP2 hsp90 proteins disassociate from the AHR and TCDD complex. The AHR nuclear translocation transcription (ARNT) factor complexes with the AHR-TCDD. This new complex is now able to bind to dioxin-responsive elements within the genome and initiate transcription of message RNA (mRNA). There are multiple DREs within a genome, so that a variety of mRNAs are formed. The mRNAs are then transported to the cytoplasm and translated into various proteins.

Cytochrome P450 CYP1A1 is the classic protein associated with the introduction of dioxin or dioxin-like compounds. A variety of other genes and their associated proteins are also induced by TCDD. These proteins include CYP1A2, CYP1A3, and NAD(P)H quinone oxidoreductase. Recent research by Sartor et al. (2009) examining the induction of AHR binding in a mouse genome indicates that the AHR has a number of regulatory functions. AHR without the TCDD ligand binds to gene clusters involved in gene expression, differentiation, and pattern specification that connect a number of different developmental and morphogenetic pathways. AHR with the ligand diverts the receptor toward promotor sites for xenobiotic degradation.

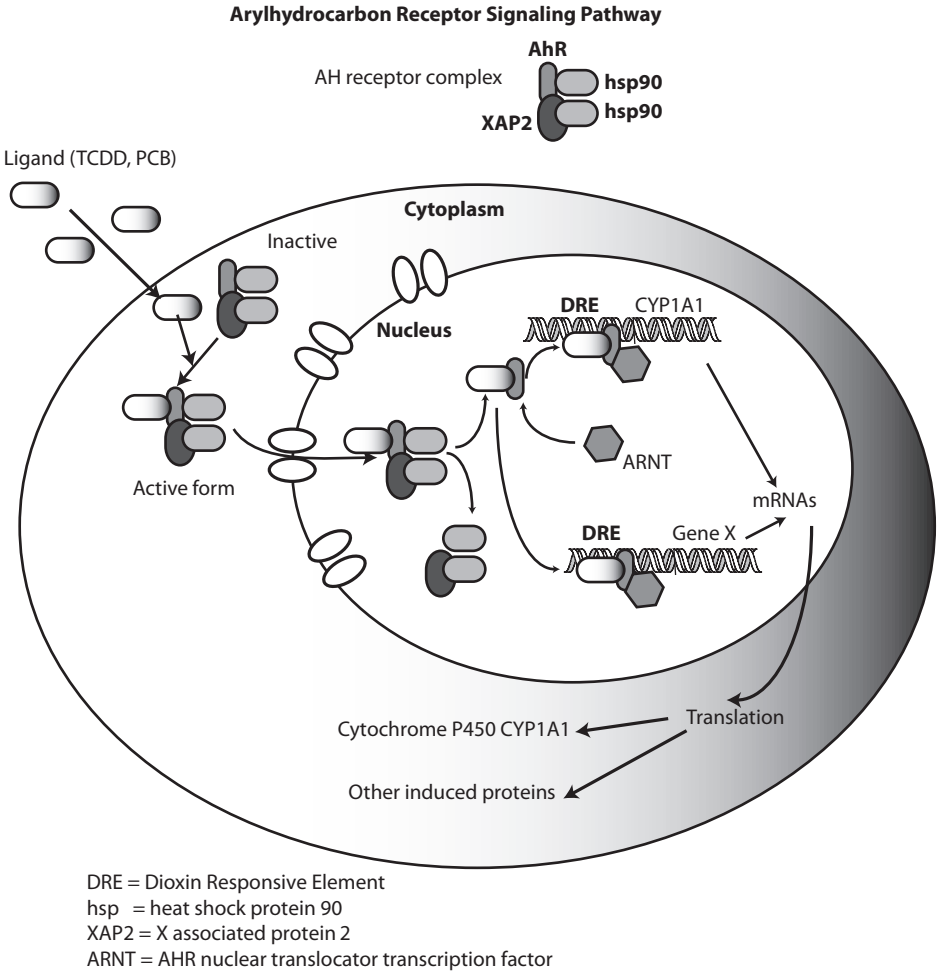


Figure 6.21 Interaction with the AHR and TCDD with the control of protein synthesis.

It is now clear that toxicity of TCDD, PCBs, and similar compounds has a broad number of effects because of their interaction with the AHR receptor. The AHR receptor, when bound to TCDD, activates a broad range of genes. Activation by the TCDD or similar ligands diverts the receptor away from other sites that are important for the regulation of a variety of developmental genes.

This model is consistent with what is known about the evolution of the AHR. In invertebrates the AHR is important in regulating a number of aspects in development. This is consistent with the typical role for the receptor in vertebrates. However, only vertebrate AHR has the ability to bind to TCDD and similar xenobiotics, initiating a variety of other gene functions (Hahn 2002; Hahn et al. 2006). The relative potencies of TCDD to invertebrates and vertebrates noted above are likely due to this evolutionary history. Given that vertebrates and invertebrates diverged more than 500 million years ago, it is ironic that an evolutionary event of the Cambrian determines the pattern of toxicity to compounds not formed until the 20th century.

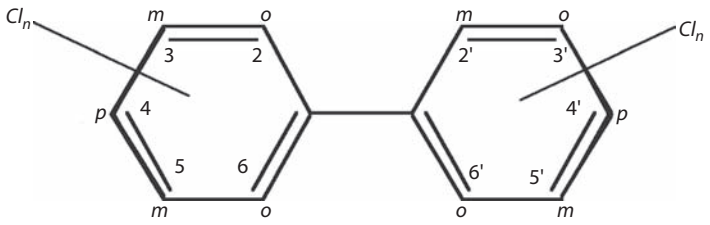


Figure 6.22 Structure and nomenclature for PCBs. A number of compounds exist with this same general structure, with varying numbers of chlorine atoms and positions along the two aromatic rings. The number of the carbons in each ring denotes the positions of these substituted chlorines. Relative positions are also denoted by the *o*, *m*, and *p*, which are shorthand for *ortho*, *meta*, and *para*.

6.5.3 The Structure-Activity Relationships of PCB and Related Compounds

First, it is necessary to review PCB structure and nomenclature (Figure 6.22). PCBs are two biphenyl rings linked by a single carbon bond. The two biphenyl rings are free to rotate unless there are ortho-chlorine substitutions at the 2,2' or 6,6' positions. A number of chlorine atoms can be substituted to each ring, although the examples used in this discussion are all hexachlorobiphenyls. The position of the chlorine substitutions, the ability of the molecule to rotate about the bridging carbon bond, and the reactivity of the chlorine atoms are all important in the final determination of toxicity. In some instances the mode of action resembles that of dioxin; in other cases the PCB may act as an estrogen analog.

The resemblance to dioxin occurs in the meta- and para-substituted PCBs that are free to rotate. Although all PCBs are essential nonplanar, one of the conformations is that the two phenyl groups exist in the same plane, or are coplanar. A compound such as 3,3',4,4',5,5'-hexachlorob iphenyl (HCB) resembles 2,3,7,8-TCDD when in the coplanar configuration (Figure 6.23). In contrast, this structure is not available to 2,2',4,4',6,6'-HCB due to the steric hindrance from the chlorine atoms. It is hypothesized that the coplanar configuration of the PCB allows these types of compounds to share the stacking and cleft type modes of action hypothesized for dioxin. In Figure 6.24 it is apparent that dioxin and 3,3',4,4',5,5'-HCB can provide a flat face to the reactive ring structure. In contrast, 2,2',4,4',6,6'-HCB cannot exhibit this same mode of action.

However, nonplanar PCBs do resemble estrogens. Figure 6.25 compares an –OH-substituted PCB to estradiol. The resemblance is common, especially when aligned along the common phenolic ring. It is hypothesized that upon hydroxylation, as part of the metabolism of PCBs, the compound becomes estrogenic (Figure 6.26). Although the ortho-substituted PCBs are less dioxin-like than other PCBs, upon hydroxylation they become more potent estrogenic compounds.

Materials other than the PCBs and dioxins described above have been found or are suspected to have estrogenic activity. Table 6.4 lists some of these compounds in addition to those already presented. Many of these compounds are derived from industrial sources including surfactants, degradation of fire retardants, plasticizers, and insecticides, or are natural products released in waste streams.

Examples of effects of these compounds upon vertebrates include intersex fish with male and female characteristics, elevated levels of the egg protein vitellogenin in male fish, and degeneration

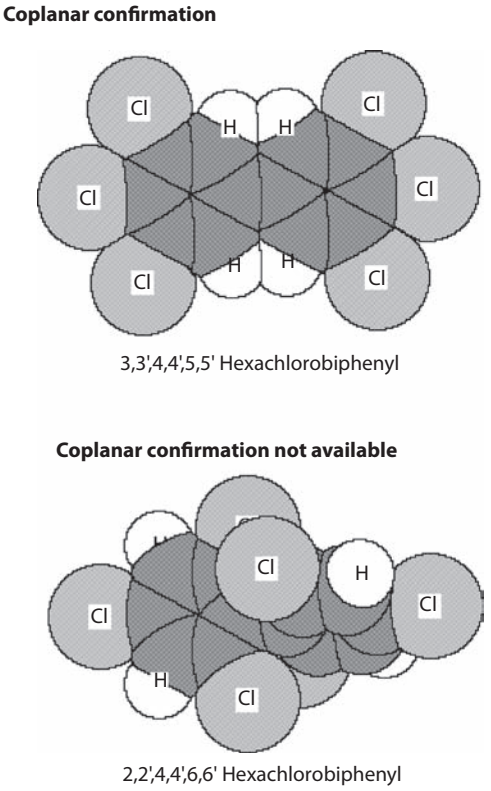


Figure 6.23 Conformations of coplanar and nonplanar PCBs.

of gonadal tissue (Pait and Nelson 2002). Similar to the estrogenic PCBs, the modes of action are mimicking the effects of estrogens and androgens, antagonizing the effects of the normal hormones, altering the synthetic pathways and metabolism of the normal hormones, or modifying the level of hormone receptors. The best studied of these materials in vertebrates are those that mimic estrogen.

One of the key diagnostic tools for estrogen activity has been the induction of vitellogenin in males of egg-laying organisms. The estrogen mimic induces the production of this protein that remains in the tissue of males instead of being absorbed into the ovaries, as in the females. Although an important biomarker, it is not clear what is the ecological significance, if any, of vitellogenin production in males. Vitellogenin is a key biomarker for exposure.

As in the case with the estrogenic PCBs, the ability to mimic estrogen is an important structural key. The next paragraphs compare some of the estrogenic-acting compounds to estradiol.

Nonylphenol (Figure 6.27), a surfactant intermediate, has an –OH-substituted ring structure similar to that of estradiol, but with a long carbon chain attached. Nonylphenol does have estrogenic activity, but only 9.0×10^{-6} that of estradiol (Pait and Nelson 2002).

Bisphenol-A and 17a-thinylestradiol both have estrogenic activity, and both have structures resembling the active portion of the estradiol molecule (Figure 6.28). b-Sitosterol has a ring structure with an –OH group, but the ring is not aromatic. Apparently the receptor can interact with ring structures with additional protons.

Tributyltin (TBT) is one of the best-studied endocrine-disrupting compounds in invertebrates (Oberdorster and Cheek 2000). Concentrations of TBT as low as 1 ng/L can lead to the

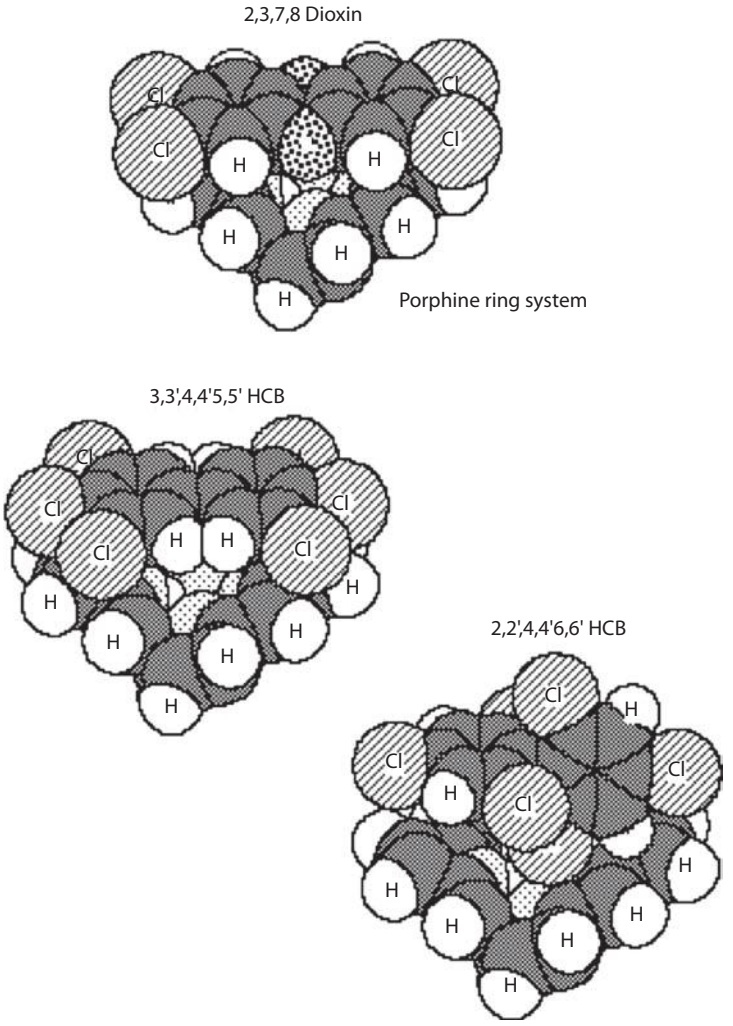


Figure 6.24 Stacking by 2,3,7,8-dioxin and PCBs.

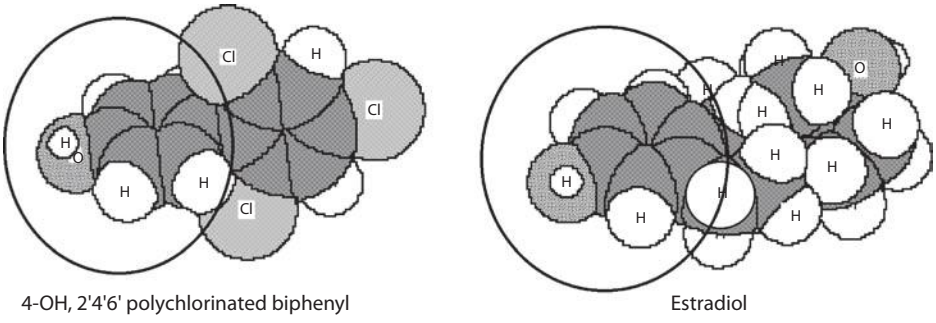


Figure 6.25 Similarity of a substituted PCB with estradiol.

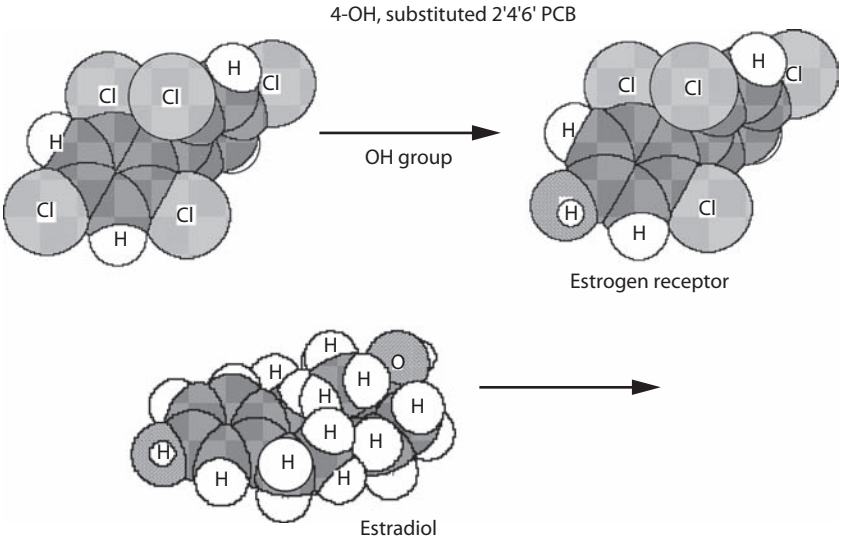


Figure 6.26 Suggested mode of action of a PCB with a substituted –OH group.

Table 6.4 Identified or Suspected Endocrine-Disrupting Compounds

Chemical/Class	*Use/Source*
Industrial Chemicals/By-Products	
4-Nonylphenol	Surfactant intermediate/degradation product
Octylphenol	Surfactant intermediate/degradation product
Bisphenol-A	Monomer of polycarbonate
4-Tert-pentylphenol	Industrial intermediate
Benzo(a)pyrene	Fossil fuel combustion product
Phenanthrene	Fossil fuel combustion product
Polychlorinated biphenyls	Transformer oil
Dioxins	Industrial and waste incineration by-products
Polybrominated biphenyl ethers	Flame retardants
Butyl benzyl phthalate	Plasticizer
Butyl benzyl phthalate	Plasticizer
Di-n-butyl phthalate	Plasticizer

(Continued)

Table 6.4 (*Continued*) Identified or Suspected Endocrine-Disrupting Compounds

Chemical/Class	*Use/Source*
Pesticides	
Atrazine	Herbicide
Carbofuran	Insecticide
Toxaphene	Insecticide
Endosulfan	Insecticide
Lindane	Insecticide
Dichlorodiphenyltrichloroethane (DDT)	Insecticide
DDE	Degradation product of DDT
Tributyltin (TBT)	Antifouling paint ingredient
Mirex	Insecticide
Metals	
Mercury	Industry
Cadmium	Industry
Lead	Industry
Natural Products	
b-Sitosterol	Plant sterol and a pulp and paper industry effluent
Genistein	Plant sterol
Daidzein	Plant sterol
Enterodiol	Plant sterol

Source: Compiled from Oberdorster, E., and Cheek, A. O., *Environ. Toxicol. Chem.*, 20, 23–36, 2001; Pait, A. S., and Nelson, J. O., *Endocrine Disruption in Fish: An Assessment of Recent Research and Results*, NOAA Technical Memorandum, NOS NCCOS SSMA 149, NOAA, NOS, Center for Coastal Monitoring and Assessment, Silver Spring, MD, 2002.

development of male sex organs in female snails. This imposex response has been identified in approximately 150 species of gastropods and is clearly due to an interference with some part of the molluskan endocrine system. The toxicity of TBT to gastropods has caused its regulation.

Endocrine disruption is a newly discovered mode of action and has encouraged a great deal of research. Compared to some of the other mechanisms described in this chapter, endocrine disruption is more subtle, with alterations in reproductive physiology and morphology often being the effects, instead of death. Because of the hormone-like activity, these compounds can have identifiable effects at very low concentrations. It is not yet clear what the overall importance of endocrine disruptors is in creating environmental impacts compared to other modes of action.

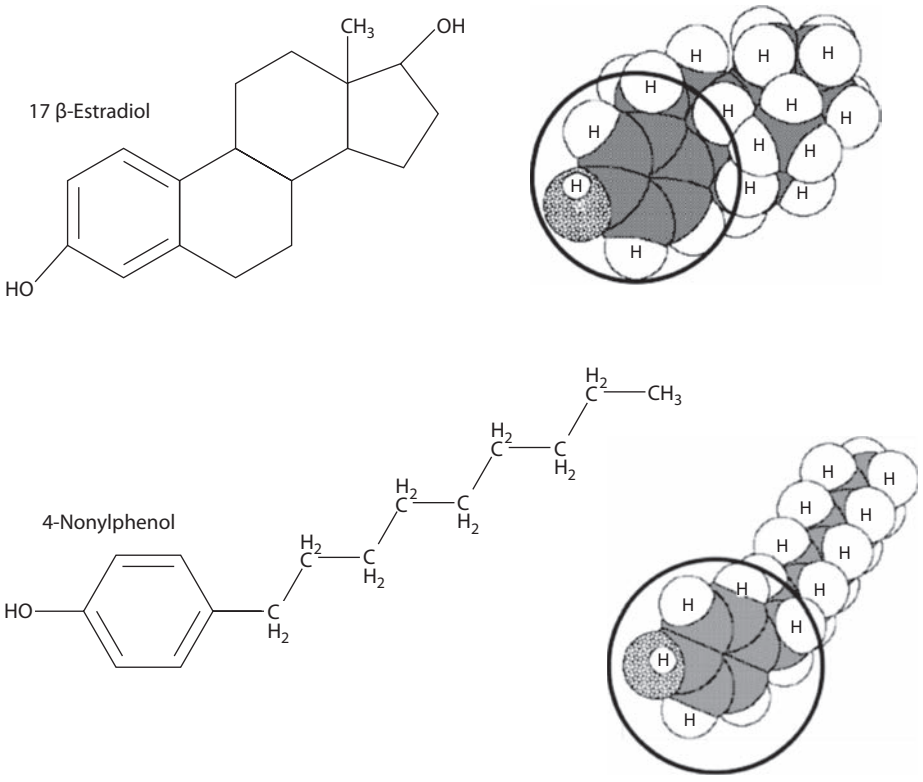


Figure 6.27 Estradiol compared to 4-nonylphenol. Similar areas are circled.

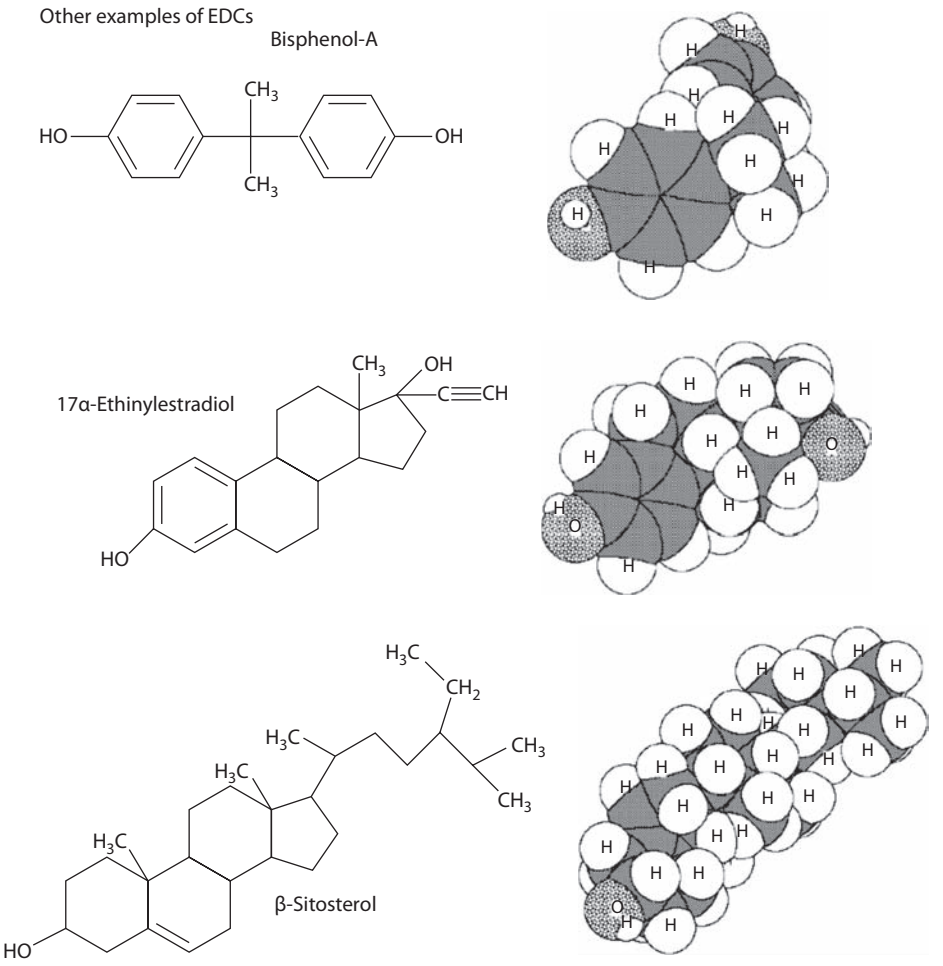


Figure 6.28 Comparisons of three other endocrine-disrupting compounds.

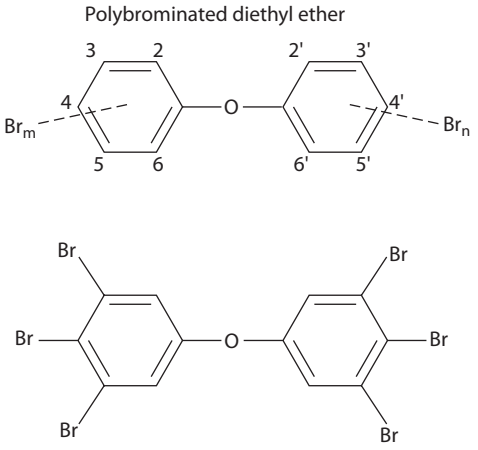


Figure 6.29 The structure of PBDE. Note the similarity in structure to PCBs.

6.5.4 Polybrominated Diphenyl Ethers (PBDEs)

Special note should be made of the toxicity of the polybrominated diphenyl ethers (PBDEs) used as flame retardants. The general structure of these compounds can be found in Figure 6.29 and should look familiar. The PBDEs have a backbone of two phenyl groups joined by a bond to a central oxygen. On the phenyl groups the halogen bromines are substituted into each ring. The rings are free to rotate around the central bond, similar to PCBs that do not have steric hindrance due to the substituted chlorines.

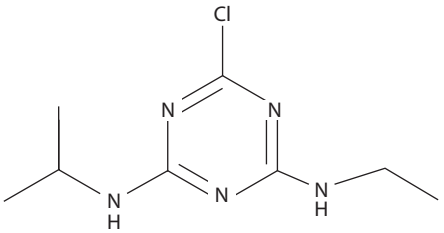
Two reviews have been published that cover the toxicity of decabromodiphenyl ether (EPA 2008a) and 2,2',4,4'-tetrabromodiphenyl ether (EPA 2008b). PBDEs accumulate in the environment in tissue and fat, similar to PCBs. Although structurally similar to PCBs and TCDD, numerous studies have only shown a weak interaction with the Ah and estrogen receptors, as much as 5 to 10% less than that of TCDD. The decabromodiphenyl ether has a lower activity than the tetrabromodiphenyl ether.

Although the PBDEs bioaccumulate in the environment in a manner similar to that of the PCBs and dioxins, it does not appear that the molecular mode of action follows this pattern. It is not clear what the differences between the PCBs and the PBDEs are that preclude the activation of the Ah receptor.

6.5.5 The Multiple Modes of Action of Atrazine

One of the major controversies in environmental toxicology has been the range of toxicity of the common herbicide atrazine (Figure 6.30). This triazine is widely used through out the United States (Solomon et al. 1996, 2008). As the time of this writing, atrazine was being evaluated by the Science Advisory Panel of the U.S. EPA as part of a Federal Insecticide, Fungicide, and Rodenticide Act review (http://www.epa.gov/pesticides/reregistration/atrazine/atrazineupdate.htm, October 8, 2009). This review has been initiated due to concerns that have been raised about the potential modes of toxicity of atrazine in addition to its plant toxicity.

In plants the mode of action of atrazine is by competing with plastoquinone II for its binding site within the photosynthesis II pathway, blocking electron transport and destroying chlorophyll. The toxicity of this mode of action varies according to type of plant, algae, or cyanobacteria. This



Atrazine (6-chloro-4-N-ethyl-2-N-propan-2-yl-1,3,5-triazine-2,4- diamine)

Figure 6.30 Structure of atrazine.

mode of action is well understood. Because of the inhibition of photosynthesis to a variety of plants, atrazine can drastically alter the plant community structure and cause indirect effects to the heterotrophic constituents. Moderate direct toxicity to a variety of organisms has been extensively documented (Solomon et al. 1996).

Hayes and colleagues (2002a, 2002b, 2003) observed gonadal abnormalities in male *Rana pipiens* exposed while larvae to atrazine at concentrations as low as 0.1 to 25 ppb in the laboratory. *R. pipiens* is a species widespread in the eastern and midwestern portions of the United States. Abnormalities were also found in field collections with exposure to atrazine (2002b, 2003). The suggestion was that atrazine also had an endocrine-disrupting mode of action that may account for observed decline in amphibian populations in many areas.

Contradicting these results, Carr et al. (2003) reported that atrazine was not a potent developmental toxicant to the reproductive systems of the frog *Xenopus laevis*, a species originating from Africa that is widely used for testing developmental endpoints. Estradiol and atrazine at 25 µg/L did increase the frequency of intersex animals, but other effects similar to that of estradiol were not observed.

It is critical to note that these studies were not replicates of each other. There were two very different species of amphibian used, different methods of exposure, and different methods of evaluating pathology. *X. laevis* is routinely used in the Frog Embryo Teratogenesis Assay–Xenopus (FETAX), a rapid screening tool. The methods used by Carr et al. (2003) were similar to the standard FETAX protocol. Both sets of studies did demonstrate gonadal abnormalities, and Carr et al. hypothesize the alteration of aromatase activity may be the mode of action. Aromatase is the enzyme that converts testosterone to estradiol and atrazine, and other triazines are known to induce activity in some vertebrate cell lines.

These studies inspired a number of researchers to investigate the potential estrogenic activity of atrazine and other triazines on fish and amphibians. Two recent and extensive reviews summarize this information. I review the papers in the order of publication.

Solomon et al. (2008) is an extensive narrative and classical style review of the literature on the environmental toxicity of atrazine. The potential modes of action, toxicity, fate, developmental toxicity, and other endpoints are evaluated, as is the evidence for each. Experiments for a broad number of species are examined as well as microcosm and mesocosm experiments. Research at a population scale was reviewed, although few field studies were conducted.

This review exemplifies both the strengths and weaknesses of such a review. One of the strengths is the exhaustive nature of the coverage. The citation list is extensive, and it is clear that every effort was made to collect the relevant literature on the toxicity of atrazine. At the end of the summary a qualitative analysis of the strengths and weaknesses of the field of study was presented,

and it is clear that there are major questions regarding experimental design, physiological effects, and potential impacts at population scales. Conversely, the nature of the narrative evaluation did not make it clear that every source was held to the same criteria for study design, such as analytical technique, statistical power, pathology technique, and data analysis. Value-laden terms such as "confusing" (p. 737), "judged to be small" (p. 733), and "are speculative" (p. 737) do not indicate what specific and quantitative criteria were used to evaluate each study.

Solomon et al. (2008) summarize the results using a weight of evidence framework based upon Koch postulates or Bradford-Hill guidelines. However, these classic guidelines for a weight of evidence are narrative, although there are more quantitative methods for assessing causality. Because of the lack of specific criteria to judge individual studies, it is not clear if negative or positive results were due to poor or excellent study design, the species tested, or other confounding factors. It is also not clear what conceptual cause–effect model was being tested in this analysis. The conclusion of the authors is that there are not lines of evidence that support estrogenic effects upon fish and amphibians at concentrations expected to be found in the field.

Rohr and McCoy (2009) use a meta-analytical approach to examine the potential environmental effects of atrazine. The starting points for the review are the studies cited in Solomon et al. (2008), with updates from a literature search. Specific criteria were used to evaluate the studies that were to be included in the analytical aspect of the study. These criteria included studies without statistics, the amount of contamination from outside sources, confounding issues from the nearness to other potential stressors, pseudoreplication, and other attributes. A vote counting method was used to tally the results for 15 response variables from 125 studies. As reported by Rohr and McCoy, a significant failing of the body of literature was the overwhelming use of hypothesis testing using an analysis of variance technique rather than the more informative concentration-response regression approach. Chapter 4 of this book reviews the issues with using hypothesis testing to derive a NOEC, as opposed to the derivation of an EC value.

The results of this meta-analysis did confirm the results of previous studies—that there is no evidence for the direct effect of atrazine on fish or amphibian survival. Other findings were not consistent with Solomon et al. (2008).

First, Rohr and McCoy (2009) delineate several aspects of the process of metamorphosis. A minimum size must be reached before the process can begin. Once the size is reached, development can be accelerated so that metamorphosis can occur earlier if the environment is not optimal, or later in optimal conditions. Metamorphosis is controlled by corticosterone and thyroid hormones. An effect of endocrine disruption will be an alteration of the timing of metamorphosis. Depending on a number of conditions, an increase or decrease in time to metamorphosis could occur due to a toxic affect. A lack of understanding of these interactions could lead to a misinterpretation of the results of toxicity tests.

The scoring systems and the conceptual model for metamorphosis were then used to evaluate the studies that met the criteria for a suitable study. The meta-analysis resulted in 13 of 21 studies demonstrating significant effects of atrazine on metamorphic timing. The results were split evenly between those that increase the time and those that decrease the time to metamorphosis. A concentration-response relationship was evident between atrazine concentration and size at metamorphosis in 19 of 19 studies, and at concentrations expected to occur in field situations. As reported by Solomon et al. (2008), Rohr and McCoy did not find studies that examined the effects of atrazine at the population level.

A number of other endpoints, such as effects on locomotor activity, antipredatory behavior, olfaction, and immune function, were also analyzed, with atrazine having effects upon many of them. The analysis also found that atrazine affected male gonadal activity in 8 of the 10 studies,

and 6 of the studies had statistically significant results. However, studies on vitellogenin induction did not indicate an estrogenic role for atrazine, and the induction of aromatase was described in one of six studies.

Rohr and McCoy conclude that atrazine is not acutely toxic at concentrations expected in the field. However, in the vast majority of studies and for a variety of endpoints, atrazine does produce effects that would be important to survival and reproduction of vertebrates at field concentrations. The impacts of these effects at the population and community scale are described further in Chapters 12 and 13. Although effects do occur, the molecular mechanism or mechanisms for atrazine toxicity are not yet clear for vertebrates.

One of the advantages of a meta-analysis such as that performed by Rohr and McCoy is that an initial screening of published reports was performed to eliminate studies that did not meet the specifications set a priori for an acceptable paper. This is a process that is also followed when quantitative structure-activity relationship models are generated from literature sources (see Section 6.6). Often, 70% of the published studies did not meet the criteria for inclusion in a QSAR database for a number of reasons. Without this screening there would be too much noise in the data to detect the structure-activity signal. A similar issue may be occurring in the atrazine effects database. Without a careful screening using preestablished criteria and clear conceptual models regarding physiological processes, the noise can overwhelm the signal. Conventional reviews of research literature performed without a meta-analysis approach may be too overwhelmed by a number of confounding issues to produce reliable results.

6.6 Introduction to QSAR

Quantitative structure-activity relationships (QSARs) are a method of estimating the toxic properties of a compound using the physical and structural makeup of a compound. These properties and the knowledge that similar compounds typically have similar modes of action make QSAR a possibility. In many instances no toxicity data are available for a compound for a variety of reasons. Perhaps the most interesting one is in the evaluation of proposed compounds of which only small amounts or none at all are available. QSAR can be instrumental in selecting compounds with the desired properties but with low toxicity to the environment.

Each substructure of a molecule contributes to its toxicity in a specific way, and the QSAR equation describes this contribution. Models of this type have proven to be successful in the estimation of carcinogenicity, mutagenicity, and rat, mouse, daphnid, and fathead minnow acute toxicity, and at establishing toxicological relationships across species boundaries.

Toxicity data are generally of two types. First, most toxicity data are continuous; that is, they may have virtually any numerical value. LD_{50}, NOEC, EC_{50}, and EC_{10} are all examples of data that are continuous. Second, discriminate data exist. These data place the result into categories such as mutagenic–not mutagenic, carcinogen–not carcinogenic, and so forth. These two basic types of toxicological determinations require models different in structure.

Continuous toxicity data can be generally described using a regression type model as depicted in Figure 6.31. This is a simple linear regression model using only one parameter to describe the toxicity. The resulting expression used to describe the relationship between toxicity and the parameter is a typical linear equation:

$$y = mx + b \tag{6.5}$$

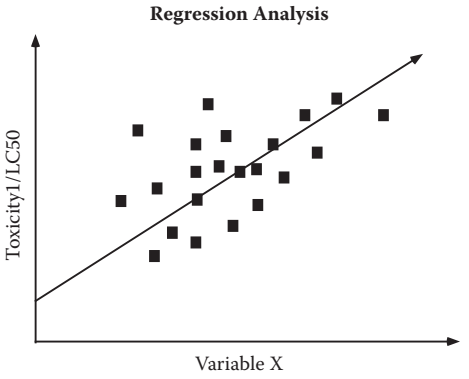


Figure 6.31 Linear regression model for continuous data in QSAR analysis. The model is a simple linear regression with toxicity plotted against the physical or structural variable being used for the estimate.

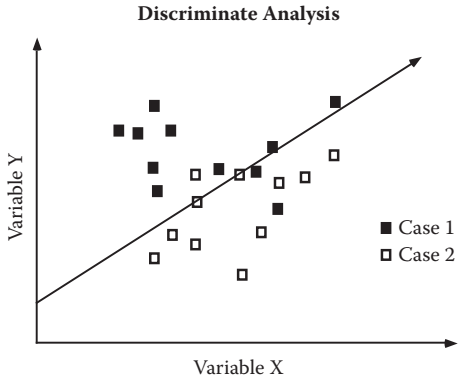


Figure 6.32 Discriminant analysis. In this case the goal is to differentiate data that are in two categories, case 1 and case 2. Case 1 could be mutagenic, and case 2 not mutagenic. Many toxicological measurements are categorical in nature.

where y is the estimate of toxicity, m is the slope of the line, x is the numeric expression of the predictive parameter, and b is the constant value that represents the y intercept of the line. This equation can be generalized to use as many dimensions as there are parameters that contribute to the estimate of toxicity. Table 6.5 portrays such an equation in tabular form, but note that the form is the basic linear equation.

Discriminate data are either/or situations and can be depicted similar to the continuous type variables (Figure 6.32). However, the black square and white square depict dichotomous data. The goal is to derive a line that separates the two groups, and this is known as a discriminant analysis. The resulting equation is similar in basic form to the linear regression depicted above.

6.6.1 Construction of QSAR Models

Three sets of traditional models for toxicity using regression and discriminate analysis are generally produced. General models are often produced relying upon chemical parameters such as log P.

Table 6.5 *Daphnia* EC_{50} Equation for Model Incorporating Molecular Connectivity Indices and Substructural Keys

Key	*Coefficient*
Primary amine bound to aromatic ring atom	1.0167
Primary amine bound to aliphatic or alicycle carbon	1.0343
Aliphatic alcohol	–0.5294
Oxygen-substituted aryl ester	–0.7801
Benzene	1.0320
Secondary or tertiary diphatic alcohol	–1.0058
1,1-Dichloro (non-beta phynyl)	0.8091
1,1-Divinyl chloride (non-beta phynyl)	1.0021
Secondary or tertiary amine bound to electron-releasing groups only	1.3375
One or more electron-releasing groups and four or more electron-withdrawing groups on a single benzene ring	0.7820
Three carbon fragments between two functional groups (electron withdrawing, electron releasing, or combination)	0.9442
NH substituted with one electron-releasing and one-electron withdrawing group	1.3467
Ethane or ethylene between two electron-releasing groups	–0.1819
Valence path MCI, order 2	0.3515
Valence path MCI, order 4	0.1198
Sum simple and valence chain MCI, order 6	0.3621
Intercept	2.2578

Source: After Enslein, K. et al., in *Aquatic Toxicology and Environmental Fate*, eds. G. W. Suter and M. A. Lewis, Vol. 11, ASTM STP 1007, American Society for Testing and Materials, Philadelphia, 1989, pp. 397–409.

Models are also often produced that attempt to describe a particular subset of compounds unique in their composition or mode of action. Third, models can be produced that incorporate toxicity data from other species or other types of biological measurements.

The first groups of models are generally constructed using molecular connectivity indices, kappa environmental descriptors, electronic charges, and substructural keys. In many instances log P has been used; however, our experience has been that models based upon log P do not model well a biological endpoint for a heterogeneous series of compounds. The attempt is made in these models to plot a broad map of the relationship between toxicity and general chemical parameters. These models have proven successful in predicting toxicity in a number of toxicity tests, including rat oral LD_{50}, *Daphnia* EC_{50}, and fathead minnow, to name a few. In addition to modeling continuous endpoints, this approach has also been found to be useful in predicting categorical endpoints such as mutagenicity, carcinogenicity, and skin irritation.

Occasionally, compounds with distinctive modes of action are better modeled apart from the general case. Examples of such compounds are the acetylcholinesterase inhibitors. These compounds are very specific in their inhibition of serine enzymes. In the instance of predicting *Daphnia* EC_{50} it was found that the organophosphates were outliers that biased the regression and were better removed from the general model and treated separately. Another class of specialized models is those grouped by chemical class. These have proven popular because of their relative simplicity, but the data sets upon which they are built are usually small.

The third set of models would be interspecies models similar to those used for the extrapolation of rat oral LD_{50} to *Daphnia magna* EC_{50}. These interspecies models have been shown to be very accurate when the size of the database is taken into account, and may prove useful when mammalian data are the only toxicity data available for a compound. Sets of these models may have a great deal of utility in interspecies estimations made necessary by the lack of data with wild species.

6.6.2 Typical QSAR Model Development

All three types of models are produced using similar methodologies. The basic methodology for the construction of a multiparameter QSAR is presented in Figure 6.33. Among the most difficult aspects is the acquisition of a reliable and consistent database. The reliability of the database cannot be overemphasized since all subsequent processes are totally dependent upon the size and quality of these data. Published open literature, government reports, contractor data, and premanufacturing notices all have been useful in supplying the raw data for the modeling process. Next, the data are evaluated according to preset guidelines to ensure the consistency of the data. Often guidelines such as those set by the American Society for Testing and Materials, the U.S. Environmental Protection Agency, and programs such as GENETOX are used to establish criteria for the inclusion of data. Data derived from mixtures, compounds with known impurities, and experiments that do not show a dose-response are eliminated from the data set. An attempt is made to include as wide a variety of classes of compounds as possible in order to describe as much of molecular space as possible. In interspecies models only the intersection of the appropriate species is used. The size of the intersection determines the accuracy of interspecies model construction. In studies conducted to date, the number of compounds in this intersection have been small; however, the power of including a toxicity endpoint increases the predictive power of the model when compared to models with chemical endpoints alone.

Because a molecule is the unit of toxicity, not mass in mg/kg, it is generally necessary and desirable to transform the LD_{50} and LC_{50} values into molar form as follows:

$$\log 1/C = \log (\text{molecular weight} \times 1{,}000/LD_{50} \text{ or } LC_{50}) \quad (6.6)$$

where C is the molar concentration.

A variety of parameters are included in the QSAR equation. Log P is a commonly used parameter and is obtained from Medchem or estimated using the CLOGP3 computer program. Molecular weight is calculated. In interspecies models the LD_{50} or LC_{50} value is incorporated as a typical parameter. Molecular connectivity indices, electronic charge distributions, and kappa environmental descriptors have been proven as powerful predictors of toxicity. The efficacy of these values lies in the fact that each of these parameters describes a molecule in a fashion similar to that actually seen by the molecular receptors that initiate a toxic response. Substructural keys are identified with the help of the MOLSTAC™ substructural key system. MOLSTAC consists of five classes of descriptors:

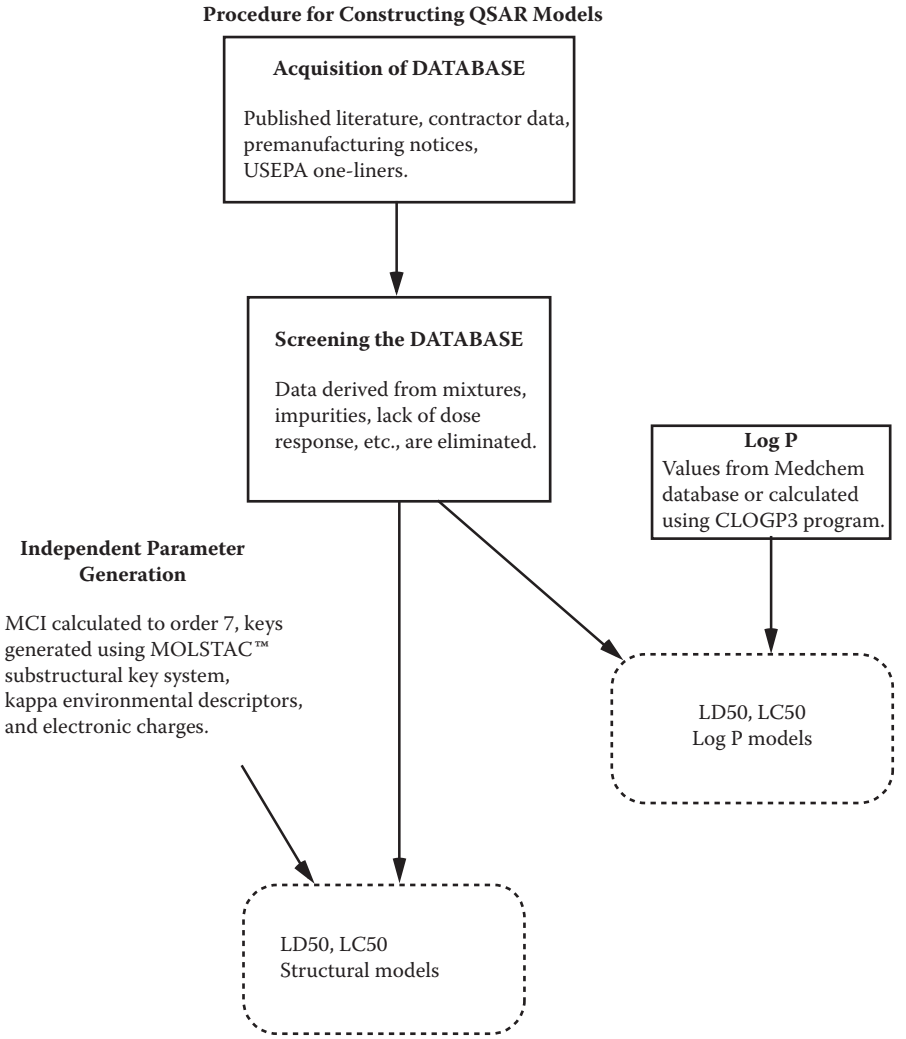


Figure 6.33 The developmental process for the construction of a structure-activity model.

1. Identification of the longest continuous chain of atoms (excluding hydrogen) in the molecule.
2. Identification of carbon chain fragments.
3. Identification of ring systems, including combinations such as the rings forming the bay region of certain carcinogens.
4. Identification of chemically or biologically, or both, functional substructural fragments.
5. Identification of electron-donating and electron-withdrawing substructural keys.

Multiple regression is used to generate the final equation. Figure 6.34 outlines the derivation of the QSAR equation. After database assembly potential parameters are examined using simple statistics for the detection of problematical distributions that may have to be transformed. Next, a stepwise regression analysis is performed. F scores of at least 1.7 are necessary for the parameter to be included in the final equation. Care is taken to avoid spurious correlations or collinearity difficulties.

The initial regression is examined for robustness from the standpoint of both influential chemicals and poorly behaved parameters. Ridge regression, Cook's distance, partial correlations, and principal components are used to evaluate the regression. After the poorly behaved parameters are removed, another analysis of the regression is performed. Usually several parameters are removed during this process.

Validation is one of the most difficult aspects of environmental QSAR development due to the comparatively small size of the database. Cross-validation has been useful in validating the

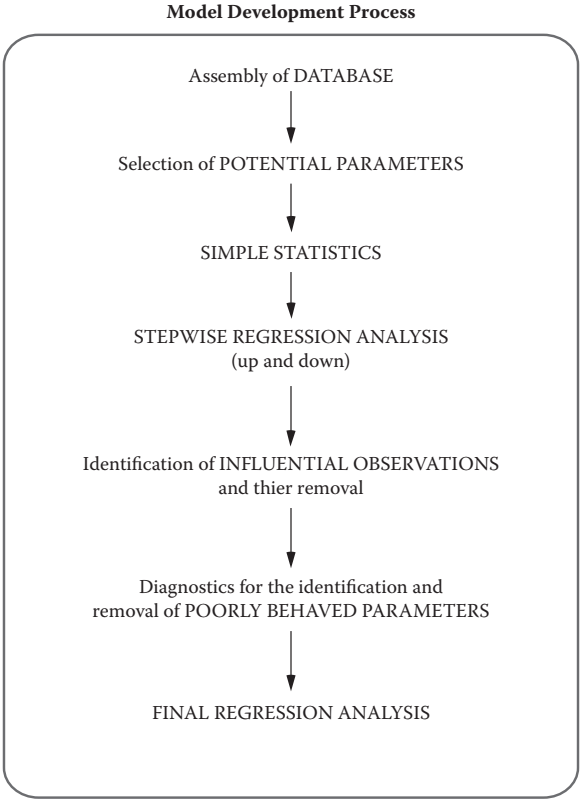


Figure 6.34 The statistical processes of QSAR model development using regression.

effectiveness of the model. In this method, one compound is removed from the database, the equation is recalculated, and the toxicity of the omitted compound is estimated. The process is repeated for all compounds in the data set, and the results are tabulated. In this manner, a calculation of the accuracy of prediction of continuous data and the rate of misclassification for categorical data can be compiled. A more useful estimate of the validity of the QSAR model is its ability to predict the toxicity of new compounds. Generally this is difficult to accomplish in a statistically significant way due to the slow accumulation of new data that meet the criteria used in the modeling process and the associated expense.

6.6.3 Estimation of Toxicity Using QSAR

The example of the toxicity estimation using QSAR is based on the TOPKAT system developed by Health Designs, Inc. and is the computer program most familiar to the authors. The process of estimation is straightforward when the equations are incorporated into the TOPKAT program. The structure to be evaluated is input using a linear notation, SMILES, for the two-dimensional structure of the compound. The model to be used is specified and loaded along with the accompanying database for validation process. The TOPKAT program searches for parameters and calculates the regression score and the resultant LD_{50} estimate. Using the TOPKAT program, an evaluation of the reliability of the estimate is made looking for similar compounds in the database. The results are reported with a comment on the terms that contributed to the estimate and a comparison of the estimate to literature values for similar compounds.

An example of the process is the estimation of the toxicity to *D. magna* of the simple organic isopropylamine. The compound was given a unique identification, and that is usually the Chemical Abstracts Service (CAS) number for easy identification. The chemical structure is then represented in SMILES and the model selected. In the case of the *D. magna* model the estimate was as follows:

Key	*Cross-Product*
Primary amine (noncyclic) r-NH2 (R = alkyl)	0.961
Valence adjusted path MCI order 1	0.437
Constant term	2.287
Total	3.685

The estimate of EC_{50} as log(1,000/molar) = 3.685, or 12.2 mg/L.

The compound was examined using the structural key and other indices to test how well the keys used in the modeling process described isopropylamine. The computer search of these keys confirmed that isopropylamine was well described by the model.

The next step is the validation process. Validation is simply an examination of the model with compounds for which toxicity data are available and that were estimated by the QSAR equation. This process provides an indication of how well the model predicts the toxicity of compounds similar to the unknown. In this estimate six compounds were used as comparisons:

Compound	*Actual EC_{50}*	*Predicted EC_{50}*
2-Ethylhexylamine	2.2	4.44

Compound	*Actual EC_{50}*	*Predicted EC_{50}*
Allyamine	110.0	14.1
Cyclohexylamine	80.0	6.9
n-Butylamine	75.0	30.8
Ethanolamine	140.0	49.6
Ethylamine	110.0	12.0

In general, the model overestimated the toxicity of these compounds. Toxicity tests performed with isopropylamine confirmed that the estimated toxicity was an overestimate. The 48-hour *D. magna* EC_{50} was found to be 89.4 mg/L with the pH uncontrolled and 195.3 mg/L with the pH adjusted to a normal range. The importance of the validation step is crucial. The performance of the model can be measured, and the overestimate of the isopropylamine toxicity was consistent with past performance.

Another crucial aspect of the validation process is the test of how well described and represented the molecule is in the map of the chemical-toxicity space that the regression equation represents. If the substructural key does not exist in the database used to build the model, then it is unlikely that the compound can be accurately estimated. In addition, if compounds similar to the test compound do not exist, then a comparison as was done above cannot be conducted, and a measure of the performance of the model with compounds similar to the test material cannot be made. This type of validation requires a large database and a substructural search algorithm, and should be included in a QSAR estimate.

Other types of QSAR models are under development. Perhaps most intriguing is the ability to actually use molecular models of proteins and the organic compound of question to examine at the molecular level the interactions giving rise to toxicity. Widespread use of such models is unlikely to occur due to the enormous amount of data necessary on protein structure, charge distribution, and the properties of the test compound, and the expense of the software and hardware necessary to perform the analysis.

The combination of toxicity information and knowledge of structure can lead to important insights into the modes of action and toxicity of chemicals. An excellent demonstration of this has been the analysis of chemicals that mimic hormones.

Study Questions

1. What is most critical to plant health when an atmospheric pollutant is introduced: ambient concentration or pollutant concentration within the leaf?
2. Describe the route by which photosynthesis and energy metabolism of a plant cell are impaired, beginning with the pollutant passing through the stomata of the epidermal tissue.
3. List six routes by which a pollutant may enter an animal. What is the most common means of entry into the body system for a toxicant?
4. What is the most important chemical property factor affecting absorption of a pollutant?
5. What role does the liver play in affecting a pollutant that has entered an animal?
6. What is the most permanent method of removing toxic substances from the body?
7. Describe the four principal mechanisms by which environmental pollutants exert toxicity.

8. How can pollutants inactivate an enzyme system?
9. Name three examples of secondary action resulting from pollutant presence.
10. What is metal shift?
11. What are the three sites of action within the membrane in narcosis?
12. The toxicity of an organophosphate is related to what chemistry?
13. Organophosphate acute toxicity is usually attributed to the ability to bind to what enzyme?
14. What is aging of a protein by an organophosphate?
15. Give an example of another binding site of organophosphates in an organism.
16. What were the first chemical warfare agents?
17. How are chemical warfare agents related to pesticides?
18. What are potential means of disposing of chemical agents?
19. What are monohaloacetic acids? Describe the mode of action of fluoroacetic acid, iodoacetic and bromoacetic acids, and chloroacetic acid.
20. What are the potential modes of action for 2,3,7,8-TCDD?
21. Describe the interaction of TCDD with the Ah receptor.
22. What kinds of pathways are initiated by the Ah-TCDD complex?
23. Explain the potential modes of action for coplanar PCBs.
24. Why is the term *coplanar PCB* really a misnomer?
25. Diagram the stacking and cleft models for describing PCB and dioxin toxicity.
26. What mode of action do PCBs without coplanarity share with synthetic estrogens?
27. Explain the difference in toxicity of TCDD between vertebrates and invertebrates.
28. PBDE resembles PCB and TCDD is what ways?
29. What are the proposed modes of action of atrazine?
30. Contrast the meta-analysis process of Rohr and McCoy with that of Solomon et al.
31. What are QSARs?
32. What are the two general types of toxicity data? How are they modeled?
33. Describe the three sets of traditional models for toxicity using regression and discriminate analysis.
34. Describe the developmental process for the construction of a structure-activity model. What is the importance of the reliability of the database?
35. What is MOLSTAC and how is it used?
36. Describe the statistical processes of the QSAR model.
37. Explain cross-validation of the QSAR model.
38. Describe the TOPKAT system.
39. What are two examples of problems that may be encountered when a compound or molecule is tested for description and representation in the map of the chemical-toxicity space represented by the regression equation.

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Chapter 7

Factors Modifying the Activity of Toxicants

7.1 Introduction

Just as there are a large number of pollutants in our environment, so are there many factors that affect the toxicity of these pollutants. The major factors affecting pollutant toxicity include physicochemical properties of pollutants, mode of exposure, time, environmental factors, interaction, biological factors, and nutritional factors. These parameters that modify the toxic action of a toxicant are examined in this chapter.

7.2 Physicochemical Properties of Pollutants

Characteristics such as whether a pollutant is a solid, liquid, or gas; soluble in water or in lipids; organic or inorganic material; ionized or nonionized; etc., can affect the ultimate toxicity of the pollutant. For example, since membranes are more permeable to a nonionized than an ionized substance, a nonionized substance will generally have a higher toxicity than an ionized substance.

One of the most important factors affecting pollutant toxicity is the concentration of the pollutant in question. Even a generally highly toxic substance may not be very injurious to a living organism if its concentrations remain very low. On the other hand, a common pollutant such as carbon monoxide can become extremely dangerous if its concentrations in the environment are high. As mentioned earlier, exposure to high levels of pollutants often results in acute effects, while exposure to low concentrations may result in chronic effects. Once a pollutant gains entry into a living organism and reaches a certain target site, it may exhibit an action. The effect of the pollutant, then, is a function of its concentration at the locus of its action. For this reason, any factors capable of modifying internal concentration of the chemical agent can alter the toxicity.

7.3 Time and Mode of Exposure

Exposure time is another important determinant of toxic effects. Normally, one can expect that for the same pollutant, the longer the exposure time, the more detrimental the effects. Also, continuous exposure is more injurious than intermittent exposure, with other factors being the same. For example, continuous exposure of rats to ozone for a sufficient period of time may result in pulmonary edema. But when the animals were exposed to ozone at the same concentration intermittently, no pulmonary edema may be observed. The mode of exposure, i.e., continuous or intermittent, is important in influencing pollutant toxicity because living organisms often can, to a certain degree, repair injuries caused by environmental agents. In addition, organisms may be able to develop tolerance so that they will be able to withstand otherwise toxic doses of chemical substances.

7.4 Environmental Factors

Environmental factors such as temperature, light, and humidity also influence the toxicity of pollutants.

7.4.1 Temperature

Numerous effects of temperature changes on living organisms have been reported in the literature (Krenkel and Parker 1969). Thermal pollution has been a concern in many industries, particularly among power plants. Thermal pollution is the release of effluent that is at a higher temperature than the body of water it is released into. Vast amounts of water are used for cooling purposes by steam-electric power plants. Cooling water is discharged at an elevated temperature, and some rivers may have their water temperatures raised so high that fish life is completely eliminated.

Temperature changes in a volume of water affect the amount of dissolved oxygen (DO) available in aquatic systems. The amount of DO present at saturation in water decreases with increasing temperature. On the other hand, the rate at which chemical reactions occur increases with increased temperatures. This leads to faster assimilation of waste and therefore faster depletion of oxygen. Fish and other aquatic life can live only within certain temperature ranges, and the range in which well-being exists is narrower than the range in which survival is possible. Subtle behavior changes in fish are known to result from temperature changes too small to cause injury or death.

Temperature also affects the response of vegetation to air pollution. Generally, plant sensitivity to oxidants increases with increasing temperature up to 30°C. Soybeans are more sensitive to ozone when grown at 28°C than at 20°C, regardless of exposure temperature or ozone doses (Dunning et al. 1974). The response of pinto bean to a 20 and 28°C growth temperature was found to be dependent on both exposure temperature and ozone dose. Hull and Went (1952) observed a positive correlation between postexposure temperature and severity of injury to five plant species within the temperature range of 3 to 36°C.

7.4.2 Humidity

Generally, the sensitivity of plants to air pollutants increases as relative humidity increases. However, the relative humidity differential may have to be greater than 20% before differences are shown. MacLean et al. (1973) found gladiolus plants to be more sensitive to fluoride as relative humidity increased from 50% to 80%.

7.4.3 Light Intensity

The effect of light intensity on plant response to air pollutants is difficult to generalize because of several variables involved. For example, light intensity during growth affects the sensitivity of pinto bean and tobacco to a subsequent ozone exposure. Sensitivity increased with decreasing light intensities within the range of 900 to 4,000 foot-candles (ft-c) (Dugger et al. 1963; Dunning and Heck 1973). In contrast, the sensitivity of pinto bean to peroxyacetyl nitrate (PAN) increased with increasing light intensity (Dugger et al. 1963). Plants exposed to pollutants in the dark are generally not sensitive. At low light intensities, plant response is closely correlated with stomatal opening. However, since full stomatal opening occurs at about 1,000 ft-c, light intensity must have an effect on plant response in addition to its effect on stomatal opening.

7.5 Interaction of Pollutants

Seldom are living organisms exposed to a single pollutant. Instead, they are exposed to combinations of pollutants simultaneously. In addition, the action of pollutants is dependent on many factors, including portals of entry, action mode, metabolism, and others described above. Exposure to combinations of pollutants will no doubt lead to manifestation of effects different from those that would be expected from exposure to each pollutant separately. The combined effects may be synergistic, potentiative, or antagonistic, depending on the chemicals and the physiological condition of the organism involved.

7.5.1 Synergism and Potentiation

These terms have been used and defined variously but nevertheless refer to toxicity greater than would be expected from the toxicities of the compounds administered separately. It is generally considered that, in the case of potentiation, one compound has little or no intrinsic toxicity when administered alone, while in the case of synergism both compounds have appreciable toxicity when administered alone. For example, smoking and exposure to air pollution may have a synergistic effect, resulting in increased lung cancer incidence. The presence of particulate matter such as sodium chloride (NaCl) and sulfur dioxide (SO_2), or SO_2 and sulfuric acid mist simultaneously would have potentiative or synergistic effects on animals.

Similarly, exposure of plants to both O_3 and SO_2 simultaneously is more injurious than exposure to either of these gases alone. For example, laboratory work indicated that a single 2- or 4-hour exposure to O_3 at 0.03 ppm and to SO_2 at 0.24 ppm did not injure tobacco plants. Exposure for 2 hours to a mixture of 0.031 ppm of O_3 and 0.24 ppm of SO_2, however, produced moderate (38%) injury to the older leaves of Tobacco Bel W3 (Menser and Heggestad 1966) (Table 7.1).

Many insecticides have been known to exhibit synergism or potentiation. The potentiation of the insecticide malathion by a large number of other organophosphate compounds is an example. Malathion has low mammalian toxicity due primarily to its rapid hydrolysis by a carboxylesterase. *O*-ethyl-*O*-*p*-nitrophenyl phenylphosphorothioate (EPN), a phosphonate insecticide, was shown to cause a dramatic increase in malathion toxicity to mammals at doses that, given alone, caused essentially no inhibition of cholinesterase. *In vitro* studies further showed that the oxygen analog of EPN, as well as many other organophosphate compounds, increases the toxicity of malathion by inhibiting the carboxylesterase responsible for its degradation.

Table 7.1 Synergistic Effect of Ozone and Sulfur Dioxide on Tobacco Bel W3 Plants

Duration (hour)	Toxicants, ppm		Leaf Damage (%)
	O_3	SO_2	
2	0.03	—	0
2		0.24	0
2	0.031	+0.24	38

7.5.2 Antagonism

Antagonism may be defined as that situation in which the toxicity of two or more compounds present or administered together, or sequentially, is less than would be expected in terms of their toxicities when administered separately. Antagonism may be due to chemical or physical characteristics of the pollutants, or it may be due to the biological actions of the pollutants involved. For example, the highly toxic metal cadmium (Cd) is known to induce anemia and nephrogenic hypertension as well as teratogenesis in animals. Zinc (Zn) and selenium (Se) act to antagonize the action of Cd. Studies show that both Zn and Se inhibit renal retention of Cd.

Physical means of antagonism can also exist. For example, oil mists have been shown to decrease the toxic effects of O_3 and NO_2 or certain hydrocarbons in experimental mice. This may be due to the oil dissolving the gas and holding it in solution, or the oil containing neutralizing antioxidants.

7.6 Toxicity of Mixtures

Evaluating the toxicity of chemical mixtures is an arduous task, and direct measurement through toxicity testing is the best method for making these determinations. However, the ability to predict toxicity by investigating the individual components and predicting the type of interaction and response to be encountered is tantamount. These mathematical models are used in combination with toxicity testing to predict the toxicity of mixtures (Brown 1968; Calamari and Marchetti 1973; Calamari and Alabaster 1980; Herbert and VanDyke 1964; Marking and Dawson 1975; Marking and Mauck 1975).

7.6.1 Simple Models of Mixture Toxicity

Elaborate mathematical models have been used extensively in pharmacology to determine quantal responses of joint actions of drugs (Ashford and Cobby 1974; Hewlett and Plackett 1959, 1964). Calculations are based on knowing the site of dosage, site of action, and physiological system, which are well documented in the pharmacological literature. Additionally, numerous models exist for predicting mixture toxicity but require prior knowledge of pairwise interactions for the mixture (Christensen and Chen 1991). Such an extensive database does not exist for most organisms used in environmental toxicity testing, precluding the use of these models.

Simpler models exist for evaluating environmental toxicity resulting from chemical mixtures. Using these models, toxic effects of chemical mixtures are determined by evaluating the toxicity

of individual components. These include the toxic units, additive (Marking 1977), and multiple toxicity (Konemann 1981) indices. These models, working in combination, will be most useful for the amount of data that are available for determining toxicity of hazardous waste site soil to standard test organisms.

The most basic model is the toxic unit model, which involves determining the toxic strength of an individual compound, expressed as a "toxic unit." The toxicity of the mixture is determined by summing the strengths of the individual compounds (Herbert and Vandyke 1964) using the following model:

$$= \frac{P_s}{P_{T50}} + \frac{Q_s}{Q_{T50}} \tag{7.1}$$

where S represents the actual concentration of the chemical in solution and T_{50} represents the lethal threshold concentration. If the number is greater than 1.0, less than 50% of the exposed population will survive; if it is less than 1.0, greater than 50% will survive. A toxic unit of 1.0 = incipient LC_{50} (Marking 1985).

Building on this simple model, Marking and Dawson (1975) devised a more refined system to determine toxicity based on the following formula:

$$\frac{A_m}{A_i} + \frac{B_m}{B_i} = S \tag{7.2}$$

where A and B are chemicals, i and m are the toxicities (LC_{50}s) of A and B individually and in a mixture, and S is the sum of activity. If the sum of toxicity is additive, $S = 1$; sums that are less than 1.0 indicate greater than additive toxicity, and sums greater that 1.0 indicate less than additive toxicity. However, values greater than 1.0 are not linear with values less than 1.0.

To improve this system and establish linearity, Marking and Dawson (1975) developed a system in which the index represents additive, greater than additive, and less than additive effects by zero, positive, and negative values, respectively. Linearity was established by using the reciprocal of the values of S, which were less than 1.0, and a zero reference point was achieved by subtracting 1.0 (the expected sum for simple additive toxicity) from the reciprocal $[(1/S) - 1]$. In this way, greater than additive toxicity is represented by index values greater than 1.0. Index values representing less than additive toxicity were obtained by multiplying the values of S that were greater than 1.0 by –1 to make them negative, and a zero reference point was determined by adding 1.0 to this negative value $[S(-1) + 1]$. Therefore, less than additive toxicity is represented by negative index values (Figure 7.1). A summary of this procedure is as follows:

$$\frac{A_m}{A_i} + \frac{B_m}{B_i} = S, \text{ the sum of biological effects} \tag{7.3}$$

$$\text{Additive index} = 1/S - 1.0 \text{ for } S \leq 1.0 \tag{7.4}$$

$$\text{Additive index} = S(-1) + 1.0 \text{ for } S \geq 1.0 \tag{7.5}$$

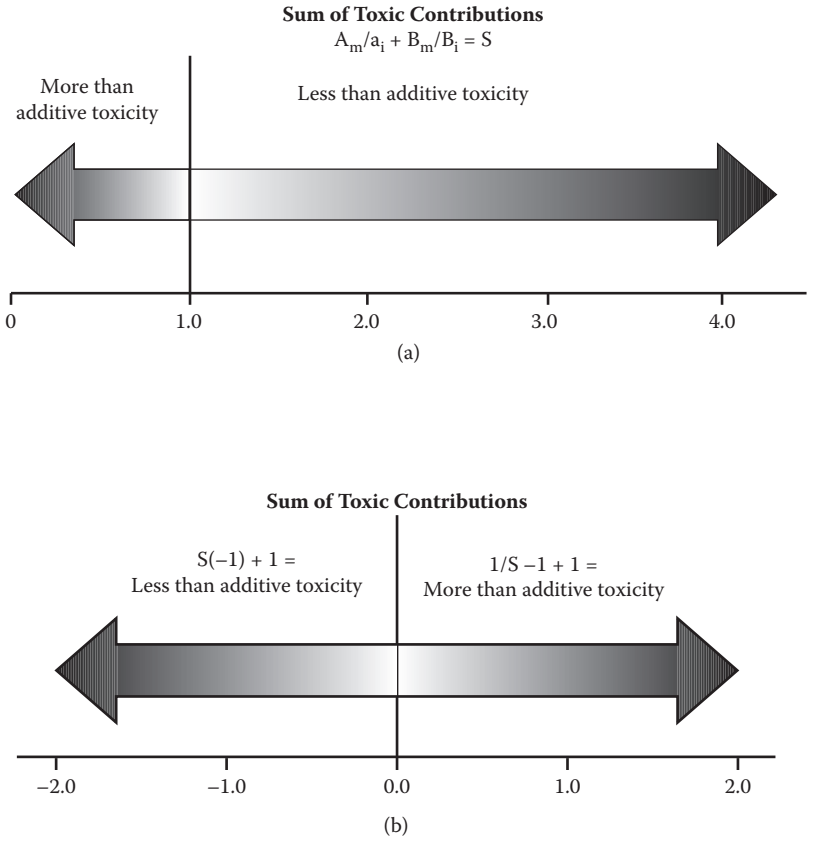

Figure 7.1 Graphical representation of the sum of toxic contributions. In the top of the figure the sum of toxic contributions is counterintuitive: The greater than additive toxicity has a ratio of less than 1 and the proportions are nonlinear. With the corrections in the corrected sum of toxic contributions the less than additive toxicity is less than 1, with the more than additive toxicity greater than 1.

Although the toxic units and additive index are useful in determining toxicity, in some cases they have disadvantages. Their values depend on the relative proportion of chemicals in the mixture. Also, because of the logarithmic form of the concentration in log-linear transformations such as probit and logit, it is desirable to have a toxicity index that is logarithmic in the toxicant concentration. For these reasons, Konemann (1981) introduced a multiple toxicity index (MTI):

$$MTI = 1 - \frac{\log M}{\log m_o} \tag{7.6}$$

where $m_o = M/f_{max}$; f_{max} = largest value of z_i/Z_i in the mixture; z_i = concentration of toxicant i in the mixture; Z_i = concentration of toxicant i, acting singly, giving the desired response (endpoint); $M = \sum_i^n = 1\ z_i/Z_i$ = sum of toxic units giving the desired response; and n = number of chemicals in the mixture.

When the concentration z_i of each chemical relative to its effect concentration Z_i, when acting alone, is a constant f for all chemicals, $f = z_i/Z_i$, the above equation reduces to

$$MTI = 1 - \frac{\log M}{\log n} \tag{7.7}$$

Even the simplest model requires prior knowledge of the LC_{50} for each compound acting singly. The additive toxicity and multiple toxicity indices require an LC_{50} for the specific mixture as well as the singular compounds. Therefore, access to a large database or the ability to estimate toxicity will be extremely important. Of these two methods, the corrected sum of toxic contributions derived by Marking and Dawson appears to be the easiest to implement and interpret.

7.6.2 Mixture Estimation System

The usefulness of these equations is (1) in the estimation of the toxicity of a mixture and (2) the setting of priorities for cleanup by establishing the major contributor to the toxicity of the mixture. The major disadvantages to the implementation are that these equations are not set up for easy use and there is a lack of environmental toxicity data. A combination of the implementation of the selected methodology into a computer program coupled to a large database and a quantitative structure-activity relationships estimation system should make these evaluations of mixture toxicity efficient and useful. The components of such a system might be:

- The front end for data input, namely, the available toxicity data for the components, Chemical Abstracts Service (CAS) numbers for the compounds with an unknown toxicity, and the toxicity of the mixture, if known. Concentrations of each material are also input.
- A system for searching the appropriate databases for toxicity data or SAR models for estimating the desired parameter. The quantitative structure-activity relationship (QSAR) system should provide adequate warnings for the appropriateness of the model and its coverage in the database from which the equation was derived.
- A processor that incorporates the data from the literature and the QSARs along with the concentration of the compounds. An estimate of the toxicity of the mixture or identification of the major contributors will be the generated output.

The difficulty in estimating the toxicity of mixtures using any of these models is establishing interaction terms. All of the models require actual toxicity tests to estimate these terms. Even in a simple mixture of four components this requires six toxicity tests of the pairwise combinations and four three-component tests to examine interactive terms. Perhaps the best that could be done in the short term is to establish interaction terms between classes of compounds and use those as models.

Initially, it would be desirable to use a simple model incorporating a linear relationship. Since the data are lacking for the determination of interactive effects, a simple additive toxic units model would make the fewest assumptions and require the minimal amount of data. Such a model would simply consist of

$$A_c/A_i + B_i/B_t + C_i/C_t = MT \tag{7.8}$$

where A_c = environmental concentration of compound A; A_i = concentration resulting in the endpoint selected, for example, EC_{50} or LC_{10}; and MT is the mixture toxicity as a fraction, with 1 equal to the mixture having the same effect as the endpoint selected.

It is certainly possible to make these estimations routine given the uncertainties in the interaction terms and the lack of toxicity data. Properly designed, such a system should allow the rapid and routine estimation of mixtures within the limitations presented above.

7.6.3 Estimating the Toxicity of Mixtures of Polynuclear Aromatic Hydrocarbons

As discussed in previous sections, there are numerous factors that can modify the toxicity of materials. The prediction of the toxicity of mixtures is also difficult. One of the best attempts at toxicity prediction has been formulated by Swartz et al. (1995): the prediction of the sediment toxicity of polynuclear aromatic hydrocarbons. The model is based on the concentration of 13 PAHs in collected sediments, the predicted concentration in the sediment pore water, and the toxicity of these concentrations as determined by a large toxicity data set.

The sediment polyaromatic hydrocarbon (SPAH) model incorporated a number of factors that can modify the toxicity of the sediment-borne PAHs. Equilibrium partitioning was used to estimate the concentration of each PAH in the pore water of the sediment. The assumption was that the pore water material is the fraction that is bioavailable. QSAR was also used to estimate the interstitial water concentration based on the octanol-water partition coefficient of several PAHs. Amphipods were used as the test organism to represent environmental toxicity. A toxic unit (TU) approach was used, and the toxicity is assumed to be additive. The assumption of additivity is justified since each of the PAHs has a similar mode of action. Finally, a concentration-response model was formulated using existing toxicity data to estimate the probability of toxicity.

The estimates of toxicity are expressed as not toxic, uncertain, and toxic. These classifications are based on the estimated percent mortality as generated by the concentration-response model. A percent of mortality less than 13% is considered not toxic. Between 13 and 24% mortality the toxicity prediction is considered uncertain. Above a prediction of 24% mortality the sediment is considered toxic.

A flowchart for estimating sediment toxicity is presented in Figure 7.2. First, a bulk sediment sample is taken and the PAH concentration and total organic carbon are measured. The equilibrium partitioning model is run to predict the concentration of each PAH in the interstitial water of the sediment. The predicted PAH concentrations are then converted to toxic units (TUs) using the 10-day amphipod LC_{50} as the toxicity benchmark. The TUs are then added up and processed through the concentration-response model. The expected mortality is then converted to nontoxic, uncertain, and toxic predictions.

The estimates of toxicity were confirmed using a variety of sediment samples with measurements of PAH concentrations and amphipod toxicity tests. At sites where the PAHs were the principal cause of contamination, the frequency of correct predictions was 86.6%. When the samples were collected from sites where PAHs were not the principal contaminant, the frequency of correct prediction was 56.8%.

Wiegers et al. (1998) have also applied the model to the concentrations of 10 PAHs (data for all 13 PAHs were not consistently available) for samples collected throughout Port Valdez, Alaska. Most of the samples were collected in the deep benthic areas, although samples from the small boat harbor in the city, and nearshore areas by Mineral Creek, the Valdez Marine Terminal, and

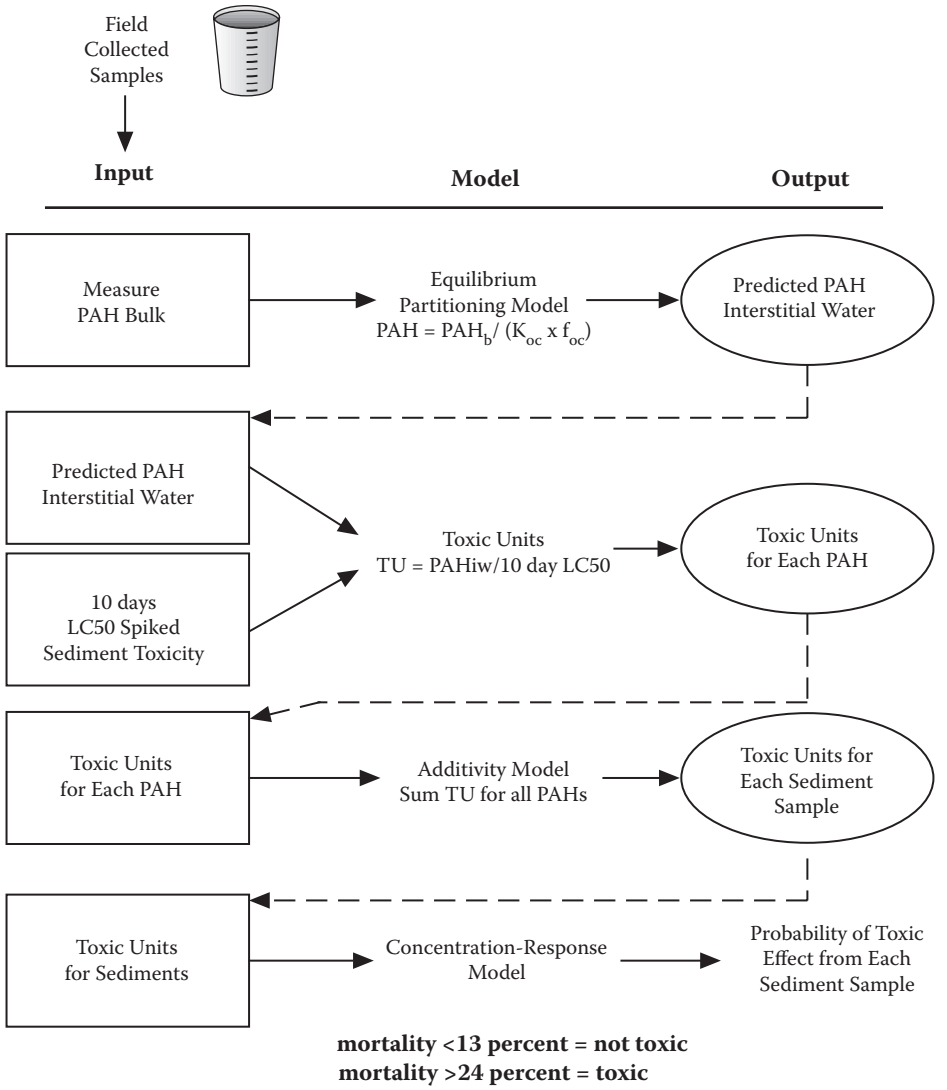

Figure 7.2 The steps in calculating the toxicity of PAHs to amphipods.

the Solomon Gulch Hatchery were also collected. All of the acute toxicity levels predicted in Port Valdez occur below the lowest levels set by the model. The sum of the toxic units (a measure of the total toxicity associated with the concentrations) is included in Table 7.2 as a comparison between samples collected from the identified subareas.

Estimating the toxicity of the sediments through use of a model develops another line of evidence to weigh against those determined by comparison of chemical level with benchmark values used to predict the toxicity of chemical contaminants. Benchmark values are based on a wide sweep of scientific studies conducted for single compounds under a variety of conditions and are applied universally to all environmental concentrations. The SPAH model described here uses effects levels derived from a number of laboratory tests, but also incorporates some site-specific information predicting bioavailability and considers multiple compounds. Compared to

Table 7.2 Acute Toxicity to Amphipods Predicted from Sediment Concentrations of 10 PAHs

Subarea	*Sum of the Toxic Units*
Mineral	0.00001 ± 0.00001
City	0.0029 ± 0.001
Hatchery	0.00001 ± 0.00001
Alyeska	0.00004 ± 0.00004
West Port	0.00001 ± 0.00002
East Port	0.00001 ± 0.00001

Note: The mean sums of the toxic units with the standard deviations are listed. In this instance, the probability of toxicity was low at each sampling site.

using set criteria for specific compounds, the SPAH offers a distinct advantage to the accurate prediction of toxicity.

7.6.4 Mixtures and Carbamate and Organophosphate Synergistic Toxicity

The importance of understanding the toxicity of mixtures has been highlighted by the study of Laetz et al. (2009) for pesticide mixtures using salmon as the test species. Pesticides exist in the environment as part of a milieu of other xenobiotics, especially in industrial, urban, or agricultural settings. In this particular study, the question was: How do two distinct classes of acetylcholinesterase (AChE) inhibitors interact *in vitro* (outside the organism) as opposed to *in vivo* (within the organism)?

The two classes of pesticides in this study are the organophosphates and the carbamates (Figure 7.3). The molecular biology of AChE inhibition and its effects are discussed in Section 6.4.2 in Chapter 6. The two classes of chemicals are structurally distinct but have very similar modes of action. Previous research had demonstrated that the toxicities are additive *in vitro* as measured by AChE inhibition. The question asked by Laetz et al. is very straightforward: Are the toxicities of these materials additive, antagonistic, or synergistic when AChE inhibition is measured *in vivo*?

The first step is to construct a model that would allow the translation of the results of the toxicity tests to an indication of the interactions of the various mixtures of AChE inhibitors. This model is presented in Figure 7.4a. The *x* axis is the EC_{50} concentration normalized so that the fraction represents the proportion of EC_{50}. A value of 1.0 along this axis represents an EC_{50} concentration. This allows the presentation of the toxicity of the various materials without concentration units. The *y* axis represents the activity of AChE. In this diagram the line representing the concentration-response curve assumes that the various combinations of the toxicants are additive. In the illustrative example, a compound with a concentration equal to 0.1 of an EC_{50} is added to a compound with a concentration equal to 0.3 of an EC_{50}. If the toxicities of the two compounds are additive,

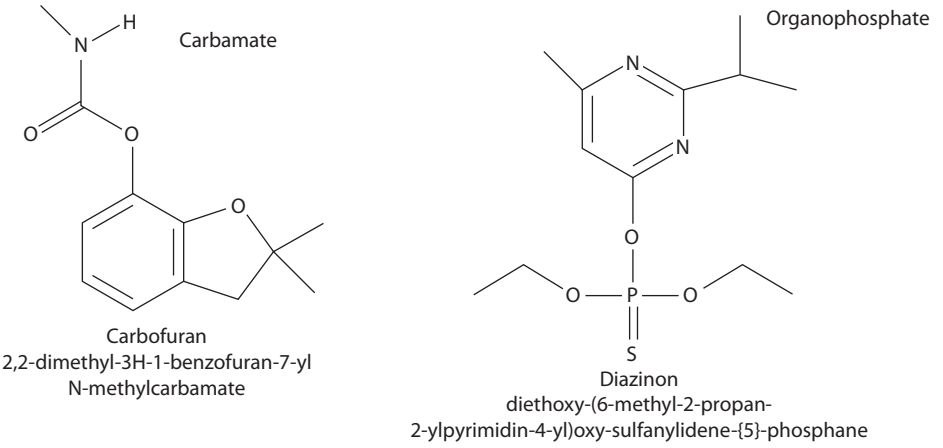

Figure 7.3 Examples of a carbamate and an organophosphate tested for synergism.

then the result of the toxicity test should be 0.4 of an EC_{50}. If the toxicities of the compounds are antagonistic, then the toxicity value would be above the line; that is, there is more AChE activity than expected. Below that line, and there would be less AChE activity than expected and the two materials would be synergistic.

The test species was Coho salmon (*Oncorhynchus kisutch*) of 4 to 7 months of age, an average size of 4.9 cm, and a weight of 1.3 g. Five compounds were examined in various combinations. The carbamates were carbaryl and carbofuran, and the organophosphates diazinon, malathion, and chlorpyrifos. Fish were exposed for 96 hours with a 24-hour static renewal and not fed for the duration of the test. After the exposure period the fish were sacrificed and the AChE activity analyzed. In three test conditions mortality was observed early in the test. These fish were removed after 24 hours of exposure and analyzed for AChE activity. The text concentrations were both with single compounds and then at concentrations to produce AChE inhibitions of 10, 29, and 50%, or 0.1, 0.4, and 1.0 EC_{50} units.

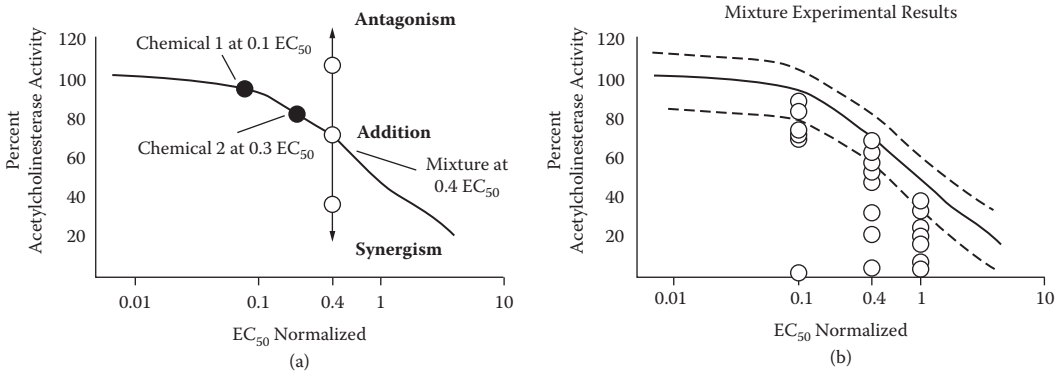

Figure 7.4 Synergistic interactions in mixtures. (a) The concentration-response model for synergism, addition, and antagonism. (b) The experimental concentration-response activities for various mixtures of OPs and carbamates. (Modified from Laetz, C. A. et al., *Environ. Health Perspect.,* 117, 348–353, 2009.)

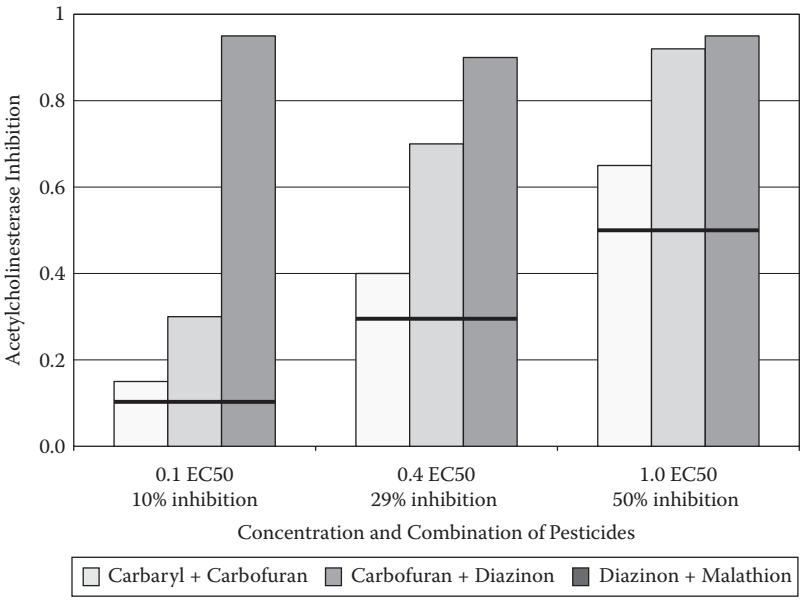

Figure 7.5 Synergistic interactions of carbamates and organophosphates. The mixtures of carbofuran + diazinon and dianzinon + malathion were synergistic at each concentration tested.

The results of the single-compound concentration-response data were plotted and a curve fitted to describe the additive concentration-response curve. A 95% confidence interval was also calculated for the additive concentration-response curve. Figure 7.4b presents these curves in the same style as our initial model. The circles are the results of the various mixture combinations. In most cases, the error bars are within the diameter of the circle symbol. At even low EC_{50} values some of the combinations exceed the confidence intervals for the additive regression line. At higher EC_{50} values the number of combinations that exceed this confidence interval increases. In a random effects expectation there should be approximately even numbers of test observations above and below the additive model curve. The pattern observed in these experiments indicates that the combinations are synergistic.

Figure 7.5 portrays these results as a conventional bar graph for three of the combinations, carbaryl and carbofuran (carbamates), carbrofuran and diazinon (carbamate and organophosphate [OP]), and diazinon and malathion (OP and OP). The bar across the columns indicates the expected toxicity given an additive model. For the OP and carbamate, and the OP and OP combinations presented in this graph, each result was statistically significant from the additive model. At the highest concentration with an EC_{50} predicted the carbamate and carbamate mixture was also different than the additive model.

In contrast to *in vitro* assays, tests of AChE inhibition using experiments with *in vivo* exposure to Coho salmon indicate that additive but most often synergistic interactions occur. This result is important. Apparently in fish there are metabolic factors that modify the toxicity of the pesticides. Combinations of OPs clearly are synergistic at the lowest concentrations tested in the study by Laetz et al. (2009). Typically, materials that share similar structure and modes of action are assumed to be additive. This assumption is not true in these experiments.

These results also demonstrate the need for experimental examination of the interactions of chemicals in mixtures. AChE inhibition is only one mode of action. PCBs have both estradiol

and dioxin-like modes of action, pharmaceuticals are designed to work on specific receptors, and materials such as atrazine have different modes of action, depending upon the receptor being a plant or vertebrate. It is not clear as of this writing how to predict the toxicity of the mixture of these materials, and the experimental evidence is not yet available.

7.7 Biological Factors Affecting Toxicity

7.7.1 Plants

In plants, the most widely studied and probably the most important factor affecting their response to air pollutants is genetic variation. Plant response varies between species of a given genus and between varieties within a given species. Such variation is a function of genetic variability, as it influences morphological, physiological, and biochemical characteristics of plants. Gladiolus has long been recognized to be extremely sensitive to fluoride. Varietal differences in fluoride response in gladiolus have also been observed. Plants show differences in their susceptibility to different pollutants. For instance, some plants may be sensitive to fluoride but resistant to SO_2, while in others the opposite may be true.

The sensitivity to O_3 of two onion cultivars has been shown to be controlled by a single gene pair. Engle and Gableman (1966) showed that the resistant gene was dominant. They reported that after exposure to O_3 the stomata of the resistant cultivar closed, with no appreciable injury, whereas the stomata of the sensitive cultivar remained open, with obvious injury.

The sensitivity of plants is also affected by leaf maturity. Generally, young tissues are more sensitive to PAN and hydrogen sulfide, and maturing leaves are most sensitive to the other airborne pollutants. According to Linzon (1966), in white pine the greatest chronic injury occurred in second-year needles exposed to SO_2.

7.7.2 Animals

Genetics, development, health status, sex variation, and behavior are among the important factors affecting the response of animals and humans to pollutant toxicity (Hodgson 1980).

7.7.3 Genetic Factors

Not all organisms, including humans, react in the same way to a given dose of a chemical or an environmental pollutant. In experimental animals, species variation, as well as variation in strains within the same species, occurs. In humans, such factors as serum, red blood cell, and immunological disorders, and malabsorption can contribute to differences in their response to environmental stresses. For instance, people with sickle cell anemia will be more susceptible to stresses than normal persons. Individuals with malabsorptive disorders are also a problem since they may suffer nutritional deficiencies, which in turn may lead to an increased susceptibility to environmental chemicals.

7.7.4 Developmental Factors

Immature immune systems, aging, pregnancy, immature detoxication systems, and circadian rhythms are included in this category. For example, lack of g-globulin to cope with invading

bacteria and viruses, decline in renal function as a result of aging, lack of receptors needed in hormonal action, greater stresses encountered by pregnant women to metabolize and detoxify foreign chemicals not only for themselves but for the fetus, and an immature hepatic mixed function oxidase (MFO) system in the young are all contributing factors to varying responses exhibited by the individuals to xenobiotics.

7.7.5 Diseases

Diseases in the heart, lungs, kidney, and liver predispose a person to more severe consequences following the exposure to pollutants. As shown previously, organs such as these are responsible for storage, metabolism, and excretion of environmental pollutants. Cardiovascular and respiratory diseases of other origins decrease the individual's ability to withstand superimposed stresses. An impaired renal function will certainly affect the kidneys' ability to excrete toxic substances or their metabolites. As mentioned earlier, the liver plays a vital role in detoxication of foreign chemicals, in addition to its role in the metabolism of different nutrients. Disorders in the liver, therefore, will not permit satisfactory detoxication to occur.

7.7.6 Behavioral Factors

Smoking, drinking, and drug habits are some examples of lifestyle that can affect human response to environmental pollutants. Research has shown that smoking acts synergistically with many environmental pollutants. A smoker may thus be at a higher risk than a nonsmoker when exposed to an additional environmental stress. For example, asbestos workers or uranium miners who smoke have been shown to exhibit higher lung cancer death rates than workers who do not smoke. Heavy drinking is widely known to cause disorders in the brain and liver. In such persons, fluoride can cause more damaging effects.

7.7.7 Sex Variation

The rate of metabolism of foreign compounds varies with the difference in sex of both humans and animals. For example, response to $CHCl_3$ exposure by experimental mice shows a distinct sex variation. Male mice are highly sensitive to $CHCl_3$; death often results following their exposure to this chemical. The higher sensitivity of male mice to certain toxic chemicals may be due to their inability to metabolize the chemicals as efficiently as the female mice. Interestingly, death rates of male mice resulting from exposure to $CHCl_3$ are affected by different strains as well (Table 7.3).

7.7.8 Nutritional Factors

The importance of nutrition as a major factor affecting the toxicity of chemicals has been recognized in recent years. Results obtained from human epidemiological and animal experimental studies strongly support the relationship between nutrition and pollutant toxicity. For example, human populations exposed to environmental fluoride may or may not exhibit fluoride toxicity, depending on their nutritional status. Laboratory animals fed low-protein diets have been reported to be more susceptible to the toxicity of chemicals. The interaction between nutrition and environmental pollutants is complex, and understanding its nature is a great challenge in the study of both toxicology and nutrition. It may be mentioned that a new area of study, called nutritional toxicology, has emerged in recent years.

Table 7.3 Effect of $CHCl_3$ Exposure on Death Rate of Various Strains of Male Mice

Strains	*Death Rate (%)*
DBA-2	75
DBA-1	51
CsH	32
BLAC	10

The relationship between nutrition and toxicology falls into three major categories: (1) the effect of nutritional status on the toxicity of drugs and environmental chemicals, (2) the additional nutritional demands that result from exposure to drugs and environmental chemicals, and (3) the presence of toxic substances in foods (Parke and Loannides 1981).

Generally, nutritional modulation can alter rates of absorption of environmental chemicals, thus affecting the circulating level of those chemicals. It can cause changes in body composition, leading to altered tissue distribution of chemicals. Dietary factors can also influence renal function and pH of body fluids, resulting in altered toxicity. In addition, responsiveness of the target organ may be modified as a result of changing nutrition.

7.7.9 Fasting/Starvation

This is the most severe form of nutritional modulation. The effect of fasting or starvation, generally, is decreased metabolism and clearance of chemicals, resulting in increased toxic effects. Studies showed that the effect of fasting on microsomal oxidase activity is species, substrate, and sex dependent; i.e., some reactions are decreased in male rats and increased in females, while others may not be affected at all. The sex-dependent effect is thought to be related to the ability of androgen to enhance binding of some substrates to cytochrome P-450. Experiments carried out with animals also showed that glucuronide conjugation was decreased under starvation.

7.7.10 Proteins

Many different chemical compounds induce the MFO in the liver and other tissues. Induction of the MFO is associated with increased biosynthesis of new protein. The most potent inducers are substrates whose rates of metabolism are low, so that they remain associated with the enzyme for long periods of time. In humans, severely limited protein intake is usually accompanied by inadequate intake of all other nutrients; thus, it is difficult to designate specific pathological conditions to protein deficiency per se. Protein deficiency causes impaired hepatic function and hypoproteinemia, resulting in decreased hepatic proteins, DNA, and microsomal P-450, as well as lowered plasma binding of xenobiotics. Conjugation is also influenced, but the effect is less consistent. Removal of pollutants from the body may be impaired, leading to an increased toxicity, although exceptions do exist.

The effects of proteins on pollutant toxicity include both quantitative and qualitative aspects. Experiments show that animals fed proteins of low biological value exhibited a lowered microsomal oxidase activity; when dietary proteins were supplemented with tryptophan, the enzyme activity

Table 7.4 Effect of Protein on Pesticide Toxicity

Compounds	Casein Content of Diet	
	3.5% LD_{50}	26% mg
Acetylcholinesterase Inhibitors		
Parathion	4.86	37.1
Diazinon	215	466
Malathion	759	1,401
Carbaryl	89	575
Chlorinated Hydrocarbons		
DDT	45	481
Chlordane	137	217
Toxaphene	80	293
Endrin	6.69	16.6
Herbicides and Fungicides		
Diuron	437	2,390
Captan	480	12,600

Note: Male rats were fed for 28 days from weaning on diets of varying casein contents, and then given an oral dose of pesticides.

was enhanced. Alteration of xenobiotic metabolism by protein deprivation may lead to enhanced or decreased toxicity, depending on whether metabolites are more or less toxic than the parent compound. For example, rats fed a protein-deficient diet show decreased metabolism but increased mortality with respect to pentobarbital, parathion, malathion, DDT, and toxaphene (Table 7.4). On the other hand, rats treated under the same conditions may show a decreased mortality with respect to heptachlor, CCl_4, and aflatoxin. It is known that in the liver, heptachlor is metabolized to epoxide, which is more toxic than heptachlor itself, while CCl_4 is metabolized to CCl_3, a highly reactive free radical. As for aflatoxin, the decreased mortality is due to reduced binding of its metabolites to DNA.

7.7.11 Carbohydrates

A high-carbohydrate diet usually leads to a decreased rate of detoxication. The microsomal oxidation is generally depressed when the carbohydrate/protein ratio is increased. In addition, the nature of carbohydrates also affects oxidase activity. Since dietary carbohydrates influence body lipid composition, the relationship between carbohydrate nutrition and toxicity is often difficult to

assess. However, environmental chemicals can affect, and be affected by, body glucose homeostasis in several different ways. For example, poisoning by chemicals may deactivate hepatic glucose-6-phosphatase by damaging the membrane environment of the enzyme. Compounds that are metabolized by the liver to glucuronyl conjugates are more hepatotoxic to fasted animals than fed animals. Low hepatic glycogen contents may also lead to a greater vulnerability of fasted animals to xenobiotics such as acetaminophen, whose metabolism is associated with depletion of the GSH component of the hepatic antioxidant defense system.

7.7.12 Lipids

Dietary lipids may affect the toxicity of environmental chemicals by delaying or enhancing their absorption. The absorption of lipophobic substances would be delayed, and that of lipophillic substances accelerated.

The endoplasmic reticulum contains high amounts of lipids, especially phospholipids, rich in polyunsaturated fatty acids. Lipids may influence the detoxication process by affecting the cytochrome P-450 system because phosphatidylcholine is an essential component of the hepatic microsomal MFO system. A high-fat diet may favor more oxidation to occur, as it may contribute to more incorporation of membrane material.

Types of lipids can also affect toxicant metabolism, as a high proportion of phospholipids is unsaturated due to the presence of linoleic acid (18:2) in the β-position of triacylglycerol. Thus, dietary linoleic acid (18:2) is important in determining the normal levels of hepatic cytochrome P-450 concentration and the rate of oxidative demethylation in rat liver.

Significant as it is, higher doses of linoleic acid decrease hepatic cytochrome P-450 and MFO activity (Hietanen et al. 1978), and unsaturated fatty acids added to rat and rabbit liver microsomes *in vitro* inhibit MFO activity with type I substrates (e.g., *p*-nitroanisole), probably because the fatty acids act as competitive substrates (Di Augustine and Fouts 1969).

Dietary lipids play a unique role in the toxicity of chlorinated hydrocarbon pesticides. Dietary lipids may favor more absorption of these pesticides, but once these chemicals are absorbed into the body, they may be stored in the adipose tissue without manifestation of toxicity. For this reason, obesity in humans is considered protective against chronic toxicity of these chemicals. Similarly, the body fat in a well-fed animal is known to store organochlorine pesticides. Fat mammals, fish, and birds are thus more resistant to DDT poisoning than their thinner counterparts. In times of food deprivation, however, organic materials such as DDT and PCB can be mobilized from mammalian fat deposits, and reach concentrations potentially toxic to the animal.

The role of dietary lipids in affecting pollutant toxicity has been fairly well defined for a few specific chemicals, including lead, fluoride, and hydrocarbon carcinogens. For example, high-fat diets are known to increase Pb absorption and retention. In addition, competitive absorption of Pb and Ca exists, and this is probably due to competition for the Ca binding protein (CaBP), whose synthesis is mediated by vitamin D, a fat-soluble vitamin. In earlier studies, a high-fat diet was shown to result in increased body burden of fluoride, leading to enhanced toxicity. This is attributed to delaying of gastric emptying caused by high dietary fat. As a consequence, enhanced fluoride absorption may result, and thus increase the body burden of fluoride. Dietary fat does not increase the metabolic toxicity of fluoride itself. As is well known, aflatoxin, a toxin produced by *Aspergillus flavus*, is a potent liver-cancer-causing agent. A high-fat diet offers protection from lethal effects of the toxin, presumably through dissolution of the carcinogen.

7.7.13 Vitamin A

Interest in vitamin A and its synthetic analogs as a potential factor in the prevention and treatment of certain types of cancer has been growing. In addition, there is evidence that vitamin A may be related to pollutant toxicity. Recent epidemiological studies in humans with a sample of 8,000 men in Chicago showed a low lung cancer incidence in those with a high vitamin A level in the diet, while the incidence was higher in those people with a low dietary vitamin A level. Experimental studies show that rats exposed to PCB, DDT, and dieldrin caused a 50% reduction in liver vitamin A store. In another study, rats deficient in retinol were shown to have a lowered liver cytochrome P-450 activity. The effect of vitamin A deficiency on MFO enzymes, however, depends on several factors, such as substrate, tissue, and animal species.

While the mechanism involved in vitamin A action in relation to carcinogenesis remains to be elucidated, several possibilities have been suggested. For example, vitamin A deficiency may act primarily on metabolic activation of carcinogens; such deficiency may facilitate interaction of ultimate carcinogen with DNA. Finally, vitamin A deficiency may affect transformation of epithelia, and thus predispose the tissue to neoplastic changes, as vitamin A is required in the differentiation of epithelial cells important in both respiratory and gastrointestinal tracts.

7.7.14 Vitamin D

The role that vitamin D plays in the prevention of rickets and osteomalacia has been well documented. Recent studies have revealed the mechanism that is involved in the conversion of vitamin D into its metabolically active form responsible for the maintenance of calcium homeostasis. Cholecalciferol (vitamin D) is first hydroxylated in the liver to 25-hydroxy-D_3; this is then converted in the kidneys to 1,25-dihydroxy-D_3, the hormone-like substance that is the active form of the vitamin. The 25-hydroxylation of cholecalciferol requires NADPH, O_2, and an enzyme whose properties are similar to those of microsomal MFO (Bjorkhelm et al. 1979). In addition, 25-hydroxy-D_3 has been shown to competitively inhibit some cytochrome P-450 reactions *in vitro*. Patients suffering from drug-induced osteomalacia show increased rates of catabolism of vitamin D_3 to 25-hydroxy-D_3.

7.7.15 Vitamin E

Vitamin E (a-tocopherol), a potent antioxidant, appears to offer protection against injuries caused by O_2, O_3, and NO_2, and nitrosamine formation. Male rats supplemented with daily doses of 100 mg tocopheryl acetate and exposed to 1.0 ppm O_3 have been shown to survive longer than vitamin E–deficient rats. The action of O_3 is attributed in part to free radical formation. In addition, there is sufficient evidence that vitamin E protects phospholipids of microsomal and mitochondrial membranes from peroxidative damage by reacting with free radicals. Because lipid peroxidation is associated with a decrease in oxidase activities, it is expected that the enzyme activity is affected by dietary vitamin E. Maximum activity has been observed when diets included both polyunsaturated fatty acids and vitamin E.

Nitrosamine is known to be carcinogenic, as it leads to liver cancer. Relationships between vitamin E and nitrosamines are attributed to the inhibitory effect of the vitamin on nitrosamine formation; i.e., vitamin E competes for nitrite, a reactant in the formation of nitrosamine.

Table 7.5 Ascorbic Acid Content of Adult Human Tissues

Tissue	*Ascorbic Acid (mg/100 g wet tissue)*
Pituitary glands	40–50
Leukocytes	35
Adrenal glands	30–40
Eye lens	25–31
Brain	13–15
Liver	10–16
Spleen	10–15
Pancreas	10–15
Kidneys	5–15
Heart muscle	5–15
Lungs	7
Skeletal muscle	3–4
Testes	3
Thyroid	2
Plasma	0.4–1.0
Saliva	0.07–0.09

7.7.16 Vitamin C

Vitamin C is found in varying amounts in almost all of our body tissues. High contents are found particularly in the adrenal and pituitary glands, eye lenses, and various soft tissues (Table 7.5). It is a potent antioxidant and participates in a large number of cellular oxidation-reduction reactions. While the role that vitamin C plays in collagen biosynthesis is well recognized, its relationship to drug metabolism as well as pollutant toxicity has attracted attention in recent years. For example, vitamin C–deficient guinea pigs have been shown to have an overall deficiency in drug oxidation, with marked decreases in *N*- and *O*-demethylations, and in the contents of cytochrome P-450 and cytochrome P-450 reductase (Parke and Loannides 1981). Administration of ascorbate to the deficient animals for 6 days reversed these losses of MFO activity. The effect of vitamin C appears to be tissue dependent (Kuenzig et al. 1977).

Recent research suggests that vitamin C may reduce the carcinogenic potential of some chemicals. It has been demonstrated that a variety of experimental tumors of the gastrointestinal tract, liver, lung, and bladder can be produced by nitroso compounds (Narisawa et al. 1976; Mirvish et al. 1975), which are produced by the reaction of nitrites with secondary and tertiary amines, amides, or others.

The nitrosation of several secondary and tertiary amines can be blocked *in vitro* by the addition of vitamin C. The vitamin appears to compete for the nitrite, thus inhibiting nitrosation. It has been demonstrated that vitamin C does not react with amines, nor does it enhance the rate of nitrosamine decomposition. However, it reacts very rapidly with nitrite and nitrous acid. It has been suggested that the vitamin decreases the available nitrite by reducing nitrous acid to nitrogen oxides, leading to inhibition of the nitrosation reaction:

$$2\ HNO_2 + \text{Ascorbate} \rightarrow \text{Dehydroascorbate} + 2\ NO + 2\ H_2O \tag{7.9}$$

Although little or no evidence is available that a similar effect occurs in humans, it has been suggested that, in view of our increasing exposure to various drugs and xenobiotics, the current Recommended Dietary Allowances (RDAs) for ascorbic acid may be inadequate (Zannoni 1977). For instance, the average American is thought to ingest approximately 70 μg Cd/day, 0.9 mg As/day, and 4.1 mg nitrite/day, in addition to exposure to ambient air containing CO, O_3, Pb, cigarette smoke, and others (Calabrese 1980). Recommendations for increasing the RDA for vitamin C to meet such additional needs, however, have not received general support. Moreover, it is known that a dietary excess of vitamin C can produce various adverse effects, based on a nutritional and clinical point of view. Furthermore, recent studies indicate that an excess intake of the vitamin might also be hazardous, since excess ascorbate is metabolized by conjugation with sulfate and excreted in the urine as ascorbate sulfate (Baker et al. 1971). Ingestion of large amounts of the vitamin may, therefore, impair conjugation reactions requiring sulfate. Certain drugs, such as salicylamide, are inactivated through sulfate conjugation; lack of sulfate could cause accumulation of the unconjugated compound in the body, leading to drug toxicity (Houston and Levy 1975).

7.7.17 Minerals

Mineral nutrition influences toxicology in different ways. Interactions are common concerning the effects of the trace nutrients on detoxication. It is recognized that trace mineral elements, like the macronutrients, can influence absorption of xenobiotics. Divalent cations can compete for chelation sites in intestinal contents as well as for binding sites on transport proteins. As is well documented, competitive absorption of Pb and Ca occurs, and this is probably due to competition for binding sites on intestinal mucosal proteins mediated by vitamin D.

Zinc is known to provide protection against Cd and Pb toxicities (Sandstead 1980). Absorption of Zn is facilitated by complexing with picolinic acid, a metabolite of the amino acid tryptophan. Although both Cd and Pb form a complex with picolinic acid, the resulting complexes are less stable than the Zn complex.

Cytochrome P-450 requires iron for its biosynthesis; thus, deficiency of Fe might lead to a decrease in MFO activity. It has been shown that the villous cells of rat duodenal mucosa rapidly lose their cytochrome P-450 content and MFO activity when dietary Fe is deficient (Hoensch et al. 1975). Selenium is antagonistic to both Cd and Hg, thus reducing their toxicity. In addition, Se enhances vitamin E function in the prevention of lipid peroxidation. The mechanisms involved in the functioning of these two trace nutrients are different, however. Whereas vitamin E is thought to function as a membrane-bound antioxidant, acting as a free radical scavenger, Se participates at the

active site of glutathione peroxidase, and thus part of the enzyme. This enzyme protects membrane lipids by catalyzing the destruction of H_2O_2 and organic hydroperoxides before they can cause membrane disruption.

Study Questions

1. Which substance will have a higher toxicity—ionized or nonionized? Why?
2. Exposure to high levels of pollutants results in _____ effects; low concentrations result in _____ effects.
3. Describe why intermittent exposure to a pollutant may not be as detrimental as continuous exposure.
4. Name two effects temperature changes (thermal pollution) have on living organisms.
5. How can humidity levels and light intensity affect pollutants' effects?
6. Describe synergistic, potentiative, and antagonistic effects resulting from the interaction of pollutants.
7. Describe the toxic unit model.
8. How is a value for additive toxicity found?
9. What is the multiple toxicity index? What are the component parts of the equation used to calculate the index?
10. What are the two uses of the toxicity equations?
11. What are the advantages of using a toxic units model for describing the toxicity of mixtures?
12. Diagram the steps for the SPAH model for estimating the sediment toxicity of mixtures of PAHs.
13. What are the modes of action of carbamates and organophosphates?
14. Describe how to determine if the interaction between carbamates and organophosphates would be additive, synergistic, or antagonistic?
15. What are plants' most important factor affecting response to air pollutants? What is another factor for plant sensitivity?
16. Name five important factors affecting the response of animals to pollutant toxicity.
17. What effects can nutritional modulation have on response to pollutant toxicity?
18. What effect does a high-carbohydrate diet have on detoxification? What effect do dietary lipids have?
19. What are several possibilities of mechanisms involved in vitamin A action in relation to carcinogenesis?
20. Discuss the relationships of vitamin E and vitamin C with nitrosamine.

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Chapter 8

Inorganic Gaseous Pollutants

In this section, four of the major gaseous air pollutants are considered, that is, sulfur oxides (SOx), nitrogen oxides (NOx), ozone (O_3), and carbon monoxide (CO).

8.1 Sulfur Oxides

Sulfur oxides include both sulfur dioxide (SO_2) and sulfur trioxide (SO_3), of which SO_2 is more important as an air pollutant. Sulfur trioxide may be formed in the furnace by reaction between sulfur and O_2, or SO_2 and O_2. Sulfur dioxide is probably the most dangerous of all gaseous pollutants on the basis of amounts emitted.

8.1.1 Sources of SO_2

Sulfur oxide emission results from the combustion of sulfur-containing fossil fuels such as coal and oil. The sulfur content of coal ranges from 0.3 to 7%, and the sulfur is in both organic and inorganic forms, while in oil sulfur content ranges from 0.2 to 1.7%, and its sulfur is in organic form. The most important sulfur compound in coal is iron disulfide (FeS_2) or pyrite. When heated at high temperatures, pyrite undergoes the following reactions:

$$FeS_2 + 3\ O_2 \rightarrow FeSO_4 + SO_2 \tag{8.1}$$

$$4\ FeS_2 + 11\ O_2 \rightarrow 2\ Fe_2O_3 + 8\ SO_2 \tag{8.2}$$

Organically bound sulfur in coal and fuel oil, when burned, also produces SO_2 as shown next:

$$S\ (\text{organic, in fuel}) + O_2 \rightarrow SO_2 \tag{8.3}$$

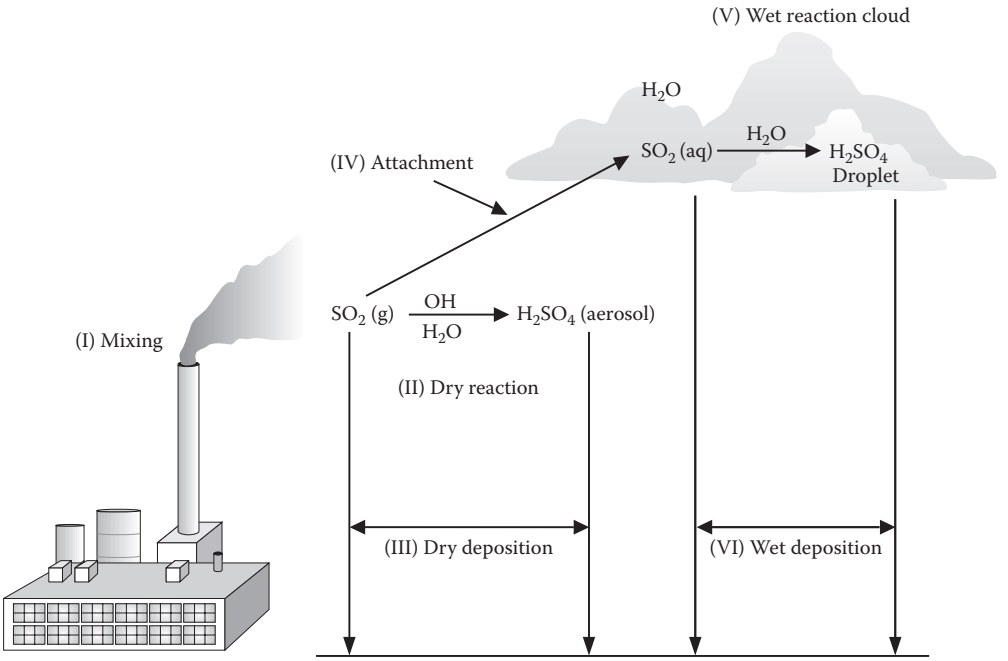


Figure 8.1 SO_2 transport, transformation, and deposition processes. Initially SO_2 is mixed into the atmosphere (I). Gaseous SO_2 may undergo oxidation in the gaseous phase with subsequent formation of H_2SO_4 aerosol (II). Both gaseous SO_2 and H_2SO_4 aerosol may be deposited at the earth's surface (III). Gaseous SO_2 may become dissolved in a water droplet (IV). The dissolved SO_2 can be oxidized in solution to form H_2SO_4 aerosol droplets (V). The H_2SO_4 aerosol and the H_2SO_4 droplet may be removed to the earth's surface by wet deposition (VI). (Adapted and redrawn from Fox, D. L., in *Air Pollution*, 3rd ed., Vol. VI, ed. A. C. Stern, Academic Press, New York, 1986, pp. 86–87.)

In the smelting process, sulfide ores of copper, lead, and zinc are oxidized (roasted) to convert a sulfide compound into an oxide. For example, zinc sulfide undergoes the oxidation process in a smelter forming ZnO and SO_2, as shown below:

$$2\ ZnS + 3\ O_2 \rightarrow 2\ ZnO + 2\ SO_2 \tag{8.4}$$

In the United States, sulfur dioxide emission from stationary sources and industry accounts for about 95% of all SO_2 emission.

8.1.2 Characteristics of SO_2

SO_2 is highly soluble in water, with a solubility of 11.3 g/100 ml. Once emitted into the atmosphere, SO_2 may undergo oxidation in the gaseous phase, forming H_2SO_4 aerosol. Gaseous SO_2 may also become dissolved in water droplets and, following oxidation, form H_2SO_4 aerosol droplets. Both forms of H_2SO_4 thus produced may be removed by deposition to the earth's surface (Figure 8.1).

Recent studies have shown that the photochemistry of the free hydroxyl radical controls the rate at which many trace gases, including SO_2, are oxidized and removed from the atmosphere. The photochemistry involving the OH radical is illustrated in Figure 8.2.

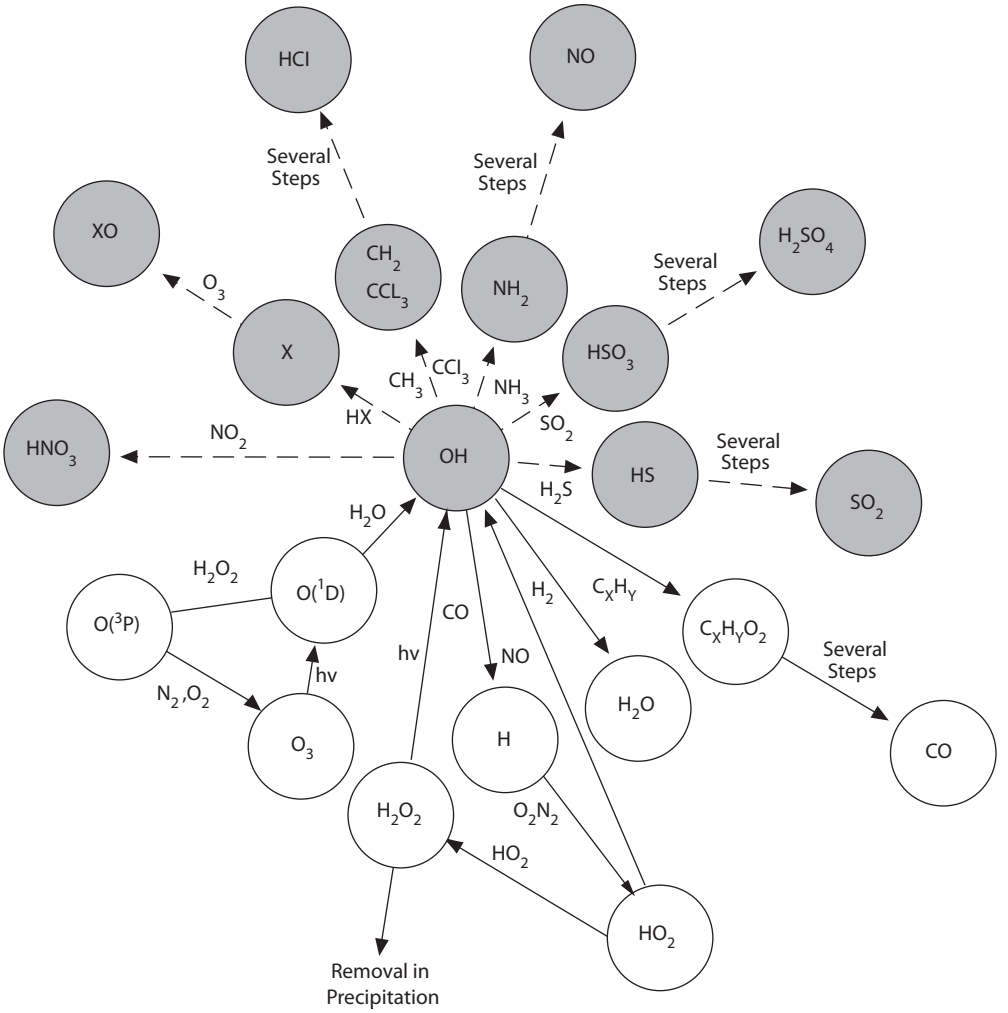


Figure 8.2 Photochemistry of the OH radical controls the trace gas concentration. The photochemistry of the free hydroxyl radical controls the rate at which many trace gases are oxidized and removed from the atmosphere. Processes that are of primary importance in controlling the concentration of OH in the troposphere are indicated by solid lines in the schematic diagram; those that have a negligible effect on OH levels but are important because they control the concentrations of associated reaction and products are indicated by shaded circles. Circles indicate reservoirs of species in the atmosphere; arrows indicate reactions that convert one species to another, with the reactant or photon needed for each reaction indicated along each arrow. Multistep reactions actually consist of two or more sequential elementary reactions. HX = HCl, HBr, HI, or HF. C_xH_y denotes hydrocarbons. (Adapted from Chameides W. L., and Davis D. D., *Chem. Eng. News*, 60, 38–52, 1982. With permission from the American Chemical Society.)

8.1.3 Effect on Plants

For SO_2, the stomatal pores are the main entry ports to the internal air spaces of plant leaves. Absorption takes place mainly by gaseous diffusion through these pores. The number of stomata and size of aperture are important factors affecting the uptake of SO_2. Other factors, such as light, humidity, wind velocity, and temperature, are also important, as these influence the turgidity of guard cells. Low concentrations of SO_2 can injure epidermal and guard cells, leading to increased stomatal conductance and greater entry of SO_2 into the plant (Black and Black 1979). Following the uptake by plant leaves, SO_2 is rapidly translocated through the plant and affects photosynthesis, transpiration, and respiration, the three major functions of plant leaves. A slight increase in both net photosynthesis and transpiration may occur at low SO_2 concentrations for short time periods, followed by a decrease in both processes. Higher SO_2 concentrations induce immediate decreases in these processes. Plant injuries may be manifested by leaf chlorosis and spotty necrotic lesions. Damage to mesophyll cells is commonly observed in microscopic studies.

Once within the substomatal air spaces of the leaf, SO_2 comes into contact with cell walls of the mesophyll cells. SO_2 readily dissolves in the intercellular water to form sulfite (SO_3^{2-}), bisulfite (HSO_3^-), and other ionic species. Both SO_3^{2-} and HSO_3^- have been shown to be phytotoxic, as they affect many biochemical and physiological processes (Malhotra and Hocking 1976). Both SO_3^{2-} and HSO_3^- have a lone pair of electrons on the sulfur atom that strongly favor reactions with electron-deficient sites in other molecules. The phytotoxicity of SO_3^{2-} and HSO_3^- can be overcome by conversion of these species to less toxic forms, such as SO_4^{2-}. Oxidation of HSO_3^- to the less toxic sulfate can occur by both enzymatic and nonenzymatic mechanisms. Several factors, including cellular enzymes such as peroxidase and cytochrome oxidase, metals, ultraviolet light, and $O_2^{-\cdot}$, stimulate the oxidation of SO_2. In the presence of SO_3^{2-} and HSO_3^-, more $O_2^{-\cdot}$ is formed by free radical chain oxidation. Other free radicals can be formed as well. These oxidizing radicals can have detrimental effects on the cell.

Plant metabolism is affected by SO_2 in a variety of ways, for instance, stimulation of phosphorus metabolism (Plesnicar 1983) and reduction in foliar chlorophyll concentration (Lauenroth and Dodd 1981). Carbohydrate concentrations were increased by low levels of SO_2 and decreased by higher levels (Koziol and Jordon 1978). Effects of SO_2 on enzyme systems have been investigated in many studies. The enzymes studied include alanine and aspartate aminotransferases, glutamate dehydrogenase, malate dehydrogenase, glycolate oxidase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, fructose-1,6-bisphosphatase, and ribulose-5-phosphate kinase. Enzyme activity may be increased or decreased by exposure to SO_2 at different concentrations. It is widely known that there are differences in tolerance of plant species to SO_2 under similar biophysical conditions. This suggests that delicate biochemical and physiological differences operating in different plants could affect the sensitivity of a particular plant to SO_2.

8.1.4 Effect on Animals

Although SO_2 is an irritating gas for the eyes and upper respiratory tract, no major injury from exposure to any reasonable concentrations of this gas has been demonstrated in experimental animals. Even exposure to pure gaseous SO_2 at concentrations 50 or more times ambient values produced little distress (Alarie et al. 1970, 1973). Concentrations of 100 or more times are required to kill small animals. Mortality is associated with lung congestion and hemorrhage, pulmonary edema, thickening of the interalveolar septa, and other relatively nonspecific changes of the lungs.

For example, mice exposed to 10 ppm SO_2 for 72 hours showed necrosis and sloughing of the nasal epithelium (Giddens and Fairchild 1972). The lesions were more severe in animals with preexisting infection. Other symptoms include decreased weight gain, loss of hair, nephrosis in kidneys, myocardial degeneration, and accelerated aging.

Many studies have demonstrated an increase in the response of animals to SO_2 in the presence of particulate matter and elevations of relative humidity. Thus, H_2SO_4 mist and some particulate sulfates enhance the reactions of animals to SO_2, suggesting that alteration of SO_2 to a higher oxidation state may increase its irritability in animals. These interactions have important implications in air pollution control, as the rate of conversion of SO_2 to acid sulfates may have greater health significance than the concentration of SO_2 in the air.

8.1.5 Effect on Humans

Sulfur dioxide is rapidly absorbed in the nasopharynx of humans. Humans exposed to 5 ppm of the gas showed increased respiratory frequency and decreased tidal volume. Similar to observations made with animals, human exposure to SO_2 alters the mode of respiration, as demonstrated by increased frequency, decreased tidal volume, and lowered respiratory and expiration flow rates. Synergism and elevated airway resistance with SO_2 and aerosols of water and saline have been demonstrated.

It was previously thought that SO_2 and black suspended particulate matter interacted, and that both had to be elevated in order to exhibit health effects. New findings and analyses have changed such perceptions concerning the health effects of this group of pollutants. Emitted SO_2 is generally thought to be oxidized slowly by atmospheric oxygen to SO_3, which readily combines with water to form H_2SO_4. Ultimately, the aerosol reacts with atmospheric particles or surfaces to form sulfates. The World Health Organization recommends that the air quality standards reflect the joint presence of SO_2 and the resulting acid sulfates. Recent experimental and epidemiological data do not provide evidence for a specific effect of sulfate aerosol. However, airway reactivity is variable among subjects. Individuals with airway hyperactivity (e.g., asthmatics) have been shown to exhibit increased pulmonary flow resistance when exposed to SO_2 by a mouthpiece, while the increase was less with nasal breathing (Frank et al. 1962). Exercise augments responses to the pollutants. Airway reactivity is also increased after acute respiratory infections.

8.2 Nitrogen Oxides

8.2.1 Forms and Formation of Nitrogen Oxides

There are six forms of nitrogen oxides that are present: nitrous oxide (N_2O), nitric oxide (NO), nitrogen dioxide (NO_2), nitrogen trioxide (N_2O_3), nitrogen tetroxide (N_2O_4), and nitrogen pentoxide (N_2O_5). Of these, NO_2 is the major toxicant because of its relatively high toxicity and its ubiquity in ambient air, while N_2O, N_2O_3, and N_2O_4 have low relative toxicity and air pollution significance. Basic chemical reactions involved in the formation of NO_2 are shown below:

$$N_2 \rightarrow O_2 \quad \overset{1210^\circ C}{\rightarrow} 2\,NO \tag{8.5}$$

$$2\,NO_2 + O_2 \rightarrow 2\,NO_2 \tag{8.6}$$

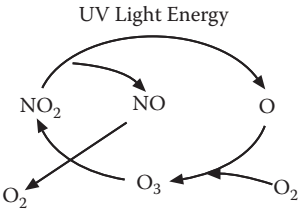


Figure 8.3 The photolytic cycle of NO_2.

The NO formed in the Equation 8.5 persists when temperature is cooled rapidly, as is the case in ambient air. The reaction shown in Equation 8.6 is one of the few that is slowed down with an increase in temperature.

8.2.2 Major Reactive N Species in the Troposphere

Several reactive N species, including NO, NO_2, and HNO_3, occur in the troposphere. Among these species, NO_2 is of particular environmental concern because it plays a complex and important role in the production of photochemical oxidants and acidic deposition. NO_2 is a unique air pollutant in that it absorbs UV light energy, whereby it is decomposed and forms NO and atomic oxygen. The energetic oxygen atom reacts with molecular oxygen to form O_3. The O_3 then reacts with NO to form molecular oxygen and NO_2, thus terminating the photolytic cycle of NO_2 (Figure 8.3). It is clear that, as far as the cycle is concerned, there is no net loss or gain of chemical substances.

However, for reasons to be described in the next section, in actuality O_3 accumulates. Several other reactions also occur, resulting in the production of photochemical smog. In addition to NO and NO_2, HNO_3 is also an important N compound in the troposphere. It is formed mainly from NO_2 and OH radicals. Nitric acid is also formed through a secondary reactive pathway, whereby NO_2 is first oxidized to NO_3 by O_3. The NO_3 then reacts with a molecule of NO_2, forming N_2O_5. The N_2O_5 thus formed combines with a molecule of water yielding HNO_3. The resultant HNO_3 may be precipitated through rainout or dry deposition. These reactions and others are shown in Figure 8.4.

8.2.3 Effect on Plants

Plants absorb gaseous NOx through stomata. NO_2 is more rapidly absorbed than NO, mainly because NO_2 reacts rapidly with water, while NO is almost insoluble. The absorbed NO_2 is then converted to NO_3^- and NO_2^- before being utilized in plant metabolism. The NO_2 injury to plants may be due to either acidification or a photooxidation process (Zeevaart 1976). Symptoms exhibited by plants exposed to NO_2 are similar to those from SO_2, but much higher concentrations are required to cause acute injury. However, decreased photosynthesis has been demonstrated even at concentrations that do not produce visible injury. The combined effect of NO and NO_2 gases appears to be additive.

Photosynthetic inhibition caused by NOx may be due to competition for NADPH between the processes of nitrite reduction and carbon assimilation in chloroplasts. NO_2 has been shown to cause swelling of chloroplast membranes (Wellburn et al. 1972). Biochemical and membrane

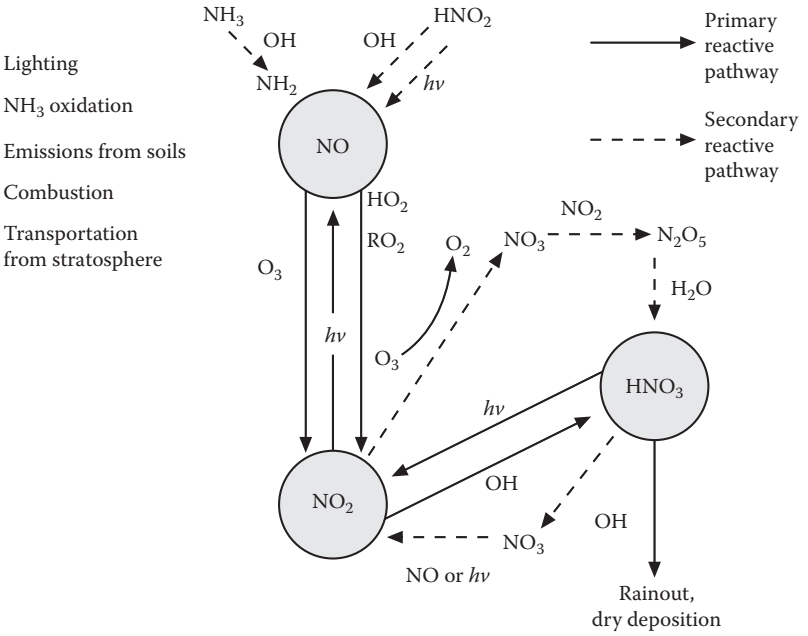


Figure 8.4 Major reactive N species in troposphere.

injury may be caused by ammonia produced from NO_3^-, if it is not utilized soon after its formation. Plants can metabolize the dissolved NOx through their NO_2 assimilation pathway:

$$NOx \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow NH_3 \rightarrow \text{amino acids} \rightarrow \text{proteins}$$

Other biochemical pathways affected by NOx include inhibition of lipid biosynthesis, oxidation of unsaturated fatty acids *in vivo*, and stimulation of peroxidase activity.

8.2.4 Effects on Humans and Animals

Studies on the pathological and physiological effects of NO_2 on animals are done at concentrations much higher than those found in ambient air. The toxic action of NO_2 is mainly on the deep lung and peripheral airway. Exposure of various species of animals to 10 to 25 ppm of NO_2 for 24 hours resulted in bits of fibrin in the airway, an increased number of macrophages, and an altered appearance of the cells in the distal airway and adjacent pulmonary alveoli. Terminal bronchioles showed hyperplasia and hypertrophy, loss of cilia, and disturbed ciliagenesis. Large crystaloid depositions also occurred in the cuboidal cells. Continuous exposure for several months produced thickening of the basement membranes, resulting in narrowing and fibrosis of the bronchioles. Emphysema-like alterations of the lungs developed, followed by death of the animals (Freeman and Haydon 1964).

8.2.5 Physiological Effects

NO_2 is rapidly converted to nitrite (NO_2^-) and nitrate (NO_3^-) ions in the lungs, and these ions are found in the blood and urine shortly after exposure to 24 ppm of NO_2 (Orehek et

al. 1976). Increased respiration was shown in some studies. Other physiological alterations include a slowing of weight gain and decreased swimming ability in rats, alteration in blood cellular constituents such as polycythemia, lowered hemoglobin content, thinner erythrocytes, leukocytosis, and depressed phagocytic activity. Methemoglobin formation occurred only at high concentrations. Methemoglobinemia is a disorder manifested by high concentrations of methemoglobin in the blood. Under this condition, the hemoglobin contains Fe^{3+} ion and is thus unable to reversibly combine with molecular oxygen. As mentioned previously, although almost all the studies done were conducted by using much higher concentrations of NO_2 than are found in ambient air, a few papers did deal with low NO_2 concentrations. Orehek et al. (1976) showed that in asthmatic subjects exposed to 0.1 ppm of NO_2 significantly aggravated the hyperreactivity in the airway. While at the prevailing concentrations of NO_2, its health effects are generally considered insignificant, NO_2 pollution may be an important aspect of indoor pollution. Evidence suggests that gas cooking and heating of homes, when not vented, can increase the exposure to NO_2, and that such exposures may result in increased respiratory problems among young children.

8.2.6 Biochemical Effects

Extracts of lung lipids from rats exposed to NO_2 have been reported to show oxidation. Lipid peroxidation was more severe in animals fed a diet deficient in vitamin E (Roehm et al. 1971). In contrast to ozone, reaction of NO_2 with fatty acids appears to be incomplete, and phenolic antioxidants can retard the oxidation from NO_2. Exposure to NO_2 may cause changes in the molecular structure of lung collagen. In a series of papers, Buckley and Balchum (1967a, 1967b) demonstrated that exposure for 10 weeks or longer at 10 ppm or for 2 hours at 50 ppm increased both tissue oxygen consumption and lactate dehydrogenase (LDH) and aldolase activity. Stimulation of glycolysis has also been reported.

8.3 Ozone

8.3.1 Sources

Ozone is a natural constituent of the upper atmosphere; trace amounts naturally exist in the lower atmosphere. Formation of O_3 in the upper atmosphere occurs in steps, i.e., a molecule of oxygen being split into atomic oxygen, and the resulting atomic oxygen reacting with another oxygen molecule to form ozone:

$$O_2 \xrightarrow{h\nu} O + O \tag{8.7}$$

$$O + O_2 \rightarrow O_3 \tag{8.8}$$

Ozone in the lower atmosphere is also produced as a result of modern technology. Equipment that produce sparks, arcs, or static discharge; ultraviolet and other ionizing radiation; commercial applications such as air purifiers and deodorizers in homes, hospitals, and offices; and closed environmental systems such as aerospace cabins and submarine chambers due to electric discharge from equipment or ionizing radiation are some examples.

By far the most important source of O_3 contributing to environmental pollution is that found in photochemical smog. As shown in Section 8.2, disruption of the photolytic cycle of NO_2 (Equations 8.9 to 8.11) by atmospheric hydrocarbons is the principal cause of photochemical smog.

$$NO_2 \xrightarrow{h\nu} NO + O \tag{8.9}$$

$$O + O_2 \rightarrow O_3 \tag{8.10}$$

$$\text{Net: } NO_2 + O_2 \leftrightarrow NO + O_3 \tag{8.11}$$

In the above equations, theoretically back reaction proceeds faster than the initial reaction, so that the resulting O_3 should be removed from the atmosphere. But free radicals formed from hydrocarbons and other species present in the urban atmosphere react with and remove NO, thus stopping the back reaction. As a result, O_3 builds up. Free radicals are noncharged fragments of stable molecules, for example, hydroxy radical, $OH^{\cdot}$; hydroperoxy radical, $HO_2^{\cdot}$; atomic oxygen, O^1D; and higher homologs, $RO^{\cdot}$ and $RO_2^{\cdot}$, where R is a hydrocarbon group. Free radicals participate in chain reactions, including initiation, branching, propagation, and termination reactions in the atmosphere. The $OH^{\cdot}$–$HO_2^{\cdot}$ chain is particularly effective in oxidizing hydrocarbons and NO. Some examples illustrating these reactions are shown below:

$$OH^{\cdot} + RH \rightarrow R^{\cdot} + H_2O \tag{8.12}$$

$$R^{\cdot} + O_2 \rightarrow RO_2^{\cdot} \tag{8.13}$$

$$RO_2^{\cdot} + NO \rightarrow RO^{\cdot} + NO_2 \tag{8.14}$$

$$RO^{\cdot} + O_2 \rightarrow R'CHO + HO_2^{\cdot} \tag{8.15}$$

$$HO_2^{\cdot} + NO \rightarrow NO_2 + OH^{\cdot} \tag{8.16}$$

It is noticeable that the process starts with an OH radical. After one pass through the cycle, two molecules of NO are oxidized to NO_2. The OH radical formed in the last step (Equation 8.16) can start the cycle again. On the other hand, O_3 can also be formed from O_2 reacting with hydrocarbon free radicals, as shown below:

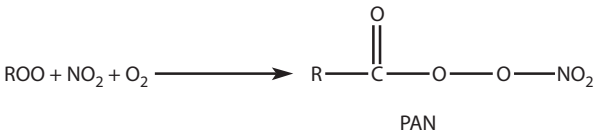
$$R^{\cdot} + O_2 \rightarrow RO_2^{\cdot} \xrightarrow{O_2} O_3 + RO^{\cdot} \tag{8.17}$$

8.3.2 Photochemical Smog

The hydrocarbon free radicals (e.g., $RO_2^{\cdot}$) formed can react further with different species, including NO, NO_2, O_2, O_3, and other hydrocarbons. Thus,

$$ROONO \rightarrow RONO_2 \tag{8.18}$$

The free radical $RO_2^{\cdot}$ can react with O_2 and NO_2 to produce peroxyacyl nitrate (PAN):



(8.19)

Peroxyacyl nitrate can also be formed from a reaction involving $RO_3^{\cdot}$ and $NO_2^{\cdot}$:

$$RO_3^{\cdot} + NO_2 \rightarrow RO_3NO_2 \tag{8.20}$$

Clearly, a large number of chemical reactions occur in the atmosphere leading to the formation of many secondary air pollutants. In areas with abundant sunshine and unique topographical conditions, as is the case in Los Angeles, accumulation of these pollutants occurs, leading to smog formation. This is a problem that many large cities in the world are facing. Principal components of photochemical smog include O_3 (up to 90%), NOx (mainly NO_2, about 10%), PAN (0.6%), free radical oxygen forms, and other organic compounds, such as aldehydes, ketones, and alkyl nitrates.

8.3.3 Effect on Plants

By far, ozone is the most important of the phytotoxic pollutants. A large volume of literature has been published dealing with the influence of O_3 on higher plants. Highlights of the experimental results include the following: (1) either an increase or a decrease in plant growth (Blum and Heck 1980); (2) reduction in size, weight, and number of fruits (Henderson and Reinert 1979; Oshima et al. 1977); (3) reduction in shoot and root growth (Grunwald and Endress 1984; Letchworth and Blum 1977); (4) reduction in seed oil (Grunwald and Endress 1984); (5) reduction in growth ring size (McLaughlin et al. 1982); (6) reduction in net photosynthesis (Blum et al. 1983); (7) reduction in unsaturated fatty acids (Perchorozicz and Ting 1974); (8) increase in membrane permeability (Pauls and Thompson 1981); (9) increase in respiration (Dugger and Ting 1970); and (10) altered intermediary metabolism.

The effect of O_3 on plant metabolism is complex, and contradictory results have been reported. However, it is well established that photochemical oxidants such as O_3 and PAN can oxidize SH groups, and such oxidation may be sufficient to cause loss of enzyme activity. For example, several enzymes involved in carbohydrate metabolism, such as phosphoglucomutase and glyceraldehyde-3-phosphate dehydrogenase, have been shown to be inhibited by O_3. The hydrolysis of reserve starch was inhibited by exposure to 0.05 ppm O_3 for 2 to 6 hours in cucumber, bean, and monkey flower (Dugger and Ting 1970), suggesting an inhibition of amylase or phosphorylase. While a decrease in glyceraldehyde-3-phosphate dehydrogenase activity suggests inhibition of glycolysis, an increase in the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, reported by some workers (Tingey et al. 1975), implies increased activity of the pentose phosphate pathway. In addition to carbohydrates, lipids are also affected by exposure to O_3. Lipid synthesis, requiring NADPH and ATP, for example, is known to proceed at a lower rate, presumably because O_3 lowers the total energy of the cell.

8.3.4 Effects on Humans and Animals

Ozone and other oxidants cause respiratory and eye irritation. The threshold limit value (TLV) for O_3 in industry is 0.1 ppm. Exposure to 0.6 to 0.8 ppm O_3 for 60 minutes resulted in headache,

nausea, and increased airway resistance. Exposure at 0.7 to 0.9 ppm in experimental animals may predispose or aggravate a response to bacterial infection. Coughing, chest pain, and a sensation of shortness of breath were shown in the exposed subjects who were exercised (Batesm and Hazucha 1973). Morphological and functional changes occur in the lung in experimental animals subjected to prolonged exposure to O_3. Such changes as chronic bronchitis, bronchiolitis, and emphysematous and septal fibrosis in lung tissues have been observed in mice, rabbits, hamsters, and guinea pigs exposed daily to O_3 at concentrations slightly above 1 ppm. Thickening of terminal and respiratory bronchioles was the most noticeable change. For example, in the small pulmonary arteries of rabbits exposed to O_3, the walls were thicker and the lumina were narrower than those of the controls. Mean ratios of wall thickness to lumen diameter were 1:4.9 for the control, while those of the exposed animals were 1:1.7 (P'an et al. 1972). Other physiological effects include dryness of upper airway passages, irritation of mucous membranes of the nose and throat, bronchial irritation, headache, fatigue, and alterations of visual response. There is suggestive evidence that O_3 exposure accelerates aging processes. Some investigators suggest that aging is due to irreversible cross-linking between macromolecules, principally proteins and nucleic acids.

Animals exposed to 0.1 ppm O_3 may have increased susceptibility to bacterial infections. Exposed mice may have congenital abnormalities and neonatal deaths.

Development of hyperreactivity following O_3 exposure in humans and dogs has been shown. The most characteristic toxic effect of relatively high level O_3 exposure is pulmonary edema (Mueller and Hitchcock 1969), a leakage of fluid into the gas exchange parts of the lung. This effect was seen at concentrations only slightly above those observed in community pollution in Los Angeles.

It has long been known that humans as well as animals develop tolerance to O_3. Tolerance refers to increased capacity of an organism that has been preexposed to the oxidant to resist the effects of later exposures to ordinarily lethal (or otherwise injurious) doses of the same agent. Rodents exposed to 0.3 ppm O_3, for example, would become tolerant to subsequent exposures of several ppm, which would produce massive pulmonary edema in animals exposed for the first time. Some human subjects exposed to 0.3 ppm at intervals of a day or so showed diminished reactivity with later exposures. This response is designated as *adaptation* (Horvath et al. 1981).

8.3.5 Biochemical Effects

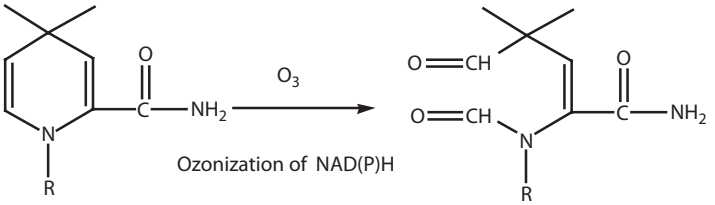
Research on the biochemical effects of O_3 has been extensive. Among the many mechanistic postulations that have been advanced concerning the toxicity of O_3, the following are noted: (1) reactions with proteins and amino acids, (2) reactions with lipids, (3) formation of free radicals, (4) oxidation of sulfhydryl compounds and pyridine nucleotides, (5) influence on various enzymes, and (6) production of more or less nonspecific stress, with the release of histamine.

Ozone interacts with proteins and some amino acids, causing alteration. For instance, the lysozyme in tears of individuals exposed to smog has been reported to be 60% less than the normal. Concentrations of protein sulfhydryl and nonprotein sulfhydryl in the lungs of rats exposed to 2 ppm O_3 for 4 to 8 hours have been shown to be decreased. Mudd et al. (1969) showed that aqueous solutions of amino acids such as tyrosine, histidine, sistine, and tryptophan were oxidized by O_3. Methionine, for example, was oxidized to methionine sulfoxide. A number of investigators have shown that O_3 could cause the oxidation of the SH group, and that addition of SH compounds was protective. The activities of several enzymes have been shown to be either enhanced or depressed in animals exposed to O_3. These include a decrease in glucose-6-phosphate dehydrogenase, glutathione reductase, and succinate-cytochrome c reductase in the lungs of rats exposed to 2

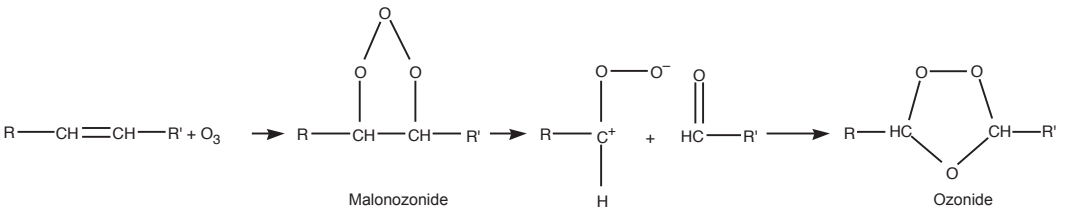
ppm O_3 for 4 to 8 hours; and an increase in glucose-6-phosphate dehyrogenase, 6-phosphogluconate dehydrogenase, and isocitrate dehydrogenase.

Balchum et al. (1971) have provided evidence supporting the concept that the peroxidation or ozonization of unsaturated fatty acids in biological membranes is a primary mechanism of the deleterious effects of O_3. The hypothesis is based on the tendency of O_3 to react with the ethylene groups of unsaturated fatty acids, resulting in the formation of free radicals. The free radicals can, in the presence of molecular oxygen, cause peroxidation of unsaturated fatty acids. In support of this hypothesis is the evidence that after O_3 exposure there was a relative decrease in unsaturated fatty acids compared to saturated fatty acids, and the more unsaturated the fatty acid, the greater the loss. Furthermore, a deficiency of vitamin E increases the toxicity of O_3 for the rat (Goldstein et al. 1970). Subsequent published reports appear to support these observations.

Another chemical pathway leading to O_3-dependent unsaturated fatty acid oxidation is through incorporation of O_3 into the fatty acid double bond, resulting in ozonide formation. This process is generally known as ozonolysis:



Ozone is also known to oxidize glutathione and pyridine nucleotides NADH and NADPH. The ozonization of NAD(P)H may proceed in the nicotinamide ring as follows:



Since the intracellular ratios of NADH/NAD^+, NADPH/$NADP^+$, and ATP/adenylates are carefully regulated by the cell, loss of the reduced nucleotide can be compensated by faster operation of the Krebs cycle. But, the cell can only make up for a net loss of all nucleotides by an increase in synthesis. The oxidation of NADPH or NADH results in elevated enzyme activity, and this permits the cell to restore the initial ratio of the nucleotides. With NADPH, its oxidation increases the activity of the pentose phosphate pathway. Such an increase also occurs following the oxidation of GSH, as shown below. Oxidation of either NADPH or GSH, therefore, may be responsible for the apparent increase in the enzymes found in the pentose phosphate pathway after repeated O_3 exposure.

$$2\ GSH + [O] \xrightarrow{\text{GSH peroxidase}} GSSG + H_2O \tag{8.21}$$

$$GSSG + 2\ NADPH \xrightarrow{\text{GSH reductase}} 2\ GSH + 2\ NADP^+ \tag{8.22}$$

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-P dehydrogenase}} \text{6-PG} + \text{NADPH} \quad (8.23)$$

8.4 Carbon Monoxide

Carbon monoxide is an odorless, colorless, and tasteless gas that is found in high concentrations in the urban atmosphere. No other gaseous air pollutant with such a toxic potential as CO exists at such high concentrations in the urban environment. Historically, early exposures began from fires and then from coal for domestic heating. Combustion associated with developing industry, explosions, fires in mines, and illumination gas prepared from coal have all been sources of exposure. The migration of agricultural populations to cities increased the proportion of the population exposed, as well as the number of persons generating CO.

With the emergence of automobiles propelled by an internal combustion engine, the CO emitted from the exhaust pipe has become the major source for human exposure. Serious problems exist with occupational exposure to increased ambient CO for firefighters, traffic police, toll booth attendants, coal miners, coke ovens, smelter workers, and transportation mechanics.

8.4.1 Formation of CO

Formation of CO usually occurs through one of the following three processes:

1. Incomplete combustion of carbon or carbon-containing compounds. This occurs when available oxygen is less than the amount required for complete combustion in which carbon dioxide is the product, or when there is poor mixing of fuel and air:

$$2\,\text{C} + \text{O}_2 \rightarrow 2\,\text{CO} \quad (8.24)$$

$$2\,\text{CO} + \text{O}_2 \rightarrow 2\,\text{CO}_2 \quad (8.25)$$

2. Reactions between CO_2 and carbon-containing materials at high temperature. This occurs at elevated temperature, common in many industrial devices such as blast furnaces.

$$\text{CO}_2 + \text{C} \rightarrow 2\,\text{CO} \quad (8.26)$$

   The CO produced in this way is beneficial and necessary in certain applications, as in the blast furnace, where CO acts as a reducing agent in the production of iron from Fe_2O_3 ores, as shown below. Some CO may escape into the atmosphere, however.

$$3\,\text{CO} + \text{Fe}_2\text{O}_3 \rightarrow 2\,\text{Fe} + 3\,\text{CO}_2 \quad (8.27)$$

3. Dissociation of CO_2 at high temperature. Carbon dioxide dissociates into CO and O at high temperature, as follows:

$$\text{CO}_2 \xrightarrow{\text{High temperature}} \text{CO} + \text{O} \quad (8.28)$$

High temperature favors the dissociation of CO_2. For example, at 1745°C the dissociation is 1%, while at 1940°C, it is 5%.

8.4.2 Human Exposure to CO

Exposure to CO comes mainly from three sources:

1. CO in the surrounding ambient environment mainly from exhaust gases (automobile, industrial machinery), suicidal and accidental intoxication (e.g., house fires, >50,000 ppm), and home environmental problems such as defective furnaces, charcoal burning in poorly vented houses, or garages connected to living quarters, and space heaters in campers.
2. Occupational exposure, such as firefighters (>10,000 ppm CO), traffic police, coal miners, coke oven and smelter workers, toll booth attendants, and transportation mechanics.
3. Cigarette smoking. Smokers have higher carboxyhemoglobin (COHb) levels than nonsmokers (Table 8.1). With a large percentage of the population smoking, particularly in the less developed countries, nonsmokers are subjected to inhalation of CO from cigarette smoke in confined places.

8.4.3 Toxicological Effects

An important physiological effect of CO is interfering with O_2 transfer brought about by the combination of CO gas with hemoglobin (Hb), forming carboxyhemoglobin, HbCO or COHb:

$$Hb + 4\ O_2 \rightarrow Hb(O_2)_4 \tag{8.29}$$

$$Hb + 4\ CO \rightarrow Hb(CO)_4 \tag{8.30}$$

$$\text{Sum: } Hb(O_2)_4 + 4\ CO \rightarrow Hb(CO)_4 + 4\ O_2 \tag{8.31}$$

CO has more than 200 times greater affinity for combining with Hb than O_2 does. A binding site on an Hb molecule cannot be occupied by both CO and O_2. Although an increase in oxygen

Table 8.1 Blood COHb Levels of Smokers

Category of Smokers	*Median Equilibrium Blood COHb (%)*
Never smoked	1.3
Ex-smoker	1.4
Pipe or cigar smokers only	1.7
Light Cigarette Smoker	
(<½ pack/day; noninhaler)	2.3
(<½ pack/day; inhaler)	3.8
Moderate smoker (½–2 packs/day; inhaler)	5.9
Heavy smoker (>2 packs/day; inhaler)	6.9

Table 8.2 COHb Levels and Demonstrated Toxicological Effects

COHb Level (%)	*Demonstrated Effects*
<1.0	No apparent effect
2–4	Impairment of visual function
5–10	Impairment of visual perception, manual dexterity, learning, and performance of certain intellectual tasks
	Increased coronary blood flow
	Impairment in response to certain psychomotor tests
	Decreased night vision and peripheral vision
20–30	Nausea, weakness (particularly in the legs), occasional vomiting
30–35	Clouding of mental alertness occurs with increasing weakness
35–45	Collapse and coma
>50	Death (in young people)

concentrations can shift the equilibrium in Equation 8.30 to the left, recovery of Hb is slow, while the asphyxiating effect of putting Hb out of business is rapid. The normal or background level of blood HbCO is about 0.5%. The CO is derived from both the CO in ambient air and the CO produced by the body during catabolism of heme (a component of Hb).

The equilibrium percentage of HbCO in the bloodstream of a person continually exposed to an ambient air CO concentration of less than 100 ppm can be calculated from the following equation:

$$\text{Percent COHb in blood} = 0.16 \times (\text{CO concentration in the air in ppm}) + 0.5 \qquad (8.32)$$

Based on COHb levels, various health effects may be expected to occur. Table 8.2 summarizes demonstrated health effects associated with COHb levels.

Carbon monoxide also inhibits function of alveolar macrophages. This can lead to weakening tissue defenses against airborne bacterial infection. Maternal CO poisoning during pregnancy has been shown to cause fetal death because of lack of O_2 in the fetal circulatory system. Carbon monoxide poisoning causing unconsciousness for 30 minutes to 5 hours does not do permanent damage to the mother but can cause brain damage, mental deficiency, or death to the fetus. Severity of damage is related to the month of pregnancy, the fetus being particularly vulnerable shortly before birth.

The half-life of COHb is 4 hours at rest at room air. It is shortened to 60 to 90 minutes if 100% oxygen is given using a face mask. Since more than 2 hours at 100% oxygen can cause pulmonary oxygen toxicity, the oxygen concentration should be reduced to 60% at 2 hours.

8.4.4 Mechanism of Action

As mentioned previously, CO competes with O_2 for binding of hemoglobin, but in addition, it also binds other proteins, such as myoglobin, cytochrome c oxidase, and cytochrome P-450. Carbon monoxide also impairs the facilitated diffusion of O_2 to the mitochondria, shifting the

oxyhemoglobin dissociation curve to the left. Alteration of the oxyhemoglobin dissociation curve by COHb occurs in such a manner that O_2 is released to tissues with great difficulty and at a lower O_2 tension.

Study Questions

1. What is the most dangerous gaseous pollutant and why?
2. How does SO_2 affect a plant's structure and function? What affects SO_2 uptake by a plant? How is plant metabolism affected?
3. At what levels does SO_2 affect experimental animals? What does it affect?
4. What condition of SO_2 might have a greater health significance than the air concentration of SO_2?
5. What effect does SO_2 have on humans?
6. Which form of nitrogen oxide is the major toxicant and why?
7. How does gaseous NO_2 affect plants?
8. How does NO_2 affect animals?
9. What is the most important source of O_3, which contributes to environmental pollution? What causes this source?
10. How do photochemical oxidants affect plant enzyme activity? Lipid synthesis?
11. Describe the effects oxidants have on humans and animals.
12. What is adaptation to O_3?
13. Discuss the five mechanisms postulated for O_3 toxicity.
14. How does CO formation occur?
15. What is an important physiological effect of CO?

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Chapter 9

Fluoride as a Contaminant of Developing Economies

9.1 Environmental Sources and Forms of Fluoride

Fluoride (F) is a ubiquitous element. It occurs naturally in the atmosphere through volcanic eruption, and in the earth's crust. It rarely occurs free in nature, but combines with a variety of elements to form fluorides that exist in minute amounts in air, water, minerals and soils, vegetation, and body tissues.

9.1.1 Minerals and Soils

The chief fluoride-containing minerals are fluorspar (CaF_2), cryolite (Na_3AlF_6), and fluorapatite [$Ca_{10}F_2(PO_4)_6$]. Whenever any of these minerals are used in industrial processes, for example, some of them are emitted into the environment. Eventually, emitted gaseous or particulate forms of fluorides are precipitated onto the ground and become absorbed in soils. The absorbed F may assume different forms depending on such factors as soil pH, organic matter, clay content, and exchangeable Ca content.

9.1.2 Natural Waters

Fluoride content in natural waters in the northeastern part of the United States ranges from 0.02 to 0.1 ppm, while in the West and Midwest river waters, it ranges from 0 to 6.5 ppm, with an average of 0.2 ppm. Groundwaters contain from 0.1 to 8.7 ppm, depending on the rocks from which the waters flow. The level of F in seawater is about 1.2 ppm.

9.1.3 Foods

Virtually all foods contain trace amounts of F. Table 9.1 shows the F contents of several kinds of foods produced in the United States. Fluoride-containing foods and beverages are, therefore, the

Table 9.1 Fluoride Content of Selected Foods

	ppm on Dry Basis
Meats	0.01–7.7
Fish	0.10–24
Cheese	0.13–1.62
Butter	0.4–1.50
Rice and peas	10
Cereal and cereal products	0.10–0.20
Vegetables and tubers	0.10–2.05
Citrus fruits	0.04–0.36
Sugar	0.10–0.32
Coffee	0.2–1.6
Tea (U.S. brands)	Average 60

Source: Adapted from Committee on Biologic Effects of Atmospheric Pollutants, *Fluorides*, National Academy of Sciences, Washington, DC, 1971.

most important sources of F intake. For an adult male residing in a fluoridated U.S. community, F intake from food and beverages is estimated to range from 1 to 3 mg/day. The intake is reduced to ≤1.0 mg/day in a nonfluoridated area (Phipps, 1996).

Plants can absorb F from soil, water, or atmosphere. Most plants contain 0.1 to 10 ppm F, while forage plants generally contain 5 to 10 ppm, on a dry basis. The contents of F in plants vary with plant species. Several species of plants are known as F accumulators. Tea leaves, for example, may contain as high as 760 ppm; camellia, 620 ppm; and elderberry, 3,600 ppm (on a dry basis). It should be noted that although tea leaves are an important F accumulator, tea beverages may contain less than 0.5 mg F per cup.

9.1.4 Air

Fluoride content in air in U.S. residential and rural communities varies markedly, and depends on the location where samples are taken, but is less than 0.04 to 1.2 ppb F (0.03 to 0.90 μg F/m^3). In many cities in developing countries, the content is much higher. In Beijing, for example, the level is 0.11 to 2.14 ppb F (0.08 to 1.61 μg F/m^3), with an average of 0.61 μg F/m^3 (Feng et al. 2003).

9.2 Industrial Sources of Fluoride Pollution

Fluoride emissions into the atmosphere are derived mainly from modern-day anthropogenic sources, particularly industrial sources. They include the steel industry, phosphate fertilizer

industry, aluminum industry, ceramics industry (brick, tile, glass, etc.), nonferrous metal foundries, welding operations, and coal-burning power plants.

Fluorides emitted into the atmosphere from different sources include both gaseous and particulate forms. Historically, most of the F pollution problems occurred as a result of emissions from anthropogenic sources. Such emissions occasionally resulted in the presence of harmful levels of F compounds in the environment as well as in body tissues. The forms of F emitted from these sources include hydrogen fluoride, cryolite, fluorospa, and silicon tetrafluoride (SiF_4). The anthropogenic sources also contribute F to surface waters.

Some heavy discharges of F into the atmosphere and waters have occurred in connection with manufacture of elemental phosphorus, phosphate fertilizer, and aluminum. In the manufacture of elemental phosphorus, ground phosphate rock, whose main component is $Ca_{10}F_2(PO_4)_6$, is mixed with silica and coke and then heated in a carbon-lined furnace with carbon electrodes. Equation 9.1 shows the chemical reaction involved in this process:

$$2\ Ca_{10}F_2(PO_4)_6 + 18\ SiO_2 + 30\ C \rightarrow 18\ CaO \cdot SiO_2 \cdot 1/9\ CaF_2 + 30\ CO + 3P_4 \uparrow \qquad (9.1)$$

Aluminum, on the other hand, is produced by dissolving alumina (Al_2O_3) in molten cryolite followed by electrolytical reduction. The net chemical change is shown in Equation 9.2:

$$Al_2O_3 + 2C \rightarrow 2\ Al + CO \uparrow + CO_2 \uparrow \qquad (9.2)$$

In this process, besides CO and CO_2, other gases, such as SO_2, SiF_4, HF, CS_2, COS (carbonyl sulfide), CS_2, hydrocarbons, and water vapor, are produced. Particulate emissions also occur, including alumina, cryolite, aluminum fluoride (AlF_3), CaF_2, chiolite ($Na_5Al_3F_{14}$), and iron oxide (Fe_2O_3).

These emissions have been associated with increased levels of F in exposed organisms, including vegetation, wildlife, and humans. Several examples are given below.

In the manufacture of phosphate fertilizer, fluroapatite is heated at high temperature in blast furnaces. This results in emissions of both gaseous and particulate forms of fluorides. In a study done in an area near a phosphate fertilizer plant in southern China, Ding et al. (1987) showed that the F concentrations of the air samples collected within 200, 400, 600, 800, and 1,600 m of the plant were inversely related to the distance from the plant. In particular, the researchers found that the concentration of F in all air samples collected within 400 m exceeded the one-time maximum concentration standard set by the Chinese government, and that the highest F concentration recorded was 0.165 mg F/m^3, which was 7.3-fold more than the standard. Furthermore, the percentages of samples with F concentrations in excess of the one-time maximum concentration standard were 45, 26, 20, 10, and 5% for the sampling sites 200, 400, 600, 800, and 1,600 m, respectively, from the emitting source (Ding et al. 1987).

As mentioned above, the manufacture of aluminum is another important source of atmospheric F pollution, which led to injuries to vegetation and wildlife. A comparative study was done in the early 1970s on a black-tailed deer killed on a road near an aluminum plant in northwestern Washington (F contaminated), and on another black-tailed deer killed on a road in an area with no industrial facilities (non-F contaminated). The F-contaminated deer manifested marked dental disfigurement and an abnormal tooth wear pattern compared to the non-F-contaminated animal. The F concentrations in the bones of the F-contaminated deer were 18 to 38 times higher than those in the bones of the non-F-contaminated deer (Table 9.2) (Newman and Yu, 1977).

Table 9.2 Fluoride Content of Bone Tissues from Black-Tailed Deer

Bone	Fluoride Concentration (ppm)[a]		F:C Ratio
	Control[b]	F-Contaminated[c]	
Rib	157	2,820	17.9
Metatarsal	89	2,475	27.8
Digit	54	2,048	37.9

[a] Fat-free basis.
[b] Male, 2.5 years.
[c] Female, 15–18 months.

Combustion of coal in power plants also emits considerable amounts of F into air. Fluoride contents in coals range from 0.001 to 0.048%, usually as fluorapatite or fluorspar. During combustion, about half of the F in coal is evolved as gaseous HF and SiF_4, and particulate matter. With the trend of increasing use of coal as an energy source, atmospheric F pollution has been increasing markedly in many cities and areas in the world. A number of cities in China, such as Chongqing and Beijing, are particularly well known.

In Beijing, coal is the dominant energy source, accounting for more than 75% of the total energy consumption. Additionally, combustion of coal for heating in winter accounts for 23% of the annual coal combustion. Furthermore, the coal consumed in the city is reported to contain 163 μg/g, more than double the mean value of 80 μg/g in coals of other parts of the world (Feng et al. 2003). In Beijing, another important source of F is soil dust resulting from fresh concrete used for building. Factors such as these have contributed to the elevated F concentrations of wet depositions in the city. For example, the annual volume-weighted average concentration of soluble F of ambient aerosol is reportedly 0.60 μg/m^3, which is 75 times higher than the concentration observed in the air sample taken in the city of Morioka, a non-fluoride-polluted city in the northern part of Japan (Feng et al. 2003).

Fluoride has also been traced to runoff from application of insecticides and weed killers. In addition to deposition into surface waters, airborne F may eventually be deposited into surface water and onto the ground, and taken up by soils, plants, and animals. Figure 9.1 shows environmental transfer of F.

9.3 Effect on Plants

9.3.1 Injuries to Leaf Tissues

Fluoride-induced effects in plants may be viewed based on four levels of biologic organization: ecosystem, organism, tissue or organ, and cellular levels. Plants growing near F-emitting sources can accumulate high levels of F in leaves. Gaseous forms of F, such as HF and SiF_4, are taken up by leaves much more rapidly than are particulate fluorides. Fluoride ions accumulate in plant leaves mainly as a result of diffusion through the stomata from the atmosphere or following root absorption from soil. In contrast to other major air pollutants, such as SO_2, NO_2, O_3, and peroxyacyl nitrate (PAN), discussed in Chapter 7, F accumulates in the leaf tips and margins of many species (Figure 9.2).

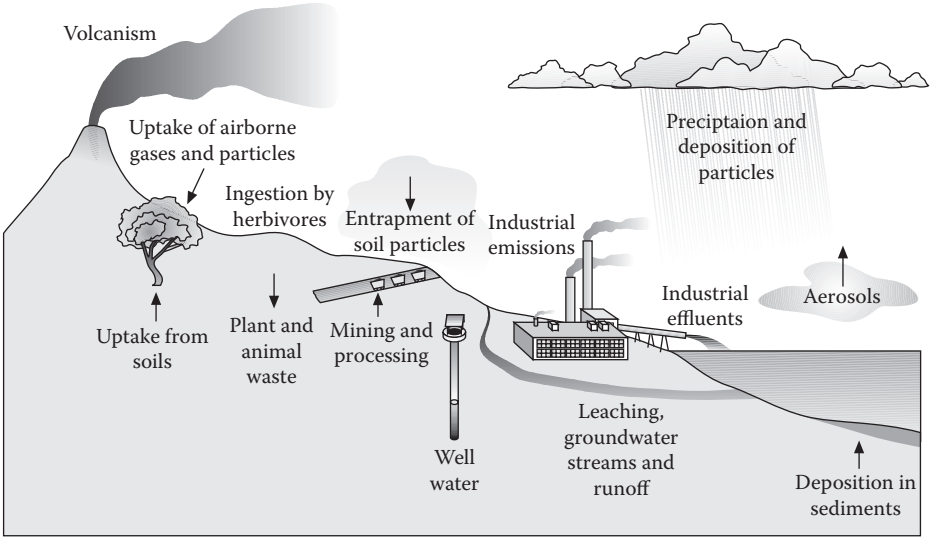


Figure 9.1 Environmental transfer of fluoride and other elemental pollutants.

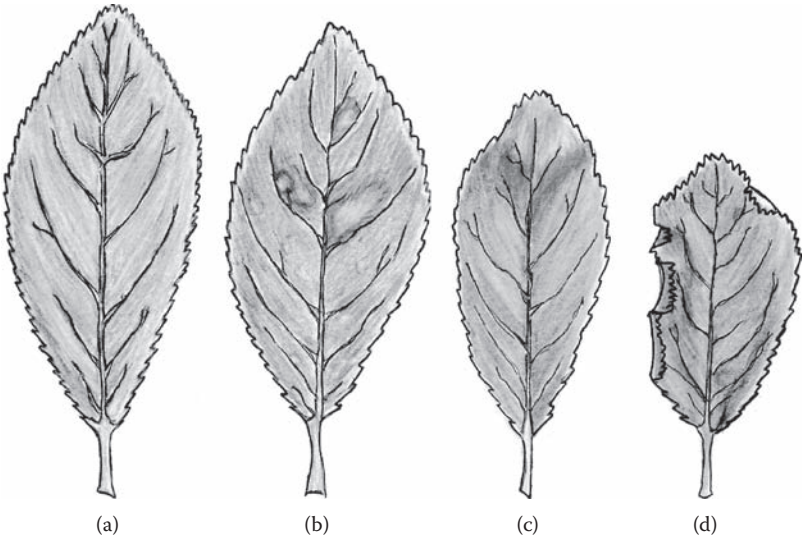


Figure 9.2 Drawing of normal and leaf tissues with chlorosis and necrotic lesions. The normal leaf tissue (a) can be compared to those with chlorosis (b, c, d) and necrosis. (Drawing by Eva Landis.) (See color insert following page 268.)

Table 9.3 Effect of Fluoride on Fresh Weight and Root Elongation in Mung Bean Seedlings Exposed to NaF

NaF (mM)	*Radical Weight (mg/seed)*	*%*	*Radical Length (mm)*	*%*
0	139 ± 8.2	100	77 ± 10.9	100
0.1	125 ± 11.2	90	73 ± 15.1	95
1.0	117 ± 16.1*	84	52 ± 8.2***	67
5.0	35 ± 5.7***	25	21 ± 4.6***	27

Note: Values are mean ± SD ($N = 15$).

* $p < 0.05$; *** $p < 0.001$.

Although plants differ widely in their susceptibility to F injury, accumulation of elevated levels of the element in leaves can lead to chlorosis or necrosis. Chlorosis represents yellowing of plant leaves resulting from partial failure to develop chlorophyll, caused by nutrient deficiency or the activities of a pathogen. Similarly, the destruction of part of the leaf exhibited by necrosis will cause a comparable reduction in photosynthesis. It is clear, then, that both chlorosis and necrosis can lead to lowered plant growth and yield.

9.3.2 Effect on Germination

A large number of studies have focused on F effects on germination and seedlings. In laboratory experiments, plants exposed to various concentrations of F generally exhibit concentration-dependent growth impairment. For example, 1 mM NaF was shown to severely inhibit germination of mung bean (*Vigna radiata*) seeds, as manifested by reduced radical length and weight (Table 9.3) (Yu, 1996). A similar observation was made recently by Gupta et al. (2009) using rice (*Oryza sativa*). These researchers exposed rice seeds to 0, 10, 20, and 30 mg NaF/L for 15 days and found, at the end of the experiment, that the seeds treated with 0 and 10 mg NaF/L showed 100% germination, but at 20 and 30 mg NaF/L germination was reduced to 92 and 96%, respectively. In addition, seeds exposed at 30 mg NaF/L resulted in decreases in root length, shoot length, and dry weight by 50, 27, and 29%, respectively (Gupta et al. 2009).

Kamaluddin and Zwiazek (2003) reported that a long-term exposure of roots of aspen (*Populus tremuloides*) seedlings to NaF markedly decreased root hydraulic conductivity and stomatal conductance. NaF absorbed from roots led to significant electrolyte leakage in leaf tissues, restricted leaf expansion, and decreased net photosynthesis. A short-term exposure of excised roots to 5 mM NaF and KF significantly depressed root water flow with a concomitant decline in root respiration and depressed stomatal conductance.

9.3.3 Biochemical Effect

Many metabolic processes, such as glycolysis, tricarboxylic acid (TCA) cycle reactions, photosynthesis, protein synthesis, and lipid metabolism, are affected by exposure to F. Much of the action of F on these processes can be attributed to F-dependent inhibition of enzymes. Examples of enzymes shown to be inhibited by F include enolase, phosphoglucomutase, phosphatase,

hexokinase, phosphoenolpyruvate carboxykinase (PEP), carboxylase, pyruvate kinase, succinic dehydrogenase, malic dehydrogenase, pyrophosphatase, phytase, nitrate reductase, mitochondrial ATPase, urease (Miller et al. 1983), lipase (Yu et al. 1987), amylase (Yu et al. 1988), invertase (Yu 1996; Ouchi et al. 1999), and superoxide dismutase (SOD) (Wilde and Yu 1998).

Fluoride inhibition of certain enzymes in leaf tissues can lead to compositional changes. For instance, soybean leaves exposed to 30 ppb of HF were shown to contain lowered sucrose, while the levels of both glucose and fructose were elevated (Yang and Miller 1963a). Similarly, there was a marked increase in several organic acids, such as malic, malonic, succinic, and citric acids (Yang and Miller 1963b). On the other hand, inhibition of SOD in seedlings (Wilde and Yu 1998) may be reflected by increased oxidative stress, leading to impaired growth and development.

9.4 Effect on Animals

Animals normally ingest small amounts of F in their rations without observable adverse effects, but excessive intake is harmful. The most common sources of excessive F intake by animals are (1) forages that have been subjected to airborne contamination from nearby industrial operations, or forages that have been contaminated with soils high in F; (2) water containing an excessive amount of F; and (3) feed supplement and mineral mixtures containing high levels of F. The effects of F on domestic animals may be acute or chronic, depending on F concentrations.

9.4.1 Acute Effects

Together with arsenic, F has caused serious effects on livestock in the United States and other countries. The sources of the pollutant are mostly limited to phosphate fertilizer manufacturing, aluminum production, fluorohydrocarbon, and heavy metal production. Safe levels of soluble F in animal rations range from 30 to 50 mg/kg for cattle and 70 to 100 mg/kg for sheep and swine. Such physiological effects as gastroenteritis, muscular weakness, pulmonary congestion, nausea, vomiting, diarrhea, chronic convulsions, necrosis of mucosa of the digestive tract, anorexia, cramping, respiratory and cardiac failure, and collapse are observed, leading to eventual death.

9.4.2 Chronic Effects

The two most conspicuous and thoroughly studied manifestations of chronic F poisoning are dental and skeletal fluorosis. Once absorbed in the animal body, F has a great affinity for developing and mineralizing teeth. Such affinity of fluorides for developing and mineralizing teeth can either enhance tooth development or induce dental lesions, depending on the amounts of fluorides ingested. Dental lesions are manifested by abnormal enamel matrix, such as chalkiness, mottling, and hypoplasia (thin enamel). An affected tooth is also subject to more rapid wear and to erosion of the enamel away from the dentine.

In skeletal fluorosis, the affected bones lose their normal, hard, smooth luster and appear rough, porous, and chalky white. A generalized hyperostosis (excessive formation of bone tissue, especially in the skull) and, in some cases, exostotic lesions of the otherwise smooth, long bones can be observed (Figure 9.3). Exostosis refers to a spur or bony outgrowth from a bone.

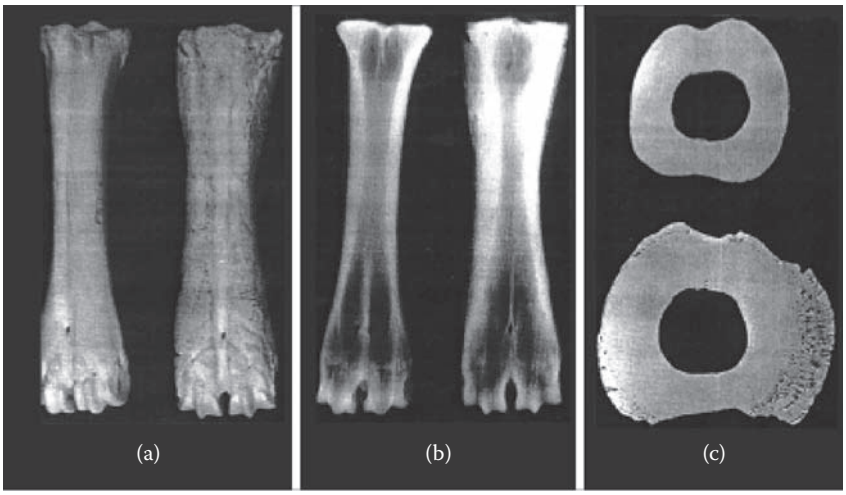


Figure 9.3 Skeletal fluorosis in bones from dairy cows. (a) Left: Metatarsal bone from a dairy cow fed 12 ppm F from 3 to 4 months to 7.5 years of age. The bone is normal. Right: Metatarsal bone from a dairy cow fed 93 ppm F for the same period. The bone shows marked periosteal hyperostosis with a roughened surface. (b) Radiographic comparison of bones in (a). (c) Upper: Cross section of a metatarsal bone from a dairy cow fed 12 ppm F from 3 to 4 months to 7.5 years of age. The bone is normal. Lower: Cross section of a metatarsal bone from cow ingesting 93 ppm F for the same period. The bone shows definite osteofluorosis. (Adapted from Greenwood, D. A. et al., *Fluorosis in Cattle*, Special Report 17, Agricultural Experiment Station, Utah State University, Logan, Utah, 1964, p. 36.)

In cattle, fluorosis can take the form of intermittent lameness, stiffness, and lesions of the bones and teeth. The clinical basis for the lameness is not well understood. Appetite is normally low, and this may result in decreased weight gain, cachexia, and lowered milk yield. Decline in milk production may be secondary to appetite impairment or other responses. Evidence that animals may be suffering chronic F effect may be obtained from chemical analysis of the feed, and elevated levels of F in urine and body tissues (Parker et al. 1979; Shupe and Olson 1983). Increased susceptibility to other environmental stresses and a decrease in longevity have also been observed.

A number of factors influence the manifestation of dental and skeletal fluorosis, for example, the amount and the bioavailability of F ingested; duration of ingestion, species of animals involved (Table 9.4), age at time of excessive F ingestion, nutritional and general health status of animals, mode of F exposure (e.g., continuous or intermittent), presence of synergistic or antagonistic substances, presence of other stress factors, such as those caused by poor management, and individual biologic response (Yu and Hwang 1986).

Certain nutrients, including proteins, Ca, and vitamin C, have been shown to influence the severity of F toxicity. These nutrients are known to alleviate the adverse effect of F. For example, both Ca and vitamin C have been shown to decrease the toxicity in guinea pigs (Hodge and Smith 1965). In laboratory experiments, it has been shown that mice fed a low-protein (4%) diet deposited five times more F in their tibia than control animals fed a regular diet containing 27% protein, and that supplemental vitamin C greatly reduced the F deposited in the bone (Yu and Driver, 1983; Yu and Hwang 1986). It should be mentioned that mice produce vitamin C as well.

Table 9.4 Fluoride Tolerances (in ppm) in Livestock Diets

	Breeding or Lactating Animals	*Finishing Animals*
Dairy and beef heifers	30	100
Dairy cows	30	100
Beef cows	40	100
Sheep	50	160
Horses	60	—
Swine	70	—
Turkeys	100	—
Chicken	150	—

Source: Adapted from Committee on Biologic Effects of Atmospheric Pollutants, *Fluoride*, National Academy of Sciences, Washington, DC, 1971.

9.5 Effect on Human Health

9.5.1 Daily Intake

Because of differences in F content of similar products and wide variations in consumption patterns, it is difficult to estimate F intake. Nevertheless, for an adult male residing in a fluoridated community, estimates of F intake from food and beverages range from 1 to 3 mg/day (Phipps, 1996). This range is reduced to ≤1.0 mg/day in a nonfluoridated area. The amount of F inhaled from air is about 0.05 mg/day for an adult residing in a non-F-polluted community.

Wang et al. (2009) recently studied the F levels in several environmental samples collected from two villages, A and B, in Shaanxi Province in China. F levels in samples from village A were high, whereas those from village B were low (control). They found that in village A, the F levels in the samples were 1,757 mg/kg in coal, 0.007 mg/L in drinking water, 1.47 mg/kg in soil, 4.78 mg/kg in corn, and 31.79 mg/kg in chili, respectively, whereas the F levels in village B were 120 mg/kg in coal, 0.008 mg/L in drinking water, 0.64 mg/kg in soil, 2.69 mg/kg in corn, and 7.98 mg/kg in chili, respectively. The incidence of skeletal fluorosis in village A was 6% for male subjects and 4% for female subjects, respectively, whereas the incidence in village B was 0% for both male and female subjects.

9.5.2 Absorption

Absorption of F from the gastrointestinal tract occurs through a passive process; it does not involve active transport. Absorption is rapid and probably occurs in the lumen. The rate of absorption is dependent on the compounds involved, e.g., NaF, 97%; $Ca_{10}F_2(PO_4)_6$, 87%; Na_3AlF_6, 77%; and CaF_2, 62%. Once taken up, about 50% of the absorbed F is excreted by the kidneys, while the remainder is stored primarily in calcified tissues. No significant F accumulation occurs in soft tissues. Almost all of the remaining 50% of absorbed F is fixed in bones. The bone has a great affinity for F and incorporates it into hydroxyapatite [$Ca_{10}(OH)_2(PO_4)_6$], forming fluorapatite. Even at

low levels of F intake, appreciable amounts of F will in time accumulate in calcified tissues. The effectiveness of low levels of F intake in reducing dental caries in humans and rats and some other species of animals has been well recognized. In the human population, water supply containing 1 ppm F has been widely known to reduce more than 50% in incidence of dental caries in individuals who consume F from infancy. Fluoride is incorporated into tooth mineral as fluorapatite at the time of calcification.

9.5.3 Acute Toxicity

The lethal dose of inorganic fluoride is estimated to be in the range of 2.5 to 5 g for a 70 kg man, or approximately 50 mg/kg, a dose similar to the LD_{50} for several animal species. The cause of death is probably related to the prompt binding of serum Ca and Mg by F. Clinical symptoms include excessive salivation, perspiration, nausea, painful spasms of limbs, stiffness, chronic convulsion, necrosis of mucosa of the digestive tract, and heart failure.

9.5.4 Chronic Toxicity

Fluoride accumulates in the skeleton during prolonged, high-level exposures. Radiological evidence of hypermineralization (osteofluorosis) is shown when bone concentrations reach about 5,000 ppm F. Coupled with other environmental factors, such as nutrition and health status, the patient may suffer severe skeletal dysfunction. In addition, vomiting and neurological complaints have been reported. Increased levels of serum and urinary F are usually observed. In parts of the world, such as India and China, where the water supply (from wells) in many villages and towns contains extremely high levels of F, osteofluorosis is commonly found. In China alone, it is estimated that about 20 million people may be suffering chronic F poisoning (Yu and Tsunoda 1988).

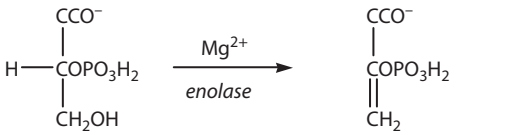
9.6 Biochemical Effect

While it is clear that the action of F on plant metabolism is complex and involves a variety of enzymes, the mode of action of F^- ion on these enzymes is not so clear. Suggested principal mechanisms include (1) formation of complex with metalloenzymes, (2) removal of a metal cofactor from an enzyme system, and (3) binding to the free enzyme or to the enzyme substrate complex (Miller et al. 1983). Studies using a model system indicate that F can disrupt the hydrogen bonding of protein molecules (Edwards et al. 1984). Because hydrogen bonding is important in the maintenance of the tertiary structure of a protein molecule, disruption of an enzyme protein by F would lead to enzyme inhibition.

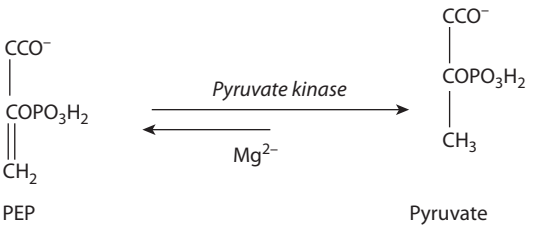
Similar to an earlier discussion of F effects on plants, F inhibits a large number of enzymes in animals and humans. The general mode of F inhibition includes direct interaction with enzymes and formation of metal-F complexes. These are explained below:

1. Direct interaction with enzymes. Most enzymes are proteins with [+] and [–] charges on the molecule. The negatively charged F^- ion can thus interact with an enzyme protein and cause its inactivation. The F^- ion can also inactivate an enzyme by disrupting the hydrogen bonds on the molecule. Such disruption leads to changes in molecular conformation of the protein, resulting in impaired enzyme activity. The inhibition of cytochrome oxidase by F is an example.

2. Formation of metal-fluoride complexes. Fluoride can inhibit metal-requiring enzymes by forming metal-F complexes. A number of enzymes require magnesium (Mg) for their activity. Fluoride inhibits such enzymes by forming a complex with Mg. Enolase is one of those enzymes. Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Equation 9.3), a key step in the glycolytic pathway. The resultant phosphoenolpyruvate, a high-energy-containing compound, is then converted to puruvate with the production of ATP, a reaction catalyzed by pyruvate kinase (Equation 9.4). F also inhibits pyruvate kinase in the same way, because it interacts with the cofactor Mg^{2+} (Garrett and Grisham 1995). This is an example showing how F inhibits oxidative metabolism, thus blocking normal metabolism. In animals and humans, enolase inhibition can lead to hyperglycemia.



(9.3)



(9.4)

The inhibition of myosin ATPase by F resembles the example shown above. Myosin is an enzyme responsible for the breakdown of ATP into ADP and inorganic phosphate (Pi), providing the free energy that drives muscle contraction (Equation 9.5). According to Park et al. (1999), Mg^{2+} is the physiological divalent cation stabilizing myosin. F and MgADP form a complex that traps the active site of myosin and inhibits myosin ATPase.

$$ATP + H_2O \xrightarrow{ATPase} ADP + Pi + H^+ \tag{9.5}$$

The interaction of F with Ca has been widely known. Many enzymes that occur in different plant tissues have been shown to require Ca for activity. Examples include amylase (Yu et al. 1986) and invertase (Yu 1997; Ouchi et al. 1999) from germinating mung bean seedlings.

In humans and animals, F is known to impair the functions controlled by Ca. Thus, subjects exposed to F often exhibit lowered plasma Ca levels (hypocalcemia). Fluoride also affects blood clotting, membrane permeability, nervous system, and cholinesterase activity, all known to involve Ca. Fluoride exposure thus can lead to cell damage and necrosis. Eventually, F produces massive impairment of the function of vital organs, particularly when F is given orally in humans and animals.

While F can inhibit a large number of enzymes in living organisms, it is also known to enhance the activity of certain enzymes. An example is adenyl cyclase (Equation 9.6), an enzyme that catalyzes the conversion of ATP into cyclic AMP (cAMP):

$$\text{ATP} \xrightarrow{\text{adenyl cyclase}} \text{cAMP} \tag{9.6}$$

Fluoride stimulates adenyl cyclase activity in all tissues thus far examined.

Interest in F-induced oxidative stress has been growing in recent years. Sun et al. (1994) observed changes in endogenous antioxidant components such as SOD, GSHPx, and GSH. They reported that aluminum plant workers exposed to F in the workplace showed marked increases in urinary F levels. Additionally, there were increases in serum lipid peroxides and the activity of SOD, compared with those of workers who were not exposed to F. These observations suggest that industrial workers chronically exposed to high levels of F may be subjected to free-radical-initiated lipid peroxidation in their body system.

A number of studies have shown that animals exposed to F also exhibited tissue lipid peroxidation in several organs and tissues. For example, Lawson and Yu (2000) studied the activity of (Cu-Zn)-SOD and the levels of GSH in the worm (*Eisenia fetida*) exposed transcutaneously to NaF at concentrations of 0.1, 1.0, and 5.0 mmol/dm^3 for 24, 48, and 72 hours. They observed that SOD was inhibited while GSH levels increased in a dose-dependent manner. These investigators suggested that F presumably is a competitive inhibitor of the SOD because F binds to its catalytic center. Increased GSH concentrations have partly been explained by reduced H_2O_2 levels due to SOD inhibition.

Chinoy and Patel (2000) administered NaF (10 mg/kg body mass) to female mice for 30 days, and observed increases in lipid peroxide levels. Furthermore, they found that the cerebral levels of GSH and ascorbic acid, as well as the activities of SOD, GSHPx, and catalase, were decreased. They also observed that administration of vitamins C and E, and Ca fully reversed these changes.

In a 2006 U.S. National Research Council (NRC) review, F (as F^- ions) is described as an endocrine disruptor, and that it has the potential to disrupt the function of many tissues that require iodine (I_2) or iodide (I^-). (NRC Press 2006). A number of researchers have implicated an association between fibrocystic breast disease and iodine deficiency. According to Clinch (2009), iodine appears to accomplish the following:

- Desensitize estrogen receptors in the breast, and reduce estrogen production in overactive ovaries
- Increase progesterone production
- Reduce or eliminate fibrocystic breast disease in women
- Provide an anticancer effect at the promotional level
- Decrease lipoperoxidation and act as an antioxidant
- Increase urinary excretion of F, mercury, and bromide

Study Questions

1. Explain chlorosis and necrosis in plants exposed to fluoride.
2. What are the most important fluoride-containing minerals?
3. How does fluoride affect seed germination?
4. What are the common sources of excessive fluoride intake by animals?
5. Explain how dental lesions are manifested in animals chronically exposed to fluoride.
6. What are the characteristic features of skeletal fluorosis in animals intoxicated by fluoride?

7. List five factors that can influence the manifestation of dental and skeletal fluorosis in animals.
8. What are the principal mechanisms suggested as the mode of action of fluoride ion on plant enzymes?
9. What are the chronic effects of fluoride accumulation in humans?
10. What is the action of fluoride on enzymes requiring Mg or Ca?
11. Explain how fluoride affects enolase.
12. How does fluoride directly interact with enzymes?
13. Explain how fluoride may be related to lipid peroxidation.
14. Explain why in humans and animals fluoride impairs the functions controlled by Ca.
15. Which nutrients are known to alleviate fluoride toxicity?
16. Explain why fluoride emissions could be of concern in the aluminum manufacturing process.

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Chapter 10

Heavy Metals

10.1 Introduction

Pollution caused by heavy metals is a worldwide phenomenon. Among the many heavy metals, lead (Pb), mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), zinc (Zn), and copper (Cu) are of most concern, although the last three metals are essential nutrients in animal and human nutrition. These metals are widely used in industry, particularly in metal working or metal plating, and in such products as batteries and electronics. They are also used in the production of jewelry, paint pigments, pottery glazes, inks, dyes, rubber, plastics, pesticides, and even in medicines. These metals enter the environment wherever they are produced, used, and ultimately discarded.

Heavy metals are very toxic because, as ions or in compound forms, they are soluble in water and may be readily absorbed into living organisms. After absorption, these metals can bind to vital cellular components, such as structural proteins, enzymes, and nucleic acids, and interfere with their functioning. In humans, some of these metals, even in small amounts, can cause severe physiological and health effects.

In this chapter, we will consider Pb, Cd, and Hg, the three heavy metals widely recognized as the most toxic in our environment.

10.2 Lead

Lead (Pb) is one of the ancient metals and has been used by humans for several thousand years. Lead plays an important role in the economy of all industrialized countries in the world. In the United States, the industrial consumption of Pb is estimated to be about 1.3 million tons per year, with a concomitant annual emission of about 600,000 tons of Pb into the environment (National Academy of Sciences [NAS] 1980). Additional amounts are added through mining, smelting, manufacturing, disposal, and recycling processes. Furthermore, until recently, huge amounts of Pb and its compounds had been emitted into the atmosphere as a result of leaded gasoline combustion. Consequently, Pb is ubiquitous in our environment.

Because Pb is toxic to humans at high doses, levels of exposure encountered by some members of the population constitute a serious public health problem (NAS 1980). The importance of Pb

as an environmental pollutant is apparent since the Environmental Protection Agency (EPA) has designated Pb as one of the six criteria air pollutants.

10.2.1 Properties and Uses

Lead has a low melting point (326°C). It is a soft, malleable metal; i.e., it can be easily formed into a variety of shapes. It can form alloys with many other metals. Other important industrial products containing Pb include pipes, paints, solders, glass, pottery glazes, rubber, plastics, and insecticides.

10.2.2 Exposure

10.2.2.1 Atmospheric Lead

Sources of atmospheric Pb include lead smelters, burning of coal and materials containing Pb, refining of scrap, wind blown from soils, and lead alkyls from gasoline. Effluents from smokestacks and other gaseous emissions from smelters and refining processes can distribute significant quantities of Pb to the air and soils and vegetation growing nearby. However, until recently the most common source of Pb contamination in ambient air was the exhaust from automobiles. Tetraethyl lead was introduced as an antiknock agent in gasoline in the 1920s and since then has played an increasingly important role as an atmospheric pollutant. Following the mandatory use of unleaded gasoline and improved industrial emission control, atmospheric Pb emission has decreased dramatically. According to an EPA report, Pb emission from major emission sources in the United States decreased from 56,000 to 7,100 metric tons per year between 1981 and 1990 (EPA 1991). While the atmospheric lead pollution problem in other developed countries has likewise been significantly reduced, a similar trend has not occurred in many developing countries.

10.2.2.2 Waterborne Lead

Surface waters may contain significant amounts of Pb when subjected to some special contamination. About 14% of representative drinking water supplies (i.e., piped drinking water) were found to contain more than 10 mg/L in a 1963–1965 survey. Less than 1% was found to be in excess of 30 mg/L. On the other hand, rainwater collected near a busy highway may contain as much as 50 mg/L.

Another serious problem related to waterborne Pb is lead shot left in North America's lakes and ponds. A large number of waterfowl in the United States are poisoned or killed following ingestion of the shot.

10.2.2.3 Lead in Food

Food has long been a major source of Pb intake for animals and humans. Animals may ingest Pb-contaminated vegetation and become intoxicated. In humans Pb may be ingested through Pb-contaminated containers or Pb pottery glazes. Researchers suggest that some Roman emperors may have become ill and even died from Pb poisoning by drinking wines contaminated with high levels of Pb.

Vegetation growing near highways has been shown to accumulate high amounts of Pb deposited from automobile exhaust (Lagerwerff et al. 1973; Khalid et al. 1996). Pica, children's

craving for unnatural foods, is thought to be responsible for the chronic Pb poisoning among many poor urban children, as they eat flaking paint from the walls of old houses. About 27 million housing units were built before 1940, when Pb was in common use (Lin-Fu 1982). Lead paint poses a major threat for children and is one of the major public health problems that many communities face.

10.2.2.4 Lead in Soils

Lead and other metals can impact soils and biota by deposition from polluted air. Stack emission from smelters (Little and Martin 1972) and emission from automobile exhaust systems along highways are examples. Pb contamination due to mine wastes is also an important problem in areas surrounding metal mines. Earlier reports indicate that about 50% of the Pb liberated from motor vehicles in the United States is deposited within 30 m of the roadways (Ryan 1976), and the remainder is scattered over large areas. Lead accumulation in soils near roads varies with traffic volume and decreases rapidly with distance from the road. For example, Pb concentrations of 128 to 700 ppm were found in soil adjacent to 12 highways in the Minneapolis–St. Paul area (Ryan 1976). These levels are much greater than the reported value of 10 to 15 ppm in unpolluted rural soils. Grass collected near an intersection of two heavily traveled highways near Denver, Colorado, contained as much as 3,000 ppm Pb, while vegetable samples from gardens less than 50 feet from roads in Canandaigua, New York, averaged 115 ppm Pb (range, <10 ppm to 700 ppm).

In an attempt to assess the effect of the mandatory use of unleaded gasoline in new automobiles on Pb concentrations in highway soils, Byrd et al. (1983) determined Pb concentrations in soils along U.S. Interstate 20 in northeast Louisiana and observed that the concentrations increased from 1973 to 1974 but decreased from 1973 to 1979. They concluded that the mandatory use of unleaded gasoline had significantly reduced the Pb concentrations in soils near highways.

10.2.3 Lead Toxicity

10.2.3.1 Effect on Plants

Plants exposed to high levels of Pb from ambient air and soils can accumulate the metal and manifest toxicity. The toxicity varies greatly among plant species as well as the presence of other trace metals. Based on *in vitro* studies, toxicity sequences have been determined for several species. Barley plants were shown to be more sensitive to Pb than Cr, Cd, Ni, or Zn (Oberlander and Roth 1978). Exposure to relatively high levels of Pb was shown to inhibit seed germination (Koeppe 1977; Yu 1991). The effect of Pb on germination, however, was found to be less severe than several other metals, such as Cd, As, and Hg (Koeppe 1977; Fargasova 1994). It is important that, following plant uptake, Pb moves into the food chain and thus can affect animals and humans.

10.2.3.2 Effect on Animals

The effect of Pb on freshwater fish varies depending on the species of fish. Goldfish, for example, are relatively resistant to lead, presumably due to their abundant gill secretion. As mentioned above, following the ingestion of expended lead shots in lakes or in the field, more than 1 million birds are estimated to be killed each year in the United States. Lead absorbed by the bird paralyzes the gizzard, leading to starvation, and death usually follows within several weeks of the exposure.

10.2.3.3 Effect on Humans

Daily intake of Pb in humans is estimated to range from 20 to 400 mg per person. The Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Expert Committee established a provisional tolerable weekly intake (PTWI) of 3,000 mg, corresponding to ca. 500 mg/day). Only half of this amount appears to be safe for children. About 5 to 15% of ingested Pb is absorbed. This amounts to 15 to 25 mg per day and represents two-thirds of the total absorbed lead. By contrast, about 20 to 40% of the inhaled Pb is absorbed, amounting to about 8 mg/day, or one-third of the total absorbed lead.

The considerably higher blood Pb levels in industrial populations reflect widespread environmental Pb pollution. However, data obtained from the Second National Health and Nutrition Examination Survey (NHANES II) indicate that there has been a reduction in the overall mean blood-lead level of the U.S. population during the period 1976 through 1980, from 15.8 mg/dL to 10.0 mg/dL (Lin-Fu 1982). It is suggested that an increased use of unleaded gasoline by the U.S. population may be responsible for the observed decrease.

Lead is one of the systemic poisons in that, once absorbed into the circulation, it is distributed throughout the body, where it causes serious health effects. Manifested effects of Pb poisoning include nausea, anorexia, severe abdominal cramps, weight loss, anemia, renal tubular dysfunction, muscle aches, and joint pains. Lead can pass the placental barrier and may reach the fetus, resulting in miscarriages, abortions, and stillbirths.

Through interaction with cellular components of brain cells, Pb also adversely affects the central nervous system (CNS). Clinical symptoms such as encephalopathy, convulsions, and delirium may occur. In severe cases, coma and death may follow. These injuries are often reflected by behavioral disturbances observed in Pb-poisoned victims.

It is estimated that approximately 90% of Pb absorbed by humans is deposited in the bone (Aufderheide and Wittmers 1992). Bone, however, is no longer considered a sink for Pb in the body. Rather, it is recognized as a two-way process of active influx and efflux of Pb to and from the bone and bloodstream (Silbergeld et al. 1993). As a result, bone acts like a reservoir for Pb, thus influencing the exposure of the metal in the body.

Although there is evidence that both inorganic and organic lead compounds are carcinogenic in experimental animals (Cherlewski 1979; Blake and Mann 1983), no conclusive evidence has been reported in humans.

10.2.3.4 Biochemical Effect

Lead is taken up and transported in plants (Cannon and Bowles 1962) and can decrease cell division at very low concentrations. Lead inhibits the electron transport in corn mitochondria, especially when phosphate is present (Koeppe and Miller 1970).

Lead, as mentioned above, is a systemic poison and can induce a deleterious effect in living organisms. The biochemical effect of Pb is complex, and in certain areas, its mode of action remains unclear. Several well-established biochemical effects are discussed here. First, as an electropositive metal, Pb has a high affinity for the sulfhydryl (SH) group. Enzymes that depend on the SH group as the active site are therefore inhibited by Pb. In this case, Pb reacts with the SH group on the enzyme molecule to form mercaptide, leading to inactivation of the enzyme. The following reaction depicts such a relationship:

$$2RSH + Pb^{2+} \rightarrow R–S–Pb–S–R + 2H^{+}$$

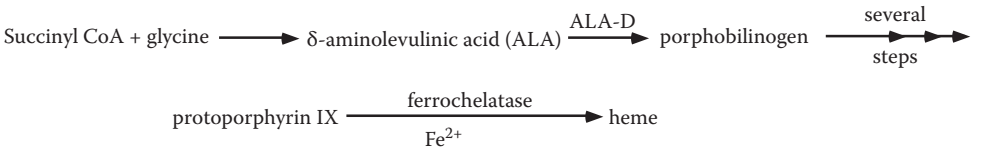

Figure 10.1 Steps in heme synthesis inhibited by lead.

Examples of the sulfhydryl-dependent enzymes include adenyl cyclase and aminotransferases. Adenyl cyclase catalyzes the conversion of ATP to cyclic AMP needed in brain neurotransmission. Aminotransferases are involved in transamination, and thus are important in amino acid metabolism.

Second, divalent Pb is similar in many aspects to Ca and may exert a competitive action in body processes such as mitochondrial respiration and neurological functions. Lead can compete with Ca for entry at the presynaptic receptor. Since Ca evokes the release of acetylcholine across the synapse, this inhibition manifests itself in the form of decreased end plate potential. The miniature end plate potential release of subthreshold levels of acetylcholine has been shown to be increased (Barton et al. 1978). The close chemical similarity between Pb and Ca may partially account for the fact that they seem interchangeable in biological systems, and that 90% or more of the total body burden of Pb is found in the skeleton.

Third, Pb can interact with nucleic acids, leading to either decreased or increased protein synthesis. Lead has been shown to reduce the ability of t-RNA to bind ribosomes. The effect of Pb on nucleic acids, therefore, has important biological implications (Barton et al. 1978).

Finally, it is widely known that Pb impairs the formation of red blood cells. The mechanism involved in the impairment is that Pb inhibits both d-aminolevulinic acid dehydratase (ALA-D) (Hernberg et al. 1970) and ferrochelatase (Tephly et al. 1978). These are two key enzymes involved in heme biosynthesis (Figure 10.1). ALA-D catalyzes the conversion of δ-aminolevulinic acid into porphobilinogen (PBG), whereas ferrochelatase is responsible for catalyzing the incorporation of Fe^{2+} into protoporphyrin IX to form heme. Lead inhibition of the two enzymes appears to be due to its interaction with Zn and Fe required in the process.

10.3 Cadmium

Cadmium (Cd) is a transition metal in Group IIb along with Zn and Hg. It is frequently associated with Zn. The United States is the world's largest producer of cadmium, with an annual output of about 5,000 short tons. Mexico is an important producer of Cd-bearing dusts and fumes, but most of these are smelted in the United States.

10.3.1 Properties and Uses

Cadmium is a silver-white metal with an atomic weight of 112.4 and a low melting point of 321°C. It is malleable and can be rolled out into sheets. The metal unites with the majority of the heavy metals to form alloys. It is readily oxidized to the +2 oxidation state, giving the colorless Cd^{2+} ion. Cadmium persists in the environment; its biological half-life is 10 to 25 years.

About two-thirds of all Cd produced is used in the plating of steel, Fe, Cu, brass, and other alloys to protect them from corrosion. Other uses include solders and electrical parts, pigments, plastics, rubber, pesticides, galvanized iron, etc. Special uses of Cd include aircraft manufacture and semi-conductors. Because Cd strongly absorbs neutrons, it is also used in the control rods in nuclear reactors.

10.3.2 Exposure

General sources of exposure to Cd include air, water, and food. Atmospheric emission of Cd may arise from such activities as mining and metallurgical processing, combustion of fossil fuel, textile printing, application of fertilizers and fungicides, recycling of ferrous scraps and motor oils, disposal and incineration of Cd-containing products (e.g., plastics), and tobacco smoke.

The major nonoccupational routes of human Cd exposure are through ingestion and inhalation. Ambient air is not a significant source of Cd exposure for the majority of the U.S. population. Nearly all airborne Cd is due to human activities, and thus the highest concentrations are found in industrialized cities and in the vicinity of smelting operations (Fleischer 1974). While aerial deposition is an important route of mobility for Cd, airborne routes of exposure are not as important as soil and water routes.

Tobacco in all of its forms contains appreciable amounts of Cd, and tobacco smoke is one of the largest single sources of Cd exposure to humans. Since the absorption of Cd from the lungs is much greater than that from the gastrointestinal tract, smoking contributes significantly to the total body burden. Each cigarette, on average, contains approximately 1.5 to 2.0 mg of Cd, of which 70% passes into the smoke.

Waterborne Cd is probably the largest problem because Cd is common in the aquatic environment. Many Cd-containing wastes end up in lakes and marine water. Wastes from Pb mines, various chemical industries, motor oils, and rubber tires are some examples.

Cadmium pollution of soils can occur from several sources, a major one being the deposition of municipal sewage sludge on agricultural soils. Other sources of Cd pollution are through rainfall and dry precipitation of Cd, as well as phosphate fertilizers.

Food consumption accounts for the largest sources of exposure to Cd by animals and humans primarily because of the ability of plants to bioaccumulate Cd at high rates. In addition, aquatic organisms can potentially accumulate large amounts of Cd.

10.3.3 Cadmium Toxicity

10.3.3.1 Effect on Plants

Cadmium is accumulated by all plants. The extent of Cd accumulation, however, varies markedly with species and variety. Soil pH is the most important factor controlling Cd uptake by plant, with lower pH favoring its uptake. Tobacco plants have been shown to absorb high levels of Cd from the soil (Bache 1985). Phytotoxicity of Cd is manifested by stunting, chlorosis, reduction in photosynthesis, wilting, and necrosis. Like lead, Cd inhibits seed germination under laboratory conditions (Koeppe 1977; Yu 1991; Fargasova 1994). Seedlings exposed to solutions of Cd salts exhibit decreased root elongation and development.

10.3.3.2 Effects on Animals/Humans

Cadmium is toxic in small amounts, and there is no evidence that Cd has any useful biological function. Among the sources of exposure to Cd mentioned above, exposure through airborne Cd is minimal to the general population, with the exception of tobacco smokers. Cadmium in drinking water, although a major source, rarely becomes a serious problem. On average, potable waters contain about 10 ppb Cd. This amounts to an uptake of about 20 to 30 μg/day, based on daily water consumption of 2 to 3 L (Friberg 1974).

Daily intake of Cd from food is estimated at 35 to 90 μg. When dietary exposure reaches critical concentrations, estimated to be about 250 to 300 μg/day, toxicity symptoms are manifested. Cadmium intakes of the Japanese farmers suffering from the widely known itai-itai disease were reported to be from 600 to 1,000 μg/day. The disease was caused by ingestion of rice highly contaminated with Cd. The rice paddies received water discharged from upstream Zn mines. Many of the victims died as a result of the disease.

Once absorbed, Cd readily shows up in the blood plasma, bound in albumin (Nordberg 1985). The bound Cd is shortly taken up by tissues, preferentially by the liver. The Cd in the liver apparently cycles, bound with metallothionein (MT), through the blood, kidneys, and to a small extent, bone and muscle tissue.

The excretion of Cd appears minimal under normal exposure. Loss in the urine accounts for a major route of Cd excretion, whereas only minute amounts are excreted in the feces. As mentioned above, absorbed Cd persists in body tissues. The long-term excretion rate of Cd is only 0.005% per day beginning after about 50 years of age (Friberg 1974).

Although dietary intake is the means by which humans are most highly exposed to Cd, inhalation of Cd is more dangerous than ingestion. This is because through inhalation, the body's organs are directly and intimately exposed to the metal. Furthermore, 25 to 40% of inhaled Cd from the air is retained, while only 5 to 10% of ingested Cd is absorbed. Inhaled Cd may cause emphysema and pneumonitis, while ingested Cd may result in disturbances in the gastrointestinal tract, vomiting, proteinuria, osteomalacia, liver dysfunction, kidney damage manifested by anemia, and hypertension. Cadmium is also known to be embryotoxic.

10.3.4 Biochemical Effect

Cadmium has been shown to impair many plant cellular functions, such as photophosphorylation, succinate oxidation, ATP synthesis, mitochondrial NADH oxidation, and electron transport (Nriagu 1980). Cadmium is a potent enzyme inhibitor, affecting a variety of plant enzymes, such as phosphoenolpyruvate carboxykinase (PEP), lipase, invertase (Yu 2003), and others. Extensive reports are available concerning Cd-dependent inhibition of enzymes from animals and humans. Alkaline phosphatase and ATPases of myosin and pulmonary alveolar macrophage cells are examples.

Two mechanisms appear to be involved in enzyme inhibition. One is through binding to SH groups on the enzyme molecule; another is through competing with zinc and displacing it from metalloenzymes. Naturally, Cd can also bind with SH-containing ligands in the membrane and other cell constituents, causing structural and functional disruptions. For instance, by inducing damage to mitochondria, Cd can uncouple oxidative phosphorylation and impair energy metabolism of the cell. At moderate levels, Cd toxicity is related to its antimetabolite activities toward essential metals such as Zn, Cu, Se, and Fe. In mammals, the impact caused by Cd is thus influenced by the relative intakes of these and other metals and vice versa (Hamilton and Valberg

1974). In addition, dietary protein has been shown to be related to the toxicity of ingested Cd. A low-protein diet results in an increased absorption of Cd, and thus increased toxicity.

10.4 Mercury

Mercury (Hg) is the only common metal that is liquid at room temperature. It is rare in the earth's crust (0.1 to 1 ppm). Although several forms occur, the principal ore is cinnabar, HgS. Elemental Hg yields as cinnabar is "roasted" and the resulting Hg vapor condensed. Some inorganic and organic Hg compounds are extremely toxic. A number of episodes leading to many fatalities occurred in different countries in recent years as a result of exposure to the metal or its compounds.

10.4.1 Properties and Uses

Mercury, atomic number 80, atomic weight 200.59, has a high specific gravity, 13.6 times that of water. Its boiling point is 357°C, which is relatively low, and this property leads to easy separation from its ores and amalgams. Its freezing point is –39°C, the lowest for any metal. Mercury has a long liquid range of 396°C, and it expands uniformly over this range. This linear expansion, together with the fact that Hg does not wet glass, makes the metal useful in thermometers. Mercury has the highest volatility of any metal. Its good electrical conductivity makes it exceptionally useful in electrical switches and relays of the sealed type. Many metals dissolve in mercury to form amalgams (alloys).

In the United States, the largest user of Hg is the chlor-alkali industry, in which chlorine and caustic soda are produced by electrolysis of salt (NaCl) solution. Mercury is widely used in barometers, Hg batteries, and other electrical apparatus. Many of its compounds are used as catalysts in industrial chemistry, and Hg vapor is utilized in UV spectrophotometers. In addition, high-pressure mercury vapor lamps are now widely installed for street and highway lighting. Mercury compounds are added to paints as preservatives. Certain Hg compounds were widely used as pesticides in agriculture also. Mercury has no known biological role and, as mentioned above, the metal and its compounds are toxic to all living organisms.

10.4.2 Sources of Mercury Pollution

Mercury contamination of the environment is caused by both natural and human-made sources. Natural sources include volcanic action and erosion of mercury-containing sediments. Humans contaminate the environment with Hg through mining and transporting mercury ores and processing; dumping industrial wastes into rivers and lakes; combustion of fossil fuels (e.g., the Hg content of coal is about 1 ppm), pulp, and paper; use of mercury compounds as seed dressings in agriculture; and exhaust from metal smelters, and so forth.

10.4.3 Toxicity

10.4.3.1 Effect on Plants

All plants appear to concentrate traces of Hg. The concentration of Hg in plants depends on deposits in the soil, plant species, and locality. Like Pb and Cd discussed previously, Hg can have a deleterious effect on different species of plants. It is particularly toxic to barley plants, more so

than Pb, Cr, Cd, Ni, and Zn (Oberlander and Roth 1978). Mercury, similar to Pb and Cd, impairs germination, as manifested by depressed root elongation and shoot growth (Yu 2003).

10.4.3.2 Effect on Animals

Freshwater and marine organisms and their predators normally contain more Hg than terrestrial animals. Levels in top predatory fish are higher. Fish may accumulate Hg in excess of the 0.5 mg/g FDA guideline, depending on various factors. This accumulation is part of a dynamic process in which an organism strives to maintain equilibrium between intake and elimination. Numerous analyses have demonstrated that a majority of the tissue Hg in most fish is in the form of methylmercury (Westoo 1973). The Hg accumulated in fish comes primarily through absorption from the water across the gill or through the food chain, although some higher species may convert inorganic Hg into methylmercury. Some Hg is also taken up through the mucous layer or skin.

The metabolic rate of the fish and the mercury concentration in the aquatic ecosystem appear to be more important factors in bioaccumulation than age or exposure rate. Since increased temperature enhances the metabolic rate, more Hg is concentrated in the summer than in the winter. The toxicity of Hg and other heavy metals to fish is increased with increase in temperature. The 96-hour LC_{50} of Hg for freshwater crayfish (*Procambarus clarkii* (*Girard*)) was found to be 0.79 mg/L at 20°C, 0.35 mg/L at 24°C, and 0.14 mg/L at 28°C (Del Ramo et al. 1987).

10.4.3.3 Terrestrial Animals

Wild birds concentrate the highest levels of Hg in the kidneys and liver, with less in the muscle tissues. Swedish ornithologists observed the first Hg-related ecological problems during the 1950s. Many species of birds declined both in numbers and breeding success, while Hg levels increased in the feathers of several species of seed-eating birds. In the United States and Canada, elevated levels of Hg were also found in seed-eating birds and their predators, presumably through eating Hg-treated seed dressings. In 1970 both countries banned alkylmercurial seed dressings, and the levels decreased in game birds that do not feed on aquatic organisms. However, where phenylmercuric seed dressings continue to be applied in the United States, pheasants and other wild birds can still accumulate relatively high levels of Hg.

10.4.3.4 Effect on Human Health

There is no indication that mercury compounds in the concentrations and forms found in either the atmosphere or drinking water supplies contribute significantly to the methylmercury burden in the human body. The available data show that almost all the methylmercury in the human diet comes from fish, other seafood, and possibly red meat.

The two major Japanese outbreaks of methylmercury poisoning, in Minamata Bay and in Niigata, were caused by industrial discharge of methylmercury and other mercury compounds into Minamata Bay and the Agano River, resulting in accumulation of methylmercury in fish and shellfish. The median total Hg level in fish caught in Minamata Bay at the time of the epidemic was estimated as 11 mg/g fresh weight. More than 700 cases of methylmercury poisoning were identified in Minamata and more than 500 in Niigata (WHO 1976).

The critical organ concentration may differ for different stages of the human life cycle. The developing fetal (and newborn) brain may be the most sensitive organ (i.e., critical organ) in terms of human methylmercury toxicity. During the Japanese Minamata outbreak, 23 infants with

severe psychomotor signs of brain damage were born to mothers who had consumed fish taken from waters known to be heavily contaminated with effluent containing methylmercury.

Perhaps the greatest source of danger in industrial and research laboratories lies in the inhalation of Hg vapor. Mercury vapor can diffuse through the alveolar membrane and reach the brain, whereby the vapor may interfere with coordination. The relative toxicity of various compounds toward tissue depends on their relative ease of formation of the Hg^{2+} ion.

The biological half-life of Hg is estimated to be 70 days. A critical daily intake was estimated to be 300 mg Hg as methylmercury for an average 70 kg man. Chronic Hg poisoning may result from exposure to small amounts of Hg over extended periods of time, such as may occur in industries, which use Hg or its salts. The symptoms include salivation, loss of appetite, anemia, gingivitis, excessive irritation of tissues, nutritional disturbances, and renal damage. Acute Hg poisoning results from ingestion of soluble Hg salts. Mercuric chloride precipitates all proteins with which it comes in contact. Vomiting usually occurs a few minutes after ingestion. The victim experiences extreme salivation and thirst, nausea, severe gastrointestinal irritation, and abdominal pain. Loss of fluids and electrolytes occurs.

10.4.4 Biochemical Effect

Similar to those of Pb and Cd, the ultimate effects of Hg in the body are inhibition of enzyme activity and cell damage. Inhibition of a large variety of enzyme systems by Hg has been reported (Boyer et al. 1959). The particular reactivity of Hg with thiol ligands has further confirmed the selective affinity of this metal to react with the SH group, as shown in the following with methylmercury:

$$RSH + CH_3Hg^+ \rightarrow R–S–Hg–CH_3 + H^+$$

Mercury is known to affect the metabolism of mineral elements such as Na and K by increasing the latter's permeability. Mercury also inhibits active transport mechanism through dissipation of normal cation gradient; destroys mitochondrial apparatus; causes swelling of cells, leading to lysis; decreases α- and γ-globulins while increasing β-globulin, suggesting liver dysfunction; decreases DNA content in cells; and adversely affects chromosomes and mitosis, leading to mutagenesis.

Metallothionein, a protein receptor present in kidney tissue, tends to bind actively with Hg. Thus, it is suggested that metallothionein exercises a protective effect (Clarkson 1972). When the metallothionein receptors are saturated with Hg, morphologic damage becomes manifest. Furthermore, metallothionein content in the kidneys increases with repeated Hg exposure, suggesting an adaptive mechanism.

It is widely recognized that dietary selenium (Se) exhibits a protective effect against Hg toxicity (Sumino et al. 1977). Reduction of the lethal and neurotoxic effects of methylmercury compounds has been noted. The reason for the protective action of Se is not very clear. The interaction of methylmercury with SH groups is considered the natural biological sink for the Hg compound. Approximately 95% of the methylmercury bound to fish protein has been shown to be part of the methylmercury-cysteinyl coordination complex. The selenohydryl group has been shown to bind methylmercury 100 times more tightly than the SH group (Sugiura et al. 1976).

In addition to Se, vitamin E is also known to protect against the toxic effect of methylmercury. However, a much higher concentration of this vitamin is required to provide the same level of protection as with Se.

Study Questions

1. Why are heavy metals toxic to organisms?
2. List four sources of lead exposure. Explain a source of the lead for each of the four major exposure pathways.
3. Characterize the mandatory use of unleaded gasoline on the extent of Pb contamination.
4. How does Pb affect plants? Nonhuman animals?
5. Which human systems are affected by Pb poisoning? Why would human bone be a tissue of interest in Pb toxicity?
6. Describe four biochemical effects of Pb.
7. Cadmium exposure to animals and plants is largest from which source? What other sources exist for cadmium exposure?
8. List several effects of cadmium on plants.
9. Why is inhaled Cd more dangerous than ingested Cd?
10. List the biochemical effects of Cd.
11. What are the biological roles of mercury?
12. What are the toxic effects of Hg on plants? On nonhuman animals?
13. What are the effects of temperature on Hg bioaccumulation in animals? Why?
14. What is the major source of methylmercury in the human diet?
15. What are the biochemical effects of Hg in animals?
16. Discuss several biochemical protective mechanisms against Hg toxicity.

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Chapter 11

Biotransformation, Detoxification, and Biodegradation

11.1 Introduction

As mentioned in the previous chapter, following entry into a living organism and translocation, a pollutant may be stored, metabolized, or excreted. When the rate of entry is greater than the rate of metabolism or excretion, storage of the chemical often occurs. However, storage or binding sites may not be the sites of toxic action. For example, lead is stored primarily in the bone but acts mainly on the soft tissues of the body. If the storage site is not the site of toxic action, selective sequestration may be a protective mechanism, since only the freely circulating form of the foreign chemical induces harmful effects.

Some chemicals that are stored may remain in the body for years without exhibiting appreciable effects. One such chemical is DDT. Accumulation or buildup of free chemicals may be prevented, until the storage sites are saturated. Selective storage limits the amount of foreign chemicals to be excreted, however. Since bound or stored toxicants are in equilibrium with their free forms, a chemical will be released from the storage site as it is metabolized or excreted. On the other hand, accumulation may result in illnesses that develop slowly, as is the case with fluorosis, or lead and cadmium poisoning.

11.2 Metabolism of Environmental Chemicals: Biotransformation

Subsequent to the entry of an environmental chemical into a mammalian organism, chemical reactions occur within the body to alter the structure of the chemical. This metabolic conversion process is known as biotransformation and occurs in any of several tissues and organs, such as the intestine, lung, kidneys, skin, and liver.

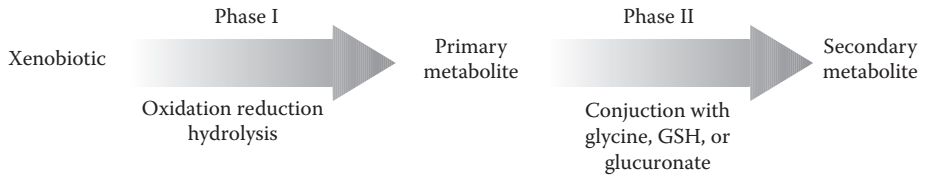


Figure 11.1 The two phases of xenobiotic metabolism.

By far most of these chemical reactions are carried out in the liver. The liver metabolizes not only drugs but also most of the other foreign chemicals to which the body is exposed. Biotransformation in the liver is thus a critical factor not only in drug therapy but also in the body's defense against the toxic effects of a wide variety of environmental chemicals (Kappas and Alvares 1975). The liver plays a major role in biotransformation because it contains a number of nonspecific enzymes responsible for catalyzing the reactions involved. As a result of the process, xenobiotics are converted to more water soluble and more readily excretable forms. While the purpose of such metabolic process is obviously to reduce the toxicity of chemicals, exceptions do occur. Occasionally, the metabolic process may convert a xenobiotic to a reactive electrophile capable of causing injuries through interaction with liver cell constituents (Reynolds 1977).

11.3 Types of Biotransformation

The process of xenobiotic metabolism contains two phases, commonly known as phase I and phase II. The major reactions included in phase I are oxidation, reduction, and hydrolysis, as shown in Figure 11.1. Among the representative oxidation reactions are hydroxylation, dealkylation, deamination, and sulfoxide formation, whereas reduction reactions include azo reduction and addition of hydrogen. With hydrolysis, such reactions as splitting of ester and amide bonds are common. In phase I reactions, a chemical may acquire a reaction group such as OH, NH_2, COOH, or SH.

Phase II reactions, on the other hand, are synthetic or conjugation reactions. An environmental chemical may combine directly with an endogenous substance, or may be altered by phase I reactions and then undergo conjugation. The endogenous substances commonly involved in conjugation reactions include glycine, cysteine, glutathione (GSH), glucuronic acid, sulfates, or other water-soluble compounds. Many foreign compounds sequentially undergo phase I and phase II reactions, whereas others undergo only one of them. Several representative reactions are shown in Figure 11.2.

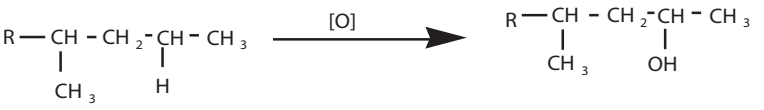
11.4 Mechanisms of Biotransformation

In the two phases of reactions noted above (Figure 11.1), the lipophilic foreign compound is first oxidized so that a functional group (usually a hydroxyl group) is introduced into the molecule. This functional group is then coupled by conjugating enzymes to a polar molecule so that the excretion of the foreign chemical is greatly facilitated.

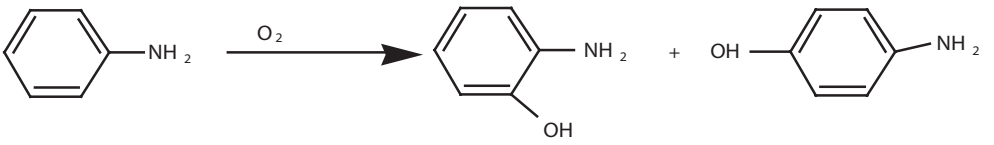
The NADPH–cytochrome P-450 system, commonly known as the mixed-function oxygenase (MFO) system, is the most important enzyme system involved in the phase I oxidation reactions. The cytochrome P-450 system, localized in the smooth endoplasmic reticulum of cells of most mammalian tissues, is particularly abundant in the liver. This system contains a number of

Phase I Reactions

Oxidation



Side-Chain Oxidation



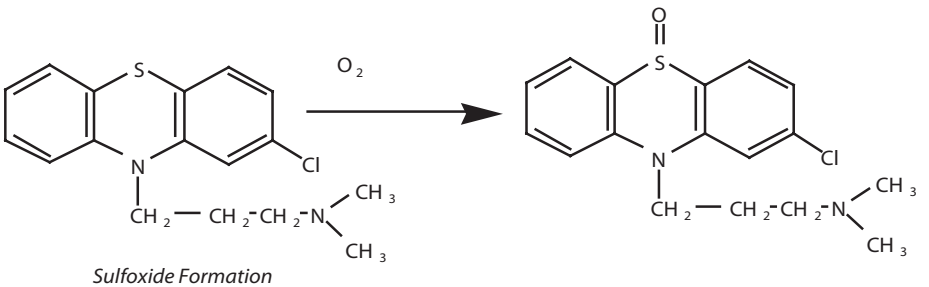
Aniline — o-Aminophenol — p-Aminophenol

Aromatic hydroxylation

N-Dealkylation

O-Dealkylation

Deamination



Sulfoxide Formation

Figure 11.2 Detoxification pathways. (***Continued***)

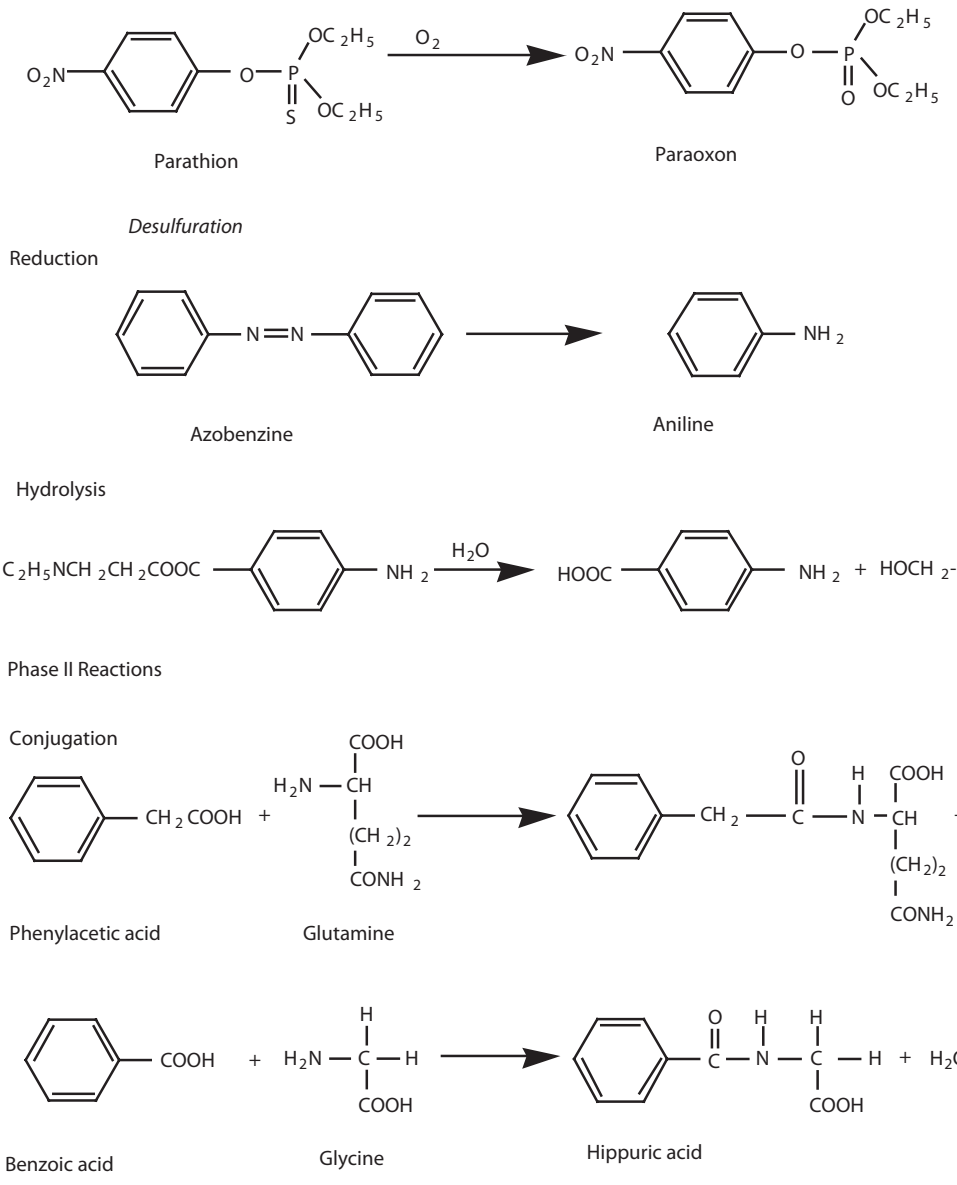


Figure 11.2 (*Continued*) Detoxification pathways. **(*Continued*)**

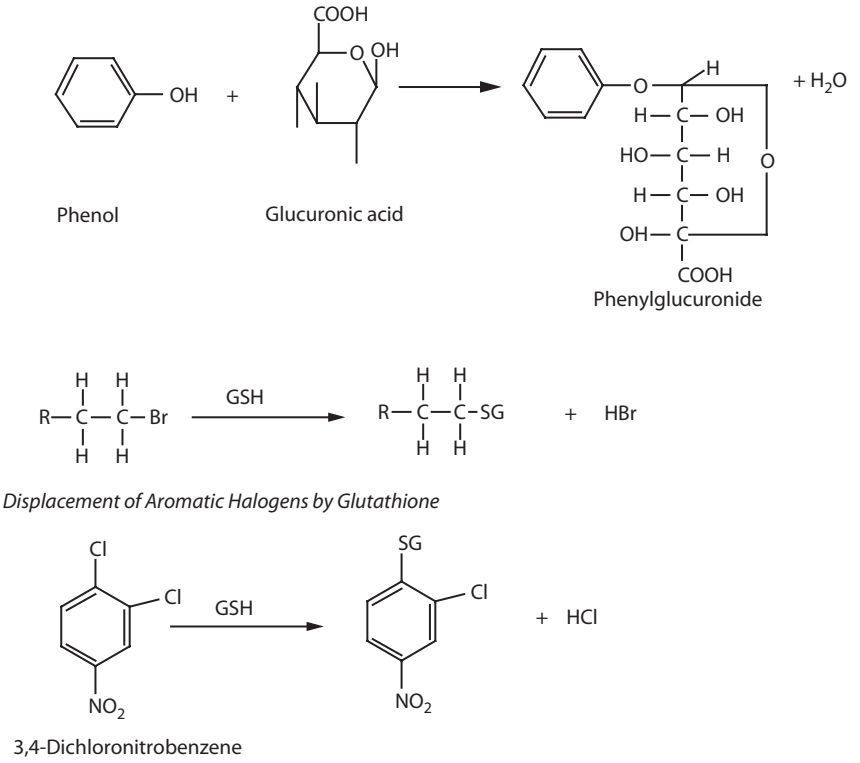


Figure 11.2 (*Continued*) Detoxification pathways.

isozymes, which are versatile in that they catalyze many types of reactions, including aliphatic and aromatic hydroxylations and epoxidations, N oxidations, sulfoxidations, dealkylations, deaminations, dehalogenations, and others (Wislocki et al. 1980). These isozymes are responsible for the oxidation of different substrates or for different types of oxidation of the same substrate. Carbon monoxide binds with the reduced form of the cytochrome, forming a complex with an absorption spectrum peak at 450 nm. This is the origin of the name of the enzyme. As a result of the complex, inhibition of the oxidation process occurs.

At the active site of cytochrome P-450 is an iron atom that, in the oxidized form, binds the substrate (S) (Figure 11.3). Reduction of this enzyme-substrate complex then occurs, with an electron being transferred from NADPH via NADPH–cytochrome P-450 reductase. This reduced (Fe^{2+}) enzyme-substrate complex then binds molecular oxygen in some unknown fashion, and is then reduced further by a second electron, possibly donated by NADH via cytochrome b_5 and NADH cytochrome b_5 reductase. The enzyme-substrate-oxygen complex splits into water, oxidized substrate, and the oxidized form of the enzyme. The overall reaction is as below:

$$SH + O_2 + NADPH + H^+ \rightarrow SOH + H_2O + NADP^+ \qquad (11.1)$$

As shown in Equation 11.1, one atom from molecular oxygen is reduced to water and the other is incorporated into the substrate (SH). The requirements for this enzyme system are oxygen, NADPH, and Mg^{2+} ions.

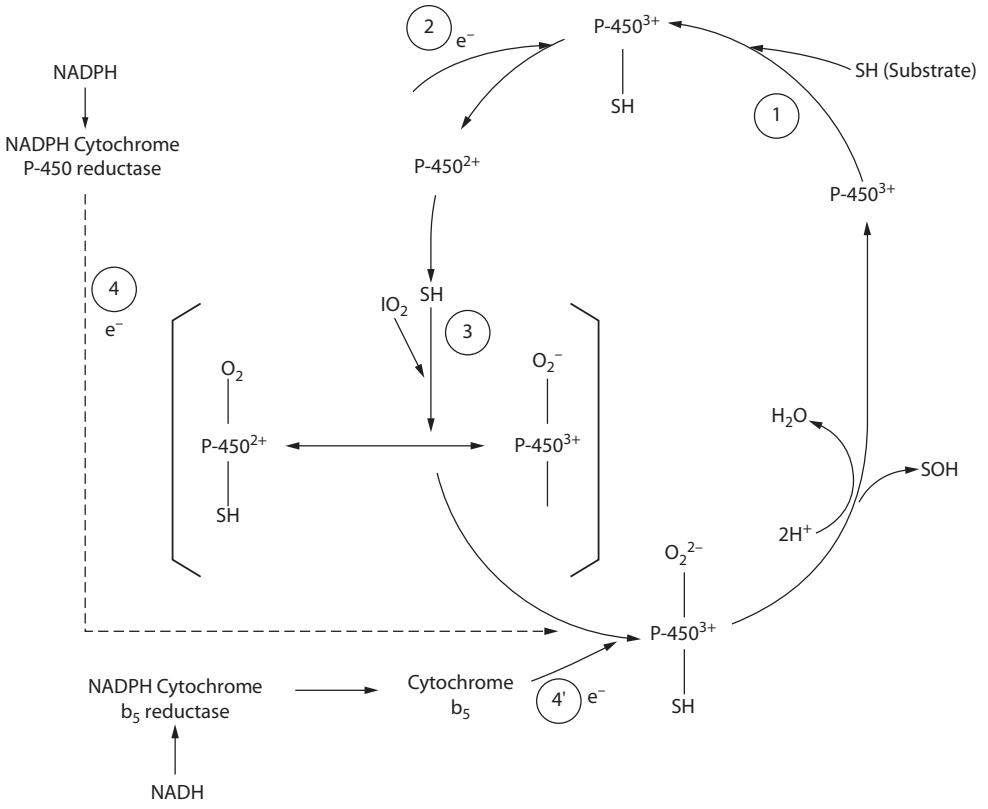


Figure 11.3 The cytochrome P-450 monooxygenase system. $P\text{-}450^{3+}$, cytochrome P-450 with heme iron in oxidized state (Fe^{3+}); $P\text{-}450^{2+}$, cytochrome P-450 with iron in reduced state; S, substrate; e, electron. (Based on Gram, T., Ed., *Extrahepatic Metabolism of Drugs and Other Foreign Compounds*, Spectrum Publications, Jamaica, NY, 1980.)

Contrary to the cytochrome P-450 system, most hepatic phase II enzymes are located in the cytoplasmic matrix. In order for these reactions to occur efficiently, adequate activity of the enzymes involved is essential. In addition, adequate amounts of intracellular cofactors such as NADPH, NADH, O_2, glucose-1-phosphate, glucuronate, ATP, cysteine, and GSH are required for one or more reactions.

11.5 Consequences of Biotransformation

Although hepatic enzymes that catalyze phase I and II reactions are primarily to detoxify xenobiotics, they also participate in the metabolism or detoxification of endogenous substances. For example, the hormone testosterone is deactivated by cytochrome P-450. The S-methylases detoxify H_2S (hydrogen sulfide) formed by anaerobic bacteria in the intestinal tract. It can be seen, then, that chemicals or conditions that influence the activity of the phase I and phase II enzymes can affect the normal metabolism of endogenous substances.

As mentioned previously, the biotransformation of lipophilic xenobiotics by phase I and II reactions might be expected to produce a stable, water-soluble, and readily excretable compound.

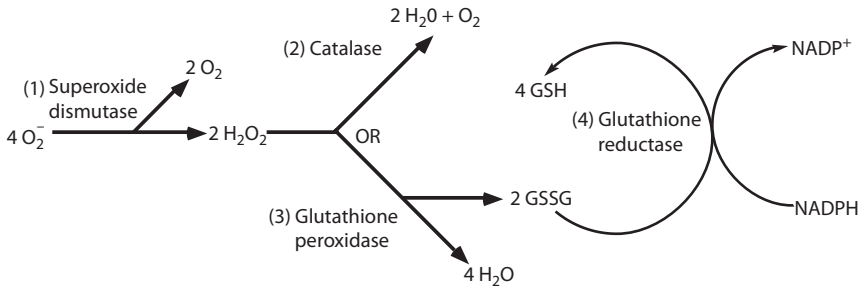


Figure 11.4 The four important enzymatic components of the cellular antioxidant defense system. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide ($O_2^{-\cdot}$) to peroxide. Catalase reduces peroxide to H_2O. GSH peroxidase also detoxifies peroxide by reducing it to H_2O. GSH reductase rereduces the oxidized glutathione (GSSG) to GSH. The NADPH required for the reduction of GSSG to GSH is primarily supplied by the oxidation of glucose via the pentose phosphate pathway. (Based on Mottet, N. K., Ed., *Environmental Pathology*, Oxford University Press, New York, 1985.)

However, there are examples of hepatic biotransformation mechanisms by which xenobiotics are converted to reactive electrophilic species. Unless detoxified, these reactive electrophiles may interact with a nucleophilic site in a vital cell constituent, leading to cellular damage. There is evidence that many of these reactive substances bind covalently to various macromolecular constituents of liver cells. For example, carbon tetrachloride (CCl_4), known to be hepatotoxic, covalently binds to lipid components of the liver endoplasmic reticulum (Reynolds and Moslen 1980). Some of the reactive electrophiles are carcinogenic as well.

Although liver cells are dependent on the detoxification enzymes for protection against reactive electrophilic species produced during biotransformation, endogenous antioxidants such as vitamin E and glutathione (GSH) also provide protection. Vitamin E (α-tocopherol) is widely known as a free radical scavenger. Its main role is to protect the lipid constituents of membranes against free-radical-initiated peroxidation reactions. Experimental evidence has shown that livers of animals fed diets deficient in vitamin E were more vulnerable to lipid peroxidation following poisoning with CCl_4 (Reynolds and Moslen 1980). Glutathione, on the other hand, is a tripeptide, and has a nucleophilic sulfhydryl (SH) group that can react with, and thus detoxify, reactive electrophilic species (Van Bladeren et al. 1980). Glutathione can also donate its sulfhydryl hydrogen to a reactive free radical ($GS\cdot$). The glutathione radical formed can then react with another glutathione radical to produce a stable oxidized form of glutathione (GSSG). The GSSG can then be reduced back to GSH through an NADPH-dependent reaction catalyzed by glutathione reductase. The NADPH, in turn, is derived from reactions in the pentose phosphate pathway.

In addition to vitamin E and GSH, there are other enzymatic systems that are also important in the defense against free-radical-mediated cellular damage. These include superoxide dismutase (SOD), catalase, and GSH peroxidase. Figure 11.4 shows the interrelationship between these enzymatic components.

11.6 Microbial Degradation

Microbial degradation of xenobiotics is crucial in the prediction of the longevity and the long-term effects of the toxicant, and may also be crucial in the actual remediation of a contaminated site.

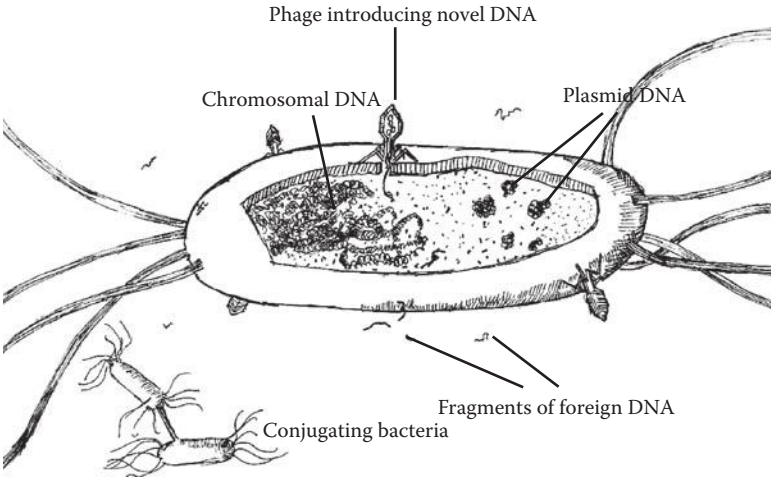


Figure 11.5 Schematic of a typical prokaryote. Genetic information and thereby coding for the detoxification and degradation of a xenobiotic may be available from a variety of sources.

Utilization of the propensity of microorganisms to degrade a wide variety of materials may actually provide an opportunity for environmental toxicologists to not only diagnose and provide a prognosis, but also prescribe a treatment to assist the ecosystem in the removal of the xenobiotic.

Microbial cell structure is varied, with a tremendous diversity in size and shape. Prokaryotic cells typically contain a cell wall, 70 s ribosomes, a chromosome that is not membrane bound, various inclusions and vacuoles, and extrachromosomal DNA or plasmids. Eukaryotic microorganisms are equally varied with a variety of forms; many are photosynthetic or harbor photosynthetic symbionts. Many eukaryotic cells contain prokaryotic endosymbionts, some of which contain their own set of plasmids. Given the variety of eukaryotic microorganisms, they have been labeled protists, since they are often a mixing of algal and protozoan characteristics within apparently related groups.

Many of these microorganisms have the ability to use xenobiotics as a carbon or other nutrient source. In some instances it may be more appropriate to ascribe this capability to the entire microbial community, since often more than one type of organism is responsible for the stages of microbial degradation.

Microorganisms often contain a variety of genetic information. In prokaryotic organisms the chromosome is a closed circular DNA molecule. However, other genetic information is often coded on smaller pieces of closed circular DNA called plasmids. The chromosomal DNA codes the sequences that are responsible for the normal maintenance and growth of the cell. The plasmids, or extrachromosomal DNA, often code for metal resistance, antibiotic resistance, conjugation processes, and often the degradation of xenobiotics. Plasmids may be obtained through a variety of processes, conjugation, infection, and the absorption of free DNA from the environment (Figure 11.5).

Eukaryotic microorganisms have a typical genome with multiple chromosomes as mixtures of DNA and accompanying proteins. Extrachromosomal DNA also exists within the mitochondria and the chloroplasts that resemble prokaryotic genomes. Many microbials also contain prokaryotic and eukaryotic symbionts that can be essential to the survivorship of the organism. The ciliate protozoan *Paramecium bursaria* contains symbiotic chlorella that can serve as a source of sugar given sufficient light. Several of the members of the widespread species complex

Paramecium aurelia contain symbiotic bacteria that kill paramecium not containing the identical bacteria. Apparently this killing trait is coded by plasmid DNA contained within the symbiotic bacteria. Protists generally reproduce by asexual fission, but sexual reproduction is available. Often during sexual reproduction an exchange of cytoplasm takes place, allowing cross-infection of symbionts and their associated DNA.

Microorganisms are found in a variety of environments: aquatic, marine, groundwater, soil, and even the Arctic. Many are found in extreme environments, from tundra to the superheated smokers at sites of sea floor spreading. The adaptability of microorganisms extends to the degradation of many types of xenobiotics.

Many organic xenobiotics are completely metabolized under aerobic conditions to carbon dioxide and water. The essential criterion is that the metabolism of the material results in a material able to enter the tricarboxylic acid (TCA) cycle. Molecules that are essentially simple chains are readily degraded since they can enter this cycle with relatively little modification. Aromatic compounds are more challenging metabolically. The 3-ketoadipic acid pathway is the generalized pathway for the metabolism of aromatic compounds with the resulting product acetyl-CoA and succinic acid, materials that easily enter into the TCA cycle (Figure 11.6). In this process the aromatic compound is transformed into either catechol or protocatechuic acid. The regulation of the resultant metabolic pathway is dependent upon the group, and basic differences exist between bacteria and fungi.

Often the coding process for degradation of a xenobiotic is contained on the extrachromosomal DNA, the plasmid, and the chromosome. And often the initial steps that lead to the eventual incorporation of the material into the TCA cycle are coded by the plasmid. Of course, two pathways may exist, a chromosomal and a plasmid pathway. Given the proper DNA probes, pieces of DNA with complementary sequences to the degradation genes, it should be possible to follow the frequency and thereby the population genetics of degradative plasmids in prokaryotic communities.

In prokaryotic mechanisms the essential steps allowing an aromatic or substituted aromatic to enter the 3-ketoadipic acid pathway are often, but not always, encoded by plasmid DNA. In some cases both a chromosomal and a plasmid pathway are available. Extrachromosomal DNA can be obtained through a variety of mechanisms and can be very infectious. The rapid transmission of extrachromosomal DNA has the potential to enhance genetic recombination and result in rapid evolutionary change. In addition, the availability of the pathways on relatively easy to manipulate genetic material enhances our ability to sequence and artificially modify the code, and perhaps enhance the degradative capability of microorganisms.

Simple disappearance of a material does not imply that the xenobiotic was biologically degraded. There are two basic methods of assessing the biodegradation of a substance. The first is an examination of the mass balance or materials balance resulting from the degradative process. This is accomplished by the recovery of the original substrate or by the recovery of the labeled substrate and the suspected radiolabeled metabolic products. Mineralization of the substrate is also a means of assessing the degradative process. Production of CO_2, methane, and other common congeners derived from the original substrate can be followed over time. With compounds that have easily identified compounds such as bromide, chloride, or fluoride, these materials can be analyzed to estimate rates of degradation. One of the crucial steps is to compare these rates and processes with sterilized media or media containing specific metabolic inhibitors to test whether the processes measured are biological in nature.

Although the specific determination of the fate of a compound is the best means to establish the degradation of a compound, nonspecific methods do exist that can be used when it is

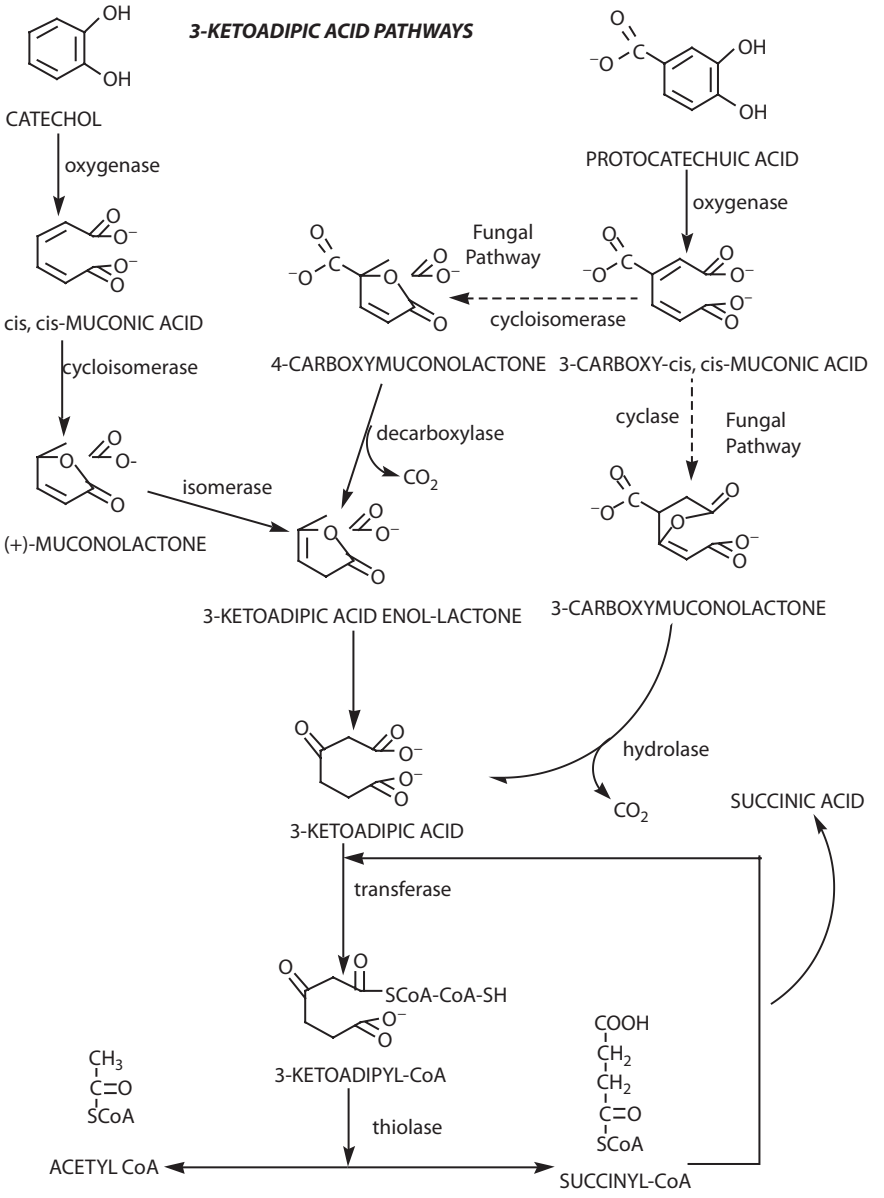


Figure 11.6 The 3-ketoadipic acid pathway. (Bottom structure: Adapted from Rochkind et al., 1986.)

difficult or impossible to label or analytically detect the substrate. Measurement of oxygen uptake as the substrate is introduced in the culture is a means of confirming the degradation of the toxic material. Biological oxygen demand as determined for wastewater samples can be used, but it is not particularly sensitive. Respirometry with a device such as the Warburg respirometer is more sensitive and can be used to measure the degradation rates of suspected intermediates. Often it is possible to grow the degradative organism using only the xenobiotic substrate as the sole carbon source, additionally confirming the degradative process. Controls using sterilized media or inhibitors are again important since microorganisms are able to grow on surprisingly minimal media, and with only small amounts of materials that may be present as contaminants.

A wide variety of aromatic organics are degraded by a variety of microorganisms. Table 11.1 provides a compilation from a review giving both the compound and the strains that have so far been found to be responsible for the degradation. Only a few examples will be discussed below.

Substituted benzenes are commonly occurring xenobiotics. In Figure 11.7 the biodegradation pathway for toluene is diagrammed. The process begins with the hydroxylation of the toluene. In one case the hydroxylation of the substituent, the methyl group, occurs to form benzyl alcohol. Additional steps result in catechol, a material readily incorporated into the 3-ketoadipic acid pathway. Another set of species hydrolyze the ring itself, producing a substituted catechol as the end process.

The degradation mechanism of materials such as naphthalene by fungi has been found comparable in a broad sense to the detoxification mechanisms found in the liver in vertebrates. Fungi use a monooxygenase system that incorporates an atom of oxygen into the ring as the other atom is incorporated into water (Figure 11.8). The resulting epoxide can be further hydrolyzed to form an intermediate ultimately ending with a transhydroxy compound. The epoxide can also isomerize to form a variety of phenols. Both of these mechanisms occur in the degradation of naphthalene by the fungus *Cunninghamella elegans.*

A particularly widespread environmental contaminant is the pesticide pentachlorophenol (PCP). PCP has been used as a bactericide, insecticide, fungicide, herbicide, and molluskicide in order to protect a variety of materials from decomposition. Although it has bactericidal properties, PCP has been found to be degraded in a variety of environments by both bacteria and fungi. In some instances, degradation occurs with PCP being used as an energy source.

A proposed pathway for the degradation of PCP by two bacterial strains is represented in Figure 11.9. Cultures of *Pseudomonas* were found to transform PCP into tetrachlorocatechol and tetrachlorohydroquinone (TeCHQ). These materials are then metabolized, and radiolabeled carbon can be found in the amino acids of the degradative bacteria. Mycobacterium methylates PCP to pentachloroanisole but does not use PCP as an energy source. Fungi also metabolize PCP to a less toxic metabolite.

11.7 Bioremediation

Given the ability of many organisms to degrade toxic materials within the environment, a practical application would be to use these degradative capabilities in the removal of xenobiotics from the environment. In the broadest sense, this might entail the introduction of a specifically designed organism into the polluted environment to ensure the degradation of a known pollutant. Other examples of attempts at using biodegradation for remediation is the addition of fertilizers to enhance degradation of oil spills and the construction of biological reactors, bioreactors, through which contaminated water or a soil slurry can be passed. In some instances these attempts have appeared successful, while in others the data are not so clear.

Table 11.1 Examples of Organic Compounds and Degradative Bacterial Strains

Organic	*Strain*
Aniline	*Frateuria* sp. ANA-18
	Nocardia sp.
	Pseudomonas sp.
	Pseudomonas multivorans AN1
	Rhodococcus sp. AN-117
	Rhodococcus sp. SB3
Anthracene	*Beijerinckia* sp. B836
	Cunninghamella elegans
	Pseudomonas sp.
	Pseudomonas putida 199
Benzene	*Achromobacter* sp.
	Pseudomonas sp.
	Pseudomonas aeruginosa
	Pseudomonas putida
Benzoic acid	*Alcaligenes eutophus*
	Aspergillus niger
	Azotobacter sp.
	Bacillus sp.
	Pseudomonas sp.
	Pseudomonas acidovorans
	Pseudomonas testosteroni
	Pseudomonas sp. strain H1
	Pseudomonas PN-1
	Pseudomonas sp. WR912
	Rhodopseudomonas palustris
	Streptomyces sp. by consortia of bacteria
2-Chlorobenzoic acid	*Aspergillus niger*
3-Chlorobenzoic acid	*Acinetobacter calcoaceticus* Bs5 (grown on succinic acid and pyruvic acid)

Table 11.1 (*Continued*) Examples of Organic Compounds and Degradative Bacterial Strains

Organic	*Strain*
	Alcaligenes eutrophus B9
	Arthrobacter sp. (grown on benzoic acid)
	Aspergillus niger
	Azotobacter sp. (grown on benzoic acid)
	Bacillus sp. (grown on benzoic acid)
	Pseudomonas aeruginosa B23
	Pseudomonas putida (with plasmid p AC25)
	Pseudomonas sp. B13
	Pseudomonas sp. H1
	Pseudomonas sp. WR912 by consortia of bacteria
4-Chlorobenzoic acid	*Arthrobacter* sp.
	Arthrobacter globiformis
	Azotobacter sp. (grown on benzoic acid)
	Pseudomonas sp. CBS 3
	Pseudomonas sp. WR912
4-Chloro-3,5-dinitrobenzoic acid	*Chlamydomonas* sp. A2
2,5-Dichlorobenzoic acid	By consortia of bacteria
3,4-Dichlorobenzoic acid	By consortia of bacteria
3,5-Dichlorobenzoic acid	*Pseudomonas* sp. WR912 by consortia of bacteria
2,3,6-Trichlorobenzoic acid	*Brevibacterium* sp. (grown on benzoic acid)
Biphenyl	*Beijerinckia* sp.
	Beijerinckia sp. B836
	Beijerinckia sp. 199
	Cunninghamella elegans
	Pseudomonas putida by consortia of bacteria
Catechol	*Pyrocatechase I*
4-Chlorocatechol	*Achromobacter* sp.
3,5-Dichlorocatechol	*Achromobacter* sp.

(Continued)

Table 11.1 (*Continued*) Examples of Organic Compounds and Degradative Bacterial Strains

Organic	*Strain*
Chlorobenzene	*Pseudomonas putida* (grown on toluene)
	Unidentified bacterium, strain WR1306
Chlorocatechol	Pyrocatechases
3,5-Dichlorocatechol	*Achromobacter* sp. (grown on benzoic acid)
Chlorophenol	*Arthrobacter* sp.
2-Chlorophenol	*Alcaligenes eutrophus*
	Nocardia sp. *(*grown on phenol)
	Pseudomonas sp. B13
3-Chlorophenol	*Nocardia* sp. (grown on phenol)
	Pseudomonas sp. B13
	Rhodotorula glutinis
4-Chlorophenol	*Alcaligenes eutrophus*
	Arthrobacter sp.
	Nocardia sp. (grown on phenol)
	Pseudomonas sp. B13
	Pseudomonas putida
2,4,6-Trichlorophenol	*Arthrobacter* sp.
2,3,4,6-Tetrachlorophenol	*Aspergillus* sp.
	Paecilomyces sp.
	Penicillium sp.
	Scopulariopsis sp.
Chlorotoluene	*Pseudomonas putida* (grown on toluene)
Gentisic acid	*Trichosporon cutaneum*
Guaiacols (*o*-methoxyphenol)	*Arthrobacter* sp.
3,4,5-Trichloroguaiacol	*Arthrobacter* sp. 1395
Homoprotocatechuic acid	*Trichosporon cutaneum*
Naphthalene	*Cunninghamella elegans*
	Oscillatoria sp.
	Pseudomonads

Table 11.1 (*Continued*) Examples of Organic Compounds and Degradative Bacterial Strains

Organic	*Strain*
Pentachlorophenol (PCP)	*Arthrobacter* sp.
	Coniophora pueana
	Mycobacterium sp.
	Pseudomonas sp.
	Saprophytic soil corynebacterium
	KC3 isolate
	Mutant ER-47
	Mutant ER-7
	Trichoderma viride
Phenanthrene	*Aeromonas* sp.
	Fluorescent and nonfluorescent pseudomonad groups
	Vibrios
Protocatechuic acid	*Neurospora crassa*
	Trichosporon cutaneum
Sodium pentachlorophenate (Na-PCP)	*Trichoderma* sp.
	Trichoderma virgatum
Tetrachlorohydroquinone	KC3
Toluene	*Achromobacter* sp.
	Pseudomonas sp.
	Pseudomonas aeruginosa
	Pseudomonas putida
4-Amino-3,5-dichlorobenzoic acid	By consortia of bacteria
2,4,5-Trichlorophenoxyacetic acid	*Pseudomonas cepacia* AC1100

Source: Compiled from Rochkind, M. L. et al., 1986, *Microbial Decomposition of Chlorinated Aromatic Compounds*, EPA/600/2-86/090, chaps. 6–10, pp. 45–98.

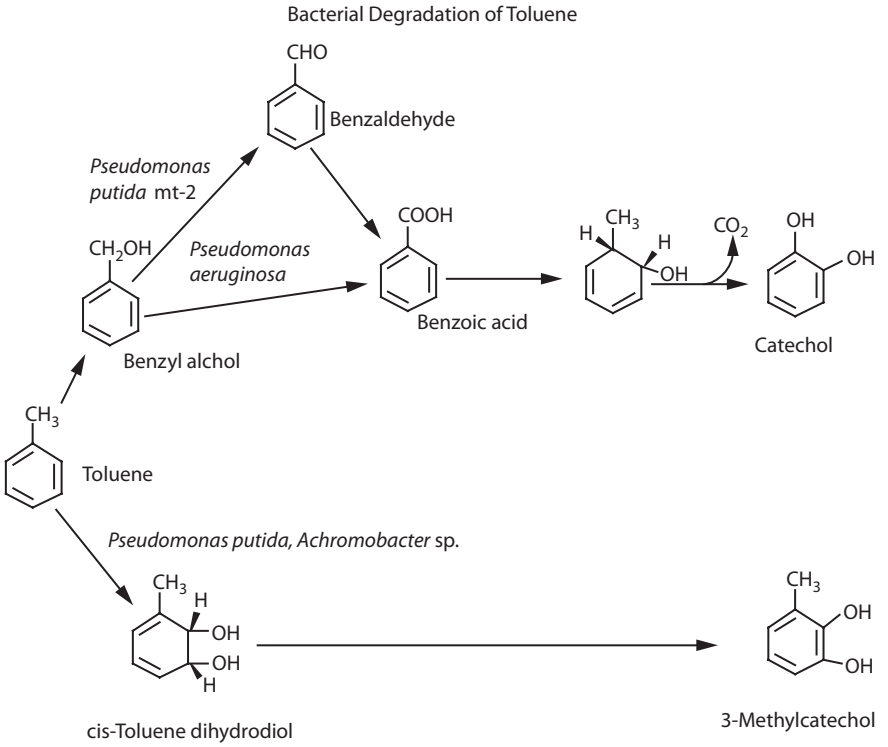


Figure 11.7 Alternate pathways for the degradation of a substituted benzene, toluene.

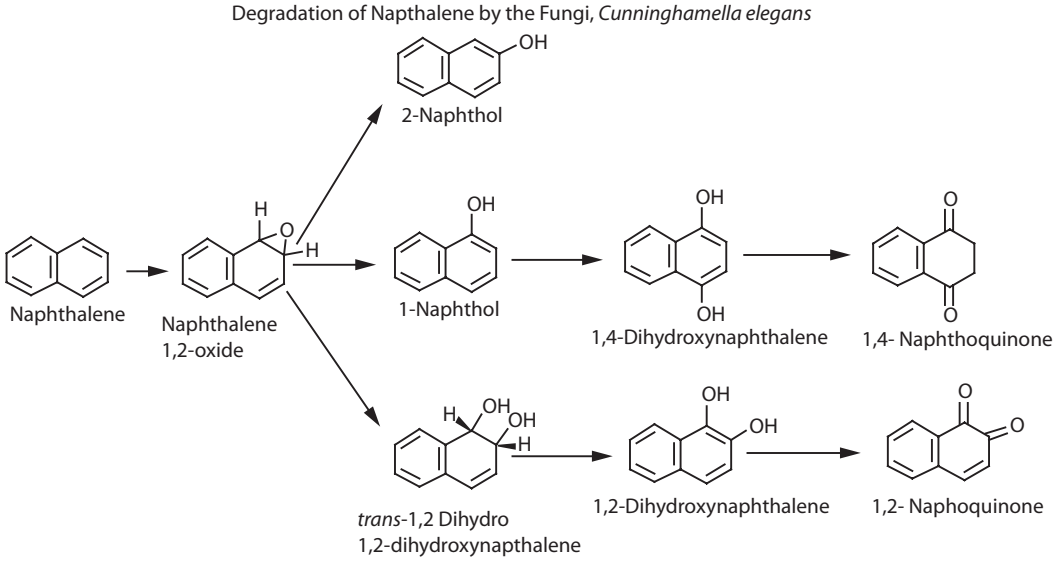


Figure 11.8 Biodegradation of naphthalene by *Cunninghamella elegans*.

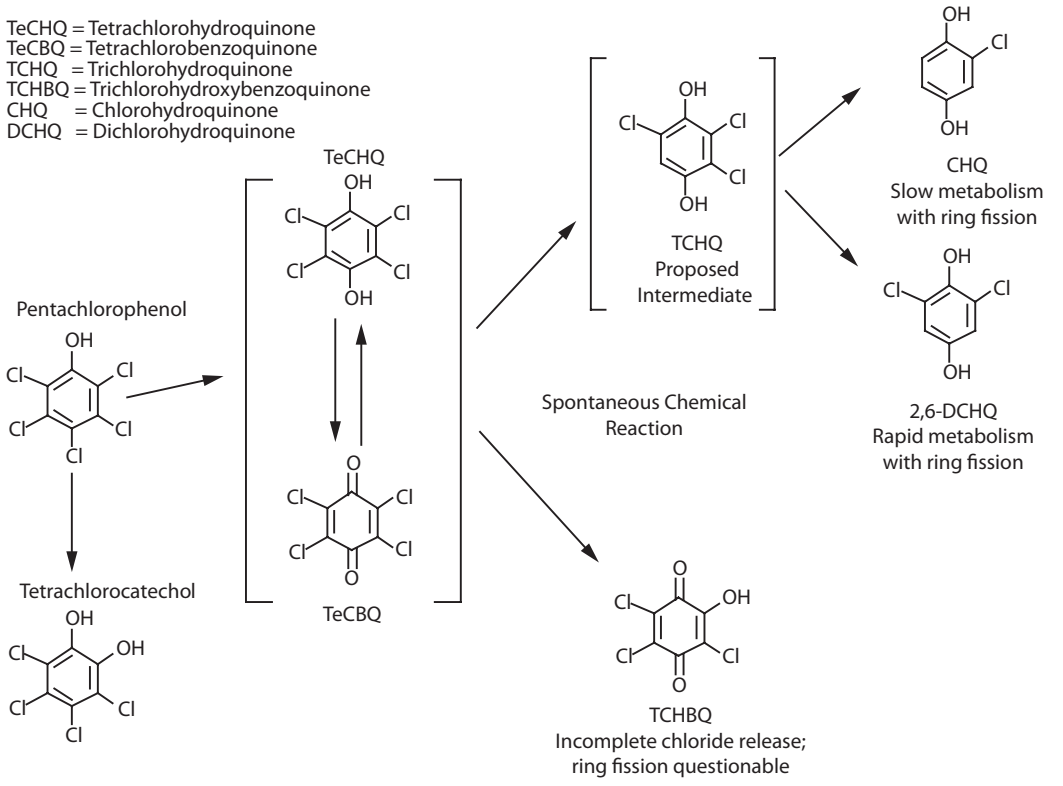


Figure 11.9 Possible mechanisms for the degradation of pentachlorophenol by *Pseudomonas* sp.

The most important design criteria for attempting bioremediation is the complexity of the environment and the complexity and concentration of the toxicants. Controlled and carefully defined waste streams such as those derived from a specific synthesis at a manufacturing plant may be especially amenable to degradation. A reactor such as the one schematically depicted in Figure 11.10 could be developed using a specific strain of bacteria or protist that has been established on a substrate. Nutrients, temperature, oxygen concentration, and toxicant concentration can be carefully controlled to offer a maximum rate of degradation. As the complexity of the effluent or the site to be remediated increases, a consortia of several organisms or of an entire degradative community may be necessary. Consortia can also be established in a bioreactor type setting.

The concentration of the toxicant is essential in determining the success of the bioremediation attempt. As shown in Figure 11.11, too low a concentration will not stimulate growth of the degradative organism. At too high a concentration the toxic effects become apparent and the culture dies. The shape of the curve is dependent not only upon the degradative system of the organism, but also upon the availability of nutrients, temperature, and other factors essential for microbial growth. One of the advantages of the bioreactor system is that all of these factors can be carefully controlled. In a situation where it may be necessary to attempt the *in situ* remediation of a toxicant, these factors are more difficult to control. Biotic factors, such as competitors and predators, also become important as the process is taken out of the bioreactor and placed in a more typical environment. Not only do the degradative organisms have to be able to degrade the toxicant, but they must also be able to compete effectively with other microflora and escape predation.

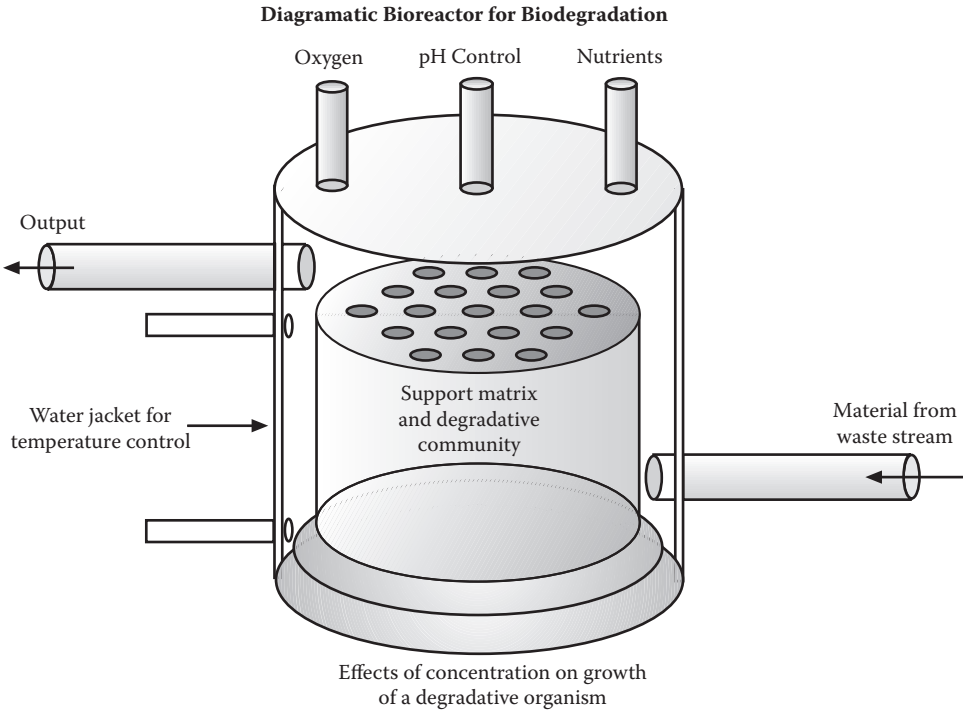


Figure 11.10 Schematic of a bioreactor for the detoxification of a waste stream or for inclusion in a pump and water treatment process.

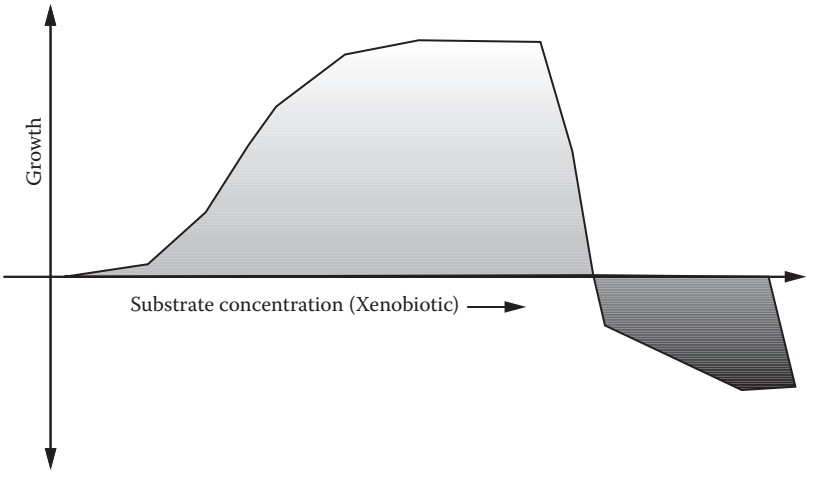


Figure 11.11 Degradative growth curve. At low concentrations, degradation may not occur due to the lack of nutritive content of the xenobiotic as substrate. Eventually, a maximal rate of degradation and also growth may occur with a plateau. The concentration of the toxic material overwhelms the ability of the organism to detoxify the material and death ensues.

To enhance degradation, frequent plowing and fertilization of a terrestrial site may be done to ensure proper aeration of the soil. Groundwater is often nutrient and oxygen limited, and both of these materials can be introduced. Often hydrogen peroxide is pumped into groundwater as an effective means of delivering oxygen as the hydrogen peroxide decomposes.

11.7.1 Isolation and Engineering of Degradative Organisms

The basic scheme of isolating degradative organisms is relatively straightforward. Samples from a site likely to contain degradative bacteria are collected. If the degradation of oil products is sought, soils and sediments near pumping stations or other sites likely to be contaminated with the materials of interest are sampled. PCP has been widely used as a preservative, so old wood processing plants may be appropriate.

The next step is to enhance the selection process for the ability to degrade the toxicant by using increasing concentrations of the material. This process can be accomplished in two related ways. First, the toxicant and sample are mixed in a chemostat. A chemostat maintains the culture at specific conditions, adds nutrients, and often has a mixing apparatus. At an initial low concentration, samples are taken in order to determine whether the xenobiotic has been degraded. It may take many months for the evolution of the degradative ability in the original microbial community. As degradation is observed, successively higher concentrations of the toxicant can be added to the chemostat to further strengthen the selection for the ability to degrade the toxicant. At very high concentrations, only a few bacterial or fungal species may survive. These survivors can then be plated and examined for the ability to degrade the toxicant. The researcher must be prepared for the possibility that no one organism may be able to completely mineralize the xenobiotic, and a consortia of several organisms may be required.

A similar process can be accomplished without access to a chemostat. Samples from a culture of an initial concentration of xenobiotic can be placed in other containers with successively higher concentrations of the toxicant, achieving the same selective pressures as found in the chemostat (Figure 11.12). Again, it may take long periods for evolution of a degradative organism or community to arise.

As the degradative organism or consortia are isolated, further studies may actually isolate a particular plasmid or even genes responsible for the degradation. It may be possible to construct organisms with several of these plasmids, or the genes may be inserted into the host chromosome. If the desire is to place the organisms into a field situation, basic survival traits must also be maintained.

11.7.2 The Genetics of Degradative Elements

Once formed, a degradative element can suffer a number of fates (Figure 11.13). Using an organophosphate degradative or *opd* gene as an example, a number of recombination and other genetic events can occur that affect the reproduction and expression of the gene.

First, the gene exists on a plasmid within the host cell. The plasmid can replicate, increasing the copy number of the plasmid that is the host of the degradative genetic element. In some instances, the plasmid can be incorporated into the host chromosome through a recombination event. The entire plasmid or sections can enter the host genome. Expression of the genes contained in the plasmid may or may not occur. Occasionally, the genetic elements can be excised from the host and again reproduce as an independent plasmid. This scenario is similar to that for the life cycle of the lambda phage.

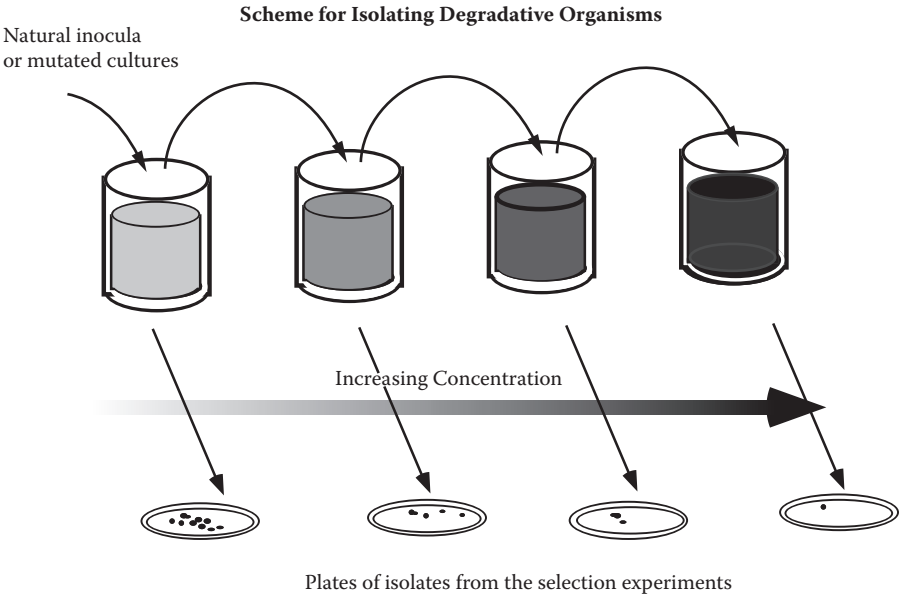


Figure 11.12 Selection protocol for the isolation of degradative microorganisms.

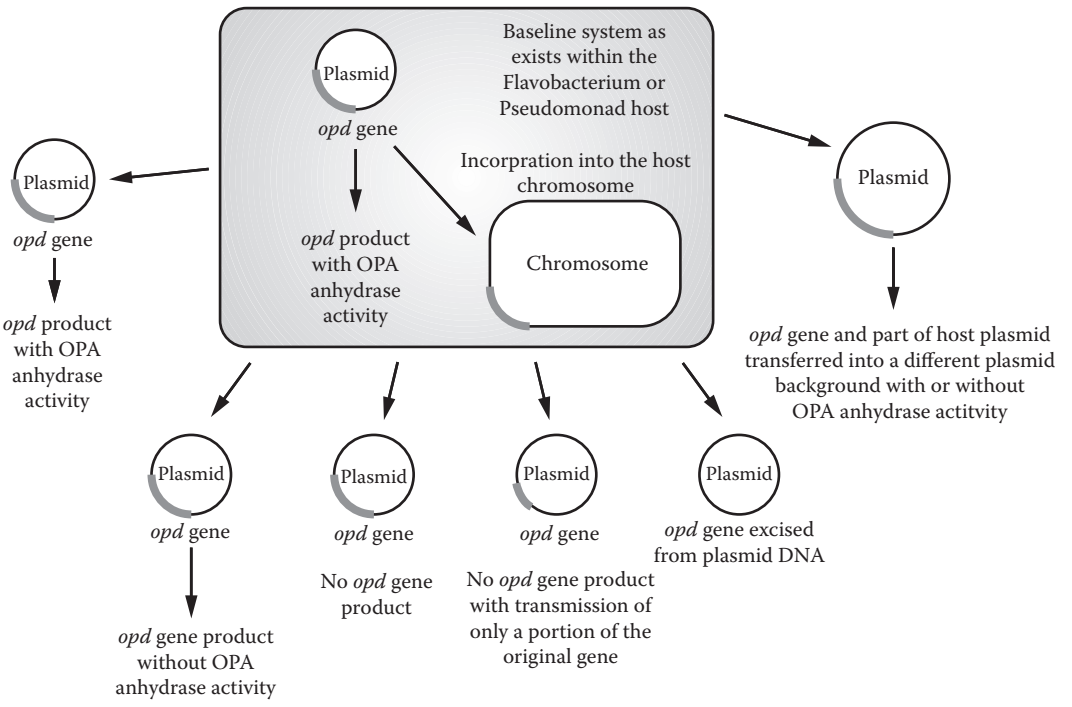


Figure 11.13 Outcomes in the evolution of a degradative element in a prokaryote.

At a conjugation event, the plasmid may be passed on in its entirety and the new host translating the genetic code into a viable degradative enzyme. However, a mistake in replication or a mismatch with the new protein-generating machinery of the new host may result in the plasmid being passed on but the activity of the gene product not being realized. In some cases a protein may be manufactured, but the degradative activity lost through mutation.

Deletions also may occur that result in only part of the degradative element remaining on the plasmid. If only a portion of the original gene is being transmitted, an inactive protein may result. If the deletion is in the base sequences that are recognized by the transcription machinery of the cell, no mRNA and the derivative protein will be produced.

A deletion event may also excise the degradative element from the plasmid, resulting in a loss of the information from the resulting host cells. In this case, the ability to degrade a xenobiotic has been lost, and will probably not recover unless recombination with a plasmid containing the degradative element occurs.

Of course, many prokaryotes contain more than one plasmid. Recombination between the plasmid containing the degradative gene and a plasmid of the same neighborhood can pass the degradative gene to a new host.

11.8 An Example of a Detoxification Enzyme: The Organophosphate Acid Anhydrolases

The examples provided above give only a brief overview of the variety of enzymatic functions that alter, biotransform, and biodegrade xenobiotics. In many instances numerous enzymes are known, as in the case of the mixed-function oxidases. In order to provide a concrete example of a system of detoxification enzymes that is widely distributed, we have chosen the organophosphate acid (OPA) anhydrolases—enzymes that may aid in the understanding of organophosphate intoxication and may also provide a means for the detoxification and bioremediation of these materials.

Interesting examples of a series of enzymes able to hydrolyze a variety of organophosphates are the OPA anhydrolases. OPA anhydrolases are a wide-ranging group of enzymes. As will be shown below, there are often several distinguishable enzymes within an organism. The ability to hydrolyze a particular substrate varies tremendously. Inhibitors have been found and cations seem to be important for activity. The enzymatic mechanism has been described for the *opd* gene product, but is still unknown for the remaining OPA anhydrolases. Currently, the natural role of these enzymes is unknown, although suggestions have been made that the OPA anhydrolases evolved for the degradation of naturally occurring organophosphates and halogenated organics (Haley and Landis 1988; Chester et al. 1988; Landis et al. 1989a,b,c).

Two categories of organofluorophosphate OPA anhydrolases have been recognized in the literature (Hoskin et al. 1984). Typically, the Mazur type is characterized as being stimulated by Mn^{2+}, hydrolyzing soman faster than DFP, nontolerant of ammonium sulfate precipitation, is usually found to be dimeric with a molecular weight of approximately 62,000 D (Storkebaum and Witzel 1975), and is competitively or reversibly inhibited by mipafox (Hoskin 1985). Mipafox is a structural analog to DFP (Figure 11.14). The Mazur type OPA anhydrase demonstrates a stereospecificity in the hydrolysis of tabun (Hoskin and Trick 1955) and soman. The archetypal Mazur type OPA anhydrase can be found in hog kidney. Typically, squid type OPA anhydrase (Hoskin et al. 1984) hydrolyzes DFP faster than soman, is stable, can be purified using ammonium sulfate, has a molecular weight of approximately 26,000 D, is usually unaffected or slightly inhibited by Mn^{2+}, experiences no inhibition of DFP hydrolysis by mipafox (Hoskin et al. 1984), and does

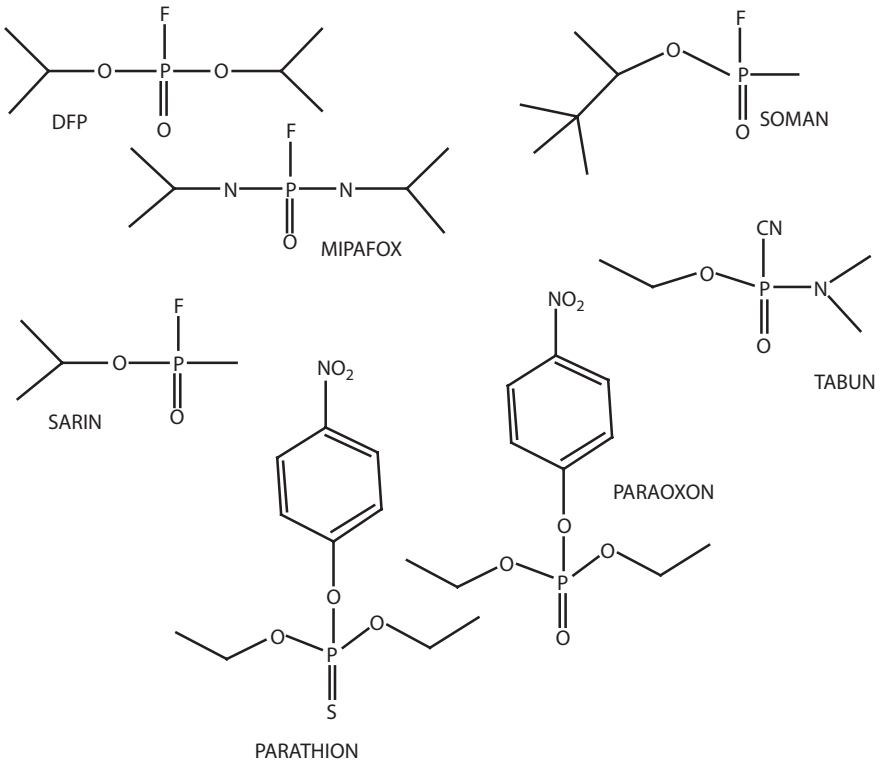


Figure 11.14 Structures of several common substrates and an inhibitor used to study the mechanisms of OPA anhydrase activity: DFP (diisopropylfluorophosphate), mipafox (N,N′-diisopropylphosphorodiamidofluoridate), tabun (N,N-dimethylethylphosphoroamidocyanidate), soman (0-1,2,2-trimethylpropylmethylphosphonofluoridate), paraoxon (diethyl 4-nitrophenyl phosphate), and parathion.

not demonstrate stereospecificity toward the hydrolysis of soman. Squid type OPA anhydrase is present in nerves (optic ganglia, giant nerve axon), the hepatopancreas, and the salivary gland of cephalopods (Hoskin et al. 1984). Cephalopods also contain OPA anhydrase resembling the Mazur type in other tissues. Table 11.2 lists the characteristics of several of the different OPA anhydrolases studied to date.

11.8.1 Characteristics of the opd *Gene Product and Other Bacterial OPA Anhydrolases*

Currently under intense scrutiny, the protein product of the *opd* gene of *Pseudomonas diminuta* is perhaps the best studied of the bacterial OPA anhydrolases. It has been shown that the *opd* OPA anhydrase (also called phosphotriesterase) has the capability to hydrolyze DFP and perhaps other organofluorophosphates (Dumas et al. 1989; Donarski et al. 1988). This activity was labeled as a phosphotriesterase and was characterized by the capability to hydrolyze materials such as paraoxon and parathion. Although not strictly aquatic, this OPA anhydrase is apparently widely distributed among bacteria, and the genetic code has been sequenced and the mechanism of hydrolysis elucidated.

Table 11.2 Comparison of Several Aquatic OPA Anhydrase Activities with Typical Squid and Mazur Type OPA Anhydrolases

Characteristic Activity	*Substrate Hydrolysis*			*Mipafox Inhibition*
	mw	*Soman-to-DFP Ratio*	*Mn^{2+} Stimulation*	
T. thermophila				
Tt DFPase-1	80,000	1.12	2.5–4.0	+
Tt DFPase-2	75,000	1.26	2.0	+
Tt DFPase-3	72,000	0.71	1.7–2.5	+
Tt DFPase-4	96,000	1.95	17–30	nt
R. cuneata				
Rc OPA-1	19,000–35,000	nt	1	—
Rc OPA-3	82,000–138,000	nt	nt	(Hydrolyzes mipafox)
Thermophile isolate OT (JD.100)	84,000	nt	+	—
Halophile isolate JD6.5				
OPAA I	98,000	nt		nt
OPAA II	62,000	0.5	3–5	nt
opd gene product (parathion hydrolase)	60,000–65,000 (35,418 subunits)	nt	+	
Squid type OPA anhydrase (*Loligo pealei*)	23,000–30,000	0.25	1	–
Mazur type OPA anhydrase (hog kidney)	62,000–66,000 (30,000 subunits)	6.5	2	+

Note: The enzymes vary in molecular weight, reaction to ions, and soman-to-DFP ratios.

The *opd* OPA anhydrase is coded by a plasmid-borne gene of 1,079 base pairs in length (McDaniel et al. 1988). The gene sequence is identical in both *Flavobacterium* and *P. diminuta*, although the plasmids bearing this gene are not. Crude preparations of bacteria containing the *opd* gene have been demonstrated to have the ability to hydrolyze a variety of phosphotriesters, such as paraoxon, fensulfothion, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphothioate (EPN), and chlorofenvinophos (Brown 1980; Chiang et al. 1985; McDaniel 1985). However, in at least the case of malathion hydrolysis, the active agent is not the *opd* OPA anhydrase. Activity that can degrade malathion exists even in *P. diminuta* cured of the plasmid containing the *opd* gene (Wild and Raushel 1988). Eighty to ninety percent of the OPA anhydrase activity apparently is associated with the pseudomonad membrane. The *opd* OPA anhydrase is insensitive to ammonium sulfate (Dumas et al. 1989). Molecular weight as determined by analysis of the gene sequence is 35,418 D

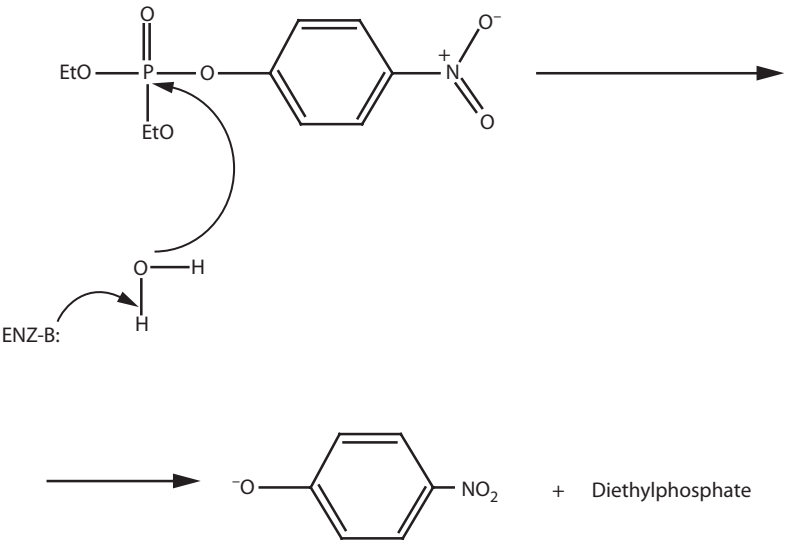


Figure 11.15 Mechanism of hydrolysis of parathion by the *opd* OPA anhydrase as determined by Lewis et al. The reaction is a single displacement using a base at the active site to activate a water molecule. The activated water attacks the phosphorus, producing diethyl phosphate and 4-nitrophenol. The same active site is able to hydrolyze DFP (Dumas et al. 1989) and related organofluorophosphates (Dumas et al. 1990). (Modified with permission from Lewis, V. E. et al., *Biochemistry*, 27, 1591–1597, 1988.)

(McDaniel et al. 1988). However, disassociated from the membrane using a Triton-X-100 or Tween 20, the apparent molecular weight is estimated to be 60,000 to 65,000 D. These data raise the possibility that the active enzyme is dimeric.

In an elegant series of experiments, the mechanism of the *opd* OPA anhydrase was elucidated (Lewis et al. 1988). Using oxygen-18 containing water and the (=) and (–) enantiomers of O-ethyl phenylphosphonothioic acid, it was determined that the reaction was a single in-line displacement by an activated water molecule at the phosphorus center of the substrate (Figure 11.15). It is significant that this same enzyme was also able to hydrolyze DFP and other related organofluorophosphates.

Attaway et al. (1987) have screened a number of bacterial isolates for OPA anhydrase activity, including strains of *Pseudomonas diminuta*, *P. aeruginosa*, *P. putida*, *Vibrio alginolyticus*, *V. parahaemolyticus*, *Escherichia coli*, and *Flavobacterium* sp. Chettur et al. (1988) and Hoskin et al. (1989) published findings on the OPA anhydrase activities of the obligate thermophile (OT) organism, also known as JD.100 from the DeFrank collection. J. DeFrank isolated the thermophilic bacteria from soil samples from the Edgewood area of Aberdeen Proving Ground, Maryland. OT has been identified as a strain of *Bacillus stearothermophilus*. The OPA anhydrase activity was purified using a Pharmacia G-100 column followed by a DEAE ion exchange column. A 5- to 10-fold purification was accomplished. Estimated molecular weight was 84,000 D. The OT OPA anhydrase hydrolyzed soman, sarin, and dimebu (3,3-dimethylbutyl methylphosphonfluoridate) but not DFP. The catalysis was markedly stimulated by Mn^{2+}. Dimebu hydrolysis was also stimulated, but less stimulation by Mn^{2+} is apparent. Sarin hydrolysis followed the pattern of dimebu. Mipafox was not inhibitory. DFP was reported to be a weak noncompetitive inhibitor of soman hydrolysis. A suggestion was made in this report that since hydrolysis and the reduction of acetylcholinesterase

inhibition coincide, the OT OPA anhydrase activity hydrolyzed all four isomers simultaneously, similar to the squid type OPA anhydrase.

Several halophilic isolates that exhibit OPA anhydrase activity have been collected and studied by DeFrank (1988). One isolate, designated JD6.5, was obtained from Grantsville Warm Springs, Utah. Two OPA anhydrase activities were present; however, 90% of the activity was represented by one of the enzymes, OPA-2. According to SDS-PAGE and gel permeation chromatography, the molecular weight has been estimated at approximately 62,000 D. OPA-2 is stimulated by Mn^{2+} and hydrolyzes soman. The optimum pH was approximately 7.2. Attempts at purification using Sepharose CL-4B indicate that the enzyme may be very hydrophobic. Isolate JD30.3 was isolated from Wilson Hot Springs, Utah, and also contained OPA anhydrase activity able to hydrolyze DFP and soman. The purified activity was stimulated by divalent cations, with Mg^{2+} being the best. Molecular weight was approximately 76,000 D, as determined by gel molecular sieve chromatography. The OPA anhydrases from JD30.3 were insensitive to ammonium sulfate.

In an often overlooked paper, Zech and Wigand (1975) demonstrated that the DFP hydrolyzing and paraoxon hydrolyzing activities in at least one strain of *Escherichia coli*, K_{12}sr, were distinct. Separated by gel filtration, the activities showed no overlap. Two peaks of DFP hydrolyzing activity were found using gel filtration, and four peaks were found at isoelectric points of 5.3, 5.7, 6.1, and 7.8. Three isoelectric points at 5.3, 5.6, and 6.2 were found for the paraoxon hydrolyzing activity. The optimal pH for DFP hydrolysis was found to be 8.3; for paraoxon hydrolysis it was 11.3. Additional bacterial OPA anhydrolases have been identified and sequenced with interesting results.

Cheng et al. (1996) have identified an enzyme from *Alteromonas* sp. that is designated OPAA2 and cloned the gene (*opaA*). This enzyme is active in hydrolyzing a variety of organophosphates. The enzyme is activated by Mn^{2+}, inhibited by mipafox, has an optimum activity between pH 7.5 and 8.5, and a molecular weight of 60 kDa. A comparison of the sequence to known *E. coli* sequences has indicated homology to the sequence of *E. coli PepQ*. The *opaA* and the *E. coli PepQ* genes have regions similar to human prolidase and *E. coli* aminopeptidase P. Although the OPAA2 enzyme and the *Flavobacterium* enzyme OPH have similar activities, no homology was found. Cheng et al. hypothesize that the natural role of the OPAA2 enzyme is bacterial peptide metabolism. A discussion of the natural role of these enzymes can be found in the following section.

Horne et al. (2002) isolated an enzyme (*opdA*) from *Agrobacterium* that hydrolyzes a variety of organophosphates. The gene (*opdA*) was sequenced and found to be 88% identical to the sequence for the *opd* gene. There are differences in substrate selectivity, with *opdA* hydrolyzing some important organophosphates more rapidly than the *opd* gene product.

Clearly, there is a diversity of related and unrelated OPA anhydrolases found in bacteria. The *opd* and *opdA* genes are clearly related sequences and share a common evolutionary ancestor. The OPAA2 enzyme is apparently quite different. The selection pressure resulting in enzymes with similar activities but quite different structures are not known. This situation clearly mimics the situation in eukaryotic organisms with at least two very different enzymes capable of hydrolyzing organophosphates.

11.8.2 Eukaryotic OPA Anhydrolases

The ability of crude extracts of the protozoan *Tetrahymena thermophila* to hydrolyze the organophosphate DFP was discovered by Landis et al. (1985). Purification of the *Tetrahymena* material was conducted with a Sephacryl S-200 and S-300 molecular sizing column using a fraction

volume of approximately half of that used in previous studies in order to increase resolution. Three repeatable peaks capable of the hydrolysis of DFP immediately became apparent. Upon the addition of Mn^{2+}, a fourth peak appeared. The activities were identified as *Tt* DFPase-1, *Tt* DFPase-2, …, *Tt* DFPase-5, and their characteristics can be found in Table 11.2. Molecular weights of the *Tetrahymena* OPA anhydrases range from 67,000 to 96,000 D. The activity of DFPase-4 is stimulated 17- to 30-fold with Mn^{2+}. *Tt* DFPase-1, *Tt* DFPase-2, and *Tt* DFPase-3 are only stimulated two- to fourfold, and part of this increase may be due to contamination by the higher molecular weight *Tt* DFPase-4. Soman-to-DFP ratios are approximately 1:1 for the *Tetrahymena* OPA anhydrases.

Mipafox is reversible and competitively inhibits *Tt* DFPase-1, *Tt* DFPase-2, and *Tt* DFPase-3 (Landis et al. 1989a,b,c). Hydrolysis of the mipafox by partially purified *Tetrahymena* extract was only 13% the rate of DFP.

Of all the conventionally recognized OPA anhydrases, the squid type as found in *Loligo pealei* is perhaps the best studied. The distribution of the squid type OPA anhydrase is relatively narrow, being found in only the nervous tissue, saliva, and hepatopancreas of cephalopods. The molecular weight of the squid type OPA anhydrase is approximately 23,000 to 30,000 D. The term *squid type* is specific to the activities found in these tissues. At times, more than one peak is apparent upon molecular sizing chromatography at this molecular weight range (Steinmann 1988). It has been estimated that the squid type OPA anhydrase constitutes approximately 0.002% of the intracellular protein (Hoskin 1989). Squid type OPA anhydrase does hydrolyze soman, although at a rate of only about 0.25 that of DFP. However, squid type OPA anhydrase apparently hydrolyzes all four stereoisomers of soman, with some stereospecificity in rates.

Mipafox is not inhibitory to the squid type OPA anhydrase. As reported by Gay and Hoskin (1979), the active site prefers an isopropyl side chain compared to an ethyl or methyl group.

The crystal structure of the DFPase from squid has been determined (Scharff et al. 2001). The gene for the enzyme was inserted into an *Escherichia coli* cloning system so that a large amount of the enzyme could be produced. The cloned enzyme has a molecular weight of 35 kD and is comprised of 314 amino acid residues. DFPase was resolved as a six-bladed propeller that has two Ca ions at its center. The active site residues were also identified and mapped.

Because of the stability of the enzyme, there has been extensive analysis of the structure of the squid type enzyme. Katsemi et al. (2005) has concluded that only specific residues in the active site are essential to the organophosphate hydrolyzing activity. Blum and Richards (2008) have detailed the current state of knowledge in an extensive review of enzymes that detoxify organophosphates and other chemical warfare agents. The squid type DFPase, and perhaps its mutants, is seen in this review as a promising tool for enzymatic detoxification of the organophosphate chemical agents.

Although the primary investigation into the OPA anhydrases of squid tissue has been of the squid type OPA anhydrase, squid does contain the more widespread Mazur type OPA anhydrase. Gill, heart, mantle, and blood tissues all exhibit OPA anhydrase activities that are Mn^{2+} stimulated and hydrolyze soman faster than DFP (Hoskin et al. 1984).

11.8.3 Characteristics of Other Invertebrate Metazoan Activities

Nervous tissue of a variety of invertebrates has been screened for OPA anhydrase activity. Other mollusks have been reported to contain OPA anhydrases, notably *Octopus*, *Anisodoris* (sea-lemon), *Aplysia* (sea-hare), and *Sepia* (cuttlefish) (Hoskin and Long 1972). *Sepia* and *Octopus* hydrolyze DFP faster than tabun, a squid type OPA anhydrase characteristic employed at that time, and now by the DFP-to-soman ratio. Conversely, *Aplysia*, *Spisula*, and *Homarus* (lobster) hydrolyze

Table 11.3 Comparison of DFP and Soman Hydrolysis Ratio and Stimulation by Mn^{2+} for Aquatic Organisms

	Soman-to-DFP Ratio		*Stimulation by Mn^{2+}*	
Enzyme Source	*Mn^{2+}*	*No Mn^{2+}*	*DFP*	*Soman*
Proteus vulgaris	19	22	1.5	1.3
Saccharomyces cerevisae	8	4	0.5	1.0
Homarus (lobster) nerve	11	9.1	2.9	3.4
Spisula (surf clam) nerve	6.1	3.0	1.0	2.0
Electrophorus electricus (torpedo fish) liver	16	14	1.7	1.8
T. thermophila (crude extract)	20	10	1.0	2.0

Source: Modified from Hoskin, F. C. G. et al., *Fund. Appl. Toxicol.*, 4, 5165–5172, 1984.

Note: Interestingly, *S. cerevisae*, *Spisula*, and *T. thermophila* do not show a stimulation in DFP hydrolysis with Mn^{2+}. Perhaps, like *T. thermophila*, the other two species have at least two enzymes, one that hydrolyzes soman and is stimulated by Mn^{2+}.

tabun faster than DFP (Hoskin and Brande 1973), a typically Mazur characteristic. Soman-to-DFP ratios and Mn^{2+} stimulation for several species are shown in Table 11.3. *Homarus* and *Spisula* were further examined by Hoskin et al. (1984). These organisms were found to have activities broadly defined as Mazur type, using Mn^{2+} stimulation and DFP-to-soman ratio as criteria. In *Spisula* (surf clam), DFP hydrolysis was not stimulated by Mn^{2+}, although soman hydrolysis was doubled. In light of research conducted since then, this result may indicate that more than one OPA anhydrase system is present.

Anderson et al. (1988) discovered an OPA anhydrase activity in the estuarine clam *Rangia cuneata*. The clams were collected from Chesapeake Bay sediment. Of the tissues examined, OPA anhydrase activity was highest in the digestive gland and lowest in the foot muscle (Anderson et al. 1988). Soman was hydrolyzed faster than DFP. Exogenous Mn^{2+} did not increase the rate of DFP hydrolysis, although soman hydrolysis was increased by 40% in the presence of 1 mM Mn^{2+}. The temperature range was determined to be from 15 to 50°C. The initial estimate of molecular weight was 22,000 D for the digestive gland, as determined by molecular sieve chromatography. Interestingly, the molecular weight for the OPA anhydrase from the visceral mass was higher, implying a different enzyme and some tissue specificity. Except for molecular weight, the clam activity appeared to more closely resemble that of Mazur type OPA anhydrase.

11.8.4 Characteristics of the Fish Activities

Hogan and Knowles (1968) examined the OPA anhydrases of liver homogenates from the bluegill sunfish, *Lepomis macrochirus*, and the channel catfish, *Ictalarus punctatus*. Initially, a 1.5% (w/v) homogenate of the excised livers from each species was determined to hydrolyze 10^{-2} M concentrations of DFP and 2,2-dichlorovinyl dimethyl phosphate (cochlorvos). Ninety percent of the activity was found in the supernatant after a 1-hour centrifugation at 100,000 G. For both species a Mn^{2+} concentration ranging from 0.3 to 1.0 mM was found to promote hydrolysis. Co^{2+} was

optimal at a concentration of 0.1 mM, but was inhibitory at concentrations greater than 1.0 mM. Mg^{2+} and Ca^{2+} had no detectable effect. For studies using other organophosphates, a 1 mM Mn^{2+} concentration was included in the reaction system.

Bluegill and catfish were both able to hydrolyze DFP, dichlorvos, and dimethyl 2,2,2-trichloro-1-*n*-butyryloxyethyl phosphonate (butonate). Catfish enzymes were also able to hydrolyze paraoxon, methyl 3-hydroxy-alpha-cronate, and dimethyl phosphate (mevinphos), although at a very slow rate. K_ms calculated for the enzymes of both species indicated that each had a greater affinity for DFP than dichorvos. Sulfhydryl reagents and Cu^{2+} were found to inhibit the enzymatic activity of both organisms. Paraoxon had no effect. Cleavage products were identified as dimethyl phosphate and 2,2-dichloroacetaldehyde from dichlorovos hydrolysis and diisopropyl phosphate from the hydrolysis of DFP.

The fish, *Electrophorus*, was examined by Hoskin et al. (1984) and found to have an activity that hydrolyzes soman faster than DFP and to be stimulated by Mn^{2+}. This activity may be similar to those of catfish and bluegill.

11.8.5 Comparison of the OPA Anhydrases

It is natural to wish to impose a classification scheme upon the OPA anhydrases that would imply a set of phylogenetic relationships. The classification scheme of squid type and Mazur type anhydrases has proven useful in that it was quickly possible to differentiate the squid type OPA anhydrase from the other forms. As will be seen below, many of the OPA anhydrase activities lie somewhere in between.

The multiple activities in *T. thermophila* share some of the characteristics of both the squid type OPA anhydrase and classical Mazur type OPA anhydrase found in hog kidney. In crude preparations, the OPA anhydrase activity has the characteristics of the hog kidney OPA anhydrase in that it hydrolyzes soman faster than DFP, is stimulated by Mn^{2+}, and is inhibited by mipafox. Further purification has revealed that the hydrolysis of soman and the stimulation of this hydrolysis by Mn^{2+} are principally due to the *Tt* DFPase-4. The *Tt* DFPase-1, *Tt* DFPase-2, and *Tt* DFPase-3 hydrolyze soman and DFP at approximately the same rates and demonstrate only moderate stimulation of soman hydrolysis by Mn^{2+}, and yet are inhibited by mipafox. The *Tetrahymena* OPA anhydrases fall within a narrow range; from 96,000 to 67,000 D. However, this range of molecular weights is larger than typically ascribed to the Mazur type enzymes. The *Tetrahymena* OPA anhydrases can be purified by ammonium sulfate precipitation, like the squid type OPA anhydrase.

Although possessing very similar kinetics and characteristics in homogenate form (Table 11.2), the OPA anhydrase activities of *T. thermophila* and *R. cuneata* are markedly different after even a simple purification. *R. cuneata* has a low molecular weight activity, *Rc* OPA-1, that is not inhibited by mipafox, and has a molecular weight close to that of the squid type OPA anhydrase. The clam also has a mipafox hydrolyzing activity that hydrolyzes mipafox faster than DFP.

The bacterial activities again point to the diversity of the OPA anhydrases. The OT strain JD.100 is able to degrade soman, sarin, and dimebu, but not DFP. The bacterial activities reported to date all seem insensitive to ammonium sulfate inhibitions and have molecular weights above that of the hog kidney OPA anhydrase.

The *opd* OPA anhydrase is smaller than the bacterial OPA anhydrase studied to date and has an apparent molecular weight of 60,000 to 65,000 D, with 35,000 D subunits. To date, the other bacterial OPA anhydrases have not been tested using paraoxon as a substrate, although JD6.5 hydrolyzes the related compound NPEPP.

Even though they are a diverse set of enzymes, some generalizations on the OPA anhydrases can be reached. Generally, the substrate range of the OPA anhydrases is quite broad. Sensitivity to ammonium sulfate is a characteristic found in only a few cases and not in those OPA anhydrases so far examined from aquatic organisms. Subunits have been demonstrated in the case of hog kidney and the *opd* OPA anhydrase, and may exist in the larger enzymes in *Tetrahymena*. A variety of OPA anhydrases seem to exist within an organism, be it a squid, *Tetrahymena*, clam, or bacteria. Differentiation among OPA anhydrases of various tissues has also been demonstrated.

To date, the active site of the Mazur type OPA anhydrases has not been mapped by x-ray crystallography, yet some indications of the topography can be made. The size of the leaving group does not seem to be important. Enzymes from *Tetrahymena*, the *opd* gene, and *R. cuneata* can hydrolyze compounds with both fluoride and nitrophenol leaving groups. It is as if the leaving group is perpendicular to the surface of the enzyme, with the remainder of the molecule inserted into the active site. If the mechanism for the *opd* OPA anhydrase can be generalized as an attack at the phosphorus by an activated water, the configuration may be important to catalytic activity. Indeed, small changes in side chains apparently make a tremendous difference: NPEPP is readily hydrolyzed by the *Tetrahymena* OPA anhydrases, but its close analog, NPIPP, is not. The squid type OPA anhydrase does not hydrolyze either the NPEPP or NPIPP. The squid type OPA anhydrase does hydrolyze the four isomers of soman at roughly comparable rates, showing a substrate tolerance of a different sort.

11.8.6 Natural Role of the OPA Anhydrases

An enzymatic activity that phylogenetically is as widespread as that of the OPA anhydrases must be important to the cellular metabolism and the survival of the organism. The strength of the selective pressure for the *opd* OPA anhydrase is evident: Divergent plasmids in *Pseudomonas* and *Flavobacterium* share identical *opd* gene sequences. The widespread nature of the OPA anhydrases also argues for a strong selective pressure over a much longer period than the last 45 years. However, the natural substrate and role(s) of the OPA anhydrases are unknown. Correlations to isethionate, pyruvate, and squid neurotoxin (Hoskin et al. 1984; Hoskin 1971; Hoskin and Brande 1973) exist, but no cause–effect relationship has been found. Generally unrecognized, however, is that many types of naturally occurring organophosphates have been identified from a variety of sources (Rosenburg 1964; Kitteridge and Roberts 1969; Rouser et al. 1963; Simon and Rouser 1967; Quin and Shelburn 1969; Neidleman and Geigert 1986). The alanine amino acid analog, 2-aminoethylphosphonic acid (AEP) (Figure 11.16), is synthesized by *Tetrahymena* (Rosenburg 1964). Other types of phosphonates are found free in cells, incorporated into glycerophosphonolipids, sphingophosphonolipids, and phosphonoproteins. The linkage of AEP to phosphonolipids appears to be covalent, although this has not been conclusively demonstrated (Rosenburg 1964; Kitteridge and Roberts 1969; Rouser et al. 1963; Simon and Rouser 1967; Quin and Shelburn 1969; Neidleman and Geigert 1986). It has been previously suggested that the OPA anhydrases are parts of a metabolic system handling the various organophosphonates incorporated into the cellular matrix and encountered in food sources (Landis et al. 1986, 1987, 1989c). That hypothesis must be expanded, as some OPA anhydrases may also be important in dehalogenation of naturally occurring halogenated organic compounds.

A wide variety of halogenated organics are also naturally occurring. Neidleman and Geigert (1986) reviewed the variety of halometabolites that naturally occur. Chlorotetracycline and chloramphenicol are two important chlorinated halometabolites. Fungi produce a variety of ringed and aromatic chlorinated organics. The richest known source of halometabolites is the

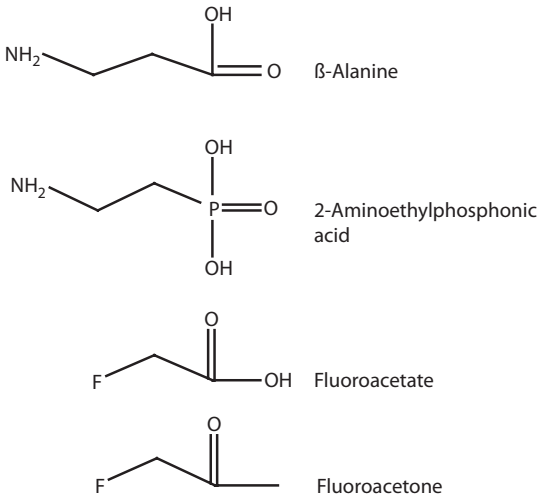


Figure 11.16 Natural substances similar to the substrates of the OPA anhydrases. AEP, naturally synthesized, is an organophosphate analog to the amino acid β-alanine. Several naturally synthesized fluorometabolites are known; fluoroacetate and fluorocitrate are two examples. OPA anhydrases may be involved in the metabolism of these or similar compounds.

marine algae, with approximately 20% of the extractable material being halogenated organics. Freshwater blue-green algae also produce halogenated molecules. The variety of halogenated molecules is amazing. The production of halogenated molecules is not restricted to microorganisms or plants, as marine animals also produce a variety of bromo-, chloro-, and iodosometabolites. Fluorometabolites are not as common but do occur, especially in higher plants. Fluoroacetate and fluorocitrate are synthesized by a number of plants (Figure 11.16). Fluorinated fatty acids are found in the seeds of *Dichapetalum toxicarium*. The fungi *Streptomyces calvus* produces the fluorinated antibiotic nucleocidin, an adenosine analog. The number of fluorinated organics may even be larger than those currently identified because of the difficulty of distinguishing a C-F bond from a C-H bond (Neidleman and Geigert 1986). With the use of F electrodes, mass spectrometry, and ion chromatography, the list of fluorinated organics and their degradation products is certain to grow.

Neidleman and Geigert (1986) also review the evidence that halometabolites are used as chemical defense in marine and perhaps other organisms. These organisms range from a green algae, *Avrainvillea longicalulis*, to the Nudibranch mollusk, *Diaulula sandiegensis*. One of the more interesting speculations of Neidleman and Geigert is the role that toxic halogenated compounds may play in prey-predator interactions. Perhaps the synthesis of active halogenated compounds is sufficiently damaging to a predator to reduce the efficiency of the predation or to kill the predator. Competitive relationships among microorganisms may also be mediated by the production of halogenated organics. Detection of the very low concentrations of these molecules appears to be the major stumbling block in further elucidating the role of halogenated organics in predator–prey and competitive relationships.

Study Questions

1. What is biotransformation of an environmental chemical? Where does it occur?
2. What are phases I and II in the process of xenobiotic metabolism?
3. Describe the NADPH–cytochrome P-450 system.
4. Hepatic enzymes that catalyze phase I and II reactions perform what functions in addition to detoxifying xenobiotics?
5. Discuss the conversion of xenobiotics to reactive electrophilic species by hepatic biotransformation mechanisms.
6. Many microorganisms have the ability to use xenobiotics for what purpose?
7. Discuss the genetic information contained in microorganisms, including their functions and origins.
8. Discuss the aerobic metabolism of organic xenobiotics.
9. How could the degradative capability of microorganisms be enhanced?
10. How is biodegradation of a substance measured? What nonspecific methods can be used as alternatives?
11. Describe the degradation of PCP by bacteria and fungi.
12. How can biodegradation be used for remediation?
13. Explain the use of a bioreactor as a bioremediation tool. What factors determine the success of the bioremediation attempt?
14. Discuss the isolation and engineering of degradative organisms.
15. What are OPA anhydrolases?
16. The squid type DFPase has had its structure determined. Why is this enzyme considered so interesting?
17. Summarize the hypothesized natural role of the OPA anhydrolases.

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Chapter 12

Ecological Effects from Biomarkers to Populations

12.1 Introduction

The next two chapters deal with perhaps the most difficult topic in environmental toxicology: how to measure and then evaluate the impact of toxicants in ecological systems at a variety of scales. This chapter starts with an evaluation of methods and ends with a discussion of the effects that can be seen at the population scale. First, we begin with a discussion of terminology and a word about context.

12.2 Terminology and Context

Occasionally, this chapter uses nonstandard terminology and does so for a reason. Words or terminology often represent implicit models that may be inaccurate and lead to unwarranted extrapolations. Much of the terminology in ecology and environmental toxicology was put in place when equilibrium-based Clementian ecology was the rule, and as discussed in Chapter 2, that is a poor model given our current understanding. Here are some examples of such terminology.

The term *ecosystem* has buried within it the implication of a system. Systems are in general designed structures, but in this case who was the designer? Phone systems, road systems, educational systems, and so forth, are human designed. The digestive system, reproductive system, and central nervous system are built upon a blueprint incorporated into the organism's DNA. That blueprint was itself derived from evolutionary process—hence a blueprint but no designer. Where is the blueprint or evolutionary process in the case of an ecosystem? What are termed ecosystems are the result of environmental constraints, history, and the evolutionary processes that led to the current interacting assemblage of organisms present in a location. A number of processes do occur—nitrogen fixing, photosynthesis, predator-prey interactions, and decomposition—but these features are not set by a blueprint. Often in this chapter, the terms *ecological system* or *ecological structure* are used as an alternative that does not imply blueprint or design.

Instead of *level* of biological organisms I often use the term *scale*. At certain scales an inherited biological blueprint or specification is clearly present and is incorporated into the organism's

genome. At other scales a blueprint does not exist and the apparent organization is an outcome of history, chance, and the features of the landscape.

This chapter concentrates on effects that are not limited to an organism or a jar but occur within an ecological context. Throughout the discussion that follows, context should always be part of the conceptual framework. Where population-scale effects are discussed, the focus of the presentation will be on population dynamics. However, what occurs is also driven by the genetics and physiology of the individual components of the population and by the surrounding organisms, physical processes, and shape of the landscape that contains that population. In an ecological context organisms and populations are subject to multiple stressors with a variety of temporal and spatial characteristics. Even when discussing biomarkers at a molecular scale, ecological context should not be forgotten.

The issue of the importance of context is well illustrated by the example of the Cherry Point site along the coast of Washington State. The scale within which most toxicological interactions take place is the individual, in this case the individual egg as seen in Figure 12.1a. At this scale the egg has been laid upon seagrass and is surrounded by millions of other eggs. At the time the photo was taken the tide was out, exposing the egg to the air and predators. The egg is also likely to contain persistent organic pollutants transferred from the female fish. However, an emphasis on this scale does not place the issue of the potential effects on Pacific herring populations or effects to the ecological structures of the Cherry Point reach into context.

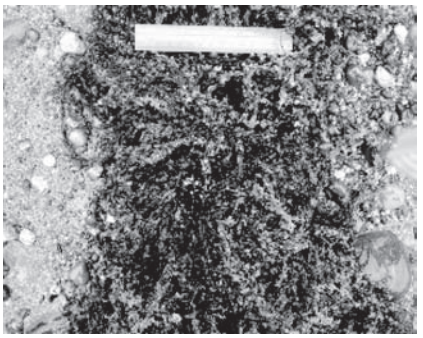
At the scale observable by a human of normal height, many more factors come into view that place the question of ecological effects into context (Figure 12.1b). In this picture the rocky nature of the beach at Cherry Point is easily visible and a road runs alongside the area. In the distance a pier is visible and a tanker ship is docked at the site. If the water were transparent, the steep slope of the coastline and the presence of rock, debris, and marine organisms would be apparent. At some areas along this coastline pipes discharging storm water runoff can be observed along with illegal modifications of the shoreline.

At 1,000 m altitude more of the context of the site can be observed (Figure 12.1c.). Three industrial facilities are visible, along with different uses of the landscape for agriculture and residential areas that can be seen in the far distance. Each of these activities and land uses can produce contaminants that may interfere with the growth of the Pacific herring eggs or alter the ecological properties of the region. However, even the view at this altitude does not incorporate all of the habitat that the Pacific herring that spawn at Cherry Point use.

The last graphic (Figure 12.1d) is a Google Earth presentation of the Salish Sea region used by the Pacific herring at Cherry Point. Parts of this graphic are in Canada; the city of Vancouver is visible as well as Bellingham. There are a number of urban and residential areas depicted. In order to understand the context of the Pacific herring at Cherry Point, along with the other ecological resources in the region, it is necessary to understand, at least to a degree, the context depicted in this map. In order to accomplish this understanding, a robust model is required to frame the questions, the collection of data, and the final analysis.

12.3 The Key to Context: The Hierarchical Patch Dynamics Paradigm

One of the reasons that I often use nonstandard terminology is because of the incorporation of the hierarchical patch dynamics paradigm (HPDP) as an overarching model. The HPDP was introduced



Pacific herring eggs
(a)

Cherry Point and tanker
(b)

Aerial photograph of Cherry Point region looking to the south
(c)

Google Earth map of the region of the Salish Sea surrounding Cherry Point
(d)

Figure 12.1 (a) Scale diagram for Cherry Point. A variety of scales need to be considered when dealing with ecological effects. At the scale of the Pacific herring eggs, a few centimeters is all that is apparent. (Photo by Amanda Seebach.) (b) As the scale becomes larger the rocky beach at Cherry Point becomes apparent, as does the tanker ship. (Photo by April Markiewicz.) (c) At a still larger scale the multiple uses of the landscape and the multiple industrial and residential sites can be observed. (Photo by Linda S. Landis.) (d) Finally, at the scale that is used by the adult Pacific herring, a number of cities, islands, and other features become apparent. A central issue in describing the effects of chemicals is the scale that is being addressed. (See color insert following page 268.)

in Chapter 2 as a potential model for understanding the impacts of chemicals or other stressors on the environment. In this chapter we are going to explore this approach in greater detail.

The initial development of the HPDP by Wu and Loucks (1995) was in reaction to dissatisfaction with the models describing the dynamics of ecological systems that were available in the mid-1990s. Table 12.1 presents a summary of each model and its predictive utility. One of the advantages of the HPDP is that it can easily incorporate individuals, populations, communities, and landscape structures and innately considers the spatial and temporal dynamics. The next several paragraphs describe in more detail the application of HPDP to environmental toxicology.

Table 12.1 A Comparison of Different Perspectives in Ecology Complexity and Stability, Indicating Criteria Met Satisfactorily by the Hierarchical Patch Dynamic Paradigm (see text for detailed discussions)

Perspective	*Balance of Nature*	*Equilibrium/Static Stability*	*Nonequilibrium/ Instability*	*Multiple Equilibria/ Homeorhesis*	*Hierarchical Patch Dynamics*
Information source	Belief and qualitative data	Theoretical and mathematical	Empirical and mathematical	Mathematical and empirical	Theoretical and empirical
Scope and generality	Broad scope and general	Broad scope but specific	Broad scope but specific	Case-by-case scope	Broad scope, probably general
Extent of testing	Untestable	Testable only recently, failing	Testable	Relatively untestable	New, testing ongoing
Notes	Model is not testable and therefore not credible; however, model is strong in popular culture and in decision making	Generally not useful as a model of ecological structures, static equilibrium not observed in nature; however, the assumption of stable equilibrium is often an assumption in environmental toxicology	Unsatisfactory, limited by scale and does not explain the persistence of some patterns within landscapes	Satisfactory but limited by scope and its ability to be tested; not clear how it would be applied to environmental toxicology	Satisfactory as a conceptual framework; high potential for explaining patterns at multiple scales; has been used as an organizing principle in a number of studies; both community conditioning and pollution-induced community tolerance would fit within this framework (see Chapter 13). Approach can be used in risk assessment (see Chapter 14)

Source: Based on Wu, J., and Loucks, O. L., *Q. Rev. Biol.*, 70, 439–466, 1995, with additional commentary.

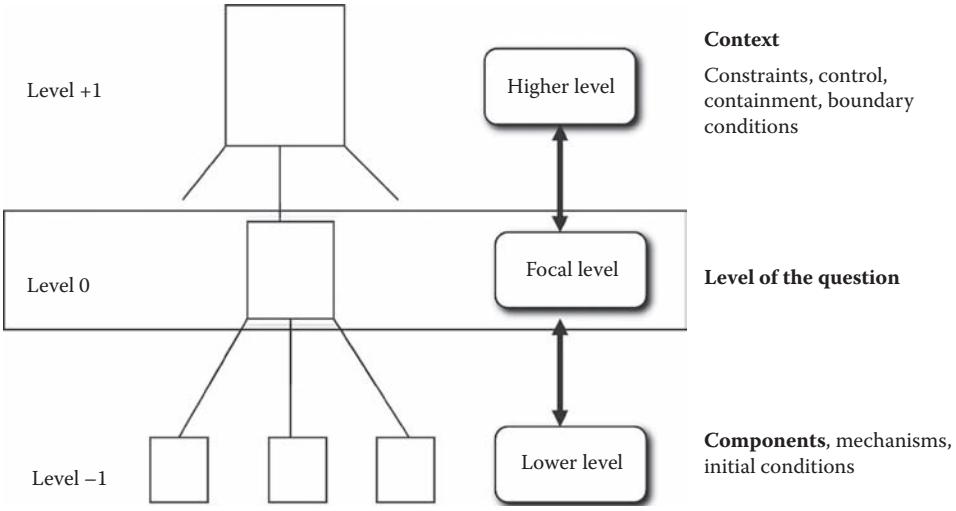


Figure 12.2 HPDP basic diagram.

The fundamental structure of the HPDP is presented in Figure 12.2. There are several hierarchical steps in this formulation. The focal level is level 0 and is the part of the structure representing the particular question at hand. If the question is focused on the persistence of a population, then that population is at the focal level. Often the focus of a study is on a particular assemblage of organisms, such as soil invertebrates or the fish species indigenous to a region. These types of items are then placed at the focal level.

Items that are level +1, or the higher level, are those that constitute constraints, controls, and boundary conditions. The organisms of interest may only be found in patches of suitable habitat within a landscape, and this constitutes a boundary condition. In my application of the HPDP I often consider level +1 variables such as long-range transport of volatile materials and persistent organic pollutants spread throughout the tissue, soil, and sediment, since both of these set certain boundary conditions for the system. Predators within an ecological structure, the availability of prey, and climate are all boundary conditions and are suitable level +1 factors.

The level –1, or lower-level, factors are mechanisms and initial conditions. Many biomarkers that indicate a mode of action may have been initiated fit into this level. The inhibition of acetylcholinesterase would be a mechanism for the death of an organism, altering the dynamics of a population, and a level –1 factor.

It should not be misunderstood that these diagrams imply a certain level of information flow. For example, let us consider the effects of carbamates and organophosphates upon fish populations in freshwater stream systems. The distribution of the carbamate and the organophosphates is a feature of the landscape and poses certain boundary conditions on the populations under consideration. These factors are at a level +1 scale. The organisms take up the organics, synergism in the inhibition of acetylcholinesterase occurs, and adult fish die or have altered behaviors precluding successful spawning. These features can be found at level –1. The decline of the population would be the effect seen at level 0, but this may also be modified by the occurrence of other patches of fish within the landscape, a level +1 feature.

The lines of connections are drawn solid in the diagrams but should not be considered permanent in either existence or strength, nor is there an implication of a purely deterministic relationship.

These interactions should be considered highly dynamic and not constant in rate. They can change vertically and horizontally and may be altered by factors external to the model. Figure 12.3 is my attempt to illustrate these relationships. The swirling arrows denote the dynamic nature of the interactions within the HPDP structure. The vertical structure is assumed to be asymmetric with a loose coupling and a variety of principles that produce the patterns observed at the various levels. The horizontal structure is assumed to be more symmetrical in its relationships, but the coupling is again loose and the strengths of the interactions changing and not symmetrical.

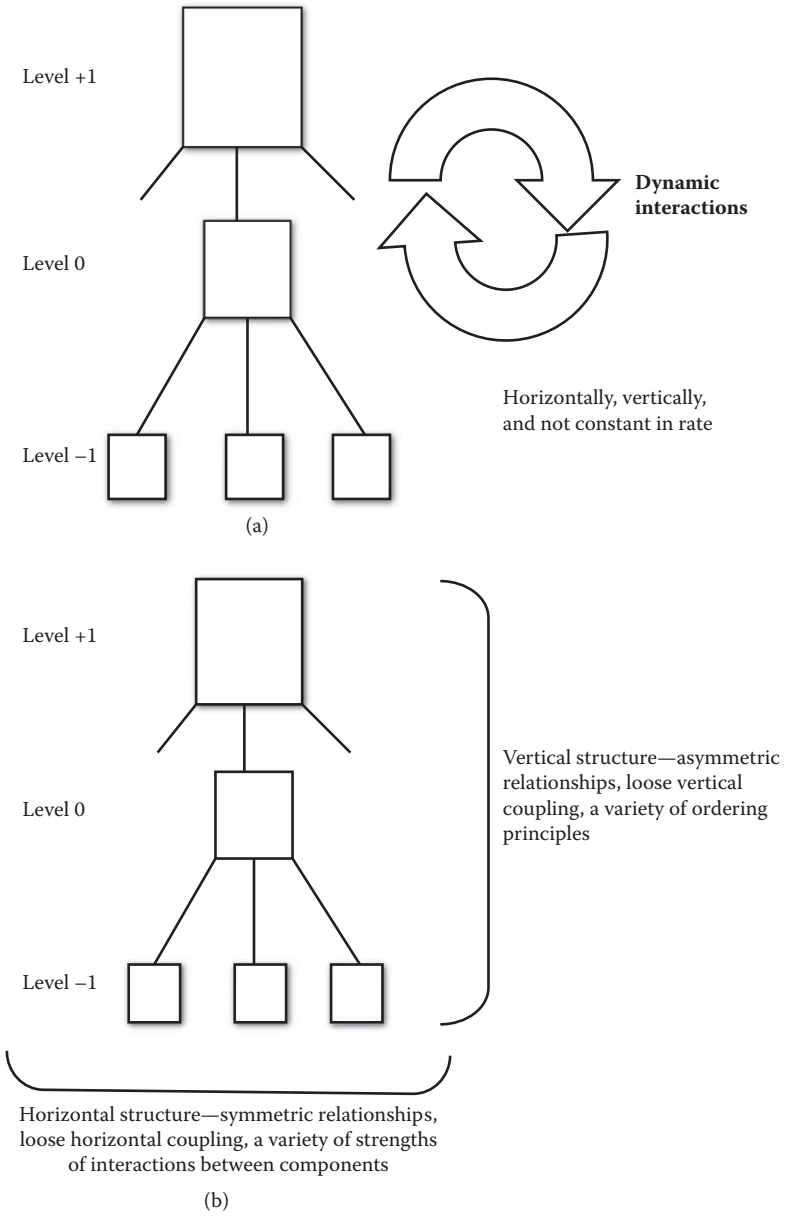


Figure 12.3 The dynamic interactions and connections of the HPDP.

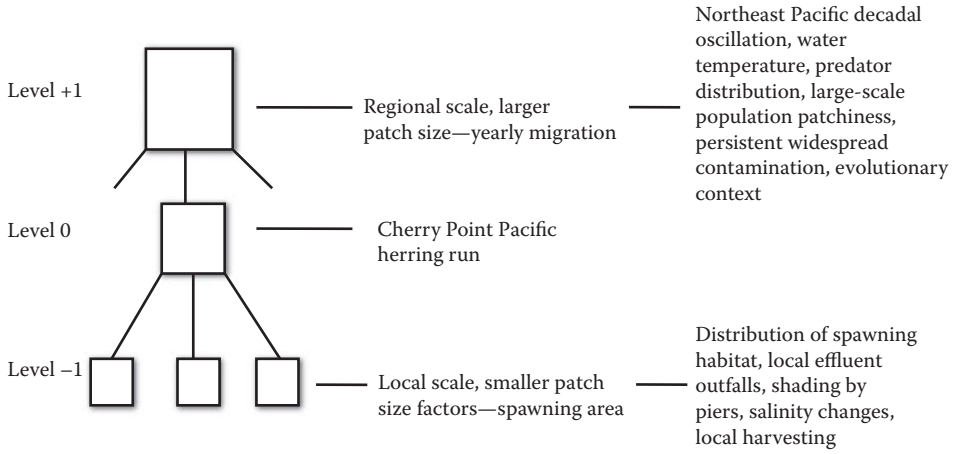


Figure 12.4 The HPDP applied to the Cherry Point Pacific herring run.

As discussed in Chapter 2, the HPDP has proven adaptable to a number of scenarios in environmental toxicology. Figure 12.4 portrays how the HPDP would be used to examine the effects of various changes to the environment to the Pacific herring at Cherry Point. Features such as boundary conditions, water temperature, persistent organic pollutants, and predator distributions are at level +1. These features also tend to be at a larger spatial scale, often covering the entire Salish Sea, as depicted in Figure 12.1d. The items at level –1 are at a smaller spatial scale, such as the location of pollution outfalls, local changes in salinity, harvesting of the fish, and local pollution events. The Cherry Point Pacific herring run is at level 0 since it is the object of our question.

The HPDP has been adapted by my research team to studies involving invasive species, the effects of fire to forests, the interaction between disease and toxicants, and the spread of infectious agents in endangered populations. As you continue in the next two chapters, one of the primary questions you should be asking is: Where does the effect or measurement discussed fit into an HPDP framework?

12.4 Measurement of Ecological Effects at Various Scales or Levels of Biological Organization

Biomonitoring is a term that implies a biological system is used in some way for the evaluation of the current status of an ecosystem. Validation of the predictions derived from the elaborate series of tests can only be done by effective monitoring of ecosystems (Landis 1991). In general, biomonitoring programs fall into two categories: exposure and effects. Many of the traditional monitoring programs involve the analytical measurement of a target compound with the tissue of a sampled organism. The examination of pesticide residues in fish tissues or polychlorinated biphenyls (PCBs) in terrestrial mammals and birds are examples of this application of biomonitoring. Effects monitoring looks at various steps of biological scale in order to evaluate the status of the biological community. Generically, effects monitoring allows a toxicologist to perform an evaluation without an analytical determination of any particular chemical concentration. Synergistic and antagonistic interactions within complex mixtures are integrated into the biomonitoring response.

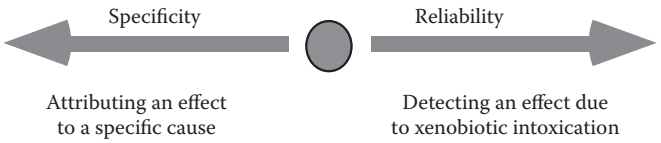


Figure 12.5 The tug of war in biomonitoring. An organismal or community structure monitoring system may pick up a variety of effects but lack the ability to determine the precise cause. On the other hand, a specific test, such as looking at the inhibition of a particular enzyme system, may be very specific but completely miss other modes of action.

In the biomonitoring process, there is the problem of balancing specificity with the reliability of seeing an impact (Figure 12.5). Specificity is important since it is crucial to know and understand the causal relationships in order to set management or cleanup strategies. However, an increase in specificity generally results in a focus on one particular class of causal agent and effects, and in many cases chemicals are added to ecosystems as mixtures. Emphasis upon a particular causal agent may mean that effects due to other materials can be missed. A tug of war exists between specificity and reliability.

There is a continuum of monitoring points along the path that an effect on an ecosystem takes from introduction of a xenobiotic to the biosphere to the final series of effects (Chapter 2). Techniques are available for monitoring at each level, although they are not uniform for each class of toxicant. It is possible to outline the current organizational levels of biomonitoring:

- Bioaccumulation/biotransformation/biodegradation
- Biochemical monitoring
- Physiological and behavioral
- Population parameters
- Community parameters
- Ecosystem effects

A graphical representation of the methods used to examine each of these levels is depicted in Figure 12.6.

Many of these levels of effects can be examined using organisms native to the particular environment, or exotics planted or introduced by the researcher. There is an interesting trade-off for which species to use. The naturally occurring organism represents the population and the ecological community that is under surveillance. There is no control over the genetic background of the observed population, and little is usually known about the native species from a toxicological viewpoint. Introduced organisms, either placed by the research or enticed by the creation of habitat, have the advantage of a database and some control over the source. Questions dealing with the realism of the situation and the alteration of the habitat to support the introduced species can be raised.

It may also prove useful to consider a measure of biomonitoring efficacy as a means to judge biomonitoring. Such a relationship may be expressed in the terms of a safety factor as

$$E = \frac{U_i}{B_i} \tag{12.1}$$

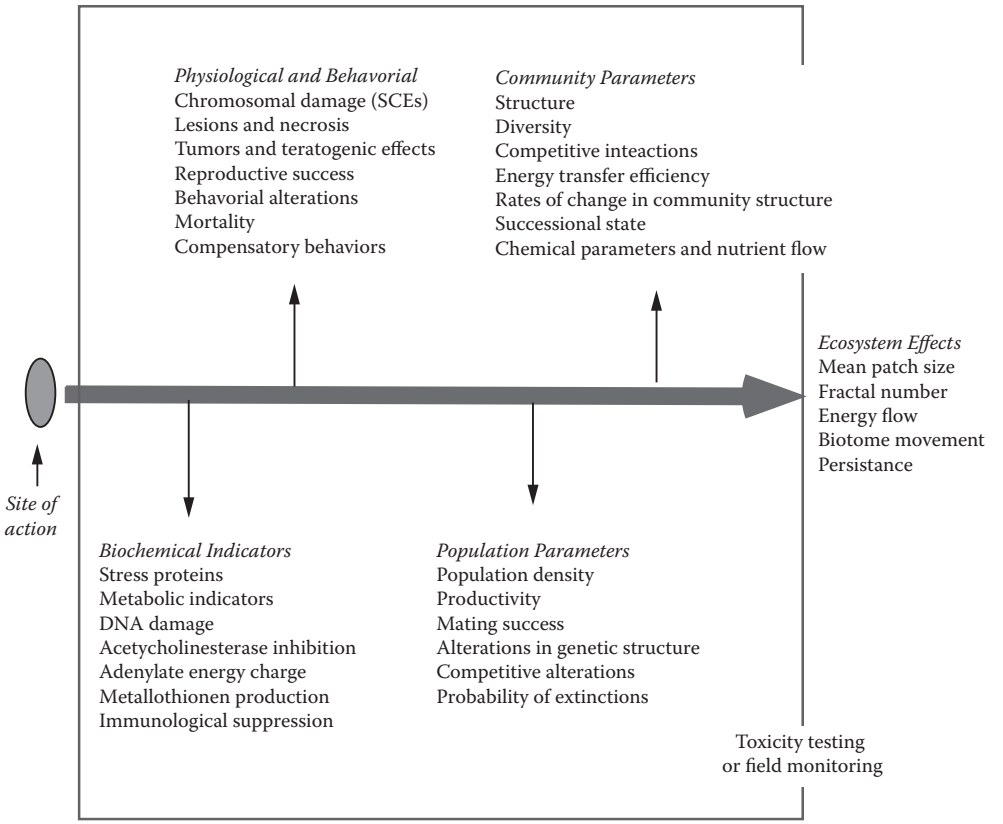


Figure 12.6 Methods and measurements used in biomonitoring for ecological effects. A number of methods are used both in a laboratory situation and in the field to attempt to classify the effects of xenobiotics upon ecological systems. Toxicity tests can be used to examine effects at several levels of biological organization and can be performed with species introduced as monitors for a particular environment.

where E is the efficacy of the biomonitoring methodology, U_i is the concentration at which undesirable effects upon the population or ecosystem in system i occur, and B_i is the concentration at which the biomonitoring methods can predict the undesirable effects in system i. The usefulness of such an idea is that it measures the ability to predict a more general effect. Methods that can predict effects rather than observe detrimental impacts are under development. Several of the methods discussed below are developments that may have a high efficacy factor.

12.5 Bioaccumulation/Biotransformation/Biodegradation

Much can occur to the introduced pesticide or other xenobiotic from its introduction to the environment to its interaction at the site of action. Bioaccumulation often occurs with lipophillic materials. Tissues or the entire organism can be analyzed for the presence of compounds such as PCBs and halogenated organic pesticides. Often the biotransformation and degradation products can be detected. For example, DDE is often an indication of past exposure to DDT. With the

advent of DNA probes it may even be possible to use the presence of certain degradative plasmids and specific gene sequences as indications of past and current exposure to toxic xenobiotics. Biosensors are a new analytical tool that also may hold promise. In this new class of sensors a biological entity such as the receptor molecule or an antibody for a particular xenobiotic is bound to an appropriate electronic sensor. A signal can then be produced as the material bound to the chip interacts with the toxicant.

One of the great advantages to the analytical determination of the presence of a compound in the tissue of an organism is the ability to estimate exposure of the material. Although exposure cannot necessarily be tied to effects at the population and community levels, it can assist in confirming that the changes seen at these levels are due to anthropogenic impacts and are not natural alterations. The difficulties in these methods lie in the fact that it is impossible to measure all compounds. Therefore, it is necessary to limit the scope of the investigation to suspect compounds or to those required by regulation. Compounds in mixtures can be at low levels, even those not detected by analytical means, yet in combination can produce ecological impacts. It should always be noted that analytical chemistry does not measure toxicity. Although there is a correspondence, materials easily detected analytically may not be bioavailable, and conversely, compounds difficult to measure may have dramatic effects.

12.6 Molecular and Physiological Indicators of Chemical Stress Biomarkers

A great deal of research has been done on the development of a variety of molecular and physiological tests to be used as indicators and perhaps eventually predictors of the effects of toxicants.

McCarthy and Shugart (1990) have published a book reviewing in detail a number of biomarkers and their use in terrestrial and aquatic environments. The collective term *biomarkers* has been given to these measurements, although they are a diversified set of measurements ranging from DNA damage to physiological and even behavioral indices. To date, biomarkers have not proven to be predictive of effects at the population, community, or ecosystem levels of organizations. However, these measurements have demonstrated some usefulness as measures of exposure and can provide clinical evidence of causative agent. The predictive power of biomarkers is currently a topic of research interest.

Biomarkers have been demonstrated to act as indicators of exposure (Fairbrother et al. 1989). Often, specific enzyme systems are inhibited by only a few classes of materials. Conversely, induction of certain detoxification mechanisms, such as specific mixed-function oxidases, can be used as an indication of the exposure of the organism to specific agents, even if the agent is currently below detectable levels. Additionally, the presence of certain enzymes in the blood plasma, which is generally contained in a specific organ system, can be a useful indication of lesions or other damage to that specific organ. These uses justify biomarkers as a monitoring tool even if the predictive power of these techniques has not been demonstrated. The following discussion is a brief summary of the biomarkers currently under investigation.

12.6.1 Enzymatic and Biochemical Processes

The inhibition of specific enzymes such as acetylcholinesterase has proven to be a popular biomarker—and with justification. The observation is at the most basic level of toxicant–active site

interaction. Measurement of acetylcholinesterase activity has been investigated for a number of vertebrates, from fish to birds to man. It is also possible to examine cholinesterase inhibition without the destruction of the organism. Blood plasma acetyl and butyl cholinesterase can be readily measured. The drawback to using blood samples is the intrinsic variability of the cholinesterase activity in the blood due to hormonal cycles and other causes. Brain cholinesterase is a more direct measure, but requires sacrifice of the animal. Agents exist that can enhance the recovery of acetylcholinesterase from inhibition by typical organophosphates, providing a measure of protection due to an organophosphate agent.

Not only are enzyme activities inhibited, but they can also be induced by a toxicant agent. Quantitative measures exist for a broad variety of these enzymes. Mixed-function oxidases are perhaps the best studied, with approximately 100 now identified from a variety of organisms. Activity can be measured or the synthesis of new mixed-function oxidases may be identified using antibody techniques. DNA repair enzymes can also be measured, and their induction is an indication of DNA damage and associated genotoxic effects.

Not all proteins induced by a toxicant are detoxification enzymes. Stress proteins are a group of molecules that have gathered a great deal of attention in the last several years as indicators of toxicant stress. Stress proteins are involved in the protection of other enzymes and structure from the effects of a variety of stressors (Bradley 1990). A specialized group, the heat shock proteins (hsps), are a varied set of proteins with four basic ranges of molecular weights: 90, 70, 58–60, and 20–30 kDa. A related protein, ubiquitin, has an extremely small molecular weight, 7 kDa. Although termed heat shock proteins, stressors other than heat are known to induce their formation. The exact mechanism is not known. Other groups of stress-related proteins are also known. The glucose-regulated proteins have a molecular weight of 75 to 100 kDa and form another group of proteins that respond to a variety of stressors.

The stress-related proteins are induced by a variety of stressors. However, other groups of proteins are induced by specific materials. Metallothioneins are proteins that are crucial in reducing the effects of many heavy metals. Originally evolved as important players in metal regulation, these proteins sequester heavy metals and thereby reduce the toxic effects. Metallothioneins are induced and, like many proteins, can be identified using current immunological techniques.

At an even more fundamental level there are several measurements that can be made to examine damage at the level of DNA and the associated chromosomal material (Shugart 1990; Powell and Kocan 1990). DNA strand breakage, unwinding of the helix, and even damage to the chromosomal structure can be detected. Formation of micronuclei as remnants of chromosomal damage can be observed. Some toxics bind directly to the DNA, causing an adduct to form. Classical mutagens can actually change the sequence of the nucleotides, causing deletions or other types of damage.

Immunological endpoints can provide evidence of a subtle but crucial indication of a chronic impact to an organism or its associated population (Anderson 1975; Anderson et al. 1981). Most organisms have cells that perform immunological functions, and perhaps the most common are the many types of macrophages. Toxicants can either enhance or inhibit the action of macrophages in their response to bacterial challenges. Rates of phagocytosis in the uptake of labeled particles can be used as an indicator of immune activation or suppression. Macrophages recently obtained from the organisms under examination can be examined as they pass through microscopic pores, as they are attracted to a bacterial or other immunological stimulus. Macrophage immunological response is widespread and an important indicator of the susceptibility of the test organisms to disease challenges.

Birds and mammals have additional immunological mechanisms and can produce antibodies. Rates of antibody production, the existence of antibodies against specific challenges, and

other measures of antibody-mediated immunological responses should prove useful in these organisms.

12.6.2 Physiological and Histological Indicators

Physiological and behavioral indicators of impact within a population are the classical means by which the health of populations is assessed. The major drawback has been the extrapolation of these factors based upon the health of an individual organism, attributing the damage to a particular pollutant and extrapolating this to the population level.

As described in earlier chapters, toxicants can cause a great deal of apparent damage that can be observed at the organismal level. Animals often exhibit deformations in bone structure, damage to the liver and other organs, and alterations in bone structure at the histological and morphological levels. Changes in biomass and overall morphology can also be easily observed. Alterations to the skin and rashes are often indicators of exposure to an irritating material. Plants also exhibit readily observed damage that may be linked to toxicant impact. Plants can exhibit chlorosis, a fading of green color due to the lack of production or destruction of chlorophyll. Necrotic tissues can also be found on plants and are often an indicator of airborne pollutants. Histological indicators for both plants and animals include various lesions, especially due to irritants or materials that denature living tissue. Cirrhosis is often an indication of a variety of stresses. Parasitism at abnormally high levels in plants or animals also indicates an organism under stress.

Lesions and necrosis in tissues have been the cornerstone of much environmental pathology. Gills are sensitive tissues and often reflect the presence of irritant materials. In addition, damage to the gills has an obvious and direct impact upon the health of the organism. Related to the detection of lesions is the detection of tumors. Tumors in fish, especially flatfish, have been extensively studied as indicators of oncogenic materials in marine sediments. Oncogenesis has also been extensively studied in medaka and trout as a means of determining the pathways responsible for tumor development. Development of tumors in fish more commonly found in natural communities should follow similar mechanisms. As with many indicators used in the process of biomonitoring, relating the effect of tumor development to the health and reproduction of a wild population has not been as closely examined as the endpoint.

Blood samples and general hematology are additional indicators of organisms with organ damage or metabolic alterations. Anemia can be due to a lack of iron or an inhibition of hemoglobin synthesis. Abnormal levels of various salts, sodium, potassium, or metals such as calcium, iron, copper, or lead can give direct evidence as to the causative agent.

Perhaps most promising in a clinical sense is the ability to detect enzymes present is the blood plasma due to the damage and subsequent lesion of organs. Several enzymes, such as lactic acid dehydrogenases (LDHs), are specific as to the tissue. The presence of an enzyme not normally associated with the blood plasma can provide specific evidence for organ system damage and perhaps an understanding of the toxicant.

Cytogenetic examination of miotic and mitotic cells can reveal damage to genetic components of the organism. Chromosomal breakage, micronuclei, and various trisomies can be detected microscopically. Few organisms, however, have the requisite chromosomal maps to accurately score more subtle types of damage. Properly developed, cytogenetic examinations may prove to be powerful and sensitive indicators of environmental contamination for certain classes of materials.

Molecular and physiological indicators do offer specific advantages in monitoring an environment for toxicant stressors. Many enzymes are induced or inhibited at low concentrations. In addition, the host organism samples the environment in an ecologically relevant manner for that

particular species. Biotransformation and detoxification processes are included within the test organism, providing a realistic metabolic pathway that is difficult to accurately simulate in laboratory toxicity tests used for biomonitoring. If particular enzyme systems are inhibited, it is possible to set a lower limit for environmental concentration given the kinetics of the site of action–toxicant interaction are known. The difficulties with molecular markers, however, must be understood. In the case of stress proteins and their relatives, they are induced by a variety of anthropogenic and natural stressors. It is essential that the interpretation is made with as much detailed knowledge of the normal cycles and natural history of the environment as possible. Likewise, immunological systems are affected by numerous environmental factors that are not toxicant related. Comparisons to populations at similar but relatively clean reference sites are essential to distinguish natural from anthropogenic stressors. Shugart (1990) has long maintained that a variety of molecular markers be sampled, thereby increasing the opportunities to observe effects and examine patterns that may tell a more complete story.

An example of using a suite of biomarkers is the investigation of Theodorakis et al. (1992) using bluegill sunfish and contaminated sediments. Numerous biomarkers were used, including stress proteins, ethoxyresorufin-*O*-deethylase activity (EROD), liver and spleen somatic indices, and DNA adducts and strand breaks, as examples. Importantly, patterns of the biomarkers in the laboratory bluegills were similar to those of the native fish taken from contaminated areas. Some of the biomarkers responded immediately, such as the ATPase activities of intestine and gill. Others were very time dependent, such as EROD and DNA adducts. These patterns should be considered when attempting to extrapolate to population or higher-level responses.

Currently, it is not possible to accurately transform data gathered from molecular markers to predict effects at the population and community levels of organization. Certainly, behavioral alterations caused by acetylcholinesterase inhibitors may cause an increase in predation or increase the tendency of a parent to abandon a brood, but the long-term populational effects are difficult to estimate. In the estimation and classification of potential effects it may be the pattern of indicators that is more important than the simple occurrence of one.

12.6.3 Toxicity Tests and Population Level Measures

Perhaps the most widely employed method of assessing potential impacts upon ecological systems has been the array of effluent toxicity tests used in conjunction with National Pollution Discharge Elimination System (NPDES) permits. These tests are now being required by a number of states as a means of measuring the toxicity of discharges into receiving waters. Often the requirements include an invertebrate such a *Ceriodaphnia*, acute or chronic tests, toxicity tests using a variety of fish, and in the case of marine discharges, echinoderm species. These tests are a means of directly testing the toxicity of the effluent, although specific impacts in the discharge area have been difficult to correlate. Since the tests require a sample of effluent and take several days to perform, continuous monitoring has not proven successful using this approach.

Although not biomonitoring in the sense of sampling organisms from a particular habitat, the use of the cough response and ventilatory rate of fish has been a promising system for the prevention of environmental contamination (van der Schalie 1986). Pioneered at Virginia Polytechnic Institute and State University, the measurement of the ventilatory rate of fish using electrodes to pick up the muscular contractions of the operculum has been brought to a very high stage of refinement. It is now possible to continually monitor water quality as perceived by the test organisms with a desktop computer analysis system at relatively low cost. Although the method has now been available for a number of years, it is not yet in widespread use.

This reaction of the fish to a toxicant has promise over conventional biomonitoring schemes in that the method can prevent toxic discharges into the receiving environment. Samples of the effluent can be taken to confirm toxicity using conventional methods. Analytical processes can also be incorporated to attempt to identify the toxic component of the effluent. Drawbacks include the maintenance of the fish facility, manpower requirements for the culture of the test organisms, and the costs of false positives. Again, the ecological relevance of such subtle physiological markers can be questioned; however as a sensitive measure of toxicity, such as the cough response has proven successful in several applications.

An ongoing trend in the use of toxicity tests designed for the monitoring of effluents and receiving waters has been in the area of toxicity identification evaluation and toxicity reduction evaluation (TIE/TRE). TIE/TRE programs have as their goal the reduction of toxicity of an effluent by the identification of the toxic component and subsequent alteration of the manufacturing or waste treatment process to reduce the toxic load. Generally an effluent is fractioned into several components by a variety of methods. Even such gross separations as into particulate and liquid phase can be used as the first step to the identification of the toxic material. Each component of the effluent is then tested using a toxicity test to attempt to measure the fraction generating the toxicity. The toxicity test is actually being used as a bioassay or a measure using biological processes of the concentration of the toxic material in the effluent. Once the toxicity of the effluent has been characterized, changes in the manufacturing process can then proceed to reduce the toxicity. The effects of these changes can then be tested using a new set of fractionations and toxicity tests. In some cases simply reducing ammonia levels or adjusting ion concentrations can significantly reduce toxicity. In other cases, biodegradation processes may be important in reducing the concentrations of toxicants. Again, questions as to the type of toxicity tests to be used and the overall success in reducing impacts to the receiving ecosystem exist; however, as a means for reducing the toxicant burden this approach is useful.

In addition to monitoring effluents, toxicity tests have also been proven useful in the mapping of toxicity in a variety of aquatic and terrestrial contaminated sites. Sediments of both freshwater and marine systems are often examined for toxicity using a variety of invertebrates. Water samples may be taken from suspected sites and tested for toxicity using the methods adopted for effluent monitoring. Terrestrial sites are often tested using a variety of plant and animal toxicity tests. Soil elutriates can be tested using species such as the fathead minnow. Earthworms are a popular test organism for soils and have proven to be straightforward test organisms.

The advantages to the above methods are that they do measure toxicity and are rather comparable in design to the traditional laboratory toxicity tests. Many of the controls possible with laboratory tests and the opportunity to run positive and negative references can assist in the evaluation of the data. However, there are some basic drawbacks to the utility of these methods. As with the typical NPDES monitoring tests, the samples project only a brief snapshot of the spatial and temporal distribution of the toxicant. Soils, sediments, and water are mixed with media that may change the toxicant availability or nutritional state of the test organism. Nonnative species typically are used since the development of culture media and methods is a time-consuming and expensive process. A preferable method may be the introduction of free-ranging or -foraging organisms that can be closely monitored for the assessment of the actual exposure and the concomitant effects upon the biota of a given site.

12.6.4 Sentinel Organisms and In Situ *Biomonitoring*

In many instances, monitoring of an ecosystem has been attempted by the sampling of organisms from a particular environment. Another approach has been the introduction of organisms that can

be readily recovered. Upon recovery, these organisms can be measured and subjected to a battery of biochemical, physiological, and histological tests.

Reproductive success is certainly another measure of the health of an organism and is the principal indicator of the Darwinian fitness. In a laboratory situation, it certainly is possible to measure fecundity and the success of offspring in their maturation. In nature, these parameters may be very difficult to measure accurately. Sampling of even relatively large vertebrates is difficult and mark-recapture methods have a large degree of uncertainty associated with them. Radiotelemetry of organisms with radio collars is perhaps the preferred way of collecting life history data on organisms within a population. Plants are certainly easier to mark and make note of life span, growth, disease, and fecundity in number of seeds or shoots produced. In many aquatic environments, the macrophytes and large kelp can be examined. Large plants form an important structural as well as functional component of systems, yet relatively little data exist for the adult forms.

It is sometimes possible to introduce organisms into the environment and confine them so that recapture is possible. The resultant examinations are used to measure organismal and populational level factors. This type of approach has been in widespread use. Mussels, *Mytilus edulis*, have been placed in plastic trays and suspended in the water column at various depths to examine the effects of suspected pollutants upon the rate of growth of the organism (Nelson 1990; Stickle et al. 1985). Sessile organisms or those easily contained in an enclosure have a tremendous advantage over free-ranging organisms. A difficulty in such enclosure type experiments is maintaining the same type of nutrients as the reference site so that effects due to habitat differences other than toxicant concentration can be eliminated.

Salazar and Salazar (1997) have developed techniques to place caged bivalves into marine or freshwater systems in order to examine toxicity in an environment. Typically the young shellfish are placed into enclosures that are then placed into the environment. Sampling is then carried out, the concentration of toxicants examined, and the growth of the organisms measured. This approach takes advantage of the filtering capabilities of the bivalves siphoning large amounts of water in the receiving environment. Although factors in addition to the toxicants can affect growth (food availability, temperature), it is possible using this method to tie exposure to a biological response.

The introduction of sentinel organisms has also been accomplished with terrestrial organisms. Starling boxes have been used by Kendall and others and are set up in areas of suspected contamination so that nesting birds will occupy the area. Exposure to the toxicant is difficult to accurately gauge since the adults are free to range and may limit their exposure to the contaminated site during foraging. However, exposure to airborne or gaseous toxicants may be measurable given these methods.

Birds contained in large enclosures in a suspected contaminated site or a site dosed with a compound of interest may have certain advantages. In a study conducted by Matz and colleagues (Matz 1992; Matz et al. 1994), bobwhite quail chicks were imprinted upon chicken hens. Both the hens and the chicks were placed in pens with the adult chicken constrained within a shelter so that the chicks were free to forage. The quail chicks foraged throughout the penned area and returned to the hen in the evening, making counts and sampling straightforward. It was found that the chicks were exposed to chemicals by all routes, and that the method holds promise as a means of estimating risks due to pesticide applications and a means of examining the toxicity of contaminated sites.

Many factors other than pollution can lead to poor reproductive success. Secondary effects, such as the impact of habitat loss on zooplankton populations essential for fry feeding, will be seen in the depression or elimination of the young age classes.

Mortality is certainly easy to assay on the individual organism; however, it is of little use as a monitoring tool. Macroinvertebrates, such as bivalves and cnidaria, can be examined, and as they are

relatively sessile, the mortality can be attributed to a factor in the immediate environment. Fish, being mobile, can die due to exposure kilometers away or due to multiple intoxications during their migrations. Also, by the time the fish are dying, the other levels of the ecosystem are in a depleted state.

In summary, sentinel species have several distinct advantages. These organisms can be used to demonstrate the bioavailability of xenobiotics since they are exposed in a realistic fashion. If the organisms can be maintained in the field for long periods, indications of the impacts of the contamination upon the growth and population dynamics of the system can be documented. Organisms that are free to roam within the site of interest can serve to integrate, in a realistic fashion, the spatial and temporal heterogeneity of the system. Sentinel organisms are also available for residue measurements; can be assayed for molecular, physiological, and behavioral changes due to chemical stress; and can serve as a genetic baseline so that effects in a variety of environments can be normalized. Introduced organisms are not generally full participants in the structure and dynamics of an ecosystem, and assessments of the native populations should be conducted.

12.6.5 Population Parameters

A variety of endpoints have been used to characterize the stress upon populations. Population numbers or density has been widely used for plant, animal, and microbial populations in spite of the problems in mark-recapture and other sampling strategies. Since younger life stages are considered to be more sensitive to a variety of pollutants, shifts in age structure to an older population may indicate stress. Unfortunately, as populations mature, often age-making comparisons become difficult. In addition, cycles in age structure and population size occur due to the inherent properties of the age structure of the population and predator-prey interactions. Crashes in populations such as that of the striped bass in the Chesapeake Bay do occur and certainly are observed. A crash often does not lend itself to an easy cause–effect relationship, making mitigation strategies difficult to create.

The determination of alterations in genetic structure, that is, the frequency of certain marker alleles, has become increasingly popular. The technology of gel electrophoresis has made this an easy procedure. Population geneticists have long used this method to observe alterations in gene frequencies in populations of bacteria, protozoa, plants, various vertebrates, and the famous *Drosophila*. The largest drawback in this method is ascribing differential sensitivities to the genotypes in question. Usually a marker is used that demonstrates heterogeneity within a particular species. Toxicity tests can be performed to provide relative sensitivities. However, the genes that have been looked at to date are not genes controlling xenobiotic metabolism, but genes that have some other physiological function and act as a marker for the remainder of the genes within a particular linkage group. Although it has some problems, this method does promise to provide both populational and biochemical data that may prove useful in certain circumstances.

Alterations in the competitive abilities of organisms can be an indication of pollution. Obviously, bacteria that can use a xenobiotic as a carbon or other nutrient source, or that can detoxify a material, have a competitive advantage, all other factors being equal. Xenobiotics may also enhance species diversity if a particularly competitive species is more sensitive to a particular toxicant. These effects may lead to an increase in plant or algal diversity after the application of a toxicant.

12.7 Assemblage and Community Parameters

The structure of biological communities has always been a commonly used indicator of stress in the community. Early studies on cultural eutrophication emphasized the impacts of pollution

as they altered the species composition and energy flow of aquatic ecosystems. Various biological indices have been developed to judge the health of ecosystems by measuring aspects of the invertebrate, fish, or plant populations. Perhaps the largest drawback is the effort necessary to accurately determine the structure of ecosystems and to distinguish pollution-induced effects from normal successional changes. There is also the temptation to reduce the data to a single index or other parameter that eliminates the dynamics and stochastic properties of the community. The variety of measurement types are diverse, each with advantages and disadvantages, as described below.

12.7.1 Species Abundance Curves

This is the plotting of the relative abundance of species, ranking from most to least abundant (Newman 1995, p. 285). These measurements may be most useful in a comparative mode, as the rankings and distribution change over time.

12.7.2 Species Richness, Diversity, and Equability

Perhaps the most commonly measured aspects of communities have been the number of species, evenness of the composition, and diversity. These are not measures of toxicant stress, but they do describe the communities. Prior judgment as to the depletion of diversity relative to a reference site due to anthropogenic causes is not warranted unless other factors that control these community level impacts are understood. Among the factors that can naturally alter these types of measures relative to a so-called reference site are history of the colonization of that habitat, catastrophic events, gene pool, colonization area, and of the substrate and the environment, and stochastic events. All of these factors can alter community structure in ways that may mimic toxicant impacts.

Many tools exist for measuring the number and evenness of the species distribution. All are summary statistics generating one number to condense the information on richness, diversity, or equability. Often these measurements are used to describe so-called healthy or unhealthy systems without regard for the limitations of the measurements or the absurdity of the health metaphor. A major disadvantage is that these summary statistics collapse a great deal of information into a single number, thereby losing most of the valuable information contained in the data set.

12.7.3 Biotic Indices

Biotic indices were developed to summarize specific aspects of community structure. As such, these indices are subject to the dominant paradigm of the time of formulation, which controls the aspects of the structure included in the measurement. It is not clear if such indices are measuring important changes in structure or leaving out critical components. When the effects of a chemical on an ecological structure are unknown, using such an index may inappropriately bias the assessment, missing important effects that can impact the critical assessment endpoints.

Perhaps the best-known biotic index in environmental toxicology is the index of biotic integrity (IBI), developed by Karr (1991). An index such as the IBI is a means of rating the structure of a community from a one-time set of samples. Standard methods can be used in the procedures set to produce the IBI, and the resulting numbers typically are used in the establishment of management programs. The IBI is based on fish taxa and is somewhat adaptable, depending on the regional and site-specific variations. A rank of 5, 3, or 1 is assigned to a group of variables selected

as correlated with increasing levels of impact. The criteria are derived from previous sampling and expert knowledge of the normal fish abundance in a particular area. The output is a single number that totals the ranks and classifies the body of water. There are several specific problems with this type of approach. As with the indices above, the single number eliminates almost all of the information contained in the data. The final score is a projection from a multivariate space into a one-dimensional format. In the current fish IBI, several species are weighted more than others, introducing bias into the accounting. In addition, a given numerical value can have many different meanings, depending on the actual values given to the various variables that comprise the index. A 35 from one measurement may not correspond to a 35 from another, because in each instance the rank of the variables that led to the score can be markedly different. The use of these numbers in correlations or in determining average water quality is inappropriate because the numbers do not always represent the same features of the ecological structure. In fact, the IBI is a crude form of classifier, not appreciably better than other, more traditional techniques (Matthews et al. 1997). The setting of an IBI does require prior detailed knowledge of the assemblage or community under study so that comparisons can be made to normal communities. The rankings require expert judgment so that components such as stream or lake type, seasonal components, and natural variation in assemblage composition can be accounted for. The components and rankings of the IBI for fish communities are presented in Tables 12.2 and 12.3.

The utility of a measure such as the IBI is that it is transferable, with modifications, to other fish assemblages and other types of organisms. Given adequate modification, the basic premise should be broadly transferable to even terrestrial communities. Dickson et al. (1992) have reported a relationship between measurements such as the IBI and biomonitoring toxicity tests. Another advantage of the index approach is that a great deal of information is condensed to a single number; this is also a disadvantage.

In a somewhat arbitrary fashion, all indices collapse the numerous dimensions that comprise them into a single number that is treated as an accurate measurement of the condition of the area or environment sampled. Of course, the variables that comprise the index and, indeed, the values assigned to the components are often based on professional judgment. Indices can be fooled, and quite different systems can result in indices of comparable scores. Interpretation of such scores should be taken with the above caveats.

Direct comparison of IBI scores lends itself to misinterpretation and misuse. It is entirely possible that a regulatory endpoint could be defined by an IBI measurement score of 55. Unfortunately, this definition leads to many possible species compositions, and the score is dependent on the assignment of values during the development of the IBI. It would be better to specify just the features of the aquatic system deemed valuable along with target populations as measurement endpoints.

12.8 Effects at the Population Scale

12.8.1 Populations

In the field of environmental toxicology and risk assessment there has been an ongoing discussion about what constitutes a population. For the purposes of this section, a population is a group of potentially interacting organisms of the same species. A population is comprised of organisms of different ages and different genders, and only part of the population is reproductively active. The organisms share a great deal of genetic material, enough so that successful breeding can occur.

Table 12.2 Index of Biological Integrity for Fish Communities

Metrics	*Rating of Metric*		
	5	*3*	*1*
Species Richness and Composition			
1. Total number of fish species[a] (native fish species)[b]	Expectations for metrics 1–5 vary with stream size and region		
2. Number and identity of darter species (benthic species)			
3. Number and identity of sunfish species (water column species)			
4. Number and identity of sucker species (long-lived species)			
5. Number and identity of intolerant species	<5	5–20	>20
6. Percentage of individuals as green sunfish (tolerant species)			
Trophic Composition			
7. Percentage of individuals as omnivores	<20	20–45	>45
8. Percentage of individuals as insectivorous cyprinids (insectivores)	>45	45–20	<20
9. Percentage of individuals as piscivores (top carnivores)	>5	5–1	<1
Fish Abundance and Condition			
10. Number of individuals in sample	Expectations for metric 10 vary with stream size and other factors		
11. Percentage of individuals as hybrids (exotics, or simple lithophils)	0	>0–1	>1
12. Percentage of individuals with disease, tumors, fin damage, and skeletal anomalies	0–2	>2–5	>5

Source: Modified from Karr, J. R., *Ecol. Appl.*, 1, 66–84, 1991.

[a] Original IBI metrics for Midwest United States.
[b] Generalized IBI metrics (see Miller et al. 1988).

Table 12.3 Index of Biological Integrity Scores with Attributes

Total IBI Score (sum of the 12 metric ratings)[a]	*Integrity Class of Site*	*Attributes*
58–60	Excellent	Comparable to the best situations without human disturbance; all regionally expected species for the habitat and stream size, including the most intolerant forms, are present with a full array of age (size) classes; balanced trophic structure
48–52	Good	Species richness somewhat below expectation, especially due to the loss of the most intolerant forms; some species are present with less than optimal abundances or size distributions; trophic structure shows some signs of stress
40–44	Fair	Signs of additional deterioration include loss of intolerant forms, fewer species, highly skewed trophic structure (e.g., increasing frequency of omnivores and green sunfish or other tolerant species); older age classes of top predators may be rare
28–34	Poor	Dominated by omnivores, tolerant forms, and habitat generalists; few top carnivores; growth rates and condition factors commonly depressed; hybrids and diseased fish often present
12–22	Very poor	Few fish present, mostly introduced or tolerant forms; hybrids common; disease, parasites, fin damage, and other anomalies regular
Not applicable	No fish	Repeated sampling finds no fish

Source: After Karr, J. R., *Ecol. Appl.*, 1, 66–84, 1991.

[a] Sites with values between classes assigned to appropriate integrity class following careful consideration of individual criteria/metrics by informed biologists.

12.8.2 Modeling of Populations Using Age Structure and Survivorship Models

Barnthouse and colleagues (Barnthouse 1993; Barnthouse et al. 1990, 1989) have explored the use of conventional population models to explore the interactions among toxicity, predation, and harvesting pressure for fish populations. These studies are excellent illustrations of the use of population models in the estimation of toxicant impacts.

Distinguishing the change in population or community structure due to a toxicant input or the natural variation is difficult. The use of resource competition models can aid in determining the factors that lead to alterations in competitive dynamics and the ultimate structure of a community. A great deal of knowledge about the system is required, and an indication of exposure is necessary to differentiate natural changes from anthropogenic effects. This categorization may be even more difficult due to the inherent dynamics of populations and ecosystems.

12.8.2.1 Population Biology, Nonlinear Systems, and Chaos

A great deal of interest has been sparked by simple models for the description of population dynamics of organisms with nonoverlapping generations. May (1974) and May and Oster (1976) demonstrated that the use of difference equations such as that for population growth:

$$N_{t+1} = N[1 + r[1 - N/K]] \tag{12.2}$$

where N = population size at time t, N_{t+1} = population size at the next time interval, K = carrying capacity of the environment, and r = intrinsic rate of increase over the time interval, can yield a variety of dynamics. At different sets of initial conditions and with varying r, populations can reach an equilibrium, fluctuate in a stable fashion around the carrying capacity, or exhibit dynamics that have no readily discernible pattern; that is, they appear chaotic.

The investigation of chaotic dynamics has also spread to weather forecasting and the physical sciences. An excellent popularization by Gleick (1987) reviews the discovery of the phenomenon, from the butterflies of Lorenz in the modeling of weather to complexity theory. What follows is only a brief introduction.

Figure 12.7 compares the outcomes. In one instance, r is set at 2.0, the carrying capacity 10,000, and N = 2,500. Within 10 time intervals, the population is oscillating around the carrying capacity in a regular fashion. It is as if the carrying capacity is attracting the system, and the system slowly but perceptively falls toward the attractor. The width of the oscillations does slowly shrink. In stark contrast is the system that is identical, except the r value is 3.0. The system

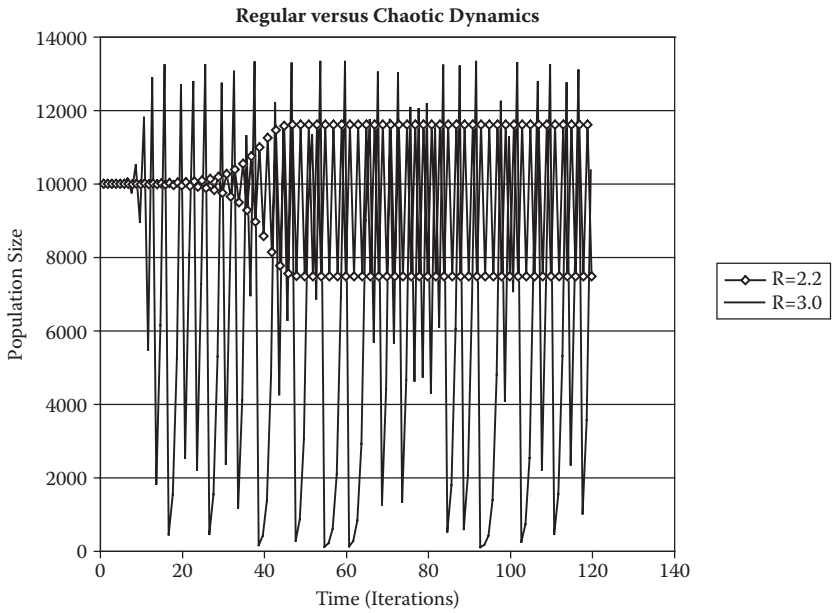


Figure 12.7 Comparison of the population dynamics of two systems that begin at the same initial conditions but with different rates of increase.

does initially climb toward the carrying capacity, but soon exhibits a complex dynamics that does not repeat itself. The system oscillates in an apparently random fashion, but is bounded. In this instance it is bounded by 13,000 and 0. The apparently stochastic pattern is, however, completely derived by Equation 12.2. The system is deterministic, not stochastic. When this occurs the system is defined as chaotic, a deterministic system that exhibits dynamics that cannot be typically determined as different from a stochastic process.

One of the characteristics of nonlinear systems and chaotic dynamics is the dependence upon the initial conditions. Slight differences can produce very different outcomes. In Equation 12.1, there are specific values of r that determine the types of oscillations around the carrying capacity. At a specific finite value of r, the system becomes chaotic. Different initial values of the population also produce different sets of dynamics. Figure 12.8 provides an example. Using Equation 12.2, the initial N in Figure 12.8a is 9,999 with a carrying capacity of 10,000. Overlaid on this figure, in Figure 12.8b, is the dynamic of a population whose initial N = 10,001. Notice that after 10 time intervals the two systems have dramatically diverged from each other. An error of 1/10,000 in determining the initial conditions would have provided an incorrect prediction of the behavior of these populations. Chaotic systems are very dependent upon initial conditions.

Can chaotic systems be differentiated from random fluctuations? Yes, even though the dynamics are complex and resemble a stochastic system, they can be differentiated from a truly stochastic system. Figure 12.9 compares the plots of N = 10,001 and a selection of points chosen randomly from 13,000 to 0. Note that after approximately 10 time intervals the dynamics of both are quite wild and would be difficult to distinguish one from another as far as one being deterministic and the other chaotic. However, there is a simple way to differentiate these two alternatives: the phase space plot.

Figure 12.10a is the phase space plot for the N = 10,001 graph. Here N and N at an arbitrary yet constant time interval are plotted against each other. For these illustrations, N is plotted versus N_{t+1}. Notice that the points fit along a simple arch; this pattern is unique to the equation and is, in fact, somewhat conserved despite the initial conditions. In Figure 12.10b, the phase space plot of the randomly generated data is present and no pattern is apparent. The phase space plot resembles a shotgun blast upon a target. This pattern is typical of a randomly generated pattern and is quite distinct from the chaotic yet deterministic pattern.

The importance of these findings is still under much debate in the biological sciences. A search for chaotic dynamics in population biology was undertaken by a variety of researchers, notably Hassell et al. (1991), Schaffer (1985), Schaffer and Kot (1985), and Tilman and Weldin (1991). Chaotic dynamics certainly are not universal, but have been found in several ecological and epidemiological contexts, as described in Table 12.4.

As can be seen, chaotic dynamics can be found in a variety of systems. Even in the classical population dynamics of the Canadian lynx, the results were demonstrably chaotic in nature. Perhaps one of the studies that has particular relevance to environmental toxicology is the demonstration that grass populations studied by Tilman and Weldin (1991) became chaotic over the period of the extended study. They hypothesize that the increase in plant litter in the experimental plots pushed the system toward chaotic dynamics.

The implications for population ecology and the interpretation of field data are important. First, these dynamics exist in nonequilibrium states. Since many of the tenets of ecological theory depend on an assumption of equilibrium, they may be misleading. Schaffer and Kot (1985) make a stronger statement: "Our own opinion is that what passes for fundamental concepts in ecology

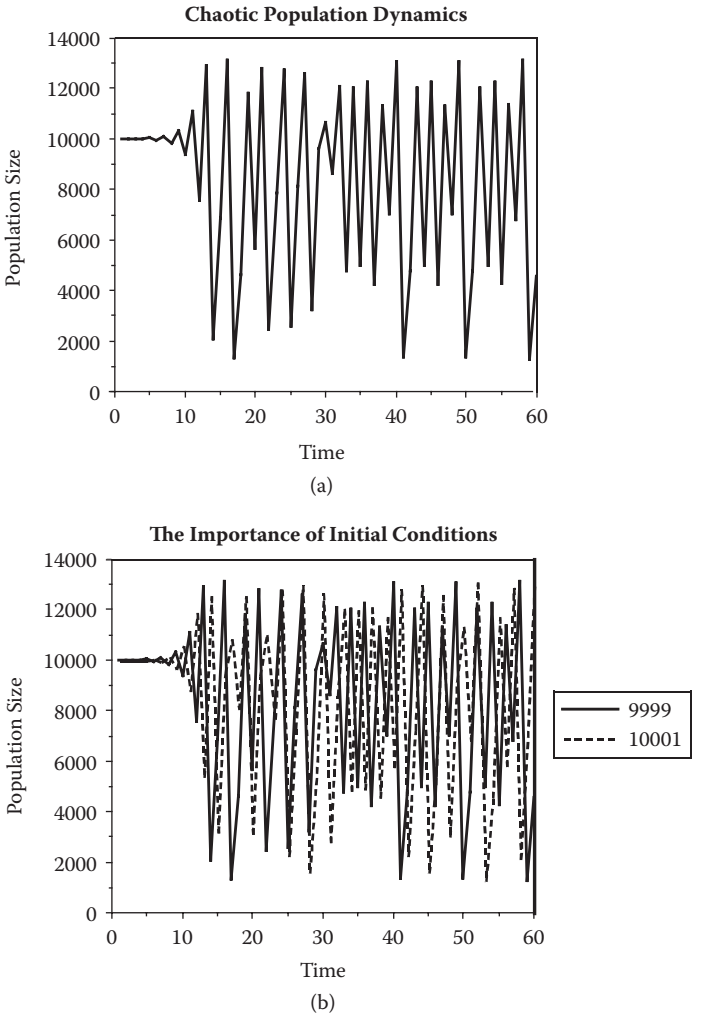


Figure 12.8 The importance of initial conditions. (a) The dynamics from starting the simulation at 9999 are plotted. (b) These dynamics compare to the dynamics of the original plot to one that had 10,001 as the starting value. Although the equations governing the populations are identical, even a 2/10,000 difference in the initial conditions results in very different dynamics.

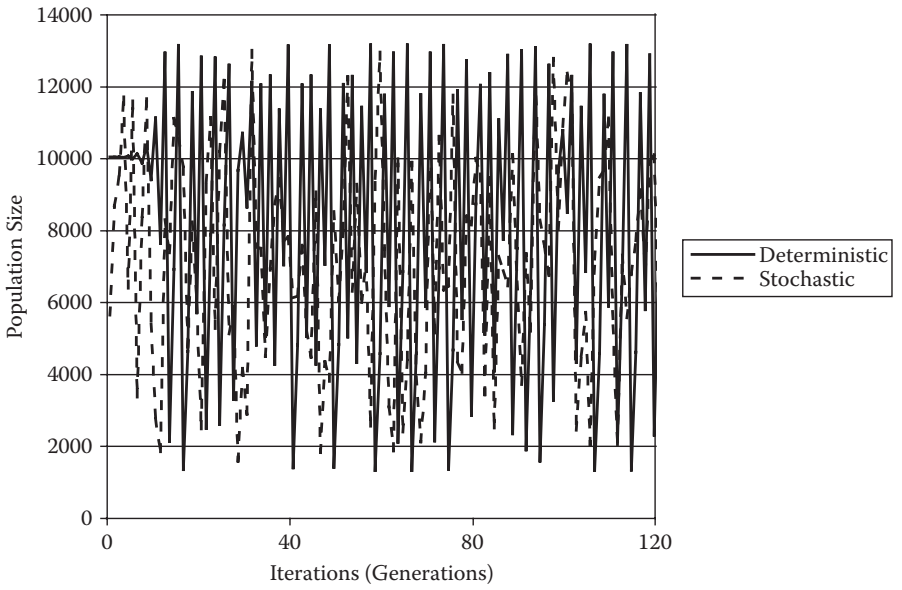


Figure 12.9 Comparison of chaotic versus random population dynamics.

is as mist before the fury of the storm—in this case, a full, nonlinear storm" (p. 349). One of the crucial recommendations of this paper is the importance of understanding the current dynamic status of the ecological system. Only then can perturbation experiments designed to elucidate interactions be considered valid.

Landis et al. (1993) have discussed the implications of nonlinear dynamics in environmental toxicology. First, if ecological systems are nonequilibrium systems, then attempts to measure stability or resilience may have no basis. In fact, it may be impossible to go back to the original state, or after a perturbation, to the state of the reference site. Second, the dynamics of the system will not allow a return to the reference state. Nonlinear systems are very sensitive to original conditions and record a history of previous alterations within the dynamics of their structure. Third, historical events give rise to dynamics that are likely unique for each situation. As stated by Schaffer and Kot (1985), unless the initial dynamics are understood, perturbation experiments, either accidental or deliberate, are impossible to interpret. Fourth, the future cannot be predicted beyond the ability to measure initial conditions. Since nonlinear systems are so sensitive to initial conditions, predictions can only be accurate for short periods of time.

The repeatability of field studies can also be seen as impossible beyond certain limits. That is not to say that patterns of impacts cannot be reproduced, but reproducibility in the dynamics of individual species is unlikely unless the initial conditions of the experiment can be made identical.

As interesting and powerful as the development of the understanding of nonlinear systems has been, it is only part of the study of system complexity. Nicolis and Prigogine (1989) have produced an excellent introduction, and the understanding of complexity theory promises to have a major impact on ecology and environmental toxicology.

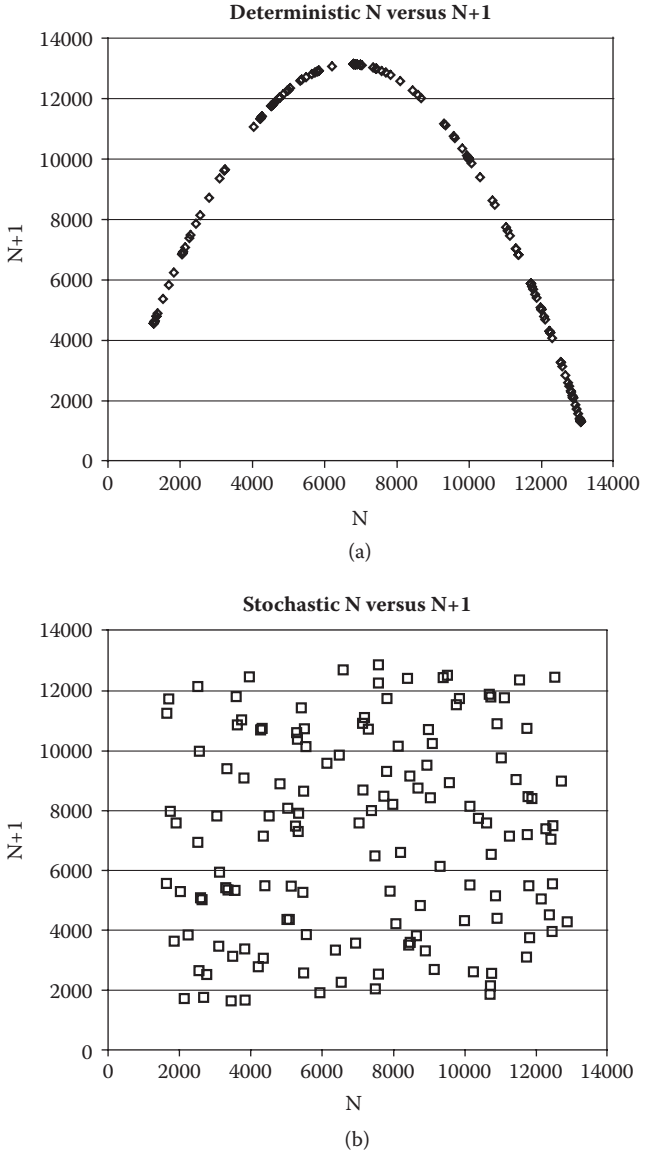


Figure 12.10 Plots of the population size versus the population size at a specific time interval reveal the structure of a chaotic system. (a) Derived from the deterministic yet stochastic-looking dynamics, a pattern readily forms that is characteristic of the underlying equation. (b) A shotgun blast or random pattern is revealed.

Table 12.4 Examples of Chaotic Dynamics in Ecological Systems

Organism (Schaffer, 1985)	*Chaotic Dynamics Observed*
Mammals	
Canadian lynx	Yes
Muskrat	No
Insects	
Thrips	Yes
Leucoptera caffeina	Yes
L. meyricki	Yes
Blowflies	Yes
Human Diseases	
Chicken pox—New York City	No
Chicken pox—Copenhagen	No
Measles—New York City	Yes
Measles—Baltimore	Yes
Measles—Copenhagen	Yes
Mumps—New York City	No
Mumps—Copenhagen	Yes
Rubella—Copenhagen	Yes
Scarlet fever—Copenhagen	No
Whooping cough—Copenhagen (Sugihara et al., 1990)	No
Measles city by city (UK)	Yes
Measles (countrywide) (Tilman and Weldin, 1991)	No-noisy 2-year cycle
Perennial grass *Agrostis*	Yes

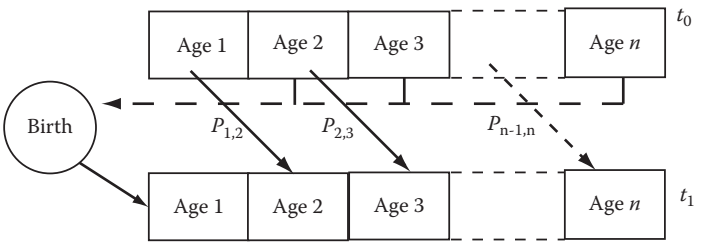


Figure 12.11 Life history diagram for an age-structured population. The numbers of organisms in the population at time t_1 is dependent on the numbers of the 1-year-younger age class of the year before and the survivorship percentage from t_0 to t_1. The numbers are also dependent upon the number of offspring from the previous year surviving to age 1. This is a general model for many plant and animal populations.

12.8.3 Age-Structured Populations

The models used above have all had simple population structures. In each case the generations were not overlapping, and each organism had the same reproductive potential. Of course this is not realistic.

A more realistic description of a population is found in Figure 12.11. At t_0 the population is comprised of organisms at age 1, age 2, age 3, …, age n. Age 1 is composed of the new organisms that are 1 year old but not able to reproduce; this varies depending upon the population. The size of the population at the next time interval, t_1, is the number of births produced by the organisms at each surviving to age 1, plus the original age 1 organisms surviving to age 2, and so forth. The number of organisms surviving from one age to another can be represented by a probability of surviving from age $n - 1$ to age n. For example, the chance of surviving from age 1 to age 2 in the next time interval can be written as $P_{1,2}$, the chance of going from age 2 to age 3 is $P_{2,3}$ and so forth. In this model there is no density dependence; neither the fertility or survivorship of the organisms are affected by an increase in numbers. Density dependence can be built in, but that is beyond the scope of the current discussion.

A toxicant can affect the population at a number of stages. A toxicant at a concentration that can cause acute mortality can decimate the population at every life stage, but older organisms may be less affected because of relative size. Materials that bioaccumulate over time may differentially affect older organisms that have had the time to acquire a high tissue concentration. This increase in tissue concentration may decrease the survivorship of older organisms. Such an increase in tissue concentration also may decrease the reproductive success of these older age classes.

Materials that are preferentially toxic to early life stages will cause a lack of age 1 organisms coming into the population, producing a population lacking the early life stages. As exposure to these toxicants persists, the population will become aged, and then as all the adults become postreproductive, collapse.

The calculations to predict effects can be set up in a spreadsheet and run iteratively, or matrix algebra can be used, and a number of programs make the computations straightforward. In the examples that follow I will use data from a Pacific herring (*Clupea pallasi*) population within the Georgia Basin off the coast of Washington as a baseline for the simulations.

Pacific herring spawn once per year at specific sites along the coast once they reach 2 years of age. The Cherry Point run has been sampled since the early 1970s. Historically, the Pacific herring

at Cherry Point did reach 9 years of age, although the censuses in the late 1990s and early 2000s indicate that the older fish have disappeared from the population. The data summarized by EVS Environmental Consultants (1999) were used to construct the life history tables for this exercise. Fecundity estimates were those of Chapman for Puget Sound stocks in the 1940s.

In the array below the top row is the number of fish (millions) estimated to exist at each age in 1983. The second row is the fraction of year 1982 fish that survived until 1983. There is no number for the year 2 fish since year 1 fish do not spawn and are not available for collection at the spawning site. The egg production from each age class as estimated from Chapman is the last row. Note again that the numbers are in millions. For the purposes of the simulation, it was assumed that the gender ratio was 50:50.

Life History Information Required for the Simulation (numbers in millions)

	Year 2	*Year 3*	*Year 4*	*Year 5*	*Year 6*	*Year 7*	*Year 8*	*Year 9*
Initial year 1982	17.6	9.6	11.4	3.4	1.4	1	0.3	0
Initial year 1983	26.2	13.4	9.2	11.4	2.8	0.7	0.9	0.2
Survivorship percentage from 1982		0.76	0.96	1.00	0.82	0.50	0.90	0.67
Egg production (millions)	0.0087	0.0142	0.0195	0.0249	0.0303	0.0358	0.0412	0.0466

The percentage survivorship from the 25+ years of collections was estimated to be 2.56087E-05, or only 0.0000256 of the eggs survive to become age 2. Survivorship from egg to age 2 was considered to be constant for each age class of fish.

Next, a series of simulations were performed to examine the effects of a toxicant that bioconcentrates in the tissue of older fish, decreasing the survivorship percentage in these age classes. There are four cases:

Case 1—The population as illustrated in the life history table above.
Case 2—A 50% reduction in survivorship from age 6 and up.
Case 3—A 0.0 survivorship fraction from age 6 and up.
Case 4—A 0.0 survivorship fraction from age 4 and up; age 4 are the oldest fish.

As can be seen in Figure 12.12, the stepwise reduction in the number of older fish decreases the increase in the population until a decrease is apparent in case 4. In the case 4 simulation a 24% decrease in the population of adults in the next generation results in a decline in the population. Since the life history table indicates that the older fish are much more fecund than the younger fish, this decline represents a much greater decrease in the reproductive success of the population.

There are two more cases to consider, both resulting in a decrease in the fertility of the fish:

Case 5—A reduction in fertility to 0.50 of the original.
Case 6—A reduction in fertility to 0.33 of the original.

The results of these simulations are seen in Figure 12.13. Compared to case 1, the baseline, these effects result in no population growth or a decline. Clearly a significant reduction in fertility can result in severe population level effects.

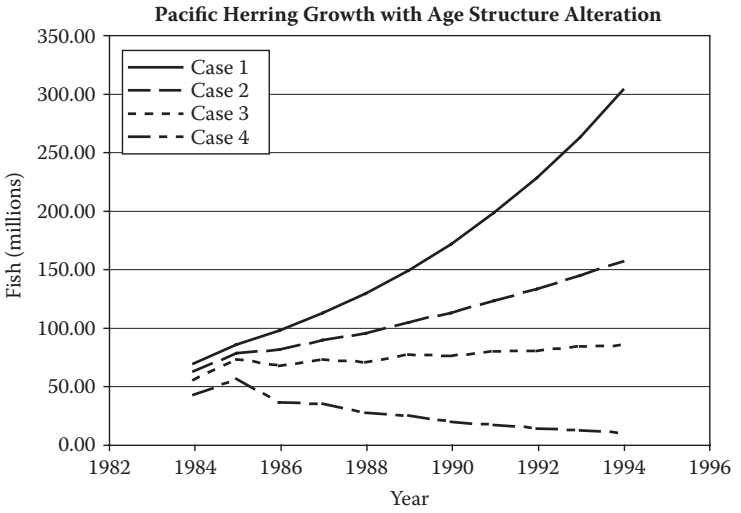


Figure 12.12 The results of four simulations when depressing survivorship of the older age classes.

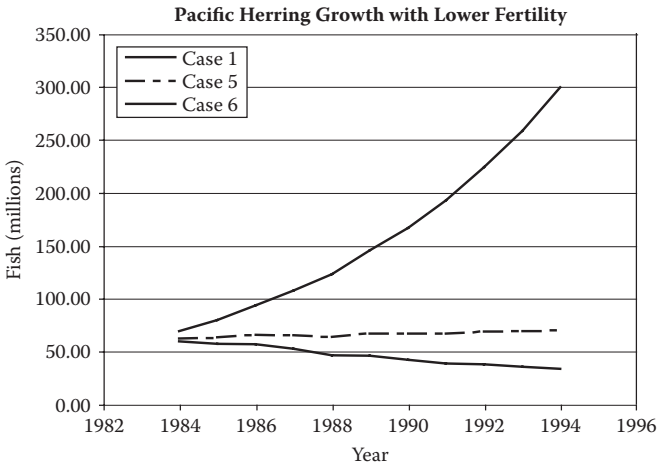


Figure 12.13 The results of four simulations when depressing fertility of the population.

At first glance, case 4 (mortality in the older age class fish) and case 6 (loss of fertility) seem to produce similar results. However, there is a diagnostic feature: the age structure of the population. Figure 12.14 compares the age structures of the two cases at similar population levels. In case 4, there are no old fish. In case 6, older fish still exist in the population, available to supply their fertility to the population once the toxicant is removed. In case 4, there are no old fish, and the population would take at least 5 years to rebuild the age structure. Now compare case 6 to case 1; there is a lower proportion of younger fish in case 6 and an increase in the proportion of older fish. This pattern is diagnostic of populations with a decrease in fertility. As fertility is reduced to near zero, the shift to an older population structure is even more dramatic.

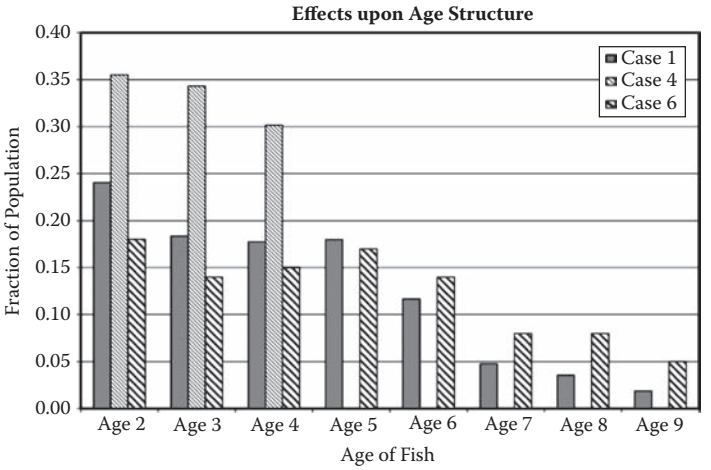


Figure 12.14 Comparison of the age structure of two different effects. Case 1 is the baseline situation. Case 4 is the instance where survivorship of older age classes is eliminated. Case 6 is a reduction in fertility to 33% of normal. Compared to the baseline there is a lower proportion of younger fish in this population due to the restriction in the fertility. There are also a larger proportion of older fish in case 6 compared to the baseline simulation.

The previous sections have demonstrated what can be seen using simple population models. However, populations exist in space, a landscape. The following sections expand this discussion to the influence of spatial relationships within and between populations and the effects of toxicants.

12.8.4 Measurement of Effects on Populations

Effects upon populations have been difficult to predict or estimate. There are four categories of measurements that are often used in the process of making these predictions:

1. Point estimates, TRVs, NOECs, LD_{50}s, and similar measures
2. Biomass, percent cover, and productivity
3. Intrinsic rate of growth r or λ
4. Change in the pattern of the age structure of a population

Each category is described below, along with a description of the strengths and weaknesses of each technique.

12.8.5 Point Estimates, TRVs, NOECs, LD_{50}s

Point estimates of regions of the concentration-response curve (LD_{50}s, EC_{20}s, etc.) have been used in order to set concentrations that should be protective of populations. However, if only mortality is used, these are not attributes at a population scale. As discussed in previous chapters, these data are taken from acute or chronic toxicity tests for an identified contaminant under conditions set to ensure the health of the organism and the repeatability of the test. Toxicity tests have been run to bracket and estimate the LD_{50} or EC_{50}, not to understand the concentration-

response relationships at concentrations typically seen in the environment. It would be useful if the concentration-response relationships at concentrations typical of environmental samples were being described with toxicity test endpoints valuable to predicting events at the population scale, such as mortality, growth, and reproduction.

The difficulties with using NOELs, LOELs, and similar derived values have been described (Moore and Caux 1997; chapter 5). These values are not part of the concentration-response curves but are derivatives of the concentration-response, experimental variability, and operator error, and choices made by the investigator of what concentrations are tested. Error terms are not associated with these estimates, so that the measurement of uncertainty is not possible. Compared to estimates derived from fitting concentration-response curves, these derived values are not useful and can misrepresent the concentration-response relationship.

The biological relevance of point estimates is also not clear since toxicity tests are typically run in laboratories as monocultures, and with only one stressor. Chronic toxicity tests are more relevant to the population scale, but require the extrapolation by models to describe the magnitude and type of effect.

Point estimates can be clearly defined, but often there is a lack of supporting information, namely, the concentration-response data upon which the estimate is based. The lack of reporting also means that the associated variability of these estimates is not documented, nor is the uncertainty due to the strain of the test species and variations in methodology or laboratory.

Point estimates are accessible to prediction and measurement. Quantitative structure-activity research has made many point estimates relatively predictable for classes of compounds with sufficient data. The derivation and fitting of a concentration-response curve and the calculation of an EC value has been refined (Environment Canada 2005) so that an objection based upon a lack of suitable techniques is not a factor.

12.8.6 Intrinsic Rate of Growth r or λ

This category is a simplification of the previous section, since only one parameter is used to summarize the potential production or biomass. Both of the parameters have a long history and look somewhat mathematical, being derived from basic population biology. The intrinsic rate of increase r is used in $dN/dt = rN$ to calculate the change in population size over the next time interval for a population with no age structure. For populations with an age structure, λ is the equivalent parameter to r and is defined as the dominant eigenvalue of the transition matrix. See this chapter's appendix for a more complete description. The transition matrix describes the reproductive output for each age class and the probability of survival from one age class to another. Both parameters, implicitly r or explicitly λ, summarize or are a projection of a large number of physiological and ecological features.

Rate of growth as measured by both parameters is a useful abstraction in the prediction of population dynamics under idealized conditions. Microbial populations in the laboratory are amenable to prediction with r as a parameter coupled to density dependence. λ is likewise useful in assessing how toxicant-induced changes in age structure and survivorship can affect growth in modeled laboratory populations (Stark et al. 2004). It has also been demonstrated by Stark et al. (2004), Spromberg and Meador (2005), and Lin et al. (2005) that slight differences in λ can have important effects upon a population. Because of this sensitivity, Naito and Murata (2007) have used λ as an endpoint in an ecological risk assessment. A further example of this sensitivity presented below, discusses modes of action and impacts on the population dynamics of a modeled Pacific herring population.

Spromberg and Meador (2005) used demographic models to examine the potential effects of chronic toxicity upon Chinook salmon populations. Small changes to λ do result in large changes in population size, but the critical analysis is in the examination of the demographics that result in the change in reproductive rate.

Both rates of growth parameters have unambiguous mathematical definitions that are operational in the laboratory or in modeling situations. Both are clearly abstractions and summaries of the numerous factors that control reproduction at the population scale. This results in a high ranking for operational definition.

Measurement of these parameters differs from the laboratory to the field. In the laboratory the intrinsic rate of increase can be measured. Data derived from chronic toxicity tests as summarized in Stark and Banks (2003) can be obtained and results that cannot be extrapolated from classic toxicity tests are observed. Many of the studies deal with arthropods that reproduce rapidly, reducing the time and cost for such studies. One of the issues discussed by Stark and Banks (2003) is the cost of conducting life table response experiments and the unrealistic nature of the exposure regimen. The estimation of λ can be difficult for species typical of those selected as endpoints in risk assessments.

In order to examine the variability of λ derived from field data, the run of Pacific herring (*Clupea pallasi*) that spawns at Cherry Point, Washington, was used as an example. Spawning surveys have been conducted from 1974 to present (Stick 2005). The characteristics of the Cherry Point Pacific herring stock and the Cherry Point region have been presented (EVS Environmental Consultants 1999; Landis et al. 2004; Hart Hayes and Landis 2004; Markiewicz 2005). Pacific herring are an important forage fish and have been observed to live to 9 years in the Cherry Point population. The stock has been in decline since the late 1970s, although periods of dramatic increases have occurred. The decline has also corresponded to a collapse of the age structure that it is dominated by age 2–4 fish. Recent papers (Landis et al. 2004; Hershberger et al. 2005, Landis and Bryant 2010) suggest that the decline is due to large-scale factors within the region and not to contamination at the spawning site.

Because of the long-term monitoring of the Cherry Point stock by the Washington Department of Fish and Wildlife, data are available on the biomass for each of the spawning populations, and they have been converted to numbers of fish for each age class (Stick 2005). These data were used to calculate survivorship of each age class since 1974. Using the population modeling program RAMAS®, λ was calculated for each year of data over this 30-year period and the variability examined.

Table 12.5 summarizes λ during the last 30 years for the Pacific herring stock at Cherry Point. During the entire period of the study the λ value averaged greater than 1, but the standard deviation is greater than half the mean. As the maximum and minimum values demonstrate, there is

Table 12.5 Summary of λ for the Cherry Point Pacific Herring Stock from 1974 to 2004 (the value is highly variable over the 30-year period)

	1974–2004	*1981–2004*	*1981–2003*
Average	1.0444	0.7908	0.7633
Standard deviation	0.6337	0.1897	0.1368
Max.	3.3846	1.4227	1.0848
Min.	0.5662	0.5662	0.5662

a wide range of values for this parameter. Using just the data from the period from 1981 to 2004 does not incorporate the λ values from the time of high population values during the early 1970s. During this period λ was below 1, but the standard deviation still remains at 24% of the average value. Some of the variability during the 1981–2004 period is due to an unexpectedly high λ value in 2004, when it exceeded 1.0 for the first time in years. Eliminating this value and calculating using the 1981–2003 data resulted in a standard deviation of 18% of the mean.

There are many reasons for this variability. The Washington State coast is affected by El Niño events, the Pacific decadal oscillation, fishing pressure, disease, contaminants, and invasive species. It is inherently difficult to sample fish populations in a coastal region, although the team sampling the Pacific herring stocks in this area has remained very consistent over the sample period.

Stark et al. (2004) has demonstrated that the parameters reflecting an intrinsic rate of increase do change when exposure occurs in the laboratory, so there is sensitivity. Barnthouse (2004) has surveyed rates of growth data for a number of species, and the impacts of toxicants and growth rates do respond to a number of stressors. Clearly the intrinsic rate of increase is a parameter that is sensitive to hazardous agents and stressors, but the sensitivity depends upon the life history characteristics of the species.

12.8.7 Biomass, Percent Cover, Productivity

Biomass, percent cover, and productivity are all population-scale measures that can be estimated by appropriate sampling methods. Biomass can be estimated by a number of techniques, from trawling for fish or counting the number of trees and the size of each. Mapping exercises, including aerial and satellite photography, can estimate percent cover. Productivity requires multiple measurements so that a change in biomass or percent cover can be estimated. Measurement of these attributes is done in fisheries and forestry management, conservation ecology, and agriculture.

This category of measurements is critical for species that provide ecological services. The amount of biomass produced each year is clearly a socially relevant parameter since it supports fisheries, forestry, agriculture, and the commercial entities that employ those resources. Ecological services are also provided by those species whose existence provides barriers to siltation as are found in riparian areas. Biological relevance is also straightforward. Salmon returning to spawn are not only an important fishery but also important nutrient sources for freshwater streams both from the spawn and from the rotting carcasses. Eelgrass coverage along the northwest coast of the United States provides an important habitat for large numbers of commercially important species (Hart Hayes and Landis 2004).

This category is also straightforward to define, as it can be the density of the organism, total population size, percent of the landscape covered, and over time, productivity can be defined. One difficulty of this category is in the measurement of each of these parameters in the field. Some species, such as herring, salmon, or cod, are commercially important, and catch records and other measures of biomass or productivity are regularly collected and the data stored. Populations that are not as commercially or recreationally important may only have limited data associated with them. Remote sensing and appropriate ground truthing can provide estimates of coverage or density with some species. Prediction is dependent upon the quantity and quality of the data on abundance and an understanding of limitations upon the population. Prediction also requires an understanding of the life history strategy of the population and its spatial extent and interactions.

As has been discussed extensively in this book, the ecological context is vital to understanding the outcome from a toxicant stressor. The productivity of a population is a result of a wide variety

of direct and indirect effects, providing a clear representation of the ecological impact upon that species. If the toxicant is not acting as a limiting factor to the population, the biomass or productivity may not respond to removal of the chemical. This result has been demonstrated in the Hudson River.

In the Hudson River Barnthouse and colleagues (Barnthouse et al. 2003, 2009) have examined the relationship between striped bass (*Morone saxatilis*) and Hudson River white perch (*Morone americana*) and PCB concentrations. Both sediment and tissue measurements over 30 years have demonstrated a decrease in PCB sediment and tissue concentrations in these species. Given the observed concentrations and information from conventional individual-based toxicity tests, the assumptions would have been that the populations should have responded to a decrease in PCB exposure. That did not happen.

In both species the population dynamics or parameters related to population structure were not correlated to the PCB concentration over the 30-year period of population sampling and chemical analysis. Of course, many other factors are important in determining the population dynamics. In the case of striped bass, a fishing moratorium would have eliminated the fishing pressure on this population. Predation and food availability can be limiting. Populations exposed to a particular stressor may have evolved tolerance mechanisms. The availability of quality habitat in the study area could also be the critical limiting factor to the fish, overriding the effects of PCB toxicity.

In order to actually understand the effect of any stressor upon a population, the stressor and population must be put into the context of the history of the site, the shape of the landscape, and the other management factors. Those features are discussed in Section 12.8.10.

12.8.8 Change in the Pattern of the Age Structure

The proportion of a population at each age class is determined by the reproductive output of the population when that age class was born and the probability of survivorship to older age classes during the intervening time span. The pattern of these proportions is a representation of the events that have occurred during the history of at least the oldest age class. Certain age classes may be extremely important in providing ecological services, such as the reproductive adults in many fisheries, or trees of a certain size in forestry. In contrast to a point estimate such as λ, the age structure contains a variety of information on historical events.

There are two types of age structure that will be discussed. The first is the realized or sampled age structure. This is the proportion at different ages as determined by sampling the population. The second type is the equilibrium age structure. In the calculation of the dynamics of an age-structured population, the calculated age structure rapidly approaches equilibrium. This equilibrium age structure can be compared to the sampled one to estimate the direction that the age structure of the population will take. In some instances the equilibrium age structure will be more heavily weighted toward younger individuals than the sampled, demonstrating an increase in the mortality of the older individuals. Conversely, an age structure that is more heavily weighted toward older individuals could be due to a lack of reproduction by the adults or an increase in survivorship of the older age classes.

12.8.8.1 Normalized Effects Vector

A normalized effects vector (NEV) may be an informative indicator of population-scale effects. If there is information about the current and historical life cycle patterns, as well as an estimate of

the age structure, modeling can be used to determine if the population is at risk. The NEV has a pattern, which is diagnostic of the scenario, contrary to the instantaneous growth rate. Given the inherent variability of many populations, a 20% reduction in population size will be difficult to measure during the 1 to 3 years of the conduct of a risk assessment. Likewise, the variability of measuring λ makes that a difficult endpoint.

The NEV is calculated as the difference between the baseline equilibrium age structure and the impaired stable age structure calculated from a projection matrix altered in some life history parameter value. The age structure is computed for the baseline matrix and for each of the matrices from the populations that have been exposed to the toxicant. These vectors are normalized, and the baseline age structure vector is subtracted from the age structure for the impaired vector. This is a computational expression of the change in age structure depicted graphically by Spromberg and Meador (2005). The simple expression is

$$\text{NEV} = i - b \tag{12.3}$$

where i is the column vector representing the impaired equilibrium age structure and b is the column vector representing the baseline equilibrium age structure. NEV is the column vector notation representing the pattern of effects of the contaminant or other stressor. Below there are a series of examples to demonstrate the potential utility of this approach.

In developing this approach Landis and Kaminski (2007) used the fecundity and survival estimates from a British Columbia, Canada, Pacific herring population, in our projection matrix (Fu and Schweigert 2004). The model is based upon Caswell (2001) and was written in MATLAB® version 7 (The Math Works, Natick, Massachusetts). The simulation starts with a baseline population that is growing exponentially, and to that we compare four impaired scenarios with five levels of effect. We used a density-independent model and the matrix projections were deterministic.

The four scenarios considered were:

1. Reduction in survival at all ages. This could represent a wide range of modes of action, including pesticides, organic solvents, and polyaromatic hydrocarbons (PAHs).
2. Reduced fecundity at all ages, representing a reproductive systems toxicant.
3. Reduction in survival in adults, representing bioaccumulative or carcinogenic chemicals.
4. Reduction in the first survival transition, representing a teratogen or a contaminant local to the spawning site.

For each of these scenarios, survival or fecundity is reduced at five levels, representing 0, 5, 10, 20, and 50% effects concentrations. All effects were considered chronic, and no variation in response within the population was incorporated.

The outcomes calculated from the projection matrix were the growth rate, the time to a 20% reduction from the baseline population size, the stable age structure of the impaired population, and the NEV. The time to 20% reduction in population size was calculated by multiplying the 5% level impaired matrix by the initial (baseline) age structure for 20 time iterations. The resulting 20-year projection was divided by the 20-year projection from the baseline matrix to find where the impaired population was not greater than 80% of the baseline population.

Table 12.6 shows that the results from these calculations demonstrate the utility of such analyses. Compared to the nondosed scenario, the EC_5 provides a time to reduction in growth of 20% of 8 years. Note that the λ for EC_5 is still above 1, denoting a positive growth rate. However, in 8 years 20% of the productivity and any ecological service will be lost. For scenario 1 an EC_{20} is

Table 12.6 The Time to 20% Reduction from the Baseline Population at the EC_5 Level and the Population Growth Rate at All Effects Levels for Each Scenario

Scenario	*Time to 20% Reduction at LC_5*	*λ (for 0, 5, 10, 20, and 50% effect respectively)*
1. Toxicant effect applied to all survival transitions (wide-ranging mode of action, pesticides, organic solvents, PAHs, etc.)	8 years	1.1167 **1.0771** 1.0370 *0.9556* *0.6965*
2. Fecundity is reduced by 5, 10, 20, and 50% (reproductive system toxicant)	16 years	1.1167 **1.1001** 1.0831 1.0477 *0.9256*
3. Toxicant effect applied to adult survival transitions (bioaccumulative, carcinogen)	12 years	1.1167 **1.0926** 1.0685 1.0201 *0.8750*
4. Toxicant effect applied to first survival transition only (teratogenic materials, contaminant local to spawning or breeding sites)	18 years	1.1167 **1.1011** 1.0851 1.0516 *0.9345*

Note: The projections of total population abundance were made using the impaired matrices and the baseline age structure, and growth rate values were calculated from the impaired transition matrices. Underlined values for λ are the baseline condition, italics are those values below 1.0, and bold values are λ for the LC_5.

required to reduce the λ to below 1. The other scenarios take longer to see a 20% reduction in the population at the EC_5 and do not result in a λ of below 1 until EC_{50}. Note, however, that it does not take a λ of below 1 to limit ecological services compared to the nondosed condition.

Note the sensitivity of λ in regard to changes in the time to a 20% reduction compared to the baseline case at the LC_5 expressed in Table 12.7. A λ value of 1.0771 produces a 20% reduction in 8 years; a λ value of 1.1011 results in the same reduction in an 18-year period. A difference of 0.024 will be difficult to measure in either a laboratory or field situation.

Examinations of the NEVs for the scenarios demonstrate how useful those patterns may be for estimating causation regardless of the magnitude of the effect. The patterns for the NEVs for scenario 1 at the EC_5-EC_{50} are presented in Table 12.8. The decrease in the older age classes is a pattern found at each concentration. The magnitude of the effect is larger at higher concentrations, but the overall pattern changes very little. Table 12.8 compares the patterns at the EC_{20} for each

Table 12.7 Normalized Effects Vectors (NEVs) for Reduced Survival at All Transitions for Each of Four Concentrations for Scenario 1

Age	EC_5	EC_{10}	EC_{20}	EC_{50}
2	0.0077	0.0157	0.0332	0.1019
3	0.0002	0.0002	*–0.0001*	*–0.0072*
4	*–0.0019*	*–0.0039*	*–0.0085*	*–0.0288*
5	*–0.002*	*–0.0041*	*–0.0085*	*–0.0254*
6	*–0.0015*	*–0.0031*	*–0.0064*	*–0.0175*
7	*–0.0011*	*–0.0021*	*–0.0043*	*–0.0109*
8	*–0.0007*	*–0.0014*	*–0.0027*	*–0.0064*
9	*–0.0004*	*–0.0008*	*–0.0016*	*–0.0036*
10	*–0.0003*	*–0.0005*	*–0.001*	*–0.002*

Note: The vectors are the impaired age structure minus the baseline age structure. Note that the population is getting proportionally younger with an increase in dose. Italics denotes those values that are negative and are used to enhance the visualization of the pattern.

scenario, and each has a different pattern. Scenario 1 demonstrates a reduction of age classes older than age 2. Scenario 2 has the opposite pattern; it is the age 2 and age 3 classes that demonstrate a reduction. Scenario 3 is similar to scenario 1 in that the older age classes are reduced, but the negative growth does not exist for age 3 at this concentration. Scenario 4 is similar to scenario 2 except that age 2 is not negative at this concentration and age 4 is negative.

The patterns revealed by the NEV also can be use to reveal which part of the ecological resource is being affected by the toxicant. In many species of fish the most desirable catch is the larger age classes. Although in scenario 1 at the EC_{20} the population is still growing, the proportion of the population that is the most desirable resource, the older, larger fish, is decreasing.

One difficulty in estimating an NEV may be that there are relatively few chronic toxicity data sets that collect the required information. However, as Stark et al. (2004) have demonstrated, the data on how toxicants affect life history factors can be routinely obtained. As demonstrated above, data on long-term changes in population structure are obtained in fisheries and in wildlife management.

12.8.8.2 So What Measure to Use?

The age structure of a population and observed or estimated changes in this structure provide useful information. Species as diverse as cod, herring, lobster, waterfowl, oysters, and spruce have life stages that are selectively harvested. The population at these valued life stages provides the ecological resource.

Age structure also has high biological relevance for age-structured populations. Knowing the age structure along with the survivorship and reproductive characteristics of each age class allows

Table 12.8 Normalized Vectors for All Four Scenarios at the EC_{20}

Age	*Scenario 1*	*Scenario 2*	*Scenario 3*	*Scenario 4*
2	0.0332	*–0.0334*	0.008	0.0243
3	*–0.0001*	*–0.002*	0.0283	*–0.027*
4	*–0.0085*	0.0075	*–0.0033*	*–0.0068*
5	*–0.0085*	0.0086	*–0.0099*	0.0004
6	*–0.0064*	0.0071	*–0.0089*	0.0024
7	*–0.0043*	0.0051	*–0.0063*	0.0025
8	*–0.0027*	0.0035	*–0.004*	0.0019
9	*–0.0016*	0.0022	*–0.0024*	0.0014
10	*–0.001*	0.0014	*–0.0014*	0.0009

Note: The patterns in the NEVs are very similar at both concentrations, but the values of the difference are in correspondence with the concentration. These patterns are a hypothesis than can be examined in further field experiments or from existing data sets. Italics denotes those values that are negative and is used to enhance the visualization of the pattern.

prediction of future dynamics. In contrast to other measurements, knowledge of age structure allows the prediction of the future quantity and type of resource provided by the population.

The age structure of a population does have an unambiguous definition for most species that are likely to serve as population-scale endpoints, so it is given a maximum score. The difficulty in using a change in age structure is the accessibility to prediction and measurement. Laboratory work such as that proposed by Stark (2005) will produce results applicable to estimating the change in age structure. Understanding the specific action of a toxicant upon different life stages will allow an estimate of the change in age structure. Examining changes in age structure with field populations requires that the appropriate sampling be performed. Such data may be available for many populations that provide ecological services derived from catch and harvest information. Compared to a derived value such as λ, the age structure can be directly measured.

Compared to λ alone, the pattern resolved in the NEV is more informative of what the cause of an impact may be and the effect on the resource. Any summary statistic such as λ is a reduction of the dimension of the state of the system. A population is much more than a mathematical representation of its growth rate. As the dimensionality of a system is reduced, so is the information content. The use of the entire age structure, as in the case of the NEV, is going to be more informative.

12.8.9 Observed Changes in Age Structure Experimental and Field Populations

The above discussion on population biology is directly related to effects seen in the field. Kidd et al. (2007) conducted a 7-year whole lake experiment dosing fathead minnow populations with

17α-ethynylestradiol (EE2). The synthetic hormone EE2 is used in birth control pills and may be a constituent of wastewater. Although there has been a great deal of evidence that estrogens feminize male fish (Chapter 6), it has not been demonstrated that this process has an effect upon populations.

Molecular and physiological effects were observed at low concentrations of EE2 (5 to 6 ng/L) and included intersex males, the production of vitellogenin mRNA and protein, and alteration of oogenesis in female fish. These are effects that indicate exposure to the EE2 was occurring, and that effects were occurring on individual fish.

These effects upon individuals did have a clear effect upon the age structure and number of the population. Figure 12.15 demonstrates the alteration of age structure due to EE2 in lake 260 of the study. In fathead minnows size corresponds to age. During the preexposure segment of the study there is an almost even proportion of fathead minnows of each age (size class). During the first year, when the EE2 was added to the system (2001), no change in the size distribution occurred. However, in the next year (2002) there was no recruitment of fish under 4.5 cm, corresponding to no age 0 fish. In the last year of dosing with EE2 the proportion of larger fish increased because there were no age 0 or age 1 fish. In 2004 no EE2 was added to the system and a few age 0 fish were collected and larger (older) fish were found. In 2005 there was some recruitment beginning to occur and no fish in the age 3–4 range. During the same period the number of fish also dramatically declined with the lack of recruitment and the loss of the older fish to aging.

From the modeling efforts presented above, it will also be some years before the even distribution of age classes and the number of fish return to predosing levels. It will take several years to grow age 3–4 fish regardless of recruitment.

The alteration of age classes within fish subject to a variety of stressors is well known. Fishing pressure can remove older age classes from the population, creating a skew toward the younger age classes. It may be that the age distribution of a population is one of the more effective measures of the impact of a stressor on that population.

So far we have treated populations as if they are not connected to any other population of the same species within a landscape or region. When that occurs there is opportunity for a variety of dynamics to occur that would not be observed in isolated populations.

12.8.10 Contaminants in Spatially Structured Populations

This section describes populations within the context of a landscape. The first part describes the types of spatial structures. The next section discusses the use of simple metapopulation models in examining the potential dynamics due to toxicants, and introduces the action at a distance hypothesis. Finally, a presentation is made that includes populations in a spatial context, chemical contamination, and the introduction of a competitor.

12.8.10.1 The Spatial Structure of Populations

Organisms that make up a population are not evenly distributed in the environment, and migrate to other patches of habitat. Five general categories of spatial structure can be identified for the purposes of investigating the effects of toxicants upon populations (McLaughlin and Landis 2000):

1. Isolated populations
2. Classical metapopulations
3. Mainland–island or source-sink metapopulations

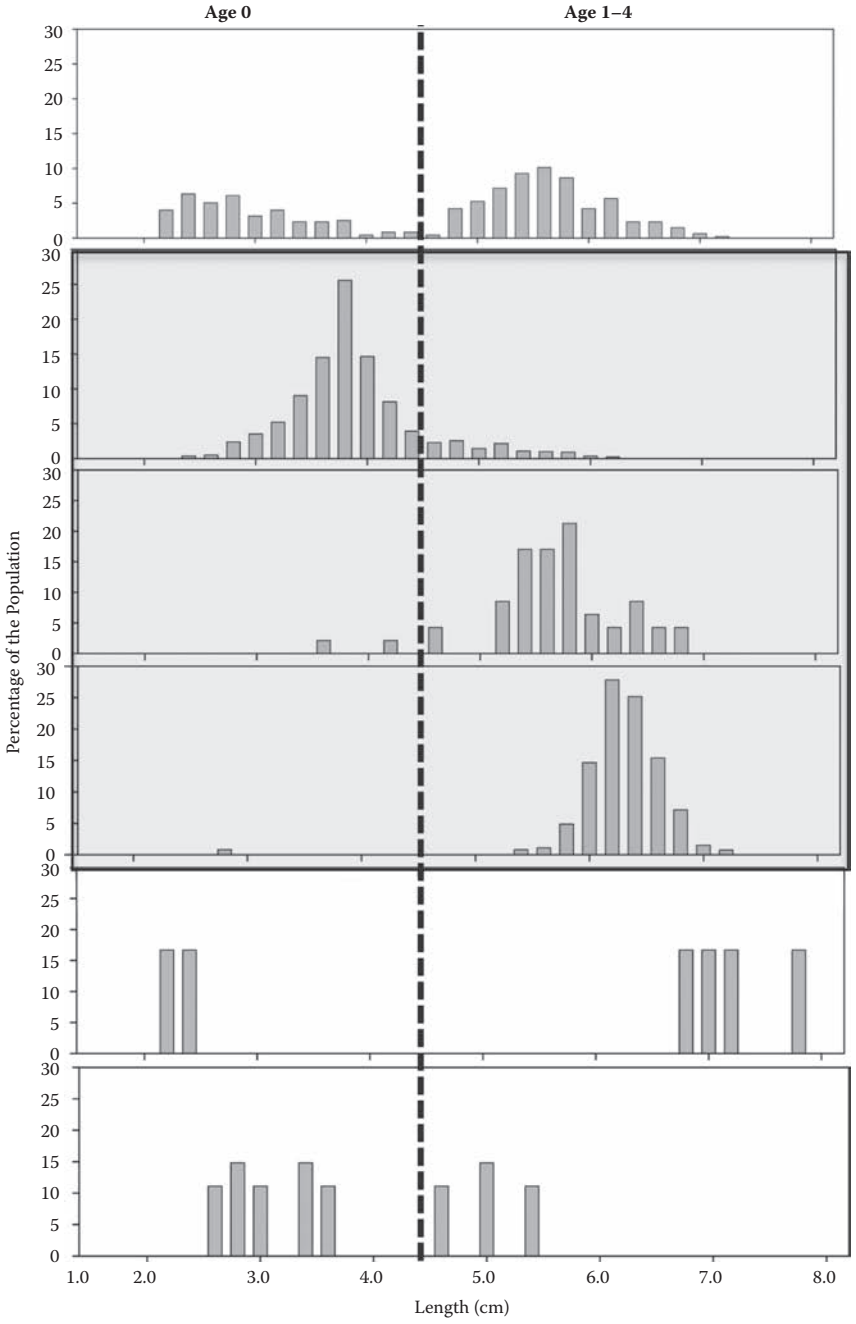


Figure 12.15 Fathead minnow age structure change due to the application of a synthetic hormone. The shaded area denotes the years dosed by the estrogen. (Data from Ken Mills, Fisheries and Oceans Canada, Winnipeg, Manitoba, Canada. Graph kindly supplied by Karen Kidd, University of New Brunswick, Saint John, New Brunswick, Canada.)

4. Patchy populations
5. Continuous populations

The first four categories are illustrated in Figure 12.16. Each of these systems has discrete habitat patches that supply the resources for survivorship and reproduction. This habitat is distinguished in this discussion between areas that provide corridors for migration between these patches. Both are important for the persistence of individuals and populations.

Isolated populations are a collection of habitats without migration or dispersal between them. These isolated populations act as if they are self-contained. Contaminants in one isolated patch do not affect dynamics in other patches. Conversely, once extinction has occurred in a patch, recolonization does not occur.

The next patterns are different types of metapopulations. A metapopulation is defined as a "population of populations" (Levins, 1969) connected through immigration and emigration. In a metapopulation, most organisms spend a majority of their life span in a single patch, but occasional migration does occur. Not all available habitats that can successfully maintain the species are always occupied.

Classical metapopulations result from low to intermediate migration between habitat patches. Not all potential habitats necessarily contain populations. Migration between patches affects the dynamics of local populations, even including recolonization following extinction. If sufficient dispersal between patches exists, then a "rescue effect" can prevent local extinctions.

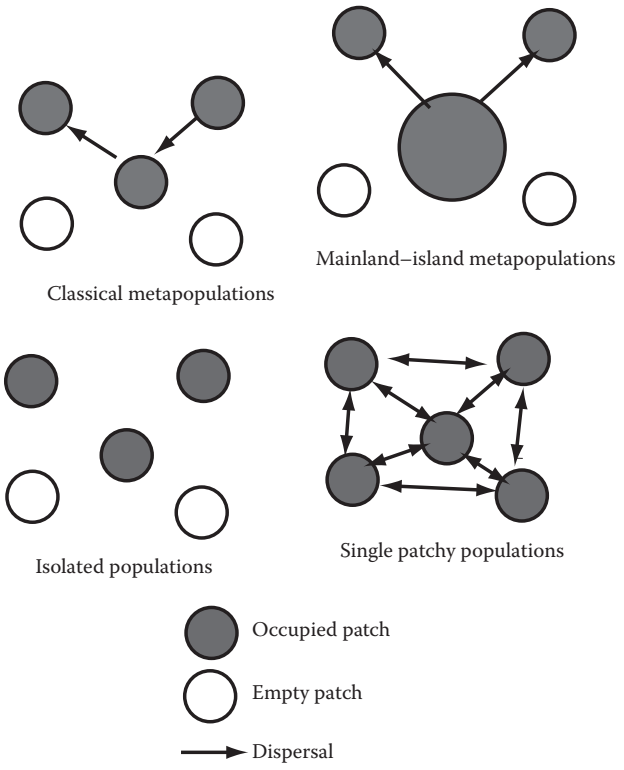


Figure 12.16 Spatial structure of populations.

Persistence of a metapopulation requires migration rates between patches sufficient to offset local extinction rates.

Mainland–island metapopulations and source-sink result when one or more of the local populations differ in the probability of local extinction. In a source-sink structure the source has excess organisms that migrate to other habitat patches. The other habitat patches, sinks, do not contain the resources to maintain a growing population. In contrast to a classical metapopulation, dispersal is not equal between patches but is from the source to the sinks. In a mainland-island metapopulation the difference is principally size, and all patches can support viable populations. Since smaller populations run a greater risk of extinction, the mainland can often provide a source for recolonization and the establishment of a new population on that patch. Conversely, islands can also act as refugia in the case when the mainland population becomes extinct.

Patchy populations are characterized by high rates of migration between habitat patches. Because of these high rates, the dynamics within the patch may be dominated by the migration instead of the local characteristics of the population. A characteristic of patchy populations is that one organism may spend its lifetime in several patches. In contrast, in a metapopulation the organism will likely spend all of its life span within one patch.

12.8.11 The Use of Metapopulation Models to Investigate Toxicant Effects

Metapopulation dynamics is a useful tool in evaluating the consequences of a stress over both time and space. The general assumption is that there is a minimum viable population (MVP) size below which patch extinction will occur. The carrying capacity is the population size that can just be maintained without a tendency to increase or decrease. A subpopulation serves as a sink if it is below the MVP and is draining immigrants. A subpopulation serves as a source for nearby patches by providing immigrants to them. Hanski and Gyllenburg (1993) derived the rescue effect: A population that is below the MVP can be rescued by organisms from a source. Wu et al. (1993) showed the importance of patch arrangement, size, and migration paths in the persistence of populations within a landscape.

Metapopulation models have been used to examine the dynamics of populations resulting from pesticide application. Sherratt and Jepson (1993) have investigated the impacts of pesticides to invertebrates using single-species and a predator-prey metapopulation models. In the case of the polyphagous predator, persistence of the population in the landscape is enhanced only if a few fields are sprayed, the application rate of the pesticide is low, or the intrinsic toxicity of the pesticide is low. There also appears to be an optimal dispersal rate that maximizes the likelihood of persistence of the predator in a sprayed field. Importantly, there are also patterns of pesticide application that would cause the prey insect population to reach higher densities than would occur otherwise. Dispersal rates of the predator and the prey are important factors determining the prey population densities. The importance of dispersal in the determination of the persistence of a population in a contaminated landscape was discovered in a subsequent study.

Maurer and Holt (1996) have used several types of metapopulation models to investigate the importance of migration and other factors in determining the impacts of pesticides. The exposure to the pesticide was assumed to decline geometrically to simulate degradation. An increase in migration rate among patches was found to decrease the persistence of the population. The more toxic the pesticide, the less persistent the population. An increase in the rate of reproduction improved the persistence of the population in the landscape. Further investigation also demonstrated that as

more of the patches became contaminated, the persistence of the population decreased by reducing the number of potential sites for colonization.

12.8.12 Patch Dynamic Models Based on Spromberg et al. (1998)

Spromberg et al. (1998) used modified versions of the Wu et al. (1993) patch dynamics model to examine the impact of toxicants on metapopulation dynamics. Since then, the models have been modified to examine the migration of degradative genetic elements (Landis et al. 2000) and the effects of toxicants and invasive species on landscapes (Dienes et al. 2005).

Using a template from Wu et al. (1993), Spromberg et al. (1998) developed a computer model of a generic animal patchy population that has at least one contaminated patch. The basic framework of the simulation model is presented in Figure 12.17. The single-species patchy population model is based upon deterministic equations for growth of the population, migration between patches, and the fate of the toxicant. In some of the simulations the toxicant is persistent and in others the toxicant degrades. In order to estimate exposure in a habitat patch, the models use a statistical distribution, the Poisson, in a stochastic (probabilistic) function. The amount of toxicant the organisms are exposed to in the habitat patch is determined by the persistence of the chemical in the patch and the chance encounter of the organism with the toxicant. The toxicological effects were determined by the incorporation of a concentration-response curve. An important assumption is that if an organism receives a toxic amount of the chemical, it dies in that patch and does not migrate to other patches and then die. The toxicant is also assumed to stay in the patch.

The models from the first formulation incorporate deterministic features (the growth, migration, and concentration-response) with a stochastic feature (exposure) and space (the arrangement of the patches).

Simulations were begun with a standard set of initial conditions with an amount of toxicant in one or more of the patches, an initial population size, and a set distance between the habitat

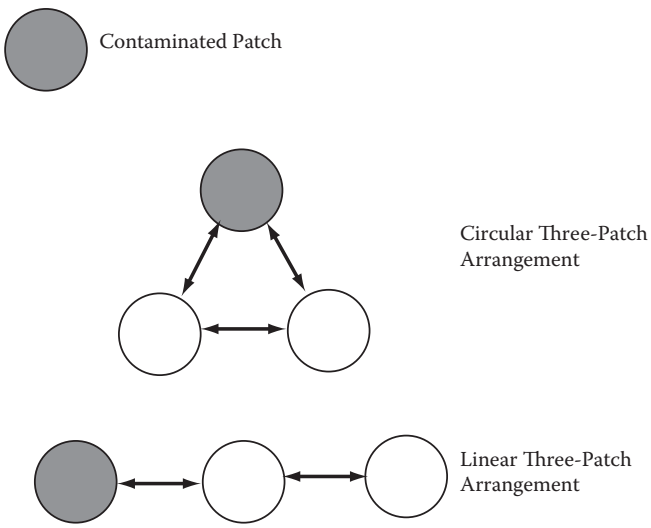


Figure 12.17 Arrangement of patches in the metapopulation model. In the discussion the distances between the patches are assumed to be equidistant. The exposure of the toxicant to the organisms is modeled using a Poisson distribution.

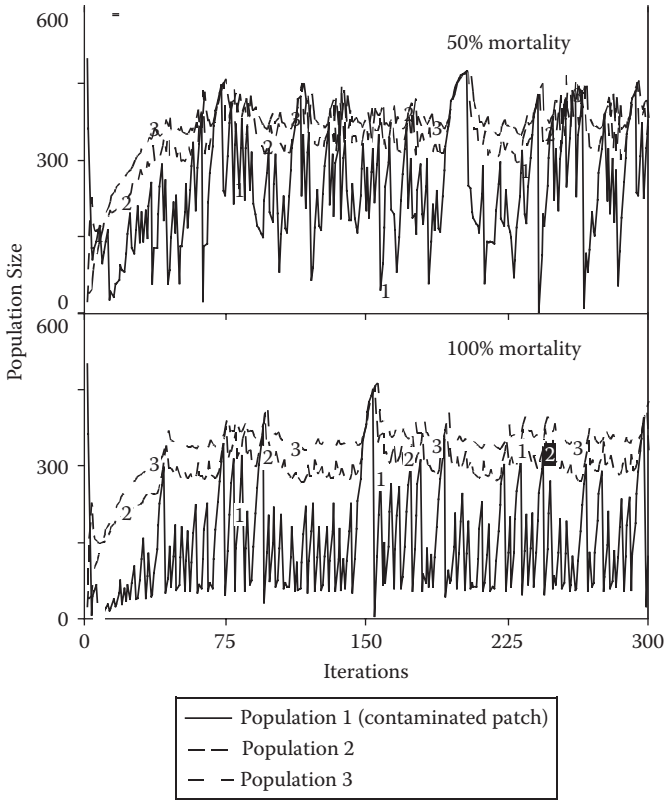


Figure 12.18 Action at a distance. In the simulation where the contaminated patch has a toxicant concentration equal to EC_{50}, the dosed patch has wide fluctuations. However, there is an occasional overlap among all three populations. The populations in nondosed patches are below the carrying capacity of 500. Even at EC_{100} in the dosed patch, organisms are still extant and occasionally reach numbers comparable to those of the nondosed patches.

patches. The model was then run through one time, and those results were used for the next iteration. Typically the models are run for 300 iterations (years), and the sizes of the populations in each habitat patch are plotted. In our first simulations a persistent toxicant was placed in the end patch, and all of the patches had the same initial population size. In some combinations of patches, starting numbers of organisms in the patches, and the patch arrangement, hundreds of replicate simulations were run to obtain information on the frequency of different outcomes.

The first models were run with the concentration of toxicant constant in the simulation. The first finding was that populations in patches removed from the contamination were affected by the presence of the toxicant. Remember that neither the toxicant nor the affected individuals migrate patch to patch. In the case of the linear, persistent toxicant model, the effects were the reduction of the population below carrying capacity and fluctuation in population size. The reduction in number and the fluctuations in the nondosed patches resulted in population sizes that were equal to those in the dosed patches, even with a dose equivalent to the EC_{50} (Figure 12.18). In the simulations when the dosed patch was at EC_{100}, organisms could still be found due to immigratation from the other patches. Due to the stochastic nature of the exposure between the toxicant and the organism, the simulations were repeatable only in type of outcome, not in the specific dynamics.

Spromberg et al. next performed simulations with a model toxicant that degrades. Three important findings resulted from these simulations. The first was that the effects of the toxicant can persist indefinitely past the presence of the toxicant. The second was that several discrete outcomes are available from the same set of initial conditions. The third finding was that not only is the chemical distribution and patch arrangement important, but the initial population sizes in each patch are critical for determining outcomes.

The range and types of outcomes depend on the specifics of toxicant concentration, initial population size, and distance between patches. The outcomes can be as varied as all three populations reaching carrying capacity, to all three becoming extinct with associated probabilities of occurrence. This simulation has three patches that are at a specified distance from each other. Only one patch contains the toxicant that is degraded halfway through the simulation. With an initial population size of 100 in each of the patches, 80% of the simulations resulted in all three of the populations in the patches reaching the minimum viable population. At MVP, one less organism and the population becomes extinct; at one more, the population can increase in size. All three populations reach carrying capacity in 20% of the simulations. In contrast are the simulations beginning with all three of the patches having initial population sizes of 140. In no instance did the populations decline to the MVP. In 82% of the simulations the outcome was all three populations reaching the carrying capacity. However, 18% of the simulations resulted in a stable oscillation, or bifurcation, of all three populations. A very different outcome, yet only the initial population sizes were altered.

In conclusion, metapopulation dynamics have several important implications for predicting the impact of chemical toxicants:

1. Effects can be promulgated between patches, even if the toxicant is not transferred. There is action at a distance between populations connected by immigration.
2. No patch is a reference if it is linked by migration in any way to the contaminated patch. If connected, the reference patch can be affected by the toxicant. The implications for the designs of field studies are dramatic. Simple upstream–downstream models where migration occurs cannot assume that the sites upstream of the contaminated area are unaffected.
3. Multiple discrete outcomes can occur from the same set of initial conditions. The differences between outcomes can be as great as from establishment of a population at carrying capacity to extinction.
4. Small differences in initial population sizes can dramatically alter the frequency of outcomes. It is not only the properties of the chemical and its interaction with an organism but also the status of the population that determines the outcome.

The simulations summarized above clearly demonstrate that a relationship exists between patch arrangement, initial population size, and the placement and amount of the toxicant in determining the number and frequencies of discrete outcomes. Changes can lead to new outcomes and alter the probabilities of each of these outcomes. Several of these findings have been confirmed by Johnson (2002) using an individual-based modeling system, a very different approach.

The findings of these modeling efforts led Spromberg et al. (1998) to hypothesize "action at a distance." The basic premise of action at a distance is that the impact of a toxicant can be transmitted to populations in other habitat patches by changes in the rate and direction of migration between patches by the individual organisms. Action at a distance does not require the direct contamination of a patch or of any organism that resides in a patch. The patterns in the resulting dynamics can be varied and nonlinear. The question is: Are these results artifacts

of the numerical simulations, or can they be expressed in simulated populations and ecological systems?

Experimental confirmation of action at a distance has been provided by the research of Louis Macovsky (1999). This study created a novel laboratory metapopulation model of the flour beetle *Tribolium castaneum*. Arranged linearly, habitat patches were linked by density-dependent dispersal of the adult morph. Patches were monitored for the indirect effects on population demographics beyond the patch that received a simulated adulticide over the period of approximately 1½ egg-to-adult cycles. It was demonstrated that indirect effects do occur in patches beyond the patch where adulticide occurred. The indirect effects were dose related and correlated with distance from the directly disturbed patch.

12.9 Interacting Populations in a Patchy Environment

In two instances the models developed by Spromberg have been expanded to include interacting populations. The first model (Landis et al. 2000) investigated the potential effects of novel genetic elements being introduced into bacterial populations. The second (Deines et al. 2005) examined the results of competition between a native and an invasive species. In both cases terms had to be added to describe the interactions of the species within each patch and migration between patches.

Further modeling using the same approach has been performed that expands the earlier findings (Landis and McLaughlin 2000). One of the questions not addressed in earlier modeling efforts was the placement of the contaminated site in the context of the landscape. To examine this aspect of the landscape a series of simulations were conducted with a linear arrangement of patches (Figure 12.19a) and a degradable toxicant. The initial population sizes were 200, 50, and 50 in patches 1, 2, and 3, respectively. In some of the simulations the toxicant was placed at the end of the linear arrangement, and in others it was placed in the middle. In every instance the patch dosed was the source patch for the simulated landscape.

When the source patch 1 was dosed (Table 12.9), the four outcomes were: (1) In 50% of the simulations only the population in patch 2 survived, and it was at the minimum viable population. MVP is the population size where the removal of one organism results in a negative growth rate for that population. (2) In 26% of the runs the populations in patches 1 and 2 survived at the MVP. (3) In 10% of the simulations all three populations survived at the MVP. (4) In only 14% of the runs did all three populations reach carrying capacity. Placing the toxicant in nonsource patches 2 and 3 in the middle and at the far end, with population densities at 200, 50, and 50 for patches 1, 2, and 3, respectively, resulted in all populations reaching carrying capacity.

In another series of simulations the arrangement was a population of 50 in patch 1, 200 in patch 2, and 50 in patch 3. Only three possible outcomes arose. (1) In 56% of the runs the populations in patches 1 and 3 existed at the MVP. (2) In 28% of the cases all three patches reached MVP. (3) In 16% of the cases all three populations reached carrying capacity. As with the first set of simulations applying the contaminant to nonsource populations (in this case at the ends of the landscape), all patches reached carrying capacity. In both series of simulations the alteration in possible outcomes and outcome frequency depended upon the location of the contaminated patch in the context of that specific landscape arrangement.

Four patch models were also examined for sensitivity to initial conditions. The arrangements examined were a linear four-patch arrangement, and a three-patch circular arrangement with the fourth patch attached to one patch of the circle as a tail (Figure 12.19b). Other than the

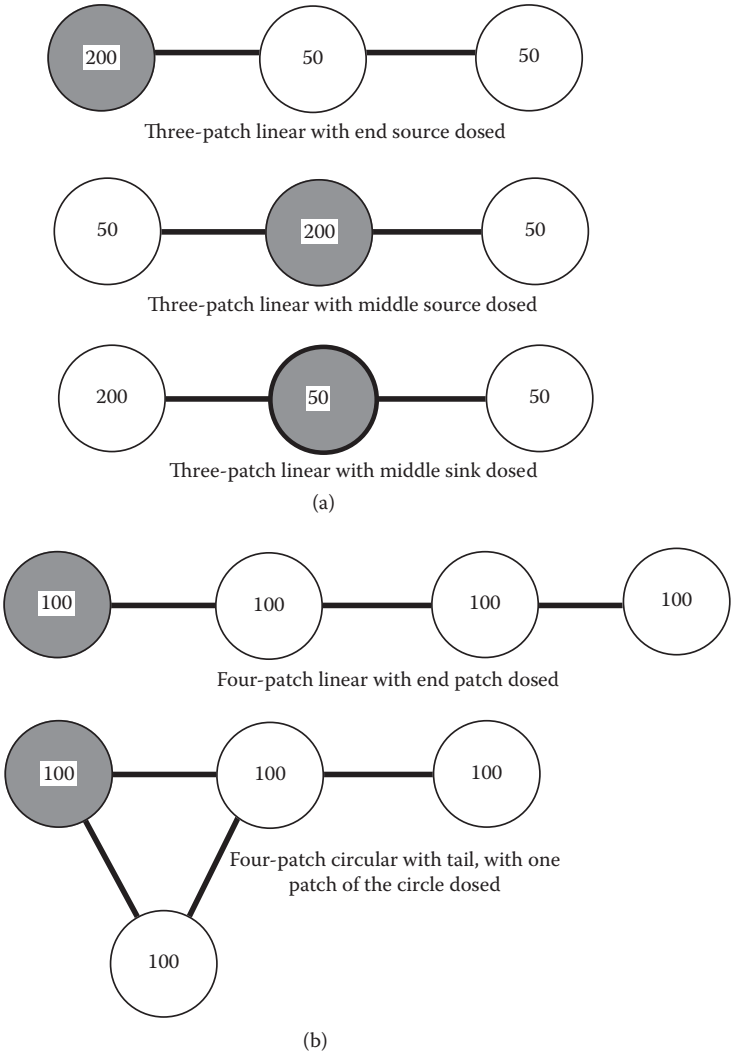


Figure 12.19 Baseline arrangement of the contaminated patches (shaded) and numbers of organisms at the start of the simulations.

Table 12.9 Frequency of Outcomes for Different Landscapes

Outcome by Patch Number	*End Dosed (source patch)*	*Middle Dosed (source patch)*
2 MVP	50%	0%
1, 2 MVP	26%	0%
1, 2, 3 MVP	10%	28%
1, 2, 3 cc	14%	16%
1, 3 MVP	0%	56%
Source Patch Not Dosed		
1, 2, 3 cc	100%	100%

Source: Adapted from Landis, W. G., and McLaughlin, J. F., *Environ. Toxicol. Chem.*, 19, 1059–1065, 2000.

Note: In each case the patch was dosed with an LD_{100}. Distance between patches is 2 units. MVP = minimum viable population, cc = carrying capacity. If not explicitly mentioned, the population in that patch is extinct.

arrangement of the patches, all other features were the same as in previous models with degradable toxicants. End patches were dosed. A series of simulations was performed to examine the importance of initial population size upon the frequencies of potential outcomes. Initial population sizes for each of the patches for simulations 1, 2, and 3 were 100, 75, and 50, respectively.

Table 12.10 summarizes the outcomes of the changes in initial population sizes upon the frequency of outcomes from these simulations. At an initial population size of 100 for each patch, each patch reached carrying capacity 100% of the time. Reducing the initial population size from 25 to 75% altered the outcomes. In 93% of the simulations patch 1 (dosed patch) went extinct and patches 2, 3, and 4 only reached the minimum viable population. In 7% of the trials patch 1 persisted at the MVP, as did the other patches. A further reduction to an initial population of 50 for each patch resulted in an outcome where patch 1 became extinct and populations in patches 2, 3, and 4 existed at the MVP. The same simulations were performed for the circle and tail arrangement, with one of the patches in the circle dosed. In the circle and tail arrangement the frequencies of the final outcomes were the same. However, the dynamics were different. Observations of the dynamics did indicate that patch 4, the tail, was more isolated from the effects of the toxicant than the end patch in the linear arrangements.

The final set of simulations examined the potential relationships in a mainland-island situation where the carrying capacity of one patch is much larger than that in the connected patches. Simulations were run in the typical fashion using a three-patch linear model, with the mainland patch being dosed. The carrying capacity of the dosed patch was 100, 500, or 1,000, with the island patches having a carrying capacity of 100. Initial population sizes were 100 for each patch.

Table 12.11 presents the results of the simulations. Three outcomes were observed in the simulations. In each case there was a probability that all three patches would reach carrying capacity no matter the initial scenario. At low initial populations in the mainland the probability was greater

Table 12.10 Frequency of Outcomes for Four-Patch Landscapes

Initial Population for Each Patch	*Outcomes*	*Percentage*
100	1 cc, 2 cc, 3 cc, 4 cc	100
75	2 MVP, 3 MVP, 4 MVP	93
	1 MVP, 2 MVP, 3 MVP, 4 MVP	7
50	2 MVP, 3 MVP, 4 MVP	100

Source: Adapted from Landis, W. G., and McLaughlin, J. F., *Environ. Toxicol. Chem.*, 19, 1059–1065, 2000.

Note: In each case, the patch was dosed with an LD_{100}. The linear and the circle and tail arrangements have the same frequency of outcomes. MVP = minimal viable population, cc = carrying capacity. If not explicitly mentioned, the population in that patch is extinct.

Table 12.11 Frequency of Outcomes for a Mainland–Island Type of Landscape

Outcome by Patch Number	*Carrying Capacities for Each Patch, Mainland First*		
	100, 100, 100	*500, 100, 100*	*1,000, 100, 100*
1 cc, 2 cc, 3 cc	21%	89%	95%
1 MVP, 2 MVP, 3 MVP	33%	9%	5%
2 MVP, 3 MVP	46%	2%	0%

Note: In each case the mainland patch was dosed with a degradable toxicant at an LD_{100}.

that either all populations existed at the MVP or patch 1, the mainland, would become extinct. As the carrying capacity of the mainland increased, so did the probabilities of all of the patches existing at MVP or reaching carrying capacity.

The metapopulation modeling clearly demonstrates that a relationship exists between patch arrangement, initial population size, and carrying capacity in determining the number and frequencies of discrete outcomes. Changes can lead to new outcomes and alter the probabilities of occurrence. Are these results artifacts of the numerical simulations, or can they be expressed in simulated populations and ecological systems?

Partial experimental confirmation of action at a distance has been provided by the research of a former graduate student, Louis Macovsky (1999). This study created a novel laboratory metapopulation model of a single insect species, *Tribolium castaneum*. Arranged linearly, habitat patches were linked by density-dependent dispersal of the adult morph. Patches were monitored for the indirect effects on population demographics beyond the patch that received a simulated adulticide over the period of approximately 1½ egg-to-adult cycles. It was demonstrated that indirect effects

do occur in patches beyond the patch directly impacted. The indirect effects were dose related and correlated with distance from the directly disturbed patch.

There is a growing recognition that populations of interest to resource managers are spatially structured. Thorrold et al. (2001) discovered that the forage fish *Cynoscion regalis* (weakfish) along the Atlantic coast of North America exists as metapopulations. Using tagging studies, it was found that individuals did stray between spawning runs along the coast at a high enough frequency to fit the definition of a metapopulation. Similarly, the Pacific herring of the British Columbia coast have been identified as a metapopulation comprised of several patches or subpopulations (Ware et al. 2000). Pacific herring are an important commercial fishery in Canadian waters. The simulation models above suggest that natural or anthropogenic impacts to one part of the metapopulation could have important effects to the apparent numbers in other parts of the range of the fish. The causes would be spatially and likely temporally displaced from the impacts, making attribution of declines or prediction of future numbers problematic without understanding the spatial construction of the population.

In two instances the models developed by Spromberg have been expanded to include interacting populations. The first model (Landis et al. 2000) investigated the potential effects of novel genetic elements being introduced into bacterial populations. The second (Deines et al. 2005) examined the results of competition between a native and an invasive species. In both cases terms had to be added to describe the interactions of the species within each patch and migration between patches.

In Landis et al. (2000) we found that the introduction of a genetic element that altered the fitness of a host within a landscape resulted in a number of different dynamics. As the rate of infection increased, the dynamics could be forced into severe oscillations that could later be damped. It also was not necessary for the movable genetic element to increase the fitness of the host; what was important was the rate of infection. The pattern of patches was important in determining the specifics of the interactions. We also found that although the specifics of the rate of infection were highly variable, it was inevitable that the infectious genetic element would be spread to the host population.

Similar in many respects to the situation for movable genetic elements is the question of the risk of invasive species. Deines et al. (2005) reported the results of our modeling efforts to describe the patch dynamics of nonindigenous (invasive) species within three patch systems. A schematic of the model is portrayed in Figure 12.20. The models included an invasive, multiple patches, differential fitness between the competitors, a toxicant, and interaction with a toxicant. As in earlier models, certain initial conditions would result in multiple outcomes in the colonization of the invasive.

The effect of the toxicant was to alter the variability of the simulations. With a persistent toxicant there was no difference in each replicate simulation until a dose of LD_{100} was present in one of the patches. If a nonpersistent toxicant was simulated, then multiple outcomes from a variety of starting conditions were the result. Toxicants had to be in high doses to influence the outcomes.

One surprise was the importance of having the invasive established in a habitat patch somewhat isolated from the other patches so that the invasive could establish a large population without the native being rescued. We termed this the "beachhead effect." The beachhead allows the invasive population to build to sufficient numbers so that it now acts as a source population for other invasions to other regions of the landscape. We found that with our models there were clear optima between distance between patches, the competitive ability of the invasive, and the percent spread of the invasive to the patches. In one example (Figure 12.21) the optimal distances between

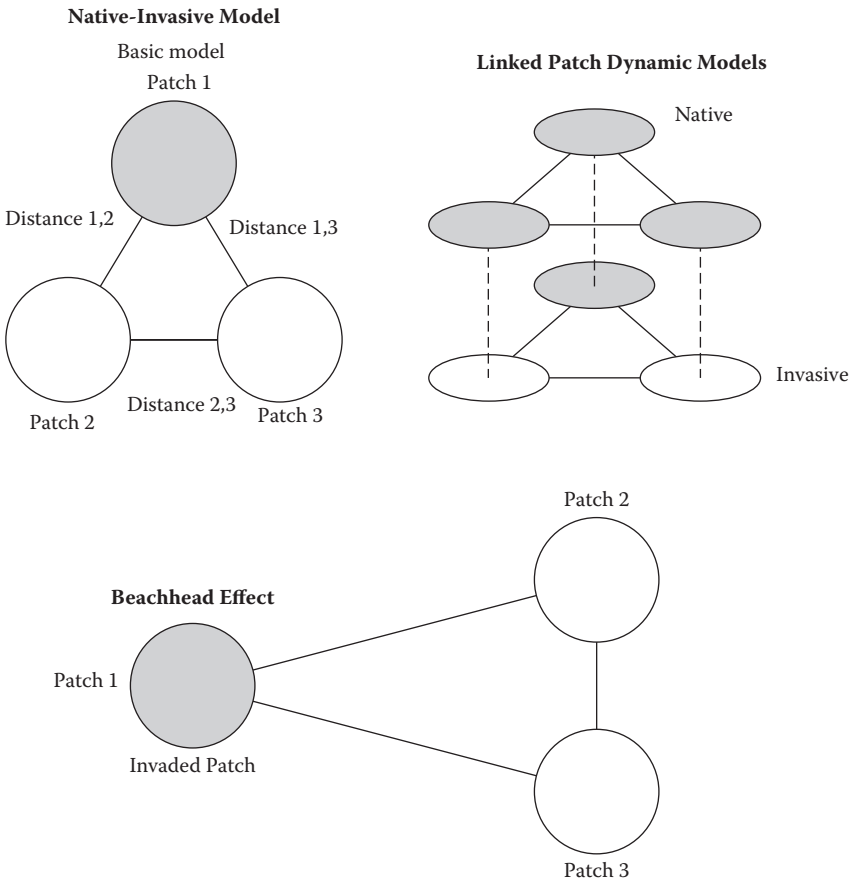


Figure 12.20 Native-invasive contaminant patch dynamics model. Basic layout of the patch dynamic model. The basic form is the three-patch circular model as found in Spromberg et al. (1998). Essentially, each layer represents the dynamics of either the native or nonindigenous species, and each layer is connected to calculate the interactions between each species. Three patches are arranged in a circular fashion to represent the three patches in the landscape. The distances between the patches can vary and relate to the relative rate of migration of the organisms between patches. The introduced species typically starts in patch 1, and the native can be found in every patch (shaded areas). The relative competitive ability of each species, the amount of toxicant, and the initial population size can be set for each patch.

patches were 10 to 50 model units. Distances longer or shorter than this resulted in no invasion. Also contrary to our expectations, at the longer distances an increase in competitive ability of the invasive was not important.

12.10 The Importance of Patch Dynamics

In the 11 years since publication of Spromberg et al., the importance of the spatial relationships of habitat patches has become recognized. First, there are now multiple examples of populations of regulatory interest that exist in patchy or metapopulations. Weakfish (Thorrold et al. 2001) and

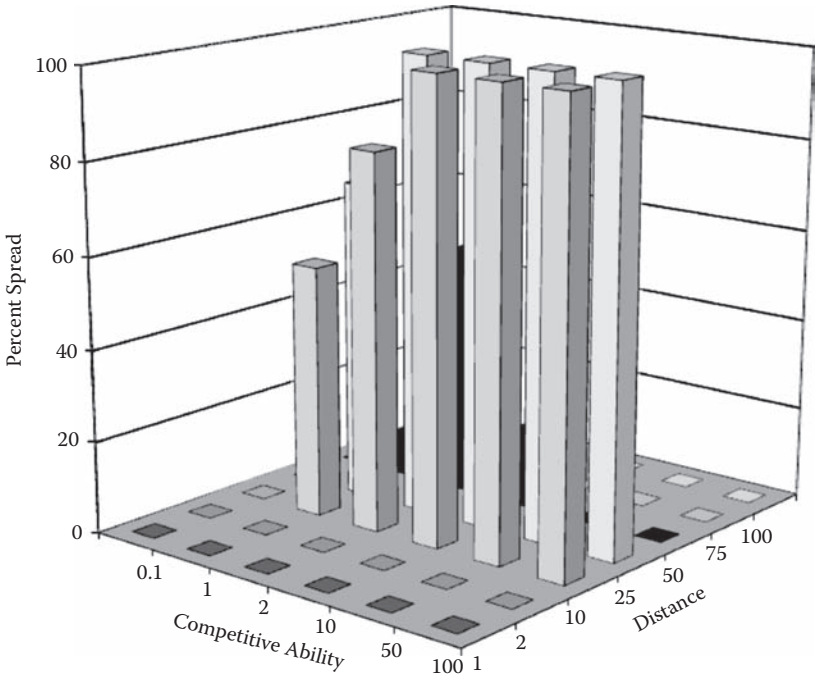


Figure 12.21 Interaction between distance between patches, competitive ability, and spread of the invasives. Note that a range of outcomes can occur depending upon the specific distances and competitive ability in this system. Even at a competitive ability far above the native species invasion does not occur if the patches are either too close or too far away. At a distance of 50 units the optimal competitive ability is actually twice that of the native.

the Pacific herring along the British Columbian coast have been identified as populations existing as interacting patches. Recent studies of common loons (Walters et al. 2008), mummichogs (Nacci et al. 2008), and the amphipod Leptocheirs (Bridges et al. 2008) have all incorporated metapopulation modeling into the assessment of toxicant effects over large spatial scales.

Ten years later Spromberg is using patch dynamics to understand how stocks of Coho salmon are impacted by toxicant inputs that result in prespawn mortality. In her latest study she has found a relationship between the number of source patches (streams) and the likelihood of extinction of the species. As the number of sinks (spawning streams with high mortality) are increased, so is the probability of extinction of the entire population. As demonstrated with our early, simple models, an entire population can become extinct, although the direct toxicant effects are localized to one area.

12.11 Implications

Agriculture and urbanization have fractured the patterns of the landscape, producing a variety of habitat patches. Aquatic environments have been modified, altering the movement of organisms by the introduction of dams, modification of channels, or contamination of water and sediment. Terrestrial environments are fragmented by shopping malls, roads, and suburbia. Clearly spatially

structured populations exist, even if they were continuous before. There are four clear implications for assessing the impacts of toxicants.

First, the spatial context of population being studied must be understood. In order to predict the effects of a toxicant upon a spatially structured population, it is important to understand the spatial structure. Impacts on one part of the population can have effects on other parts through changes in migration patterns across the landscape. A change in migration patterns or sources and sinks within a landscape can change the occupancy of the patches. Placement of the same amount of toxicant in different parts of the landscape changes the ranges of potential outcomes for the populations. To understand the dynamics of a population in one habitat patch, the spatial context of that population needs to be clearly understood. Impacts to a distant population may have important effects on a local population.

Second, multiple outcomes are likely from the same set of initial conditions. Our simulations often resulted in more than one outcome being realized by the same set of initial conditions. This is due to the fact that a probabilistic function was incorporated to describe the dosing of the individuals within a patch. Outcomes as divergent as possible for a population, from extinction to reaching carrying capacity, can result from the same set of initial conditions. Only a clear knowledge of the properties of the organisms as individuals, the distribution of the toxicant, and the spatial arrangement of the populations will allow an accurate prediction of the range of outcomes.

Third, indirect effects can cause extinction over an area broader than the occurrence of the contaminant. Action at a distance dictates that there does not have to be exposure to a contaminant by individuals of a habitat patch for impacts to be realized upon the population. Measurement of contaminant levels in organisms of that patch will not indicate any exposure, although there is an impact due to contamination in another part of the landscape. Exposure and effect need to be understood in a landscape context.

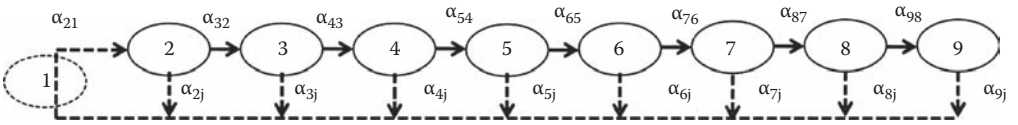
Fourth, the idea of a reference site is archaic when patch dynamics are understood. If one habitat or patch is connected to another contaminated site by migration, then it cannot serve as a reference site. Changes in one part of a landscape can be transmitted throughout. So what about using areas clearly not linked by migration? Then there is the problem of ensuring sufficient genetic and community similarity. The bottom line is that there is no such thing as a reference site when it comes to populations and landscapes.

Appendix 12.1: Age-Structured Population Modeling in Detail

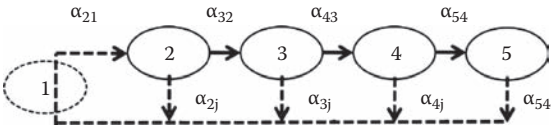
Age-structured population models are used to describe the observed and potential population dynamics of the Pacific herring within Puget Sound. The projection matrix methods of Caswell (2001) as exemplified by the RAMAS GIS program are used to characterize and simulate the population dynamics of the Puget Sound Pacific herring stock (PSPHS).

The differential growth, survivorship, and reproduction of individuals within a population were expressed in a life history graph. Such a graph for CPPHS within the study area is presented in Figure 12.22. The early collections of CPPHS had fish up to age 9 (Figure 12.22a), but in many other locations and in the latter collections of CPPHS the age structures were compressed (Figure 12.22b). A specific life history graph was made for each PSPHS for which data were available.

The horizontal arrows represent survivorship from one age to another within the population. For example, the term α_{32} is the proportion of age 2 fish that survive to age 3. The dashed lines represent the contribution of each of the age classes to the fish that hatch and survive to age$_j$, or in



(a) Life history graph for the CPPHS during the 1970s with age 9 as the oldest age class.



(b) Life history graph for a PSPHS population with age 5 as the oldest age class.

$$A = \begin{bmatrix} 0 & \alpha_{2j} & \alpha_{3j} & \alpha_{4j} & \alpha_{5j} \\ \alpha_{21} & 0 & 0 & 0 & 0 \\ 0 & \alpha_{32} & 0 & 0 & 0 \\ 0 & 0 & \alpha_{43} & 0 & 0 \\ 0 & 0 & 0 & \alpha_{54} & 0 \end{bmatrix}$$

(c) Transition matrix representation of graph b.

Figure 12.22 The depiction of the life cycle for Pacific herring as a transition matrix. (a) The life cycle for CPPHS during the 1970s is depicted. Age 9 fish were observed. Age 1 is depicted as a dotted circle since these fish are not counted during spawning events. (b) A life cycle graph for the PSPHS during the 1990s. Age 5 fish were the oldest age class observed in several stocks. If older age classes were observed, the life history graph would be modified accordingly. (c) Life history graph. In the simulations of population dynamics for the various stocks, mean values and a standard deviation were used to represent the variability in the transition terms of the life history graphs and the transition matrixes.

this case, age 2. The life history graphs can be represented by a square matrix (a) containing terms for survivorship and reproduction. Because PSPHS are only counted during spawning, age 1 is represented by a dotted eclipse in the diagram. Reproductive output for each life stage is assumed to be constant for each stock for each year. No information on differential survivability from egg to spawning age exists, so it is assumed to be the same for each age class. The contribution of each age class to the next age 2 class is then the age-specific egg production times the age-specific number of fish at that age times the constant that represents the survivorship of those eggs to age 2. In the stage matrix this would be the proportion of each age that survives to age 2. In order to accommodate the RAMAS environment, the calculated survivorship was input as if it were to age 1 and the transition value for age 1–2 fish was set at 1. No reproductive rate was included for age 1 fish.

Age-structured populations simulated in the fashion described in this paper will reach an equilibrium in the number of organisms at each age class within a few iterations. This is the equilibrium age structure; it is written as a vector and is usually normalized to the proportion of the population. As this stable age structure is reached in the simulation, there will be a single value that, multiplied by the number of each age class, will result in the increase in each age class for that population. This value is the intrinsic rate of growth, or λ. These features of a population can be derived directly from the transition matrix. The intrinsic rate of growth of the population equals the dominant eigenvalue of the transition matrix and was calculated by the RAMAS software for

Table 12.12 Transition Matrix for Squaxin Pass 1996 Derived from 1994 to 1996 (standard deviations are in parentheses)

	Age 1	*Age 2*	*Age 3*	*Age 4*	*Age 5*	*Age 6*
Age 1	0.0	0.133	0.218	0.299	0.382	0.465
Age 2	1.0	0.0	0.0	0.0	0.0	0.0
Age 3	0.0	1.9 (1.67)	0.0	0.0	0.0	0.0
Age 4	0.0	0.0	0.36 (0.33)	0.0	0.0	0.0
Age 5	0.0	0.0	0.0	0.04 (0.07)	0.0	0.0
Age 6	0.0	0.0	0.0	0.0	0.11 (0.13)	0.0

each year for each PSPHS. Likewise, the equilibrium age structure of the population, the right normalized eigenvector corresponding to the dominant eigenvalue λ, was calculated as part of the same RAMAS analysis. These values were calculated for all years and stocks for which data were available.

A series of simulations were run based upon the sampling data from Cherry Point, Squaxin Pass, Quartermaster, Port Orchard/Madison, Port Gamble, Port Susan, Skagit, Samish, and Semiahmoo Bay. These simulations incorporated the observed variability (as standard deviations) in the terms that described the survivorship between each age class in the transition matrix. The standard deviations for some of the parameters of the stocks were large compared to the mean values. This likely was due to the intrinsic variability of the population, but also the sample error.

As an example, the transition matrix for Squaxin Pass for the 1996–2006 simulation (Figure 12.22a) is below (Table 12.12). Similar matrixes were generated for each set of simulations for all of the stocks. One of the uncertainties was that many of the stocks had gaps of varying length from which to calculate average values and standard deviations.

Study Questions

1. Describe the importance of context in environmental toxicology.
2. Outline the HPDP and its key features.
3. What are the advantages of the HPDP compared to other models attempting to describe ecological systems?
4. What are the two categories of biomonitoring programs?
5. List the six current organizational levels of biomonitoring and explain.
6. Discuss some examples of means by which past and current exposures to toxic xenobiotics are detected.
7. Of what value are biomarkers as predictors of the effects of toxicants?
8. Discuss the inhibition of specific enzymes, enzyme synthesis induction, stress proteins, DNA and chromosomal damage, immunological endpoints, and nutritional state as biomarkers of exposure to xenobiotics.
9. Describe physiological and behavioral indictors of toxicant impact.
10. What are toxicity identification and reduction evaluations?
11. What are the advantages and disadvantages to the toxicity tests given as examples in the text?

12. Define and discuss sentinel organisms as ecosystem monitors. What are the advantages of using this method?
13. Discuss alternations in genetic structure as a means of measuring xenobiotic effects on a population.
14. How can species diversity indicate stress on an ecosystem? What drawbacks does the structure of biological communities have as an indicator of stress?
15. What questions should biological diversity raise if it is used as an indicator of xenobiotic impacts upon biological communities?
16. Population dynamics are a key in understanding toxicological effects. Outline the key features of an age-structured population.
17. What are the different types of effects that a toxicant may have on the age structure of a population?
18. What is an NEV and how might it be diagnostic?
19. How does the fathead minnow population dosed with EE2 respond, and how long does the effect last?
20. What are the different kinds of patch dynamics that may exist?
21. Describe action at a distance and its implications.
22. Why are multiple outcomes possible for some of the patch dynamic models?
23. What are the three major implications of patch dynamics for understanding pollutant effects to populations?

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Chapter 13

Ecological Effects: Community to Landscape Scales of Toxicological Impacts

13.1 Introduction

Chapter 12 covered the basics of biomonitoring and reviewed ecological effects that become apparent at the scale of the population. Putting populations within a spatial context also has implications as far as the existence of reference sites. So far we have not discussed competition between species and the interactions that define a community. In this chapter we start with communities, competition, and eventually discuss effects upon landscapes.

13.2 Community Effects

13.2.1 Competition and Indirect Effects

The impact of toxicants upon the structure of communities has been investigated using the resource competition models of Tilman. Species diversity may be decreased or increased, and a rationale for studying indirect effects emerges.

13.2.2 Resource Competition as a Model of the Direct and Indirect Effects of Pollutants

Resource competition as modeled by David Tilman and adopted for toxicological purposes by Landis may assist in putting into a theoretical framework the varied effects of toxicants on biological systems. Detailed derivations and proof can be found in Tilman's (1982) excellent monograph. This brief review is to demonstrate the utility of resource competition to the prediction, or at least explanation, of community level impacts. Landis (1986) applied these ideas to toxicant impacts.

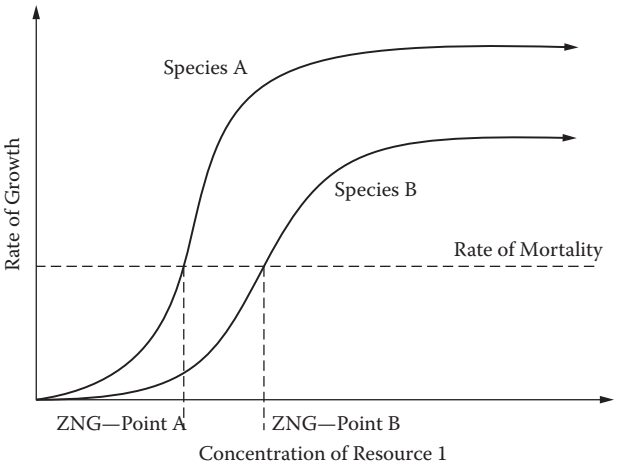

Figure 13.1 Rate of growth and resource supply. As the supply of resource increases, so does the reproductive rate of an organism until a maximum is reached. At one point the rate of growth exceeds the rate of mortality and the population increases. As long as the resource concentration exceeds this amount, the population grows; below this amount, extinction will occur.

The basis for the description of resource competition is the differential uptake and utilization of resources by species. The use of the resource, whether it is space, nutrients, solar radiation, or prey species, can be described by using growth curves with the rate of growth plotted on resource concentration or amount.

Figure 13.1 illustrates growth curves for species A and species B as plotted against the concentration of resource 1. At a point for each species, the rate of growth exceeds mortality at a certain concentration of resource 1. Above this concentration the population grows, and below this concentration extinction occurs. A different zero net growth point, the point along the resource concentration where the population is at break even, differs for the two species unless differential predation forces coincidence. These curves, at least for nutrients, are easily constructed in a laboratory setting.

To describe the uptake of the toxicant by the organism, a resource consumption vector is constructed. Figure 13.2 diagrams a consumption vector for the two species case. This vector is the sum of the consumption vectors for each of the resources, and the slope is the ratio of the individual resource vectors. Although it is certainly possible that the consumption vector can change according to resource concentration, it is assumed in this discussion to be constant unless altered by a toxicant.

The zero net growth point (ZNG) expanded to the two-dimensional resource space produces a zero net growth isocline (ZNGI), as illustrated in Figure 13.3. At the ZNGI, the rate of reproduction and the mortality rates are equal, resulting in no net growth of the population. In the shaded region the concentration or availability of the resource results in an increase in the population. In the clear area, the population declines and ultimately becomes extinct.

The shape of the ZNGI is determined by the utilization of the resource by the organism. If the resources are essential to the survivorship of the organism, then the shape is as drawn. Eight different types of resources have been classified according to the ZNGI.

The eventual goal in the single-species case is the prediction of where the equilibrium point on the ZNGI will be with an initial concentration of resources. A supply vector U1 can be derived

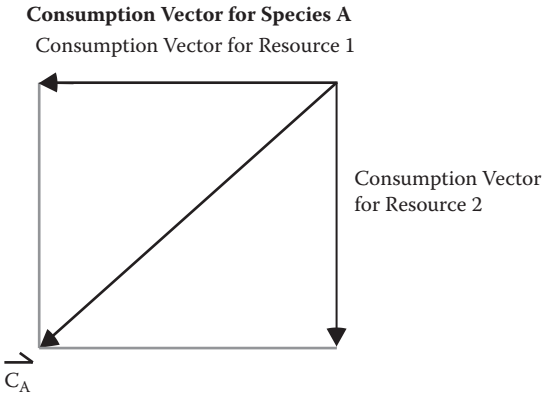

Figure 13.2 Consumption vector. Consumption vector for species A. C_A is the sum of the vectors for the rate of consumption of resource 1 and resource 2. The consumption vector determines the path of the concentrations of resources as it moves through the resource space. In the one-species case, the eventual equilibrium of resources occurs where the sum of the utilization vectors and the C_A is zero.

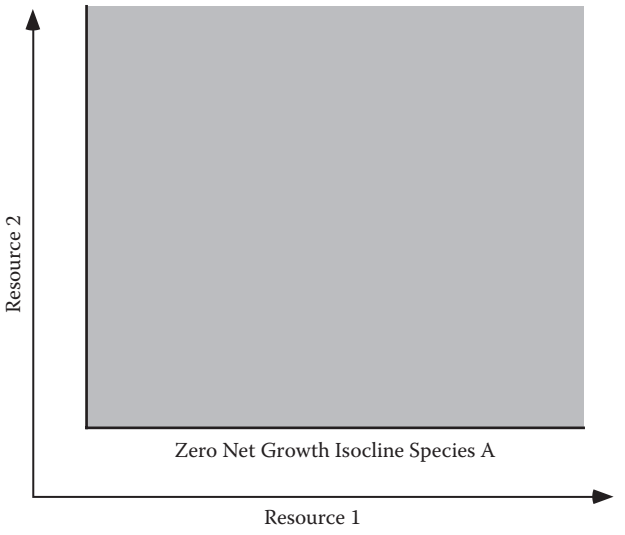

Figure 13.3 Zero net growth isocline (ZNGI). The ZNGI is the line in the resource space that represents the lowest concentration of resources that can support a species. In an equilibrium situation, the equilibrium will eventually be drawn to a point along the ZNGI. In the shaded area of the resource space, the population will grow. In the whiter area, extinction will eventually occur.

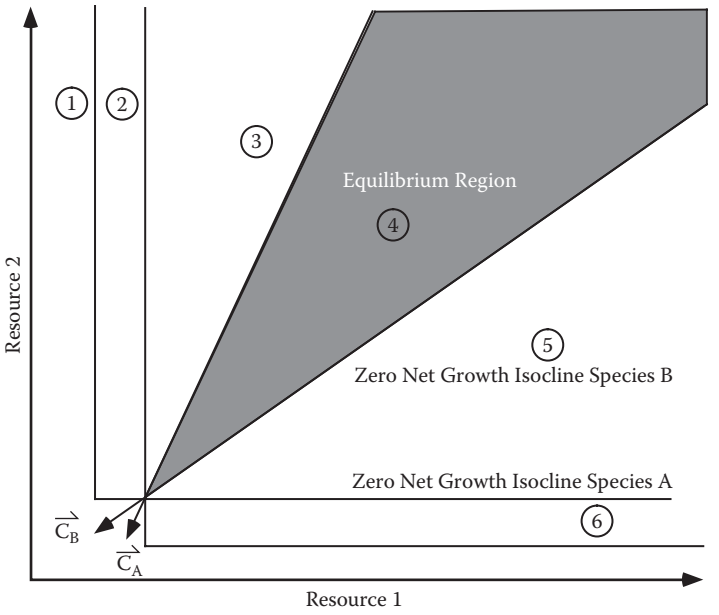

Figure 13.4 Two-species graph. The $\vec{C}_A$ and ZNGI for each species are incorporated into the graph. Six regions of the resource space are created. In region 1, neither species can exist; in region 2, only species A can survive; in region 3, species A and species B can survive but B is driven to extinction; region 4 is the equilibrium region; in region 5, both species A and species B can survive but A is driven to extinction; and in region 6, only species B can survive. In the case illustrated, if the original resource point, S1,S2, lies within the shaded equilibrium region, both species will exist.

that describes the rate of proportion of supply from the resource supply point. At equilibrium in a one-species case, the resources in a habitat will be at a point along the ZNGI where

$$\vec{C}_A + \vec{U}_{1,2} = 0 \tag{13.1}$$

Tilman has shown that this point exists and is stable. Metaphorically speaking, the $\vec{C}1$ pulls the equilibrium point along the ZNGI until the consumption of the two resources is directly offset by the rate and proportion of the supply of the resources. Although the description is for two essential resources, the same holds true for other resource types.

The two-species case can be represented by the addition of a new ZNGI and consumption vector to the graph of the resource space. In the case of essential resources, six regions are defined (Figure 13.4). Region 1 is the area in which the supply of resources is too low for the existence of either species. In region 2, only species A can survive since the resource concentration is too low for the existence of species B. In region 3, coexistence is possible for a time, but eventually species A can drive the resources below the ZNGI for species B. Region 4 is the area in which an equilibrium is possible and the consumption vectors will drive the environment to the equilibrium point. The equilibrium point lies at the intersections of the two ZNGI. In region 5, coexistence is possible for some period, but eventually species B can drive the resources below the ZNGI for species A. Finally, within region 6, only species B can survive.

An unstable equilibrium can exist if the consumption vectors are transposed. However, since any perturbation would result in the extinction of one species, this situation in unlikely to be persistent.

The basic assumptions made in order to model the impacts of toxicants on the competitive interactions discussed above are (1) the toxicant affects the metabolic pathways used in the consumption of a resource, and (2) this alteration of the metabolism affects the growth rate vs. resource curve. In the terms of resource competition, the consumption vector is changed and the shape and placement of the ZNGI are altered. In the following discussions the implications of these changes on examples using essential resources are depicted.

Case 1—In the first example, the initial conditions are the same as those used to illustrate the two-species resource competition model with essential resources (Figure 13.5). The toxicant alters the ability of species B to use resource 1. The slope of $\bar{\mathbf{C}}_\mathbf{B}$ increases and the ZNGI and the $\bar{\mathbf{C}}_\mathbf{B}$ shift the equilibrium point and reduce the area of the equilibrium region. The resource supply point A, which was part of the original equilibrium region, is now in an area that will lead to the eventual extinction of species B. Conversely, point B is now contained within the equilibrium region. However, the overall reduction of the size of the equilibrium region will decrease the likelihood of a competitive equilibrium.

Case 2—In this example the toxicant affects species A, increasing the slope of the vector $\bar{\mathbf{C}}_\mathbf{A}$ as the ability of species A to use resource 1 is altered. In Figure 13.6a the toxicant has forced the $ZNGI_A$ to a near overlap with the $ZNGI_B$ in the utilization of resource 1. In only a small region can species A drive species B to extinction. As the $ZNGI_A$ and $ZNGI_B$ overlap in regards to resource 1, the equilibrium region would be at a maximum. The addition of

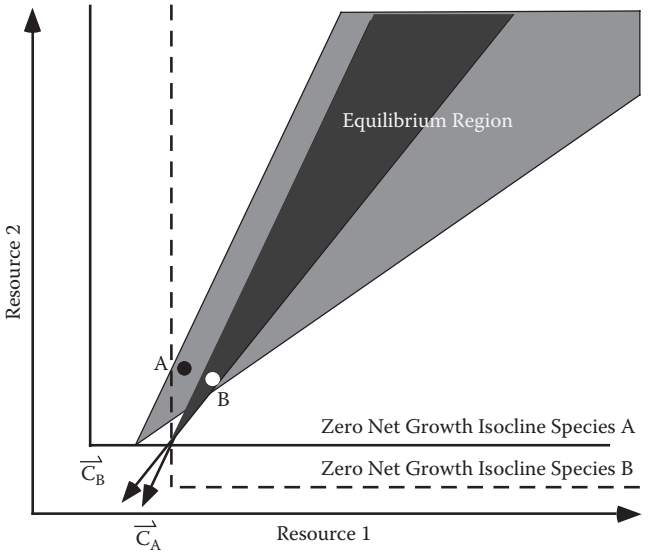

Figure 13.5 Case 1: Toxicant impacts on species B. The introduction of a toxicant alters the ability of species B to use resource 1. The slope of the consumption vector is altered and the ZNGI shifts compared to the initial condition. The equilibrium point moves and the equilibrium region shifts and shrinks. With a smaller equilibrium region, the probability of coexistence of the two species also is decreased.

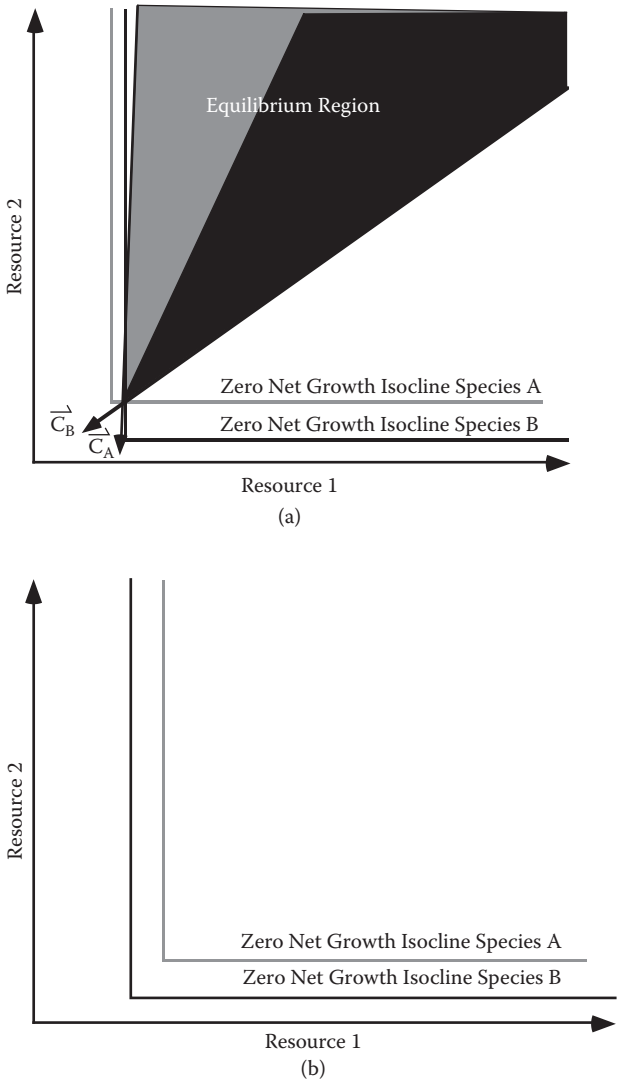

Figure 13.6 Case 2: Toxicant impacts on species A. The delivery of the toxicant impacts upon the ability of species A to use resource 1. In this case, the equilibrium point has not moved but the equilibrium region has greatly increased, thus increasing the opportunities for a coexistence of the two species (a). However, an increase in the equilibrium and an increase in species diversity do not mean that the system is less stressed. (b) The addition of a toxicant has forced the $ZNGI_A$ inside the $ZNGI_B$, resulting in the eventual extinction of species A.

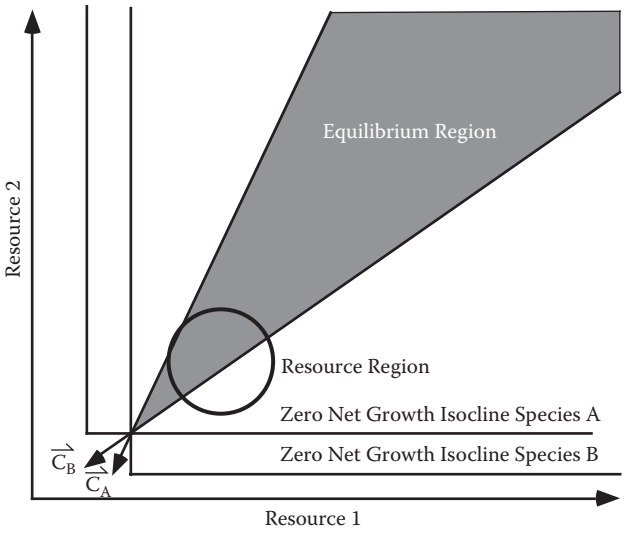

Figure 13.7 Resource heterogeneity. The heterogeneity of the resource can be represented by two-dimensional 95% intervals projected upon the graph. The placement of the circle can help to predict the dynamics of the system and describe the occasional extinction of one species and the coexistence of the two.

more toxicant (Figure 13.6b) would drive the $ZNGI_A$ inside the $ZNGI_B$, and in all regions of the resource space, species B can drive A to extinction. Coexistence over any protracted time is now impossible. Interestingly, the situation that produces the greatest likelihood of a competitive equilibrium also borders on extinction.

In the examples presented above, resource heterogeneity was not incorporated. Resources in nature are variable in regards to supply over both time and space, and this does much to explain the coexistence of competing species. Tilman represents this by projecting a 95% bivariate confidence interval, a circle, upon the resource space (Figure 13.7). In this case, the dynamics of the competitive interactions between the two species change depending upon the resource availability. In part of the confidence interval, a competitive equilibrium is possible. In other parts of the confidence interval, competitive displacement of species A is possible.

The significance of this result cannot be overlooked. If the confidence interval is based on time, competitive relationships differ on a seasonal basis and the lack of a species at certain times may not be due to an increase or decrease in pollutants but may be attributable to yearly changes in resource availability. Seasonal changes in species composition are expected and the limitations of one-time sampling are well known. However, the confidence interval can also be expressed over space. Slight differences in resources ratios that are part of the normal variation within a stream, lake, or forest can result in different species compositions unrelated to toxicant inputs.

Conversely, toxicants that do not directly affect the competing species but instead alter the availability of resources can alter the species composition of the community. In Figure 13.8, the case of the moving resource confidence interval is presented. In this case, the ratio of resource 2 has been increased relative to resource 1. This could be the alteration in microbial cycling of nutrients or the alteration in relative proportions of prey species for a predator, to name two examples. The

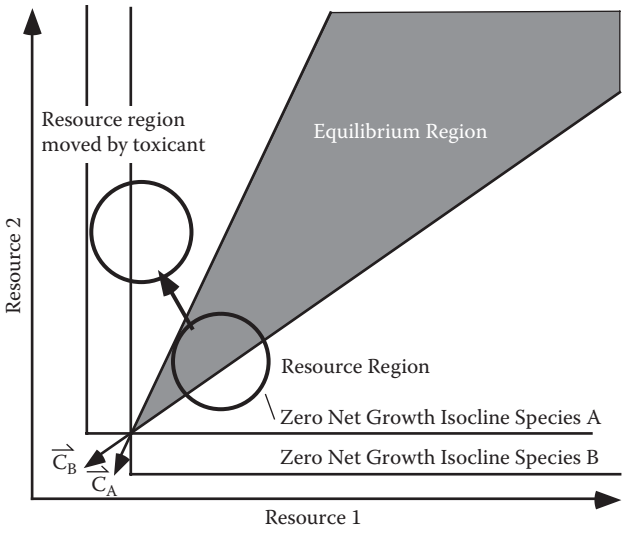

Figure 13.8 Shifting of the confidence interval of resources. The addition of a toxicant that impacts organisms that act as resources for other organisms can have dramatic effects without any direct impact upon the consumers. A shift in the resource region due to a shift in competitive interactions at other energetic levels can alter the competitive relationships of the consumers. Structure of the community is then altered even more dramatically. In this case, a situation with a general competitive equilibrium is shifted so that species A can be driven to extinction with the movement of the resource area.

confidence region is now outside the equilibrium region and species B becomes extinct. Indirect effects clearly are represented in this modeling system.

Even more subtle differences in populations may occur. The genetic variation within a population can be rather substantial. The two-dimensional ZNGIs can be expanded to demonstrate the fact that the ability of organisms to consume and use resources is not a point but a continuum dictated by the genetic variation of the population. Figure 13.9 illustrates this idea.

The lines representing the ZNGIs have become bars, and the equilibrium point has now been transformed into a confidence region. Depending upon the amount of variation within a population relating to the physiological parameter impacted by the toxicant, resource competition could also occur between the various phenotypes within the population.

The use of resource competition models also leads to a classification or a flow diagram describing the potential impacts of toxicants upon competitive interactions (Figure 13.10). The toxicant can directly or indirectly alter every aspect of the competitive interaction except the nonspecific or density-independent mortality.

- *Genetics*—The effects of the toxicant can be both long lasting and severe. Since the genome ultimately controls the biochemical, physiological, and behavioral aspects of the organism that set the consumption vector and the ZNGI, alterations can have a major impact.
- *Predation*—Often a toxicant affects more than one species. Perhaps the predators, disease organisms, or herbivores that crop a food resource are affected by the toxicant. Predation is an important aspect of mortality.

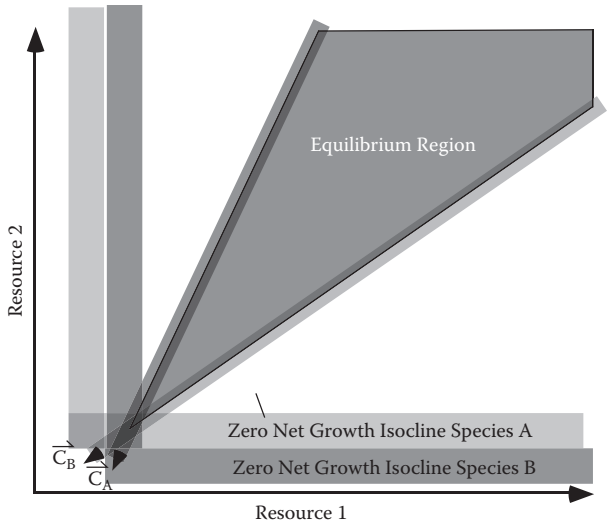

Figure 13.9 Genetic diversity. The genetic diversity of a population will alter the sharp lines of the ZNGIs into bars representing 95% confidence intervals. The consumption vectors can be similarly altered, although for this diagram they are still conventionally represented. The equilibrium point and equilibrium region then become probabilistic.

- *Reproduction*—Teratogenicity and the reduction of reproductive capacity are well-known effects of toxicants, especially in vertebrate systems.
- *Mortality*—An increase in mortality moves the minimal amount of resource necessary to maintain a population. The combination of mortality and reproduction determines the ZNGI for that population.
- *Consumption vectors*—The consumption vectors express the relative efficiencies of the uptake and utilization of resources. An alteration in the metabolic activity of even one resource will shift the slope of the vector. In conjunction with the ZNGI, the consumption vector fixes the equilibrium region within the resource space.
- *Biotic components of the resource region*—The confidence regions describing the supply of resources are dependent on the biotic components in both the temporal and spatial variability. The organisms that compose the resources can be affected as presented above. A population boom or bust can shift the confidence interval of the resource supply. Excessive production of a resource can affect other resources. An algal bloom can lead to oxygen depletion during darkness.

Since the organisms that are competing at one level are resources for other trophic levels, the effects can be reverberated throughout the system. Therefore, these models have the potential for describing a variety of interactions in a community.

One of the major implications of these models is the importance of resources and initial conditions in the determination of the outcome of a toxicant stressor. Depending upon the resource ratio, three different outcomes are possible given the same stressor. History of the system, therefore, plays a large part in determining the response of a community to a stressor.

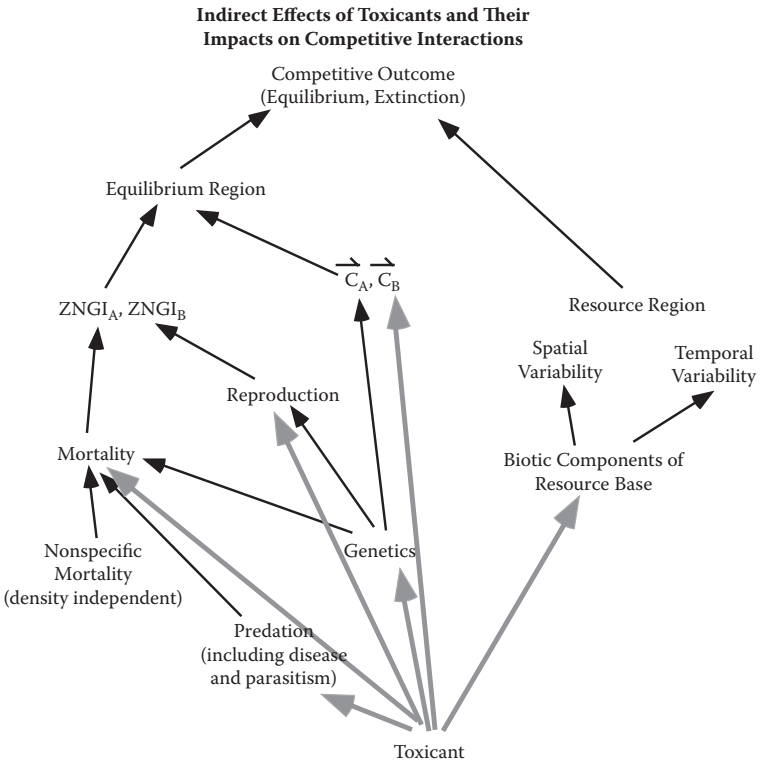

Figure 13.10 Impacts of toxicants upon the components of resource competition. The relationships among the factors incorporated into resource competition models can be affected in several ways by a toxicant. Only the density-independent factors governing mortality escape.

The difficulty of measuring community and ecosystem effects has been extensively discussed in the literature (Suter and Barnthouse 1993). Ecological systems can be perceived as mechanisms for energy flow, materials cycling, and as assemblages of species. Ecosystem properties may also be examined.

13.3 Effects on Ecosystems or Ecological Structures

Ecosystems are multidimensional constructs, and they have been seen in that fashion for a number of years. An example is the Hutchinsonian idea of organisms and populations residing in an *n*-dimensional hypervolume—the basis of current niche theory (Hutchinson 1959). The *n*-dimensional hypervolume is the ecosystem with all its components as perceived by the population. The variability of these parameters over time as well as the quantity and quality of nutrient inputs to the system are used to account for the diversity of species within this system (Hutchinson 1961; Richerson et al. 1970; Tilman 1982). An accurate description of an ecosystem should in some fashion correspond to its multidimensional nature.

Often impacts are quantified using a reference site as a negative control for comparison to other sites under question. Similarly, multispecies toxicity tests and microcosms and mesocosms attempt to detect differences between the control treatment and the dosed treatment groups.

A number of methods have been developed to attempt to measure these differences. Analysis of variance is the classical method to examine single-variable differences from the control groups. However, issues with type II error and the difficulty of graphically representing the data set have been problematic. Conquest and Taub (1989) developed a method to overcome some of the problems of classical analysis of variance (ANOVA), intervals of nonsignificant difference. This method corrects for the likelihood of a type II error and produces intervals that are readily graphed to ease examination. This method is routinely used in the examination of data derived from the standardized aquatic microcosm (SAM) and is applicable to other data sets. The major drawback to these methods is, again, the examination of only one variable at a time over the course of the experiment. In many instances the interactions may not be as straightforward as the classical predator-prey or nutrient limitation dynamics usually picked as examples of community level interactions.

13.3.1 Similarity Measures

Perhaps a more useful means of quantifying structural data is to use a similarity measurement. These are reviewed by Ludwig and Reynolds (1988) and form the basis of multivariate clustering and ordination. Similarity measures can compare the presence of species in two sites or compare a site to a predetermined set of species derived from historical data or as an artificial set comprised of measurement endpoints from the problem formulation of an ecological risk assessment. The simplest similarity measures are binary in nature, but others can accommodate the number of individuals in each set. Related to similarity measurements are distance metrics. Distance measurements, such as Euclidean distance, have the drawbacks of being sensitive to outliers, scale, transformations, and magnitudes. Distance measures form the basis of many classification and clustering techniques.

13.3.2 Classification

Ordination, classification, and clustering techniques are among the most useful methods for examining changes in structural components and may also include abiotic factors. Classifier systems attempt to fix rules that discriminate among points in data sets. Classification is a two-step process. First, a training data set is used to derive algorithms for determining which point in a data set belongs to which group. Second, unseen data are classified according to group. Such algorithms can be used not only to distinguish between groups, but also to discover the important variables in the process. Discriminant functions are a commonly used type of classifier technique. The primary difficulty is that data from typical environmental toxicology studies are underdetermined. For example, we may have measured the presence and abundance of 50 species per replicate in a mesocosm or field study, but only three to six replicates are available for three to four treatments. Given the large number of variables and low sample size, it is likely that a discriminant function can be found by chance that perfectly classifies the treatments. Our research group (Matthews, Landis, Matthews, unpublished results) has found this to be the case in microcosm data sets.

Ludwig and Reynolds (1988) provide an excellent introduction to the assumptions, derivations, and use of several multivariate classification techniques commonly used for the analysis of ecological structures. Perhaps the most common are principal components analysis (PCA) and its derivatives. PCA attempts to find orthogonal linear combinations of variables that account for the variance within a data set. Assuming that ecological structures are complex, nonlinear relationships may be the norm. PCA also emphasizes the explanation of variance, and the corresponding theory that variables may be highly variable but only contain noise (Matthews and Hearne 1991;

Matthews et al. 1995). Detrended principal components (DPCs) use a polynomial expression to remove the nonlinear relationships from the PCA axes. DPC is useful for data sets of moderate nonlinearity. Detrended correspondence analysis uses a more complex algorithm to eliminate the nonlinearity, but requires a more complex computation. Nonmetric multidimensional scaling (NMDS) is a robust method that deals with nonlinearities by using ranks.

A technique derived from a principal components approach is the coupling of PCA with redundancy analysis (RDA) (van der Brink et al. 1996; Van Wijngaarden et al. 1995). The utility of the technique is that it provides a depiction of the treatment trajectories in an ecological space, and the statistical significance can be examined using a permutation test. One of the proposed benefits of the technique is that it can determine recovery, a dubious distinction in light of our previous discussion. Like other PCA techniques, the method does assume a linear response.

Note that previously described techniques all are based on knowing the treatment groups, which introduces a strong bias into the search for patterns and explanations. Such a bias also makes it difficult to discern new patterns that may be due to other environmental gradients present in the testing facility or that are part of an outdoor setting. Most of the models assume a linear response, and that the variables with the greatest variance are by definition the most important.

13.3.3 Clustering

Clustering attempts to find natural groups as determined by the metric used, Euclidean distance, cosine distance, or categorical attributes, which are blind as to treatment. These types of techniques are particularly useful for discovering new relationships among variables and for the derivation of measurement data based on natural differentiations and not the bias of the observer. The use of these techniques to determine assessment and measurement endpoints has been extensively discussed (Landis et al. 1994).

Many clustering algorithms are based on the metrics described in the section on classification, and the drawbacks of a metric approach are also relevant to this discussion. Other techniques, such as COBWEB and RIFFLE, use machine learning techniques to derive clusters. RIFFLE has been used in conjunction with metric clustering methods and association analysis in the study of structure in field situations and in microcosms (Landis et al. 1995a; Matthews et al. 1995). These methods have proven particularly useful not only in determining statistically significant differences between groups, but also in finding new relationships between these complex data sets (Matthews et al. 1996).

Ideally, a multivariate statistical test used for evaluating complex data sets will have the following characteristics:

1. It does not combine counts from dissimilar taxa by means of sums of squares, or other mathematical techniques.
2. It does not require transformations of the data, such as normalizing the variance.
3. It works without modification on incomplete data sets.
4. It can work without further assumptions on different data types (e.g., species counts or presence/absence data).
5. Significance of a taxon to the analysis is not dependent on the absolute size of its count, so that taxa having a small total variance, such as rare taxa, can compete in importance with common taxa, and taxa with a large, random variance will not automatically be selected to the exclusion of others.

6. It provides an integral measure of how good the clustering is, i.e., whether the data set differs from a random collection of points.
7. It can, in some cases, identify a subset of the taxa that serve as reliable indicators of the physical environment.

The remainder of this section details the potential application of multivariate methods in the selection of endpoints and in the evaluation of exposure and effects of stressors in ecosystems. Particular reference is made to the application of these methods to the current framework for ecological risk assessment. Examples of the use of multivariate methods in detecting effects and in selecting important measurement variables is covered using both field surveys and multispecies toxicity tests.

13.4 Application of Multivariate Techniques

Multivariate methods have been applied to numerous field studies and multispecies toxicity tests. These examinations have demonstrated the power and usefulness of multivariate techniques in elucidating patterns in biological communities of varying complexity.

Several researchers have attempted to employ multivariate methods to the description of ecosystems and the impacts of chemical stressors. Perhaps the best-developed approaches have been those of K. Kersting, A. R. Johnson, and a new approach by Matthews et al.

13.4.1 Normalized Ecosystem Strain (NES)

NES (Figure 13.11) was developed by Kersting (1984, 1988) as a means of describing the impacts of several materials to the three-compartment microecosystems containing autotrophic, herbivore, and decomposer subsystems. These variables in the unperturbed control systems are used

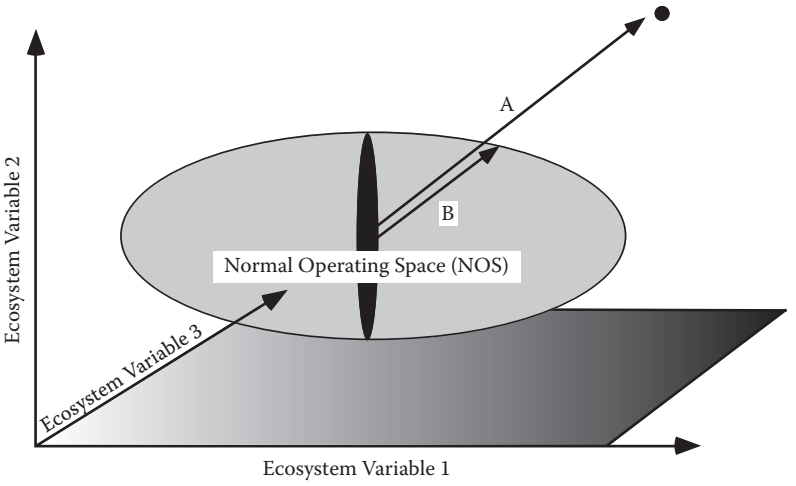

Figure 13.11 Normalized ecosystem strain. The vector of A–B provides the distance that the point is outside the previously observed range of dynamics of the system under observation.

to calculate the normal operating range (NOR) of the microecosystem. The NOR is the 95% confidence ellipsoid of the unperturbed state of a system. The center of the NOR is defined as the reference point for the calculation of the NES. The NES is calculated as the quotient of the Euclidean distance from a state to the reference state divided by the distance from the reference state to the 95% confidence (also called tolerance) ellipsoid, along the vector that connects the reference state to the newly defined state. A value of 1 or less indicates that the new state is within the 95% confidence ellipsoid; values greater than 1 indicate that the system is outside this confidence region.

Originally limited to ellipsoids, the use of Mahalonobis distances allows the use of more variables, as the confidence ellipsoid can be transformed to a confidence or tolerance hypersphere. These ideas were examined using the microecosystem test method developed by Kersting for the examination of multispecies systems. These three-compartment microecosystems are comprised of autotrophic, herbivore, and decomposer subsystems that are connected by tubing and pumps. Although relatively simple and small, these systems are operable over a number of years.

Several variable measurements are obtained weekly for these experiments:

- Algal biomass in the autotrophic and herbivore systems
- Number of *Daphnia magna*
- pH of the autotrophic and herbivore subsystems
- Molybdate-reactive phosphorus in the autotrophic subsystem and in the return flow between the decomposer and autotrophic subsystems

These variables allow for the determination of the NOR and, after dosing with a toxicant, the NES. In some instances impacts that are not significant using univariate analysis are detectable using NES. The sensitivity of the NES increased as the number of variables used to describe the system increased (Kersting 1988). Another interesting observation was the increasing distance from the normal space of the system after a perturbation, as measured by NES, as time increased. This increasing distance indicates that the perturbed system is drifting from its original state. Kersting hypothesized that the system may even shift to a different equilibrium state or domain, and that the system would remain there even after the release of the stressor.

13.4.2 State Space of Ecosystems

Apparently as an independent development, Johnson (1988a) proposed the idea of using a multivariate approach to the analysis of multispecies toxicity tests. This state space analysis is based upon the common representation of complex and dynamic systems as an *n*-dimensional vector. In other words, the system is described at a specific moment in time as a representation of the values of the measurement variables in an *n*-dimensional space. A vector can be assigned to describe the motion of the system through this *n*-dimensional space to represent successional changes, evolutionary events, or anthropogenic stressors. The direction and position information form the trajectory of the state space, and this can be plotted over time.

In the *n*-dimensional hypervolume that describes the placement and trajectory of the ecosystem it is possible to compare the positions of systems at a specified time. This displacement can be measured by literally computing the distance from the systems, and this displacement vector can be regarded as the displacement of these systems in space (Figure 13.12). The displacement vectors can be easily calculated and compared. Using the data generated by Giddings et al. (1980) in a series of classic experiments comparing results of the impacts of synthetic oil on aquarium and

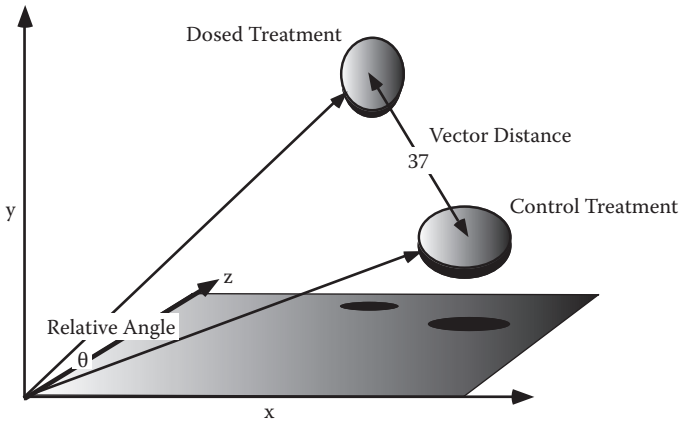

Figure 13.12 Measures of distance between clusters. Two of the commonly used measures of separation of clusters in an *n*-dimensional space are the cosine of the angle and the vector distance. Each method has advantages and disadvantages. In order to visualize the data as accurately as possible, several measures should be employed.

small pond multispecies systems, Johnson was able to plot dose-response curves using the mean separation of the replicate systems. These plots are very reminiscent of dose-response curves from typical acute and chronic toxicity tests.

As summarized by Johnson, the strengths of this methodology are the objectivity for quantifying the behavior of the stressed ecosystem and the power of the methodology to summarize large amounts of data. As with the work of Kersting, this methodology allows the investigator to examine the stability of the ecosystem and the eventual fate of the system relative to the control treatment.

Another important application proposed by Johnson (1988b) was the use of multivariate analysis to identify diagnostic variables that can be applied in the monitoring of ecosystems. Diagnostic variables, if reliable in differentiating anthropogenically stressed systems from control systems, would be extremely valuable in monitoring for compliance and determining cleanup standards. In a follow-up publication, Johnson (1988b) detailed the derivation and use of these diagnostic variables. The use of such variables is justified due to the fact that decisions often have to be made with incomplete data sets due to technical difficulties, cost, and a general lack of knowledge. Techniques proposed for the determination of these variables included linear regression, discriminant analysis, and visual inspection of graphed data. Johnson conducted a cost-benefit analysis using an ecosystem model that demonstrated, under the condition of that model, the benefits of diagnostic variables. In the discussion, Johnson proposes simulation modeling to attempt to find generalized diagnostic variables that best describe the state space and trajectory of an ecosystem.

One of the difficulties in the past of using multivariate methodologies such as those proposed by A. Johnson and Kersting was the computational effort required. Computational requirements are not the limiting factor that they may have once been, even for large data sets.

The major difficulty with the methods detailed above is the reliance on conventional metric statistics. Vector distances in an *n*-dimensional space, including such disparate variables as pH, cell counts, and nutrient concentrations, are difficult to compare from one experiment to another. Another consideration is the fact that many of the variables may be compilations of others. Algal biomass is often calculated by multiplying cell counts by an appropriate constant for each species.

Species diversity and many indices of ecosystem health are similarly composited variables. As discussed in the pervious sections, the use of metric methods with nonmetric clustering may prove a useful combination.

The attempt by Johnson to derive diagnostic variables is an interesting approach. However, our current research indicates that the variables that contribute the most to separating control treatment from dosed treatment groups change from sampling period to sampling period. The variables change in the SAM experiments no doubt in response to the successional trajectory of the system as nutrients become depleted. As nutrients become limiting and the ability of the system to exhibit large differences in community structure becomes less, the metric measures do not exhibit the same magnitudes of separation.

13.4.3 Nonmetric Clustering and Association Analysis

Multivariate methods have proved promising as a method of incorporating all of the dimensions of an ecosystem. Both of the methods presented above have the advantage of examining the multispecies test systems as a whole and can track such processes as succession, recovery, and the deviation of a system due to an anthropogenic input. The disadvantage to these systems and to conventional multivariate techniques is that all of the data are incorporated without regard to the metric (unit of measurement) or the contribution of a variable to the separation of the clusters. It can be difficult to reconcile variables such as pH with a 0 to 14 metric to the numbers of bacterial cells per milliliter, where low numbers are in the 10^6 range. Random data indiscriminately incorporated with large metrics may overwhelm important variables with a different metric. Developed for the analysis of ecological data is a multivariate derivative of artificial intelligence research, nonmetric clustering, that has the potential of circumventing many of the problems of conventional multivariate analysis.

Unlike the more conventional multivariate statistics, nonmetric clustering is an outgrowth of artificial intelligence and a tradition of conceptual clustering. In this approach, an accurate description of the data is only part of the goal of the statistical analysis technique. Equally important is the intuitive clarity of the resulting statistics. For example, a linear discriminant function to distinguish between groups might be a complex function of dozens of variables, combined with delicately balanced factors. While the accuracy of the discriminant may be quite good, use of the discriminant for evaluation purposes is limited because humans cannot perceive hyperplanes in highly dimensional space. By contrast, a conceptual clustering will attempt to distinguish groups using as few variables as possible, and by making simple use of each one. Rather than combining variables in a linear function, for example, conjunctions of elementary yes-no questions could be combined: species A greater than 5, species B less than 2, and species C between 10 and 20. Numerous examples throughout the artificial intelligence literature have proven over and over again that such conceptual statistical analysis of the data provides much more useful insight into the patterns in the data and, indeed, is often more accurate and robust. Delicate linear discriminants and other traditional techniques chronically suffer from overfitting, particularly in highly dimensioned spaces. Conceptual statistical analysis attempts to fit the data, but not at the expense of a simple, intuitive result.

However, one of the most difficult analytical challenges in ecology is to identify patterns of change in large ecological data sets. Often these data are not linear, they rarely conform to parametric assumptions, they have incommensurable units (e.g., length, concentration, frequency, etc.), and they are incomplete (due to both sample loss and sampling design, whereby different parameters are collected at different frequencies). These difficulties exist regardless of whether

there are toxicants present; the only difference is that with the presence of a toxicant, we must try to separate the response to the toxicant from the other changes that occur at the site due to geology, other stressors, and historical events. A strategy for designing studies to reconcile these issues is depicted in Section 13.8.

13.4.4 Projections for Visualizing Ecosystem Dynamics

A major difficulty in the analysis of data from microcosm/mesocosm experiments and field research is the understanding of the large amount of data available. Conventional techniques involve the plotting of individual variables over time, then examining each of these plots in order to elucidate relationships and patterns. Unfortunately, the problems of seeing in more than three dimensions reappear. Clustering and other multivariate techniques assist in the discovering of patterns, but are typically limited to only one sampling date. As we have discussed extensively in this chapter, ecosystems are dynamic and may exhibit a variety of patterns.

A technique for visualizing the dynamics should allow for the comparison of dynamical relationships. These comparisons should include factors such as inherent variability between replicates or samples, and an indication of the rate of change in variables. A method that we have developed we call space-time worms.

Space-time worms (STW) were developed to more easily visualize the dynamic relationships between the variables in microcosm experiments (Landis et al. 1997). G. Matthews and M. Roze developed the software that enables a three-dimensional viewing of the microcosm experiments.

The basis of this projection is a two-variable plot. Figure 13.13 portrays such a plot. For a plot for one sampling date, two variables are selected as axes based on their importance as demonstrated by multivariate analyses or the researcher's intuition. The mean of the replicates is then plotted and the standard deviation along each axis represented (Figure 13.13a). Whiskers to the box may be added to represent minimum and maximum values or other characteristics of the data set. The position and variability within a treatment group can then be compared to those within another treatment group. The two-dimensional plot does not give any sense of the dynamics of the systems. It is possible to plot more than one sampling date on the two-dimensional graph (Figure 13.13b). The movement of the experimental system through ecosystem space can then be portrayed. However, this soon can become complicated, and changes in rates are difficult to represent.

Time can be added as a third axis (Figure 13.14a). The different box plots can then be added to the figure and the vertices connected to form a slab-sided extrusion. This process can be further expanded to include other treatment groups or field sites. Figure 13.14b portrays the dynamics along the small daphnid, pH, and time axes of a microcosm experiment. The changes in the position and variability of the four treatment groups can be easily distinguished over the 91 days. In the early part of the experiment groups of the worms move apart. This corresponds to a treatment with a turbine fuel on two of the four treatment groups. After 35 to 40 days the four treatments occupy approximately the same part of the ecosystem (pH and small daphnid) space and form a braid as they move around and through each other. However, after a second treatment a new set of two worms is formed and the process begins again.

Other methods exist for visualizing the movement of systems through ecosystem space. Often two-dimensional graphs are comprised of axes from a PCA analysis and each sampling date plotted. The dates can then be joined in a manner similar to that portrayed in Figure 13.14. A newer method has been to use a redundancy analysis to construct the axes. Kersting and van den Brink (1997) have used this method to present the results from a series of ditch experiments (Figure 13.15). Using such a projection, the convergence of divergence of the treated systems can

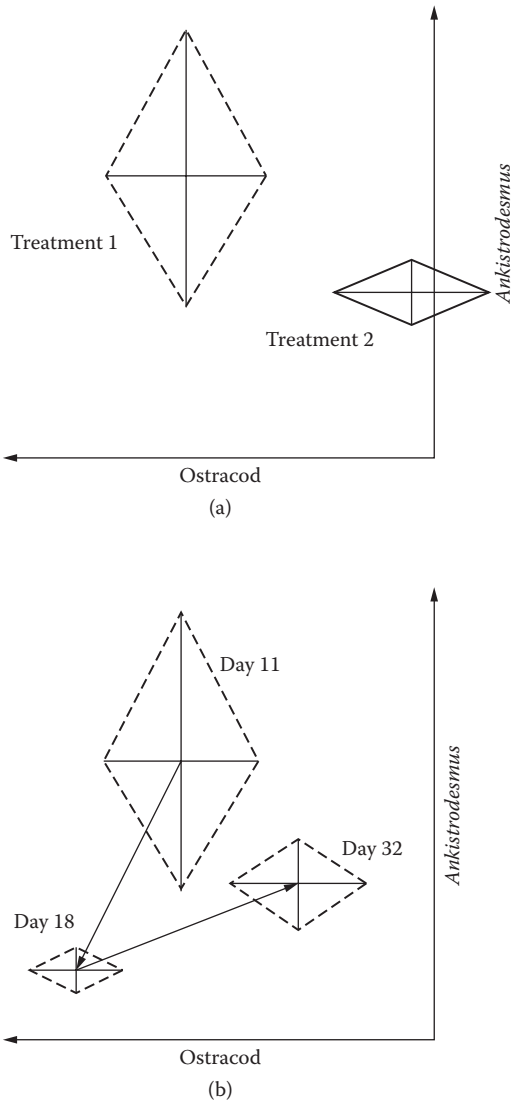

Figure 13.13 Construction of a space-time worm (STW). (a) The average values for the variables *Ankistrodesmus* and Ostracod are plotted along with a box plot to represent one standard deviation. Each treatment group can be represented and compared. If time is added (b), then a plot for each sampling date can be represented, but the diagram becomes much harder to interpret.

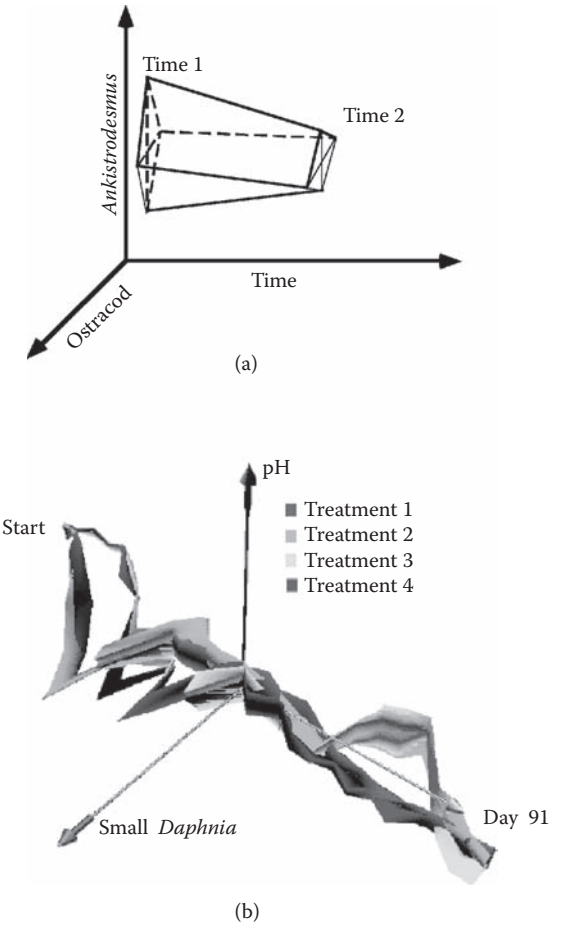

Figure 13.14 A space-time worm (STW) construction. (a) Time is added as a third axis and the measurements for the two sampling days connected. When this is done for an entire experiment (b), the relative dynamics of the systems becomes readily apparent. (See color insert following page 268.)

be observed. The drawback to these methods is that the best PCA or RDA axis likely changes for each sampling date, making interpretation difficult. However, a sense of the relative dynamics of the treatment groups can be determined.

A major contribution of these projection techniques is the realization of the importance of dynamics and trajectories in understanding the impacts of toxicants upon ecosystems. This understanding is critical if we are to correctly interpret, predict, and manage the changes due to anthropogenic stresses.

13.4.5 Examples of the Use of Multivariate Methods in Multispecies Toxicity Tests and Field Studies

The following examples demonstrate the usefulness of multivariate methods in the evaluation of field ecological data and laboratory multispecies toxicity tests. In each of the examples several

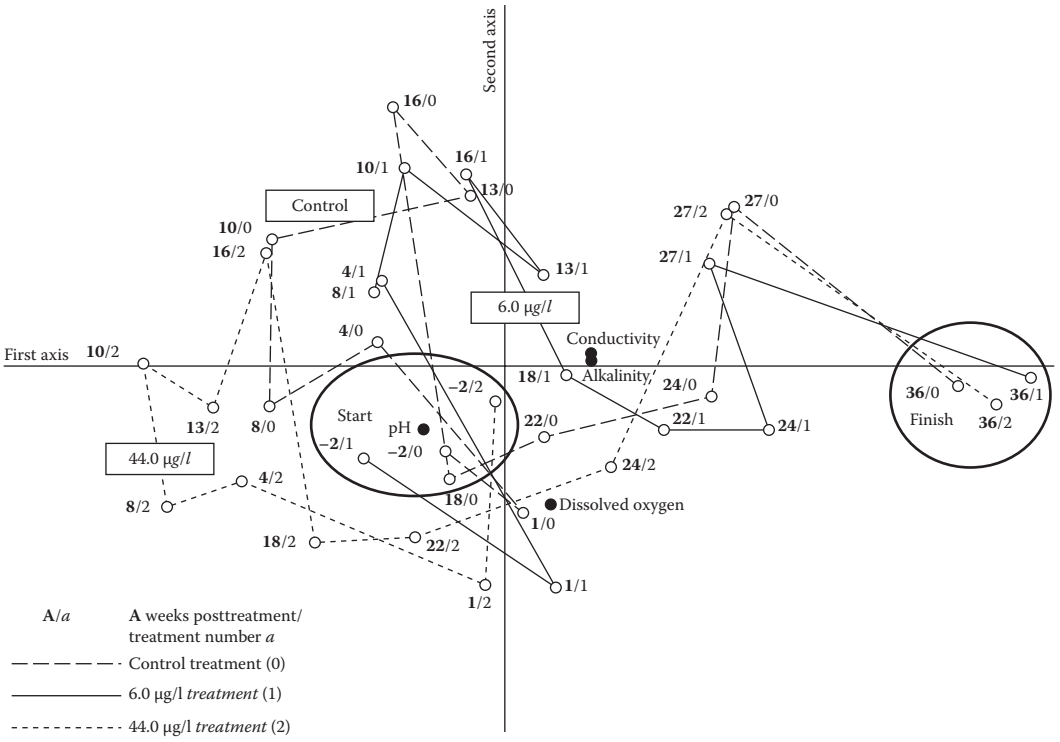

Figure 13.15 An RDA projection of an artificial stream experiment. The trajectories of the three treatments can be followed throughout the course of the experiment. (Modified from Kersting, K. and van den Brink, P. J., *Environ. Toxicol. Chem.*, 16, 251–59, 1997.)

multivariate techniques were used, generally Euclidean and cosine distances and nonmetric clustering and association analysis.

R. A. Matthews et al. (1991) and G. B. Matthews et al. (1991) have compared several types of multivariate techniques to evaluate two types of ecological data, a limnological data set that included spatial and temporal changes in water chemistry and phytoplankton populations, and a stream data set that included spatial (longitudinal) and temporal changes in benthic macroinvertebrate species assemblages. Their objective was to see whether the multivariate tests could identify obvious patterns involving the influences of stratification in the lake and the effects of substrate and water quality changes on stream macroinvertebrates. We used principal components analysis, hierarchical clustering (*k* means with squared Euclidean or cosine of vectors distance measures), correspondence analysis, and nonmetric clustering to look for patterns in the data.

In both studies, nonmetric clustering outperformed the metric tests, although both principal components analysis and correspondence analysis yielded some additional insight on large-scaled patterns that was not provided by the nonmetric clustering results. However, nonmetric clustering provided information without the use of inappropriate assumptions, data transformations, or other data set manipulations that usually accompany the use of multivariate metric statistics. The success of these studies and techniques lead to the examination of community dynamics in a series of two multispecies toxicity tests.

The multivariate methods described above have been used to examine a series of multispecies toxicity tests. Described below are the data analyses from two published tests using methodology

derived from the standardized aquatic microcosm. The method is described in some detail in Chapter 4.

In the first example, the riot control material 1,4-dibenz oxazepine (CR) was degraded using the patented organism *Alcaligenes denitrificans denitrificans* CR-1 (*A. denitrificans* CR-1) (Landis et al. 1993a). *A. denitrificans* CR-1 was obtained using a natural inoculum set in an environment containing the microcosm medium T82MV containing the toxicant CR. After demonstrating the ability of the organism to degrade the toxicant CR, a microcosm experiment was set up to investigate the ability of the microorganisms to degrade CR in an environment resembling a typical freshwater one. Toxicity tests of the riot control material demonstrated that although *A. denitrificans* CR-1 eliminated the toxicity of a CR solution toward algae, toxicity did remain to *Daphnia magna*.

The SAM experiment was set up with a control group without the toxicant or *A. denitrificans* CR-1, a second group with only CR, a third group with only *A. denitrificans* CR-1, and the fourth group containing both the toxicant CR and the bacterium *A. denitrificans* CR-1. Conventional analysis demonstrated that the major impact was the increase in algal populations since both CR and the degradative products of the toxicant both inhibited the growth of the major herbivore, *D. magna*. The control group and the microcosms inoculated initially with *A. denitrificans* CR-1 were not distinguishable using conventional analysis.

As a first test of the use of multivariate analysis in the interpretation of multispecies toxicity tests, the data set used to analyze the CR microcosm experiment was presented in a blind fashion for analysis. Neither the purpose of the experiment nor the experimental setup was provided for the analysis. Nonmetric clustering was used to rank variables in terms of contribution and to set clusters. Surprisingly, the analysis resulted in only two clusters being recognized: control and *A. denitrificans* CR-1 treatments, and the CR and CR plus *A. denitrificans* CR-1 treatments. Variables important in assigning clusters were *D. magna*, *Ankistrodesmus*, *Scenedesmus*, and NO_2. Obviously, the inclusion of the principal algal species in these experiments and the *Daphnia* was not a surprise, but NO_2 had not been demonstrated as a significant factor in previous analyses. However, the species *A. denitrificans denitrificans* is classified for its denitrification ability (Matthews and Matthews 1991).

The second major application of nonmetric clustering to the analysis of SAM data has been the investigation of the impact of the complex Jet-A (Landis et al. 1993b) (Figure 13.16). The major modification to the SAM protocol was the means of toxicant delivery. Test material was added on day 7 by stirring each microcosm, removing 450 ml from each container, and then adding appropriate amounts of the water-soluble fraction (WSF) of Jet-A to produce concentrations of 0, 1, 5, and 15% WSF. After toxicant addition the final volume was adjusted to 3 L.

All of the multivariate tests (cosine distance, vector distance, and nonmetric clustering) agree that a significant difference between treatment groups was observed through day 25. From day 28 to day 39, the effect diminished until there were no significant effects observable. However, significant effects were again observable from day 46 through day 56, after which they again disappeared for days 60 and 63.

Also of interest are the variables that best described the clusters and the stability of the importance of the variables during the course of the experiment. In general, the number of variables that were important was larger during the start of the test and lower at the end. In addition, a great deal of variability in rankings is apparent during the course of the SAM.

Conventional analysis using such techniques as the intervals of nonsignificant difference (IND) plot (Conquest and Taub 1989) was unable to detect the second oscillation. The only leads were statistically significant deviations from the control for one sampling date for the variables pH

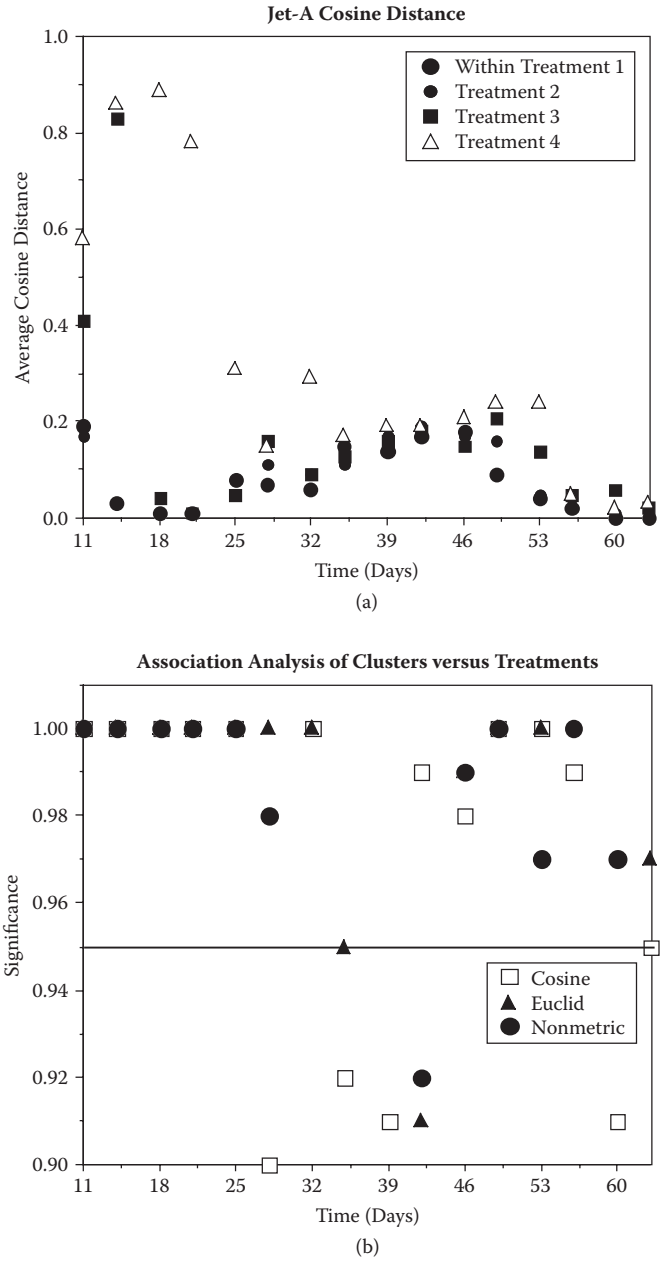

Figure 13.16 Multivariate analysis of the impact of Jet-A in the SAM test system. (a) The cosine distance from the control group to each of the treatments for each sampling day. Note that large differences are apparent early in the SAM. During the middle part of the 63-day experiment the distances between the replicates of treatment 1, the control group, are as large as the distances to the treatment groups. However, later in the experiment the distances from the dosed microcosms to the control again increase. (b) Significance levels of the three multivariate statistical tests for each sampling day. Note that there are two periods, early and late, where the clustering into treatment groups is significant at the 95% confidence level or above.

and the photosynthesis-to-respiration ratio. These deviations were considered cases of type II error until confirmation of effects using multivariate analysis.

Analysis of the toxicant concentration using purge and trap gas chromatography indicated that few of the constituents of the WSF were present in the water column at the end of the SAM experiment.

Examination of individual parameters provided only a limited, and somewhat distorted, view of the SAM response to Jet-A. The univariate data analysis did indeed show that there were some significant responses to the toxicant by individual taxa and chemistry; however, the responses were scattered over time, and did not present a logical, coherent pattern. Furthermore, the individual responses detected were typified by wild swings in a taxon's population density over time.

The repeated oscillation of the dosed replicates compared to the controls can be accounted for in two basic ways:

1. A reflection of the functioning of the community best described by parameters not directly sampled by the SAM protocol
2. A repeated fluctuation in community structure initiated by the initial stress and visible as an undampened movement in the systems

Until more data can be obtained, the cause–effect of the second oscillation cannot be determined. However, the use of multivariate analysis detected an unexpected result, one providing a new insight into the dynamics of even the relatively simple laboratory microcosm.

However, the search for diagnostic measures to indicate the displacement of an ecosystem may not be fruitless. Although the relative importance of the variables in the SAM experiments may change, there are often variables that are more critical during the earlier stages of the development of the microcosm, and those that are more crucial in the earlier stages. The variable Ostracod is generally more important in the latter half of the experimental series than in the latter stages. The crucial aspect is that the clustering algorithm is able to select ecosystem attributes that are the best in differentiating stressed vs. nonstressed systems. Although expert judgment may be able to predict, in some cases, variables that could be considered important to measure, the clustering approach is rapid, consistent, and not biased.

13.4.6 SiZer and the Detection of Thresholds

Recently, Sonderegger et al. (2009) have applied to ecological data sets a method that examines the rates of change to an ecological community using nonparametric models. The issue, as we have discussed in this and the previous chapters, is that ecological structures are subject to nonlinear dynamics. A particular issue is identifying thresholds, or where a dramatic change can occur, when only changes in conditions occur. The method applied by Sonderegger et al. is called significant zero crossings (SiZer) and is based upon looking at the derivatives of the curve of the changes of the system. A detailed description of the use of SiZer is presented in Sonderegger et al. (2009), and a program in the statistical framework R is available.

The data set used for this demonstration is that from the long-term study of the metal-contaminated Arkansas River (Clements 2004). In 1993 a reclamation activity was initiated to reduce metal contamination to reconstruct the ecological system of the river. Characterization of the water chemistry and the biotic community of the river have been examined since 1989 until present.

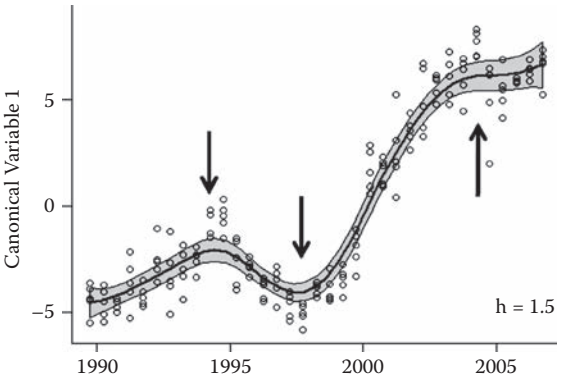

Figure 13.17 SiZer and the Arkansas River. The graph depicts the change in dynamics of the macroinvertebrate community as described by canonical variable 1 over time. The arrows point to areas along the curve when the change of the slope changes direction or magnitude as determined by SiZer. The value *h* = 1.5 refers to the bandwidth used for the analysis (see Sonderegger et al. 2009). (Courtesy of William Clements.)

An example of the use of this method is presented in Figure 13.17. In this graph the canonical variable 1 (a multivariate axis similar in concept to a principal components analysis) is plotted against time for the Arkansas River. The canonical variable is comprised of the macroinvertebrate data taken five times per year from 1990 until 2006. There are three thresholds identified in the plot. The first threshold occurs just after the control methods have been implemented and actually shows a change in direction of the trend. Then in 1997 the trend reverses and the values along the canonical variable start to increase. In the mid-2000s the slope of the curve flattens and the change in macroinvertebrate community structure is reduced along the canonical variable 1 axis. The shaded area in the graph is the 95% confidence interval for the plot.

The plot in Sonderegger et al. is similar to the space-time worms illustrated earlier in that it provides a visualization of the dynamics of the system. An additional feature is the rate of change of the system is also provided with the application of SiZer.

Another general method has been proposed to detect pollution impacts by examining a broad range of variables that describe ecological communities. The method employs the assumption that toxicants force directional selection toward tolerance.

13.5 Pollution-Induced Community Tolerance

Blank et al. (1988) proposed that an evaluation of the tolerance of the biological communities to toxicants would be a useful indicator of toxicant impacts. Pollution-induced community tolerance (PICT) has been developed further and used in a number of situations (Blank 2002; Grant 2002; Boivin et al. 2002).

The fundamental premise of PICT is that under toxicant stress natural selection occurs for organisms that are more tolerant to the pollutant. This increase in tolerance can occur at the level of the population by the induction of tolerance mechanisms by individuals or by selection for tolerant individuals. The biological community increases its tolerance to change by the pollutant by the elimination of sensitivite individuals, populations, or species, and the addition of tolerant organisms.

PICT can be determined by a variety of means. An increase in number of organisms tolerant to specific toxicants can be enumerated. The presence of biodegradative genes in prokaryotic organisms can be used as an indicator of selection. A resistance to change at the community level upon subsequent toxicant stressors is an indication of PICT. This is a measure easily examined in microcosm systems.

The difficulty in applying PICT is the difficulty of attributing causality to the observed correspondence in the field. The attribution of causality can be accomplished by (1) measuring the concentration of the pollutant, (2) using specific markers that are indicative of the mode of action, and (3) using multiple lines of investigation to connect exposure and effect.

There are a number of methods that can be used to examine the dynamics of ecological systems as they are modified by the addition or elimination of toxicants. These tools provide a powerful approach to understanding how ecological systems change without the use of indices that minimize the visualization of sample error or the use of simple hypothesis testing statistics such as analysis of variance. One of the challenges is now to fit the current knowledge of the dynamics of ecological systems into a modern model of ecological structures.

13.6 Interpretation of Ecosystem Level Impacts

The measurement of the current status of an ecosystem and the assumption that recovery is the likely outcome once the stressor is removed may not hold up to careful scrutiny given new developments in the study of population dynamics and ecosystems. First, it is crucial to know the dynamical aspects of the systems we are studying, and second, as with the weather, it may prove inherently impossible to predict the futures of ecosystems.

First, the apparent recovery or movement of a dosed system toward the reference case may be an artifact of our measurement systems that allow the *n*-dimensional data to be represented in a two-dimensional system. In an *n*-dimensional sense, the systems may be moving in opposite directions and simply pass by similar coordinates during certain time intervals. Positions can be similar, but the *n*-dimensional vectors describing the movements of the systems can be very different. One-time sampling indices are likely to miss these movements or incorrectly plot the system in an arbitrary coordinate system.

The apparent recoveries and divergences may also be artifacts of our attempt to choose the best means of collapsing and representing *n*-dimensional data into a two- or three-dimensional representation. In order to represent such data, it is necessary to project *n*-dimensional data into three or less dimensions. As information is lost when the shadow of a cube is projected upon a two-dimensional screen, a similar loss of information can occur in our attempt to represent *n*-dimensional data. The possible illusion of recovery based on this type of projection is diagrammatically represented in Figure 13.18. In Figure 13.18a the dosed and the reference systems appear to converge, i.e., recovery has occurred. However, this may be an illusion created by the perspective chosen to describe and measure the system. Figure 13.18b is the same system, but viewed from the "top." When a new point of view is taken, divergence of the systems occurs throughout the observed time period. As the various groups separate, the divergence may be seen as a separate event. In fact, this separation is a continuation of the dynamics initiated earlier upon one aspect of the community. Eventually, the illusion of recovery may simply be the divergence of the replicates within each treatment group becoming large enough, with enough inherent variation, so that even the multivariate analysis cannot distinguish treatment group similarities. Not every divergence

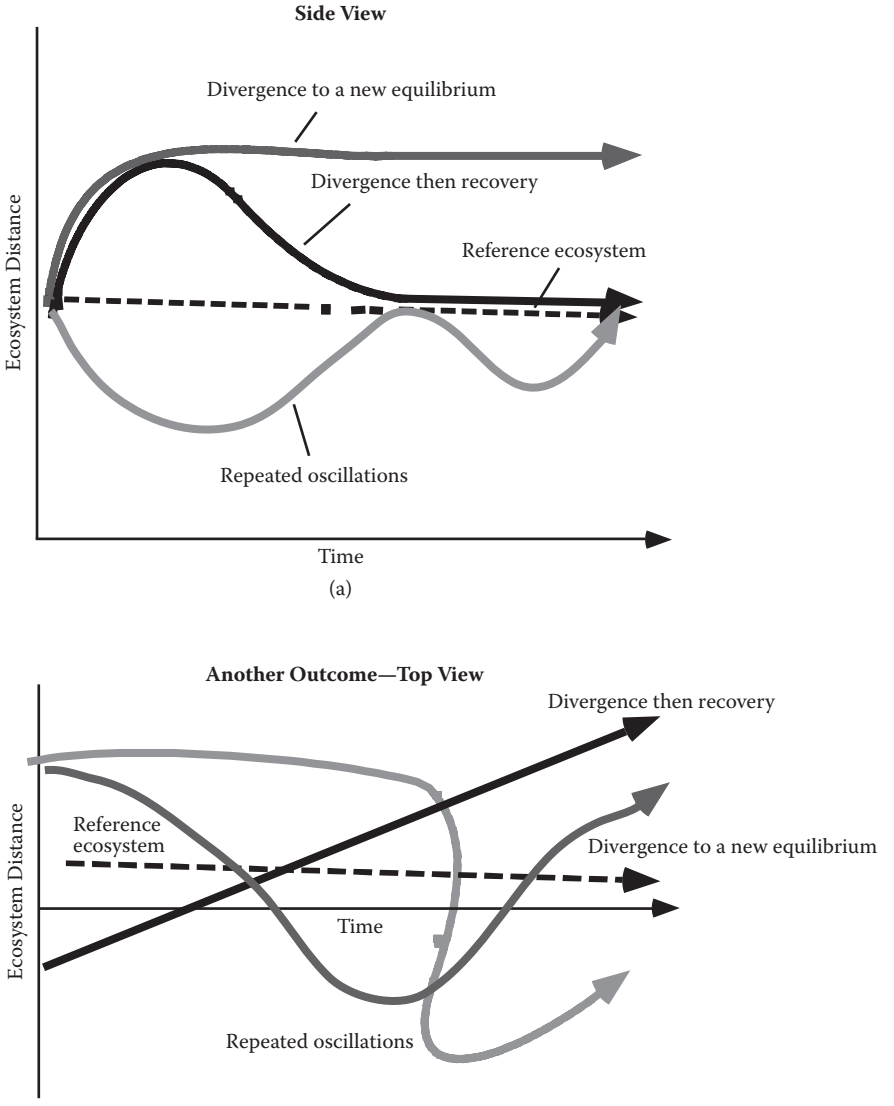

Figure 13.18 Two views of the dynamics of ecosystems. (a) It appears that in some instances the system returns to a control state or is in a stable oscillation. (b) Looking at the same system from the top indicates that the systems are moving in quite different directions.

from the control treatment may have a causal effect related to it in time; differentiating these events from those due to degradation products or other perturbations will be challenging.

Not only may system recovery be an illusion, but also there are strong theoretical reasons that seem to indicate that recovery to a reference system may be impossible, or at least unlikely. Systems that differ only marginally in their initial conditions and at levels probably impossible to measure are likely to diverge in unpredictable manners. May and Oster (1976), in a particularly seminal paper, investigated the likelihood that many of the dynamics seen in ecosystems that are generally attributed as chance or stochastic events are in fact deterministic. Simple deterministic models of populations can give rise to complicated behaviors. Using equations resembling those used in population biology, bifurcations occur, resulting in several distinct outcomes. Eventually, given the proper parameters, the system appears chaotic in nature, although the underlying mechanisms are completely deterministic. Biological systems have limits, extinction being perhaps the most obvious and best recorded. Another ramification is that the noise in ecosystems and in sampling may not be the result of a stochastic process but the result of an underlying deterministic chaotic relationship.

These principles also apply to spatial distributions of populations as reported by Hassell et al. (1991). In a study using host–parasite interactions as the model, a variety of spatial patterns were developed using the Nicholson–Bailey model. Host–parasite interactions demonstrated patterns such as static crystal lattice patterns, spiral waves, chaotic variation, and extinction with the appropriate variation of only three parameters within the same set of equations. The deterministically determined patterns could be extremely complex and not distinguishable from stochastic environmental changes.

Given the perhaps chaotic nature of populations, it may not be possible to predict species presence, population interactions, or structural and functional attributes. Kratz et al. (1987) examined the spatial and temporal variability in zooplankton data from a series of five lakes in North America. Much of the analysis was based on limnological data collected by Brige and Juday from 1925 to 1942. Copepods and cladocera, except *Bosmina*, exhibited larger variability between lakes than between years in the same lake. Some taxa showed consistent patterns among the study lakes. They concluded that the controlling factors for these taxa operated uniformly in each of the study sites. However, in regards to the depth of maximal abundance for calanoid copepods and *Bosmina*, the data obtained from one lake had little predictive power for application to other lakes. Part of this uncertainty was attributed to the intrinsic rate of increase of the invertebrates, with the variability increasing with a corresponding increase in r_{max}. A high r_{max} should enable the populations to accurately track changes in the environment. Kratz et al. suggest that these type of taxa be used to track changes in the environment. Unfortunately, in the context of environmental toxicology, the inability to use one lake to predict the nondosed population dynamics of these organisms in another eliminates comparisons of the two systems as measures of anthropogenic impacts.

A better strategy may be to let the data and a clustering protocol identify the important parameters in determining the dynamics of and impacts to ecological systems. This approach has been suggested independently by Dickson et al. (1992) and Matthews and Matthews (R. A. Matthews et al. 1991; G. B. Matthews et al. 1991). This approach is in direct contrast to the more usual means of assessing anthropogenic impacts. One classical approach is to use the presence or absence of so-called indicator species. This assumes that the tolerance to a variety of toxicants is known, and that chaotic or stochastic influences are minimized. A second approach is to use hypothesis testing to differentiate metrics from the systems in question. This second approach assumes that the investigators know a priori the important parameters. Given that, at least in our relatively simple SAM systems, the important parameters in differentiating nondosed from dosed systems

change from sampling period to sampling period, this assumption cannot be made. Classification approaches such as nonmetric clustering or the canonical correlation methodology developed by Dickson et al. eliminate these assumptions.

The results presented in this report and the others reviewed above, and the implications of chaotic dynamics suggest that reliance upon any one variable or an index of variables may be an operational convenience that may provide a misleading representation of pollutant effects and the associated risks. The use of indices such as diversity and the index of biological integrity have the effect of collapsing the dimensions of the descriptive hypervolume in a relatively arbitrary fashion. Indices, since they are composited variables, are not true endpoints. The collapse of the dimensions that are composited tends to eliminate crucial information, such as the variability in the importance of variables. The mere presence or absence and the frequency of these events can be analyzed using techniques such as nonmetric clustering that preserve the nature of the data set. A useful function was certainly served by the application of indices. The new methods of data compilation, analysis, and representation derived from the artificial intelligence tradition can now replace these approaches and illuminate the underlying structure and dynamic nature of ecological systems.

The implications are important. Currently, only small sections of ecosystems are monitored, or a heavy reliance is placed upon so-called indicator species. These data suggest that to do so is dangerous, and may produce misleading interpretations resulting in costly error in management and regulatory judgments. Much larger toxicological test systems are currently analyzed using conventional statistical methods on the limit of acceptable statistical power. Interpretation of the results has proven to be difficult.

The importance of viewpoint and the apparent chaotic nature of ecological systems make discussion of such parameters as ecosystem stability difficult to accurately determine. In Figure 13.19 a system that hits a perturbation is depicted. Although the distances that each has traveled are the same in a two-dimensional picture, from the viewpoint of the observer, one system moves farther than the other, and by some definitions is less stable. Conversely, if the chaotic nature of systems prevents a return to the original state, recovery cannot be considered an inherent property of the system.

The dynamics in the research discussed above make a metaphor such as ecosystem health inappropriate and misleading. In a classic critical evaluation, Suter (1993) dismissed ecosystem health as a misrepresentation of ecological science. Ecosystems are not organisms with the patterns of homeostasis determined by a central genetic core. Since ecosystems are not organismal in nature, health is a property that cannot describe the state of such a system. The urge to represent such a state as health has led to the compilation of variables with different metrics, characteristics, and casual relationships. Suter suggests a better alternative would be to evaluate the array of ecosystem processes of interest, with an underlying understanding that the fundamental nature of these systems is quite different than that of organisms.

13.7 An Alternative Model: The Community Conditioning Hypothesis

In order to incorporate the features that we have discussed in the latter half of this chapter, Matthews et al. (1996; Landis et al. 1996) have proposed the community conditioning hypothesis. The community conditioning hypothesis is an explicit recognition of the historical and thereby nonequilibrium nature of ecological structures. The basic precept is that ecological communities

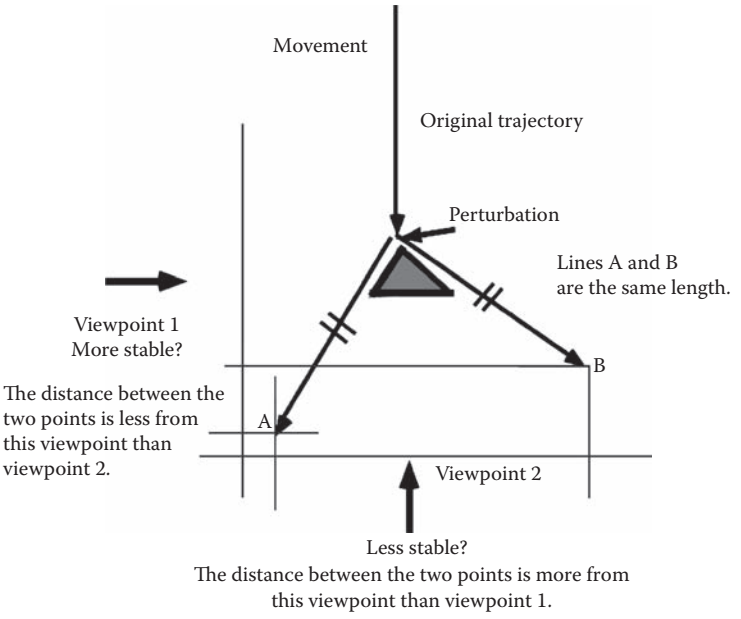

Figure 13.19 Apparent change in an ecosystem depends upon the point of view of the observer. An observer at viewpoint 1 sees the system moving steadily along until the perturbation occurs. At the perturbation two outcomes may occur, A and B. Both arrows are the same length, indicating that the change in the system is the same. However, not having the overhead point of view, the observer sees the change to be greater with line A rather than B. An observer at viewpoint 2 sees the same events very differently. To this observer the system does not change until the perturbation. Then B moves farther away than A. The observer at viewpoint A would conclude that B was impacted greater than A, although both are identical.

retain information about events in their history. The information can be contained in a variety of formats, from the relative frequencies of alleles or mitochondrial DNA, to the dynamics of predatory-prey and competitive interactions. Recovery is seen as an illusion of perception, as in the observer in Figure 13.19. Community conditioning also ties in the physiological, organismal aspects of environmental toxicology with the ecological. Much of the information about toxicological effects may only be read using molecular and physiological tools. On the other hand, indirect effects as can occur with resource competition may only be observed by noting the dynamics of populations.

The community conditioning hypothesis places critical importance on the historical aspects of an ecological structure in the determination of stressor impacts. Therefore, no two ecological structures will ever be the same. A corollary is that almost all stressors leave lasting impacts, and that the information is located in a variety of biotic and abiotic components. The hypothesis states that ecological structures are historical, unique, and complex. The hypothesis explicitly recognizes the importance of indirect effects in retention of information within systems, and in impacting the outcomes of future stressor events. These features place community conditioning in opposition to equilibrium-based or threshold models prevalent in ecological risk assessment and environmental toxicology.

The historical nature of ecological systems has been confirmed in experiments performed by our research team and other investigators using a variety of microcosm systems. The historical

information can be stored in a variety of layers, from the genetic and molecular, to the patterns and dynamics of interspecies interactions (Landis et al. 1996).

Community conditioning has been tested in a series standardized aquatic microcosms using the turbine fuel JP-8 in a series of two back-to-back replicated experiments (Landis et al. 2000). The first experiment was for the typical 63-day duration of the protocol, but the second was extended to 126 days. As much as possible, the experiments were replicated with organisms taken from the same cultures, conducted in the same rooms, with the same lot of toxicant and with the same basic staff. As in previous microcosm experiments, the organisms were counted, the toxicant quantified, and the oxygen content and pH measured. Graphical methods and three multivariate clustering methods were used to follow the patterns within both experiments.

Both experiments demonstrated similar but not identical patterns of invertebrate and algal dynamics. In each experiment the treatment group of each microcosm replicate could be identified in a statistically significant manner by at least one of the clustering methods. The strength of the clustering did fall off with time, but still corresponded to treatment effects.

The significance of community conditioning can be summarized by initially paraphrasing Tom Wolfe:

- You can never go home again. Ecosystems do not recover to the previous state, and they cannot be expected to. The history of the disturbance has changed the initial conditions of the system, resulting in one that may be superficially similar, but that is different even in its genetic makeup.
- You cannot even try. If the underlying dynamics are nonlinear, the system is unlikely to recur. Even if regular cycles are possible, the chance of the systems being in phase may be low. Even if major efforts, such as fertilization or selective colonization, force the system into a final outcome, the trajectories, the road getting there, are likely to be quite different and may take unexpected turns.
- History is important. The bulleted items above outline some of the reasons. Evolution and the history of speciation point to a nonlinear system both in the numbers of species generated and in the rate of speciation. History of colonization and the differing interactions that are caused by these events are to a large degree stochastic. Overlapping stochastic events occur and influence in important ways the dynamics of the nonlinear deterministic systems.
- You cannot predict the future exactly, no matter how much you know. If the dynamics are nonlinear and 1, 2, and 3 occur, then predicting the future and hence the impact of the system may be impossible beyond a certain time span. The prediction of another chaotic event, weather, has proven recalcitrant, even with the massive resources put into research and data collection. It may be that ecosystems and ability to predict impacts may bear a similar fate.
- Patterns should be in common. Although exact prediction may be problematic, and the idea of recovery an illusion, certain patterns should be detectable. The increase in tolerance often observed as pollution-induced community tolerance is such an example. Several potential outcomes may be possible, but not every outcome. Perhaps as a better understanding of the assembly of ecosystems is developed, we can even predict the probabilities of the outcomes. Prediction of ecological impacts will resemble more the weather forecast than the Newtonian dynamics.

Duarte et al. (2009) have observed features in aquatic systems that help to support the basic suppositions of the community conditioning hypothesis. In this study the authors test the assumption that ecological systems impacted by human activities return to their original

condition once the stressor has been removed. This is the return to Neverland (from the story of Peter Pan) scenario, where things return as if the impact had not occurred and time does not pass. Four marine coastal systems that had been impacted by nutrients were extracted from the literature that had both pre- and postremediation stages for up to 30 years of data. The systems were Marsdiep, The Netherlands; Helgoland, Germany; Odense Fjord, Denmark; and the Gulf of Riga, Latvia/Estonia.

The trajectories of each system plotted by examining nitrogen input from the watershed by chlorophyll a demonstrated a series of complex trajectories, each unique to the system. In no case did the system return to the preeutrophication state even years following reduction in nutrient levels. Apparently in each case the system could not go home again.

Although not derived from the pollution or toxicological literature, Hubbell (1997, 2005) has formulated the neutral hypothesis. The neutral hypothesis assumes that many species are functionally equivalent to many other species. After a disturbance the composition of the next system is the result of partly stochastic events, with who got there first being an important component of determining the final species composition of the system. This neutral hypothesis is in contrast to a model that assumes that competition among species will result in a species composition predetermined and consistent for a particular set of environmental conditions.

One of the implications of the neutral hypothesis is that one of the major factors determining the species composition of a community following a disturbance is the distribution of organisms in similar environments within the landscape. The colonizers from these ecological communities will form the basis of the new community within the disturbed landscape. Essentially a metapopulation (Chapter 12) of communities, or a metacommunity, is formed within the landscape. Testing of the neutral hypothesis is under way.

These results and ideas are consistent with the community conditioning hypothesis, the HPDP, and the understanding that ecological systems are complex and historical. The information from the past remains within the system, altering the dynamics and the potential future outcomes. The key to understanding the impacts of toxicants upon ecological systems is not relying upon simple tests of how similar the system is to a so-called unimpacted site, or to its return to an original condition. Current ideas and results should exterminate the idea or assumption of returning to an equilibrium or Neverland state. These ideas also mean that the design of field studies should change dramatically in the near term.

13.8 The Design of Field Studies

The HPDP, community conditioning, and other nonequilibrium models of the structure and function of ecological systems have dramatic implications for conducting field studies in environmental toxicology. The old model of comparing so-called reference sites to a contaminated site is now problematic because that reference site is likely to be linked to the contaminated site. The idea of removing the human-derived stressor and expecting a return to an original or preexisting state is also as likely as returning to Neverland. The system may be engineered to provide the ecological services desired by its managers, but a return to original condition is not an option.

A series of studies by Clements (2005), Clements and Rohr (2009), and Sonderegger et al. (2009) on the Arkansas River and the Long-Term Receiving Water Study (LTRWS) (Hall et al. 2009a, 2009b) are examples of field studies with clearly defined goals and that have been conducted over long time periods. As discussed previously in the chapter, the Arkansas River study has been an ongoing program of sampling and data analysis since 1989 on a metal-contaminated

site. The LTRWS was designed to measure the long-term changes and dynamics of four waters receiving pulp or paper mill effluent, Willamette River and McKenzie River of Oregon, Leaf River of Mississippi, and Codorus Creek of Pennsylvania. The LTRWS has now completed 10 years of investigation and is ongoing. Both sets of studies used a number of sampling techniques and data analysis tools, and each have sufficient sampling to examine the variability inherent to each system. In the next section I attempt to synthesize the lessons from each in setting a series of design goals for field studies investigating the ecological effects of toxicants.

13.8.1 The Question

The most important aspect of the field research is the specification of the exact question to be addressed. In the case of the Arkansas River, the questions revolved around the remediation of the river and the changes that would follow. The LTRWS was concerned about changes in the receiving waters due specifically to the effluent point sources from the paper mills. Nutrients and toxic materials in the waste stream were both considered in causing effects.

The timeframe for answering a specific question is also an important consideration. In a field study, a 1- or 2-year program of sampling and analysis will be limited as to the number of questions that can be addressed. Given the natural variability of many environments, it may not be possible to detect toxic effects. For example, at the Leaf River study site for the LTRWS, Hurricane Katrina struck early in the sampling period and was a major stressor event.

What is the assumed model for the mode of action and the likely effects that will be caused by the toxicant? It is not practical or likely affordable to sample all aspects of a system so that enough statistical power is available to see effects. In the case of metals and mill effluents, the researchers could rely upon the extensive literature in both fields to narrow the list of potential effects and concentrate on those aspects of each system.

Budget is a fundamental factor in describing the question. It may be that the budget is not large enough to answer questions and the appropriate scale. The kinds of questions may have to be narrowed if funding is an issue. It may be tempting to save money by reducing the number of samples, reducing the data analysis effort, or not fully characterizing the context of the site. However, it may prove foolish when not enough statistical power or confounding features of the landscape prevent conclusive interpretation of the data.

13.8.2 Context

Field sites exist within the context and history of other activities within the surrounding landscape. It is important to identify and map these activities within a study area. Effluents from a specific industry are often just one of many point sources of wastewater and other contaminants. Residential and industrial sites can have a variety of greases, metals, solvents, and pesticides that can run off as a nonpoint source. Agricultural areas can be sources of pesticides, herbicides, petroleum products, and nutrients. Terrestrial and aquatic sites are subject to long-range transport of pollutants from the wind or ocean currents. In areas where mining has been common, there may be natural outcroppings that are rich in heavy metals. Historical mining sites and small-scale smelter sites can contribute contaminants.

The sites may have also been extensively modified for a variety of other ecological services. Rivers are often channelized or dammed for flood control, water supply, or electrical power. For example, in my research group's study of the Androscoggin River, over 30 dams were present within the watershed. Because of flood control, the part of Codorus Creek passing through York,

Pennsylvania, was channelized, producing little aquatic habitat for several kilometers. In order to design a model of the potential confounding factors for a specific study, each of these factors should be considered.

13.8.3 Conceptual Model

Before designing a sampling strategy, a conceptual model should be constructed to account for all of the factors that may alter the ecological system. Because the specific question has been nominated and the context of the system documented, the conceptual model will focus on these factors. Ideally, the conceptual model will include the cause–effect pathways from the sources of the stressors to the potential effects that are to be evaluated. The conceptual model needs to include all of the sources of pollutants, the physical attributes of the surrounding landscape, and other stressors that may affect the aspects of the system under investigation. The model should incorporate what has been covered during the previous chapters of this book.

Endpoints covered in the conceptual model usually are species or ecological services of interest to the regulator or the stakeholder community. The habitats and ranges of the species of interest need to be understood and mapped. Of critical importance is documenting the location of each of these factors in the study area and the route of transport to the endpoints being considered.

A great deal of description of the formulation of conceptual models and their application to landscapes is presented in Chapter 14. Field studies are often conducted in concert with a risk assessment or damage assessment process. It is much more efficient to ensure that these models are compatible before initiating the research.

13.8.4 Sampling Design

One of the most frustrating aspects of reviewing field studies has been the inadequacy of the design of the measurement and sampling strategy. Many studies were designed on the premise that the upstream or upwind site was a control or reference for the downstream or downwind site. The discussions in this chapter and Chapter 12 have provided adequate evidence that such models of how ecological systems work are insufficient to describe cause–effect relationships. Instead, sampling should be designed to put the area most likely to be exposed to the contaminants or contaminants of interest into the context of patterns within the watershed or terrestrial landscape.

If possible, studies before and after the change to a system can be very illuminating. The Arkansas River study was able to capture the changes before and after the reclamation activities. The LTRWS also had the opportunity to examine changes to the receiving waters as processes were upgraded or volumes changed. It is important to design the sampling so that those temporal alterations can be described.

Sampling should also be able to describe the spatial and temporal changes in the landscape that may contribute to the changes in the endpoints chosen for the study. An increase or decrease in riparian areas, an increase in stormwater volume, forestry, fire, and the application of a new herbicide are all factors that may alter ecological endpoints. Adequate sampling will allow a more complete understanding of the contribution of the input that is the focus of the study to the changes observed over time and space in the endpoints being characterized.

An implicit goal of the sampling design is to understand the broad scope of patterns within a study region. This is a very different mentality than that focused only on hypothesis testing of upstream–downstream effects or the use of indices. The goal is to understand as much of the context as possible, including the variability of the patterns within the study area. Intrinsic variability

within the system and the measurement error of the sampling techniques will determine the power of the statistical tools used to examine patterns.

A useful approach for examining the patterns in the system is to select sample sites that fall along the various gradients of the study area. The gradients may be an increase or decrease in the toxicant, but may also include areas with increases and decreases of non-point-source pollution, different amounts of stream channelization, or fire damage. As described below, a goal should be to describe each of the cause–effect pathways found in the conceptual model.

It is frustrating to examine the results of a study and then to do a power calculation that says only huge and dramatic effects could be detected, if at all. It is often necessary to conduct a series of preliminary studies to examine the inherent variability of the measurement techniques and the variability of the study system to perform a power analysis. Then the number and frequency of samples can be modified. It is also useful to have documented study goals. Perhaps a 10 or 20% change in a parameter would be acceptable, and the study designed to detect that change. On the other hand, the dynamics of the system may make such a detection goal not possible and an alternative detection goal negotiated.

Do not be wed to hypothesis testing as the only method of observing changes in the system. There are many alternatives described in this and previous chapters.

13.8.5 Sampling Techniques

The actual methods of sampling are critical. Does the technique adequately measure the aspect of the system that pertains directly to the question being asked? Is the inherent variability of the technique so large that adequate statistical power cannot be obtained? Are the techniques applicable to the species found within the study system?

A key requirement is that the parts of the system sampled and the techniques used are able to inform the cause–effect model. Which tools can be used to examine the various pathways and effects that describe the segments of the conceptual model? Without adequate planning, a pathway that exists in the conceptual model may be lacking any information to confirm its presence or its effect.

13.8.6 Laboratory Studies

Both the Arkansas River and LTRWS laboratory studies were conducted to confirm the range of effects likely to be observed in the field. The laboratory is a great setting to derive potential dose-response relationships or to test the hypothesis that a particular pathology could be caused by a specific toxicant. In an ideal case the field studies should be able to generate specific questions for laboratory analysis. The laboratory should also be able to provide the field study other kinds of endpoints to be measured.

13.8.7 Data Analysis

Adequate data analysis is a critical part of the study, yet is often left as an afterthought. In the case of this discussion I assume that adequate sampling has taken place so that concerns regarding statistical power have already been addressed.

The first step in any data analysis is an exploratory examination for the patterns that exist in the data set. The conceptual model should already have produced hypotheses regarding patterns that should exist in the system, and those can be evaluated. A lookout must also be kept for patterns

that were unexpected. Often, simple scatter plots are the best place to start. Then the range of multivariate tools described earlier in this chapter are but a few of the techniques available.

The second step is confirmatory analysis. A variety of tools are available, but beware: Hypothesis testing tools such as ANOVA have severe limitations in field studies where samples cannot be assumed to be independent. The use of permutation tests, confidence intervals, and other tools is more robust given the nature of field studies. A presentation of the error terms or the calculation of a minimum significant difference associated with an analysis is also useful.

Also beware of performing analyses such as a regression of chemical concentration on a biotic index. The correlation will always look better than it really is because the index collapses and hides the inherent variation in the measurement.

13.8.8 Drawing Conclusions

The final step is reporting the patterns found in the study and how they relate to the conceptual model and the questions that initiated the study. It is perfectly acceptable to report that no effects due to the toxicant were observed as long as an indication of the statistical power of the analysis is included. It may be that a remediation effort may not have resulted in an increase in water quality or the population of an important species. The remediation effort may simply be overwhelmed by other stressors within the area.

It is also acceptable to identify effects that appear due to the toxicant, and then to state the uncertainties associated with that analysis. Consideration should be given to the likelihood that the pattern could be due to other stressors in the watershed, or that it is a rare event.

Finally, it is important that the conclusions remain within the bounds of the study. It is often tempting to report trends that are not statistically significant. If the imagined trend is not statistically significant, then it does not exist. There is also the tendency to dismiss statistically significant trends or results as not ecologically significant. Such a statement is one of the investigator's value system and is not a scientific analysis. It may be that the statistically significant effect or relationship is too small to be managed or does not affect a valuable part of the system, but both being too small or not important are valuation statements.

13.9 Making Decisions

Other than for curiosity, the reason that environmental toxicology exists is to manage ecological systems. Toxicology does not really provide decisions but supplies the scientific context in which decisions are made.

Chapter 14 provides a framework for integrating the science of environmental toxicology into the decision-making process. Ecological risk assessment is designed to include social values, regulatory context, and the description of nature that science provides in a process where choices can be made.

Appendix: Multivariate Techniques—Nonmetric Clustering

In the research described above, three multivariate significance tests were used. Two of them were based on the ratio of multivariate metric distances within treatment groups vs. between treatment groups. One of these is calculated using Euclidean distance, and the other cosine of vectors

distance (Good 1982; Smith et al. 1990). The third test used nonmetric clustering and association analysis (Matthews and Matthews 1990). In the microcosm tests there were four treatment groups with six replicates, giving a total of 24. This example is used to illustrate the applications in the derivations that follow.

Treating a sample on a given day as a vector of values, $x = \langle x_1 \ldots x_n \rangle$, with one value for each of the measured biotic parameters, allows multivariate distance functions to be computed.

Euclidean distance between two sample points x and y is computed as

$$\sqrt{\sum_i (x_i - y_i)^2}$$

The cosine of the vector distance between the points x and y is computed as

$$1 - \frac{\sum_i x_i y_i}{\sqrt{\sum_i x_i^2 \sum y_i^2}}$$

Subtracting the cosine from 1 yields a distance measure, rather than a similarity measure, with the measure increasing as the points get farther from each other.

The within-between ratio test used a complete matrix of point-to-point distance (either Euclidean or cosine) values. For each sampling date, one sample point, x was obtained from each of six replicates in the four treatment groups, giving a 24 × 24 matrix of distances. After the distances were computed, the ratio of the average within-group metric (W) to the average between-group metric (B) was computed (W/B). If the points in a given treatment group are closer to each other, on average, than they are to points in a different treatment group, then this ratio will be small. The significance of the ratio is estimated with an approximate randomization test. This test is based on the fact that, under the null hypothesis, assignment of points to treatment groups is random, the treatment having no effect. The test, accordingly, randomly assigns each of the replicate points to groups, and recomputes the W/B ratio, a large number of times (500 in our tests). If the null hypothesis is false, this randomly derived ratio will (probably) be larger than the W/B ratio obtained from the actual treatment groups. By taking a large number of random reassignments, a valid estimate of the probability under the null hypothesis is obtained as $(n + 1)/(500 + 1)$, where n is the number of times a ratio less than or equal to the actual ratio was obtained (Noreen 1989).

In the clustering association test, the data are first clustered independently of the treatment group, using nonmetric clustering and the computer program RIFFLE (Matthews and Hearne 1991). Because the RIFFLE analysis is naive to treatment group, the clusters may or may not correspond to treatment effects. To evaluate whether the clusters were related to treatment groups, whenever the clustering procedure produced four clusters for the sample points, the association between clusters and treatment groups was measured in a 4 × 4 contingency table, with each point in treatment group i and cluster j being counted as a point in frequency cell ij. The significance of the association in the table was then measured with Pearson's χ^2 test, defined as

$$\chi^2 = \sum_{ij} \frac{(N_{ij} - n_{ij})^2}{n_{ij}}$$

where N_{ij} is the actual cell count and n_{ij} is the expected cell frequency, obtained from the row and column marginal totals N_{+j} and N_{i+} as

$$n_{ij} = \frac{N_{+j} N_{i+}}{N}$$

where $N = 24$ is the total cell count, and a standard procedure for computing the significance (probability) of χ^2 is taken from Press et al. (1990).

Study Questions

1. What is resource competition? What is a resource consumption vector? What is a ZNGI?
2. Describe a two-species resource space graph.
3. Describe how resource heterogeneity can be incorporated into a two-species resource space graph.
4. How can toxicant input vs. natural variation be evaluated when community structure has altered?
5. Discuss nonlinear systems and their role in modeling xenobiotic impacts to ecological systems.
6. Describe the characteristics necessary in multivariate statistical tests used for evaluating complex data sets.
7. What is a normalized ecosystem strain? What did Kersting find occurring with the NES as time increased after a perturbation?
8. Explain A. P. Johnson's state space of ecosystems.
9. What is the major difficulty with the A. P. Johnson and Kersting methods?
10. What is nonmetric clustering and what statistical importance does it have for ecosystem analysis?
11. What is one of the most difficult analytical challenges in ecology?
12. Describe the fundamental basis of SiZer and how it can be used to assess the trajectories of ecological systems.
13. Discuss the benefits evolving from the use of multivariate techniques.
14. What illusions could give rise to a concept of recovery in ecosystems?
15. What are the main components of community conditioning?
16. Describe the importance of Neverland in regards to the community conditioning hypothesis.
17. List the steps in designing and implementing a field study.
18. What is a conceptual model?
19. What is the relationship of laboratory tests to the sampling being conducted in the field?
20. How are exploratory and confirmatory statistics used during data analysis?
21. Why would a control or reference site not be useful in a modern understanding of conducting a field study?

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Chapter 14

Ecological Risk Assessment

14.1 Introduction

A great deal of environmental toxicology is performed with the eventual goal of performing a risk assessment. Much of the research performed in the field is geared toward the determination of the risk of producing a new product or releasing a pesticide or effluent to the environment. Because of the interaction between environmental toxicology and risk assessment, a basic and clear understanding of ecological risk assessment in necessary. The U.S. EPA document *Framework for Ecological Risk Assessment* is a relatively clear review of the basics of ecological risk assessment as perceived in the early 1990s. Since the original publication of this framework, additional case studies and a guidance document have been published (U.S. EPA 1992, 1998). This chapter reviews the structure of ecological risk assessment, and introduces some current developments. The latter sections also provide a suggested approach for the risk assessment of wide-area sites with multiple stressors.

Two points should be considered carefully as regards the relationship between environmental toxicology and risk assessment. First, environmental toxicology should not be seen as dependent upon risk assessment for its justification. Risk assessment is a management tool used for making decisions, often with a great deal of uncertainty. The science of environmental toxicology, as with any science, attempts to answer specific questions. In the case of environmental toxicology the question is primarily how xenobiotics interact with the components of ecological systems. Second, risk assessment is not a strictly scientific pursuit. The assessment endpoints of risk assessment are often set by societal perceptions and values. Although the scientific process may be used in the gathering of information in the assignment of risks, unless a testable hypothesis can be formulated, the scientific method is not being applied. As a management tool risk assessment has certainly demonstrated its worth in the past 15 years.

14.2 Basics of Risk Assessment

Perhaps the easiest definition of ecological risk assessment is the probability of an effect occurring to an ecological system. Note that the word *probability* is key here. Important components of a risk assessment are the estimations of hazard and exposure due to a stressor.

Table 14.1 Comparison of Hazard Assessment with Risk Assessment

Characteristic	*Hazard Assessment*	*Risk Assessment*
Probabilistic results	No	Yes
Scales of results	Dichotomous	Continuous
Basis for regulation	Scientific judgment	Risk management
Assessment endpoints	Not explicit	Explicit
Expression of contamination	Concentration	Exposure
Tiered assessment	Necessary	Unnecessary
Decision criteria	Judgment	Formal criteria
Use of models	Deterministic fate	Probabilistic exposure and effects

Source: After Suter, G. W., II, in *Aquatic Toxicology and Risk Assessment*, eds. W. G. Landis and W. H. van der Schalie, Vol. 13, ASTM STP 1096, American Society for Testing and Materials, Philadelphia, 1990, pp. 5–15.

Note: The primary distinguishing characteristic of risk assessment is its emphasis upon probabilistic criteria and explicit assessment endpoints. Both methods of assessing the impact of toxicants are in use, but with risk assessment becoming the current standard.

A stressor is a substance, circumstance, or energy field that causes impacts, either positive or negative, upon a biological system. Stressors could be as wide ranging as chemical effects, ionizing radiation, or rapid changes in temperature.

Hazard is the potential of a stressor to cause particular effects upon a biological system. The determination of an LD_{50} and the mutagenicity of a material are attempts to estimate the hazard posed by a stressor.

Exposure is a measure of the concentrations or persistence of a stressor within the defined system. Exposure can be expressed as a dose, but in environmental toxicology it is often possible to measure environmental concentration. One of the values of determining tissue concentrations in fish and mammals is that it is possible to estimate the actual dose of a chemical to the organism. Biomarkers may also provide clues to dosage.

A stressor poses no risk to an environment unless there is an exposure. This is an extremely crucial point. Virtually all materials have as a characteristic some biological effect. However, unless enough of the stressor interacts with biological systems, no effects can occur. Risk is a combination of exposure and effects expressed as a probability. In contrast, hazard assessment does not deal with concentration and is not probabilistic in nature. Table 14.1 compares the two assessments as outlined in Suter (1990).

14.3 Ecological Risk Assessment

Two basic frameworks for ecological risk assessment have been proposed over the last 10 years. The first was based upon the National Academy of Sciences report detailing risk assessments for federal agencies. It is simple, yet this framework forms the basis of human health and ecological

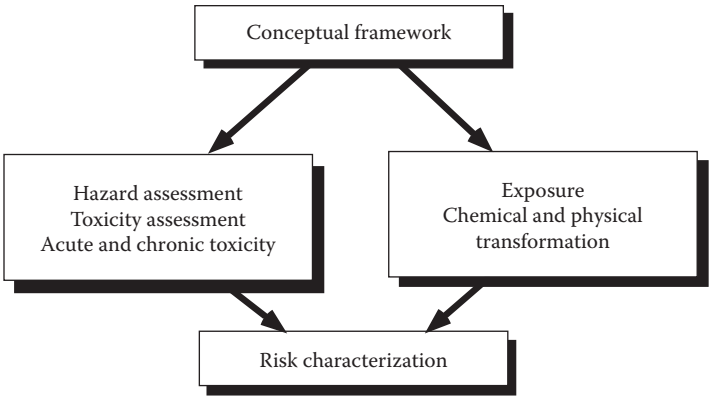

Figure 14.1 Classical risk assessment paradigm. Originally developed for human health risk assessment, this framework does not include the close interaction between effects and exposure in ecosystems.

risk assessments. Even later refinements owe a great deal to this basic description of the risk assessment process. A diagram of the basic format is presented in Figure 14.1. Basically, four boxes contain the critical steps in the risk assessment. First, problem formulation determines the specific questions that are to be asked during the risk assessment process. Second, the hazard assessment details the biological effects of the stressor under examination. Simultaneously, the exposure potential of the material to the critical biological components is calculated as part of an exposure assessment. Lastly, the probabilistic determination of the likelihood of an effect is formalized as risk characterization.

The original framework was updated to specifically apply to estimating the risks of stressors to ecological systems. Perhaps of singular importance is the fact that exposure and hazard are not easily separated in ecological systems. When considering effects upon single organisms it is usually easy to separate exposure and effect terms. However, since ecosystems are comprised of many populations, the single-species example is a subset of ecological risk assessment. For instance, once a chemical comes out of the pipe it has already entered the ecosystem. As the material is incorporated into the ecosystem biological and abiotic components transport or alter the structure of the original material. Even as the chemical affects the ecosystem, the ecosystem is altering the material. In light of this and other considerations, a revised framework was presented in 1992.

14.4 Ecological Risk Assessment Framework

The ecological risk assessment framework attempts to incorporate refinements to the original ideas of risk assessment and apply them to the general case of ecological risk assessment. The overall structure is delineated in Figure 14.2.

As before, a problem formulation process, analysis containing characterizations of exposure and effects, and a risk characterization process characterize the ecological risk assessment. Several outlying boxes serve to emphasize the importance of discussions during the problem formulation process between the risk assessor and the risk manager, and the critical nature of the acquisition of new data, verification of the risk assessment, and monitoring. The next few sections detail each aspect of this framework.

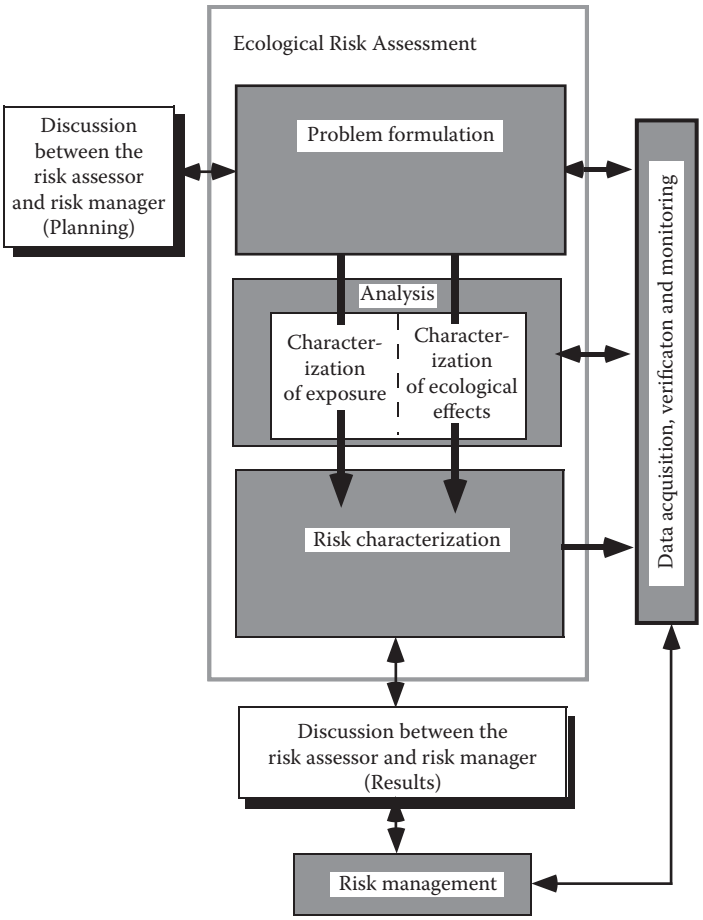

Figure 14.2 Schematic of the *Framework for Ecological Risk Assessment*. Especially important is the interaction between exposure and hazard and the inclusion of a data acquisition, verification, and monitoring component. Multivariate analyses will have a major impact upon the selection or assessment and measurement endpoints, as well as play a major role in the data acquisition, verification, and monitoring phase. (Adapted from U.S. EPA, *Framework for Ecological Risk Assessment*, EPA/630/R-92/001, Risk Assessment Forum, U.S. Environmental Protection Agency, Washington, DC, 1992.)

14.4.1 Problem Formulation

The problem formulation component of the risk assessment process is the beginning of a hopefully iterative process. This critical step defines the question under consideration and directly affects the scientific validity and policy-making usefulness of the risk assessment. Initiation of the process can begin due to numerous causes, for example, a request to introduce a new material into the environment, examination of cleanup options for a previously contaminated site, or as a component of examining land use options. The process of formulation is itself comprised of several subunits (Figure 14.3), a discussion between the risk assessor and risk manager, stressor characteristics, identification of the ecosystems potentially at risk, ecological effects, endpoint selection, conceptual modeling, and input from data acquisition, verification, and monitoring.

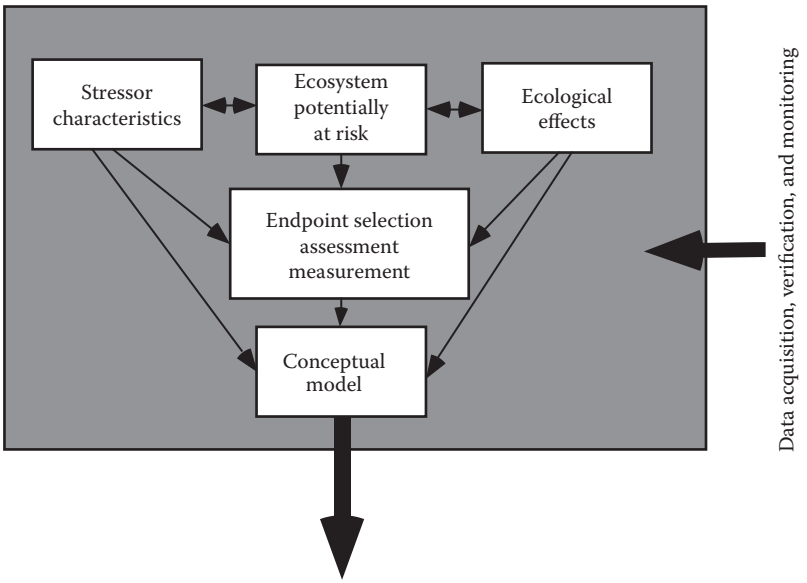

Figure 14.3 Problem formulation. This part of the risk assessment is critical because of the selection of assessment and measurement endpoints. The ability to choose these endpoints generally relies upon professional judgment and the evaluation of the current state of the art. However, *a priori* selection of assessment and measurement endpoints may block the risk assessor from consideration of unexpected impacts.

The discussion between the risk assessor and risk manager is crucial in helping to set the boundaries created by societal goals and scientific reality for the scope of the risk assessment. Often, societal goals are presented in ambiguous terms: protection of endangered species, protection of a fishery, or the even the more amorphous preserve the structure and function of an ecosystem. The interaction between the risk assessor and the risk manager can aide in consolidating these goals into definable components of a risk assessment.

Stressor characteristics form an important aspect of the risk assessment process. Stressors can be biological, physical, or chemical in nature. Biological stressors could include the introduction of a new species or the application of degradative microorganisms. Physical stressors are generally thought of as a change in temperature, ionizing or nonionizing radiation, or geological processes. Chemical stressors generally constitute such materials as pesticides, industrial effluents, or waste streams from manufacturing processes. In the following discussion chemical stressors are used as the typical example, but often different classes of stressors occur together. Radionucleotides often produce ionizing radiation and also can produce toxic effects. Plutonium is not only radioactive but is also highly toxic.

Stressors vary not only in their composition but also in other characteristics derived from their patterns of use. These characteristics are usually listed as intensity (concentration or dose), duration, frequency, timing, and scale. Duration, frequency, and timing address the temporal characteristics of the contamination as the characteristic scale addresses the spatial aspects.

Ecosystems potentially at risk can be one of the more difficult characteristics of problem formulation to address. Even if the risk assessment was initiated by the discovery of a problem in a particular system, the range of potential effects cannot be isolated to that locale. Given

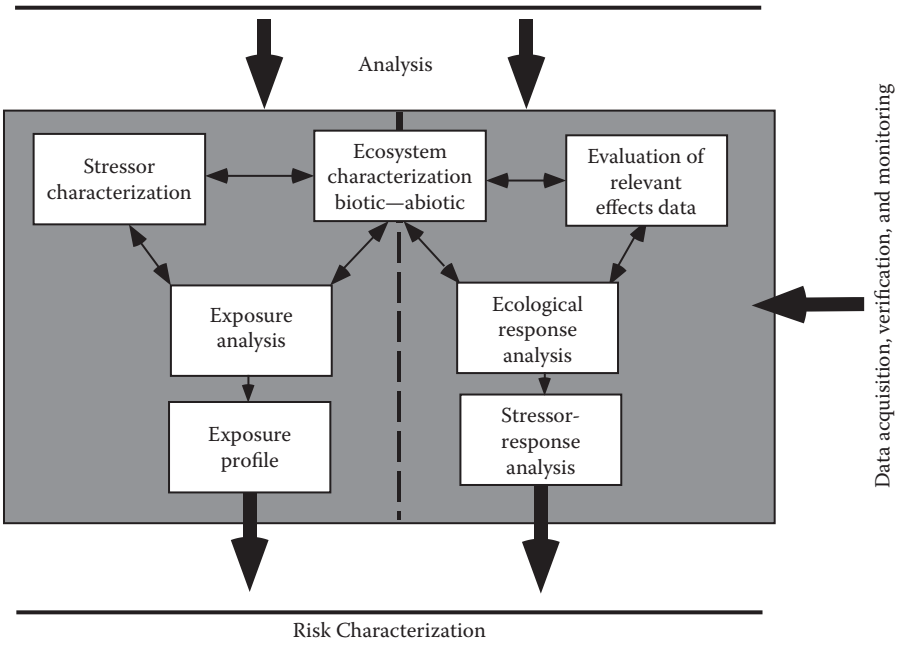

Figure 14.4 Analysis. Although separated into different sides of the analysis box, exposure and ecological responses are intimately connected. Often the biological response to a toxicant alters the exposure for a different compartment of the ecosystem.

atmospheric and waterborne transport, materials can impact a range of aquatic and terrestrial ecosystems. Pesticides, although applied to crops, can find their way into ponds and streams adjacent to the agricultural fields. Increased UV intensity may be more damaging to certain systems, those at higher latitudes or elevations, but the ramifications are global. For instance, the microlayer interface between an aquatic ecosystem and the atmosphere receives a higher exposure to chemical contamination or UV radiation due to the characteristics of this zone. However, alterations in the microlayer affect the remainder of the system since many eggs and larval forms of aquatic organisms congregate in this microlayer.

Ecosystems have a great number of abiotic and biotic characteristics to be considered during this process. Sediments have both biotic and abiotic components that can dramatically affect contaminant availability or half-life. History is an often overlooked characteristic of an ecosystem, but it is one that directly affects species composition and the system's ability to degrade toxic materials. Geographic relationship to nearby systems is another key characteristic influencing species migration, and therefore recovery rates from stressor impacts. Size of the ecosystem is also an important variable influencing species number and system complexity. All of the characteristics and others are crucial in accurately describing the ecosystem in relationship to the stressor.

Ecological effects are broadly defined as any impact upon a level of ecosystem organization. Figure 14.4 lists many of the potential interactions between a xenobiotic and a biotic system. Information is typically derived as part of a hazard assessment process but is not limited to detrimental effects of the toxicant. Numerous interactions between the stressor and the ecological system exist, and each should be considered as part of the potential ecological effects. Examples of

such interactions include biotransformation, biodegradation, bioaccumulation, acute and chronic toxicity, reproductive effects, predator-prey interactions, production, community metabolism, biomass generation, community resilience and connectivity, evolutionary impacts, genetics of degradation, and many other factors that represent a direct impact upon the biological aspects of the ecosystem. The effects of the ecosystem upon the toxicant are crucial if an accurate understanding of ecological effects is to be reached.

Endpoint selection is perhaps the most critical aspect of this stage of risk assessment, as it sets the stage for the remainder of the process. Any component from virtually any level of biological organization or structural form can be used as an endpoint. Over the last several years two types of endpoints have emerged: assessment and measurement endpoints.

Assessment endpoints serve to focus the thrust of the risk assessment. Selection of appropriate and relevant assessment endpoints can ultimately decide the success or failure of a risk assessment. Assessment endpoints should describe accurately the characteristic of the ecosystem that is to be protected as set by policy. Several characteristics of assessments should be used in the selection of relevant variables. These include ecological relevance, policy goals as defined by societal values, and susceptibility to the stressor. Often, assessment endpoints cannot be directly measured and must be inferred by the use of measurement endpoints.

Measurement endpoints are measurable factors that respond to the stressor and describe or measure characteristics that are essential for the maintenance of the ecosystem characteristic classified as the assessment endpoint. Measurement endpoints can be virtually any aspect of the ecosystem that can be used to provide a more complete picture of the status of the assessment endpoint. Measurement endpoints can range from biochemical responses to changes in community structure and function. The more complete the description of the assessment endpoint that can be provided by the measurement endpoints, the more accurate the prediction of impacts.

The design and selection of measurement endpoints should be based on the following criteria:

- Relevant to assessment endpoint
- Measurement of indirect effects
- Sensitivity and response time
- Signal-to-noise ratio
- Consistency with assessment endpoint exposure scenarios
- Diagnostic ability
- Practicality

Each of these aspects is discussed below.

The relevance of a measurement endpoint is the degree to which the measurement can be associated to the assessment endpoint under consideration. Perhaps the most direct measurement endpoints are those that reflect the mechanism of action, such as inhibition of a protein, or mortality of members of the species under protection. Although correlated functions can and are used as measurement endpoints, correlations do not necessarily imply cause and effect.

Consistency with assessment endpoint scenarios simply means that the measurement endpoint be exposed to the stressor in a manner similar to that of the assessment endpoint. Consistency is important when an organism is used as a surrogate for the assessment endpoint or if a laboratory test is being used to examine residual toxicity. However, this is not consistent with the approach that secondary effects are important. Other components of the ecosystem essential to the survivorship of the assessment endpoint may be exposed by different means.

Diagnostic ability is related to the relevance issue. Mechanistic scenarios are perhaps the most relevant and diagnostic.

Finally, the practicality of the measurement is essential. The gross physical and chemical parameters of the system are perhaps the easiest to measure. Data on population dynamics, genetic history, and species interactions tend to be more difficult to obtain, although they often are the more important parameters. Trade-offs must also be considered in the methods to be used. In many cases in ecological systems the absolute precision and accuracy of only a few of the measurement endpoints may not be as important as obtaining many measurements that are only ranked high, medium, or low. Judgment calls such as this require input from the data acquisition, verification, and monitoring segment of the risk assessment process.

The conceptual model of the risk assessment is the framework into which the data are placed. Like the selection of endpoints, the selection of a useful conceptual model is crucial to the success or failure of the risk assessment process. In some cases a simple single-species model may be appropriate. Typically, models in ecological risk assessment are comprised of many parts and attempt to deal with the variability and plasticity of natural systems. Exposure to the system may come from many different sources. The consideration of organisms at risk depends upon the migratory and breeding habits of numerous organisms, many rare and specialized.

As crucial as the above steps are, they are all subject to revision based upon the acquisition of additional data, and verification that the endpoints selected do in fact perform as expected, and that the process has proven successful in predicting ecosystem risks. The data acquisition, verification, and monitoring segment of risk assessment is what makes this a scientific process as opposed to a religious or philosophical debate. Analysis of the response of the measurement endpoints and their power in predicting and corroborating assessment endpoints is essential to the development of better methodologies.

14.4.2 Analysis

As the problem formulation aspect of the risk assessment is completed, an analysis of the various factors detailed above comes into play (Figure 14.4). Central to this process is the characterization of the ecosystem of concern.

Characterization of the ecosystem of concern is often a most difficult process. In many cases involving restoration of damaged ecosystems, there may not be a functional ecosystem and a surrogate must be used to understand the interactions and processes of the system. Often the delineation of the ecosystem is difficult. If the protection of a marine hatchery is considered the assessment endpoint, large areas of the coastal shelf, tidewater, and marine marsh systems have to be included in the process. Even many predominantly terrestrial systems have aquatic components that play a major role in nutrient and toxicant input. Ecosystems are also not stagnant systems, but under succession, and respond to the heterogeneity of climatic inputs in ways that are difficult to predict.

In addition to the gross extent and composition of the system, the resource undergoing protection and its role in the ecosystem need to be understood. Behavioral changes due to the stressor may preclude successful reproduction or alter migratory patterns. Certain materials with antimicrobial and antifungal properties can alter nutrient cycling. It is also not clear what part ecosystem stability places in dampening deviations due to stressors, or if such a property as stability at the ecosystem level exists.

In the traditional risk assessment, exposure and biological response have been separated. In the new framework for ecological risk assessment each of these components has been incorporated

into the analysis component. However, as has been detailed in preceding chapters, organisms degrade, detoxify, sequester, and even use xenobiotics as resources. Conversely, the nature and mixture of the pollutants and the resources of the ecosystem affect the ability of organisms to modify or destroy chemical stressors. Although treated separately, this is as much for convenience, and the reality of the intimate interaction between the chemical and the physical and biological components of the ecosystem should not be forgotten.

14.4.3 Exposure Analysis

Characterization of exposure is a straightforward determination of the environmental concentration range or, if available, the actual dose received by the biota of a particular stressor. Although simple in concept, determining or predicting the environmental exposure has proven to be difficult.

First, there is the end of pipe or deposition exposure. This component is determined more by the use patterns of the material or the waste stream and effluent discharges from manufacturing. In some cases, the overall statistics as to production and types of usage, such as the fluorohydrocarbons, are well documented. Manufacturers often can document processes and waste stream components. Effluents are often regulated as to toxicity and composition. Problem areas often occur due to past practices, illegal dumping of toxic materials, or accident events. In these instances the types of materials, rate of release, and total quantities may not be known.

However, as the material leaves the pipe and enters the ecosystem it is almost immediately affected by both the biotic and abiotic components of the receiving system. All of the substrate and medium heterogeneity as well as the inherent temporal and spatial characteristics of the biota affect the incoming material. In addition to the state of the system at the time of pollution, the history of the environment as contained in the genetic makeup of the populations plus the presence, in the past or present, of additional stressors impact the chemical-ecosystem interaction. The goal of the exposure analyses is to quantify the occurrence and availability of the stressor within the ecosystem.

Perhaps the most common way of determining exposure is by the use of analytical chemistry to determine concentrations in the substrates and media as well as the biological components of the ecosystem. Analytical procedures have been developed for a number of chemicals, and the detection ranges are often in the microgram per liter range. Analytical procedures, however, have difficulty in determining degradation products due to microbial activity and do not quantify the exposure of a material to the various biological components. The analysis of tissue samples of representative biota does give a more accurate picture of exposure to materials that are not rapidly detoxified or eliminated. Molecular markers such as DNA damage or enzyme induction or inhibition can also provide useful clues as to actual exposure. Since exposure can occur through different modes and at varying rates through those modes, the total burden upon the organism is difficult to estimate.

It should not be forgotten that a great deal of biotransformation does occur, especially for metals such as mercury and for many organics. In many cases the result is a less toxic form of the original input, but occasionally more toxic materials are created.

Lastly, models attempting to predict the fate and resultant exposure to a stressor can be used, and often they are applied in a variety of scenarios. Models, however, are simplifications or our imperfect understanding of exposure and should be tested whenever possible against comparable data sets. The reader should refer to the brief introduction of models found in Chapter 1.

As the temporal and spatial distribution of the stressor has been quantified in the exposure analysis step, it should prove possible to provide the distribution curve for exposure of the biotic

components of interest to the stressor. Dose and concentration probabilities are the typical units used in environmental toxicology.

14.4.4 Characterization of Ecological Effects

The characterization of ecological effects is perhaps the most critical aspect of the risk assessment process. Several levels of confidence exist in our ability to measure the relationship between dose and effect. Toxicity measured under set conditions in a laboratory can be made with a great deal of accuracy. Unfortunately, as the system becomes more realistic and includes multiple species and additional routes of exposure, the ability to even measure effects is decreased.

Evaluation of relevant effects data has long been left to professional judgment. Criteria typically used to judge the importance of the data usually include the quality of the data, number of replicates and repeatability, relevance to the selected endpoints, and realism of the study compared to the ecosystem for which the risk assessment is being prepared.

Toxicity data from several sources are usually compiled and compared. Generally there are acute and chronic data for the stressor on one or several species. Toxicity data are usually limited as to species, and the species of interest as an assessment endpoint may not have appropriate data available. This situation often occurs with threatened or endangered species since even a small-scale toxicity test involves relatively large numbers of animals to acquire data of sufficient quality.

Field observations and controlled microcosm and large-scale tests can provide additional data on which to base the risk assessment. Only in these systems can an indication of the importance of indirect effects become apparent. Field research also has limitations. No two fields are alike, requiring extrapolation.

14.4.4.1 Ecological Response Analyses

The combining of the exposure analysis with the ecological effects data results in the stressor-response profile. This profile is an attempt to match ecosystem impacts at the levels of stressor concentration under study. Relationships between the xenobiotic and the measurement endpoint are evaluated with a consideration of how this interaction affects the assessment endpoint. Rarely is this process straightforward. Often some model is used to specifically state the relationship between the measurement and assessment endpoint; when this relationship is not specifically stated, it is then left to professional judgment.

The EPA framework lists the relationships between assessment and measurement endpoints:

1. *Phylogentic extrapolation*: Relationship of toxicity data from one species to another, or perhaps more often, class to class. Often only a 96-hour green algal toxicity test is available to use as a representative of all photosynthetic eukaryotes.
2. *Response extrapolation*: Relationship between two toxicity endpoints such as the NOAEL (no observed adverse effect level) and the EC_{50}.
3. *Laboratory-to-field extrapolation*: Relationship of the estimate of toxicity gathered in the laboratory to the effects expected in the field situation. Laboratory situations are purposefully kept simple compared to the reality of the field, and are designed to rank toxicity rather than to mimic the field situation. Laboratory tests have limited the route of exposure and behavior. In the field these restrictions are not in place, often leading to unexpected results.

4. *Field-to-field (or habitat-to-habitat) extrapolation*: Relationship of one field or habitat to another. It may be highly unlikely that any two habitats can be identical. Streams on one side of a continental divide tend to have different flora and fauna than a comparable stream on the other side. Even controlled field studies exhibited differences in the replicates. The effect of a toxicant in the streams may be the same in a qualitative fashion, but quantification may not be possible.
5. *Indirect effects*: Does the toxicant have impacts due to the disruption of the ecosystem apart from direct impacts upon the ecosystem components? The elimination of photosynthetic organisms in a pond by an herbicide will eventually eliminate the invertebrate herbivores and the fish that rely upon them as a food source.
6. *Organizational levels*: Examine the transmission of effects up and down levels of biological organization. An alteration in fecundity at the organismal level will generally decrease the rate of growth of a population. Conversely, the decrease and elimination of an herbivore population, eliminating much of the top-down control at the community level, will allow the plant populations to grow in an exponential fashion, even if the toxicant has some effect upon maximum rate of growth.
7. *Spatial and temporal scales*: Exist in a variety of dimensions relating to the life span and size of the organisms and systems under investigation. One day and 10 m represent several generations and the entire world of many microorganisms, but this level of temporal and spatial scaling is relatively insignificant to a redwood of the Northwest. Not only is the size of the scale important, but so is the heterogeneity. Heterogeneity of both of these variables apparently contributes to the diversity of species and genotypes found in a variety of systems. Maintaining heterogeneity of these scalars may be as important as any other environmental variable in a consideration of impacts to the assessment endpoints.
8. *Recovery*: The rate at which a system can be restored to its original state. Recovery in the sense of a stable system returning to its original state is what is generally meant, and this may be difficult if not impossible to accomplish. If recovery does occur, it generally depends upon the ability of colonizing organisms to become established upon the impacted site, and therefore the isolation of the damaged ecosystem is important. Community conditioning and complexity theory also suggests that initial conditions are extremely important, and that several new stable points may be reached given similar initial conditions. Recovery to the initial state may in fact be of a low likelihood, and a more realistic goal may be a new dynamic that involves the factors selected as valuable in the choice of assessment endpoints.

In the evaluation of the ecological response consideration must often be given to the strength of the cause–effect relationship. Such relationships are relatively straightforward in single species.

14.4.4.2 Stressor-Response Profile

The stressor-response profile is in some ways analogous to a dose-response curve in the sense of a single-species toxicity test expanded to the community and ecosystem level. Since many of the responses are extrapolations and based on models from the molecular to ecosystem levels, it is important to delineate the uncertainties, qualifications, and assumptions made at each step.

One of the difficulties in the quantification of the stressor-response profile is that many of the extrapolations are qualitative in nature. Phylogenetic extrapolations are rarely quantified or

assisted with structure-activity relationships. Quantification of population level effects is likewise difficult, and in some cases probabilities of extinction have been used as the quantified variable, not a subtle population endpoint.

Perhaps the greatest difficulty is evaluating the stressor-response relationship for an ecological risk assessment and the fact that systems are under the influence of many other stressors. Laboratory organisms are generally healthy, but laboratory conditions do not mimic the ration of micronutrient, behavioral opportunities, and many other factors contained within an ecosystem. Field studies include many climatalogical and structural stressors that are separate from the introduced toxicant. Additionally, there is unlikely to be an ecosystem within range of a laboratory that has not been subjected to an anthropogenic stressor, again confounding even the best-designed study.

14.4.5 Data Acquisition, Verification, and Monitoring

Input from this block is most critical at this stage. Basic research on the effects of stressors to ecosystems, improvement in test methods, molecular mechanisms, and improvements in modeling provide critical input to this stage of the risk assessment.

14.5 Risk Characterization

Risk characterization is the final stage of the risk assessment process (Figure 14.5). This aspect of a risk assessment is comprised of a risk estimation and a risk description compartment. The overall process is a combining of the ecological effect with the environmental concentration to provide a likelihood of effects given the distribution of the stressor within the system. This process has proven to be difficult to accomplish in a straightforward manner. The probability of toxic impacts is analogous to the weather forecaster's prediction of rain. For instance, today there is a 50% chance of rain in the local area. This means that given the conditions observed, a prediction is made, generally from experience, that the chance of rain is 50 out of 100 trials. Notice that this is not a prediction that it will only rain over half of the forecast area. Toxicology attempts to make similar predictions regarding the probability of an effect given the conditions of chemical type, concentration, and ecosystem type. This predictive process is still as much an art as weather forecasting.

14.5.1 Integration

The integration of exposure with toxicity has been problematical. As we have previously discussed, environmental toxicology deals with a variety of effects at various levels of biological organization. A fish LD_{50} value is difficult to compare with loss of nitrogen fixation from an ecosystem. Perhaps the most widely used method of estimating risk is the quotient method.

The quotient method is simple and straightforward. The method simply divides the expected environmental concentration by the hazard:

$$\text{Quotient} = \frac{\text{Expected environmental concentration}}{\text{Concentration producing an unacceptable environmental effect}}$$

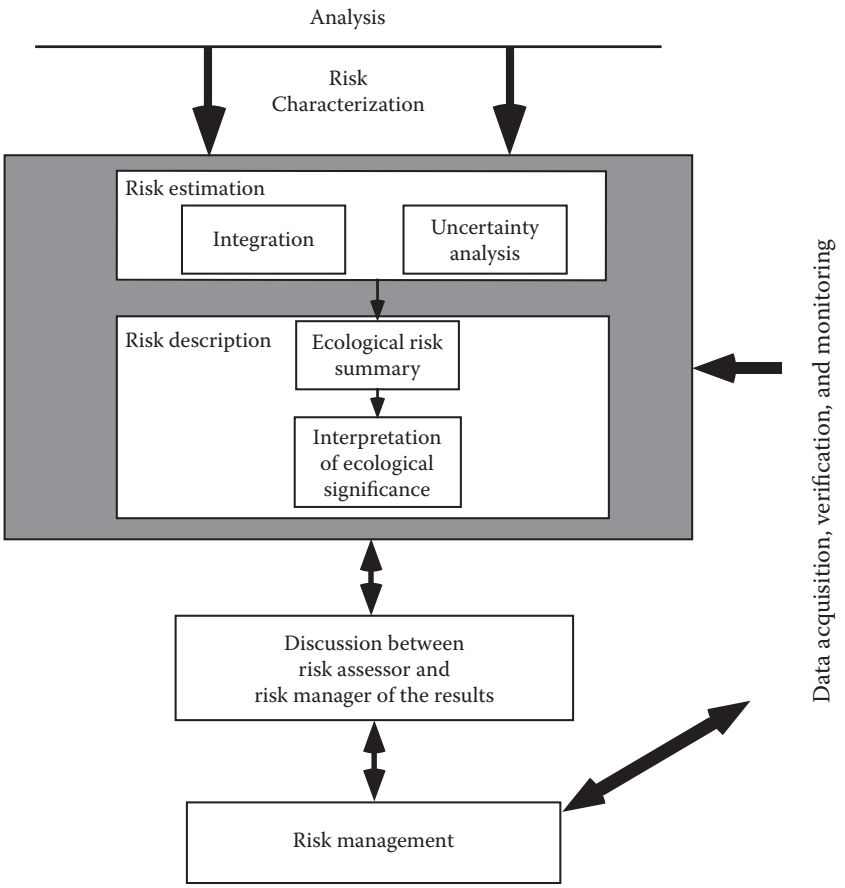

Figure 14.5 Risk characterization. This compartment is comprised of the risk estimation and risk description boxes. The integration of the exposure and effects data from the analysis compartment is reconciled in the risk estimation process.

Of course, the equation produces a ratio that is generally judged by the criteria below:

Quotient	*Risk*
>1	Potential or high risk
≈1	Potential risk
<1	Low risk

The difficulty with such an analysis is that it is a qualitative expression of risk without regard to the probability distributions of the chemical concentrations or the effects. Distributions of each can be plotted, and the distribution of expected effects calculated. In this example, although the probability of a high concentration is low, the probability of the effect is high, and at low concentrations, the probability of the effect is significantly reduced, but the likelihood of the concentration is much higher. Analyses such as these may prove more accurate, although more difficult to

calculate and perhaps interpret. Time and spatial factors should also be included, complicating the functions but better modeling the interaction between xenobiotic and biota.

Uncertainty analysis goes hand in hand with the integration process and has many points of origin. In some instances the conceptual model and the assessment and measurement endpoints associated with it may be inaccurate descriptions of the system under investigation. Only with rigorous monitoring and follow-up validation of the risk assessment is it likely that these types of errors will be routed out. Fundamental misunderstandings or ignorance of ecosystem processes and interactions may be corrected in this manner.

The quality and source of the data incorporated into the risk assessment again contribute to the uncertainty associated with the risk assessment. Toxicological data routinely vary according to the strain or test organism used. Quantitative structure-activity relationships (QSARs) have an associated uncertainty, although this is not routinely quantified. Field studies are noteworthy for the difficulty of interpretation. One of the most perplexing areas of uncertainty is the necessity of using data from studies that were not originally designed to address the question specific to the risk assessment.

Many multispecies tests and field studies are designed to look at only certain populations or other attributes of the ecosystem. This is not the fault of the study per se, since the funding, personnel, and physical resources are usually limited. The danger lies in the picking and choosing of secondary results from these studies. For example, the standardized aquatic microcosm contains 16 species that are initially inoculated into the system. However, in the reporting of the results for publication, the dynamics and interactions of all species and the combinations are not reported; to do so would be cumbersome and expensive. Only the dynamics of the organisms and interactions that are the apparently critical components are reported. Assuming that the other components are not affected because of their omission or lack of space in the article could be erroneous. Anecdotal data from field or multispecies tests are similarly difficult to interpret. Omission or inclusion of a report may reflect more the nature of the researcher than the presence or absence of the effect.

14.5.2 Risk Description

The next step in this framework is risk description. The two aspects of this segment include an ecological risk summary and the interpretation of ecological significance. Although this division is somewhat artificial, it can be paraphrased as: What are the potential effects and do I believe them? And how big a problem is this really going to be?

The ecological risk summary summarizes the risk estimation results and the uncertainties. The crucial aspect to this section is the decision making regarding the accuracy of the risk estimation. These decisions revolve around three general aspects of the analysis:

1. Sufficiency of the data
2. Corroborative information
3. Evidence of causality

Sufficiency of the data relates to the quality of the data and their completeness. Much of the discussion revolves around the quality and appropriateness of the research conducted or cited in the formation of the risk assessment.

Corroborative information are data derived from similar studies with similar stressors that tend to support the conclusions of the risk assessment. Science is inherently conservative, and similarity to data and conclusions of related studies enhances the credibility of the current risk assessment.

However, similarity to previous conclusions or ecological theory does not mean that the current study is flawed; it may mean that the previous work is not as similar as originally thought, or that the overall paradigm is incorrect.

Evidence of causality is perhaps the most concrete, and also the most elusive, aspect of the data assessment process. At the single organism or species level it is often possible to assign specific mechanistic connections for mortality or other impact. Unfortunately, it is not well understood how prevalent and pervasive these impacts are at the community level. Correlational data may be all that are available for impacts at the level of interspecies interactions. Correlations are difficult to assess because correlation does not denote cause and effect. In a system as complex as an ecosystem, multiple correlations may occur simply due to chance. It may also be difficult to separate cause and effect without firmly established criteria.

Perhaps the most critical aspect of the analysis above is the realization that additional data or even a reformulation of the conceptual model is required. In this case the assessment process is rerouted to the data acquisition, verification, and monitoring stage. An iterative process can then occur to obtain a usable and perhaps accurate risk assessment.

14.5.3 Interpretation of Ecological Significance

Finally, an interpretation of ecological significance is produced that details probable magnitudes, temporal and spatial heterogeneity, and the probability of each of these events and characteristics. One of the judgments that is usually called for is the recovery potential of the affected ecosystem. Given that recovery to the initial state may not be probable or even biologically possible, the question is perhaps dubious. Perhaps a better question is: Can the system exhibit at some future time the properties that initially made it valuable in terms of the assessment endpoints?

Recently, the idea of ecological services has become useful. Ecological services are those segments of the ecological structure that support the general human welfare. Human welfare in this context can mean human health, economics, subsistence food supply, spiritual or religious significance, or other factors considered valuable.

14.5.4 Discussion between the Risk Assessor and Risk Manager

Lastly, a report is made to the risk manager detailing the important aspects of the risk assessment. Of crucial importance are the range of impacts, uncertainties in the data and the probabilities, and the stressor-response function. These factors are then taken into consideration with social, economic, and political realities in the management of risks. An approach to risk assessment as outlined above, however, does not include a risk-benefit type of analysis. Such considerations are the purview of the risk manager.

14.5.5 Data Acquisition, Verification, and Monitoring

In the above outline the importance of the data acquisition, verification, and monitoring process in the development of accurate risk assessments has been emphasized. The importance of this aspect, often overlooked, is crucial to the development of risk assessments that reflect ecological reality. Models, no matter how sophisticated, are simply attempts to understand processes and codify relationships in a very specific language. Ptolemaic (earth-centered) astronomy accurately predicted many aspects of the stars and planets and served to make accurate predictions of celestial events. However, the reversing of direction in the celestial sphere of the planets was difficult

to account for given the earth-centered model. Eventually, the Copernican (sun-centered) model replaced the Ptolemaic model as the descriptions of solar system dynamics and the insights from the new framework led to other discoveries about the nature of gravity and the motion of the planets. How many of our current models are earth centered? Only the reiteration of the predictive (risk assessment) and experimental (data acquisition, verification, and monitoring) process can answer that question.

One of the difficulties of ecosystem level analysis has been our inability to accurately present the dynamics of these multidimensional relationships. Conventional univariate statistics are still prevalent, although the shortcomings of these methods are well known. Several researchers have proposed different methods of visualizing ecosystems and the risks associated with xenobiotic inputs.

14.6 Techniques in Ecological Risk Assessment

The previous sections of this chapter introduced some of the basics of ecological risk assessment. The first segment of this section presents an approach to making the estimates of ecological effects from laboratory data more realistic. The second segment discusses an approach for estimating ecological risks to regions that have a variety of stressor and habitat types.

14.6.1 New Methods for Calculating Ecological Risk

The risk quotient (RQ) for each combination of contaminant and receptor (plant or animal) of concern is calculated by dividing the estimated environmental concentration (EEC) by the toxicity reference value (TRV):

$$\text{RQ} = \frac{\text{EEC}}{\text{TRV}} \tag{14.1}$$

This has been the classical model for calculating risk. This basic equation requires two factors, the expected environmental concentration and the toxicity reference value. The EEC can be determined by a number of means, direct measurement in the field being the best. The advantage of the field measurement is that not only is it a direct indication of exposure, but spatial and temporal variability can be assessed. A variety of values have been used as TRVs, NOELS, and maximum allowable toxicant concentrations (MATCs), but we prefer using a regression method to obtain a specified effective concentration, or an EC_x.

An attempt to improve the RQ method has been made by using the 95% upper confidence limit (UCL) of the mean for all of the measured values for each medium or the maximum measured concentration, whichever is lowest. This will result in a conservative estimate of risk, particularly for a small site with relatively few environmental sampling points or a site with one or more small areas of high contamination. This approach should only be used as a screening tool. If the RQ exceeds 1, the value gives no indication of where on the site or at what time the exceedence occurred.

A simple quotient value as in the above example does not take into account the variability in exposure due to the movement of an animal, or the variability in exposure due to the uneven distribution of contaminant in the environment. Although spatial variability is not expressed in the classical quotient method above, two methods can incorporate spatial variability into the risk calculations.

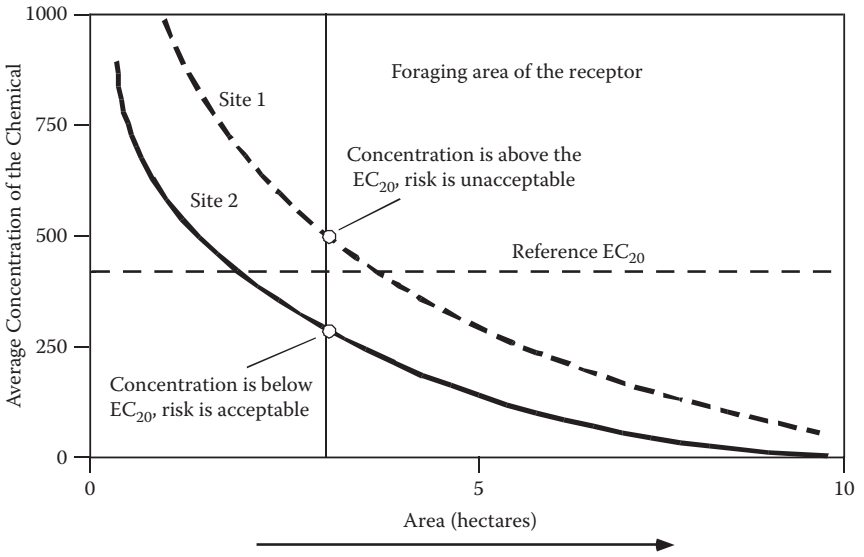

Figure 14.6 Curve exposure model. Site 1 exceeds the EC_{20}. Site 2, with a slightly different average concentration curve, is now below the EC_{20} when it crosses the size of the foraging area.

14.6.2 The Curve Model

The curve model (Freshman and Menzie 1996) is used to describe the risk to wildlife that forage over the contaminated site. The model is based off of grids or areas of sampling in the site map. If the organisms are sessile, then the model reduces to the spatially distinct risk quotient calculation presented above. Freshman and Menzie (1996) present the entire derivation, and an adapted step-by-step progression is presented below (Figure 14.6). The steps are straightforward and easily accomplished using a spreadsheet format.

1. Plot the first data point as the highest environmental concentration for a site (c_1) by its associated area (a_1).
2. Plot the next data point as the average concentration for the two highest contaminated areas ($c_1 + c_2$)/2 vs. the associated area ($a_1 + a_2$).
3. Plot additional data points by progressively including lesser contaminated areas until the entire site is included.
4. Add to the graph horizontal lines that represent the EC_x values appropriate for the particular species involved.
5. Plot the foraging area of the organism as a vertical line.
6. Compare the intersection of the area line to the line representing the average environmental concentration. If this intersection is below the horizontal line representing the EC_x, then the risk is low. If the intersection is above the EC_x line, then the risk is above the cutoff limit for effects.

This approach can also be used to estimate cleanup goals. A cleanup would ensure that the intersection of the concentration curve is below the EC_x value for the proposed land use. As sites or concentrations are proposed for cleanup, the model can be computed to examine the intersection of the foraging area with the EC_x value. Decisions can then be made to clean up sites with a few

very contaminated areas versus sites that are not as contaminated or are of a larger surface area. Such a plan can be used in the mitigation section of the final report.

14.6.3 Spatially Distinct Risk Quotients

RQs should be calculated using Equation 14.1 for each site from which an environmental sample was collected, for each plant or animal species of concern. The RQs should be plotted on the site map in order to determine if there are areas where risk is high (RQ > 100), areas of low risk (RQ < 1), or areas of intermediate risk (1 < RQ < 100). If several samples were taken in close proximity to each other, use the average concentration and plot it as a single value at that location.

The *probability* of exceeding an RQ of 1 (or 100) anywhere on the site can also be estimated from this information by

$$\frac{\text{Number of RQs} > 1 \text{ or } 100}{\text{Total number of RQs}} \times 100 \tag{14.2}$$

A common assumption is that RQs can be added together to get a total risk. Since each is calculated for a species-specific toxicity value, the units for each RQ will be different. Therefore, RQs calculated for different species should *never* be added together, as they are not equivalent values. However, the probability of exceedence over all species over all locations will be an approximation of overall risk.

14.6.4 A Ranking Approach to Multiple Stressors, Wide-Area Ecological Risk Assessment

One of the emerging problems in environmental toxicology and ecological risk assessment (EcoRA) is the problem of multiple receptors and multiple stressors over a broad region or landscape. Over the region the quality of data on exposure to the variety of stressors may be quite different. Likewise, for some species there may be extensive toxicity data, and for others no data may be extant. Coupled with the classical data issues of exposure and effects are the variety of landforms, habitats, and anthropogenic uses that occur within a region. Landis and Wiegers (1997) have published a method for investigating the risk within sites that contain a broad range of habitat types and stressors. The method was derived because of the necessity for calculating risks within Port Valdez, Alaska. This fjord is the home of port facilities for the Alaska pipeline as well as having a fishing fleet, a refinery, fish processing plants, a fish hatchery, and the town of Port Valdez. The relief of the land is spectacular, with mountains, glaciers, and a deep water port. The necessity of attempting to evaluate risk in such a diverse environment, with data availability ranging from detailed to nonexistent, led the research team to use a ranking approach to the assessment process.

EcoRA methods traditionally evaluate the interaction of three environmental components (Figure 14.7a): *stressors* released into the environment, *receptors* living in and using that environment, and the receptor *response* to the stressors. Measurements of *exposure* and *effects* represent the interactions between the components. At a single contaminated site, especially where only one stressor is involved, the connection of the exposure and effects measurements to the assessment endpoints may be fairly simple. Conventional EcoRA depends on measurements of exposure and effects to calculate an estimate of risk. Exposures occur, and are measured, between the stressor and the receptor, while effects are a measure of the receptor response.

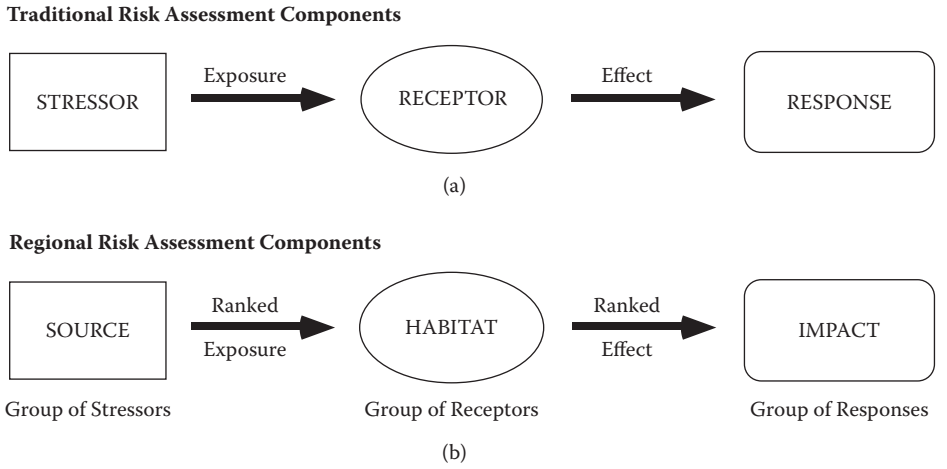

Figure 14.7 Comparison of risk components applied at the traditional and regional levels. At the regional level, the source releases the stressor to the habitat. The habitat is explicitly and spatially defined within the region. If one of the organisms that constitute an assessment endpoint or other ecological properties of concern exist within that habitat, then an impact can occur. (After Landis, W. G., and Wiegers, J. A., *Hum. Ecol. Risk Assess.*, 3, 287–297, 1997).

Expanding an assessment to cover a region requires additional consideration of scale, complexity of the structure, and the regional spatial components: *sources* that release stressors, *habitats* where the receptors reside, and *impacts* to the assessment endpoints (Figure 14.7b). The three regional components are analogous but not identical to the three traditional components. However, in a regional, multiple-stressor assessment, the number of possible interactions increases combinatorially. Stressors are derived from diverse sources, receptors are often associated with a variety of habitats, and one impact can lead to additional impacts. These interactions are painted upon a complex background of natural stressors, effects, and historical events. At the regional level stressors and receptors are represented as groups: A source is a group of stressors; a habitat is a group of receptors. These groupings are usually too indistinct to obtain overall measurements of exposure and effects. However, comparisons are possible. Exposures from a continuous source are greater than exposures from a similar, but infrequent source. Likewise, effects to a salmonid population are different in intertidal areas where they spawn than in the open water where the adults travel. At the regional scale, exposures and effects have to be evaluated on a habitat, and then receptor basis with emphasis placed upon the spatial and temporal heterogeneity of both.

The proposed approach for a regional assessment is to evaluate the risk components at different locations in the region, rank the importance of these locations, and combine this information to predict the relative risk among these areas. The numbers of possible combinations that can result from this approach depend on the number of categories that are identified for each risk component. If two types of sources (point discharges and fish wastes), two habitats (the benthic environment and the water column), and two possible impacts (a decline in the sportfish population and a decline in sediment quality) are identified, there are eight possible combinations of these components that can lead to potential environmental risk (Figure 14.8).

Each identified combination establishes a possible pathway to a hazard. For this hazard to result in an environmental impact, the risk components must overlap (Figure 14.8). If a source generates stressors that affect habitats important to the assessment endpoints, the ecological

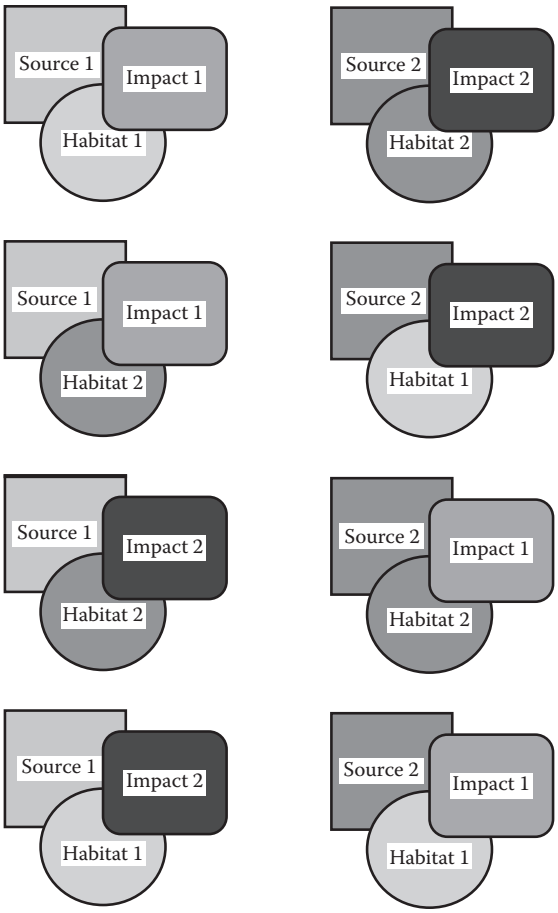

Figure 14.8 Possible combinations characterizing risk from two sources, two habitat types, and two potential impacts to assessment endpoints. Eight potential combinations are possible, and each needs to be evaluated. (After Landis, W. G., and Wiegers, J. A., *Hum. Ecol. Risk Assess.*, 3, 287–297, 1997).

risk is high (Figure 14.9a). A minimal interaction between the components results in a low risk (Figure 14.9b). If one component does not interact with one of the other two components, there is no risk (Figure 14.9c). For example, a discharge piped into a deep water body is not likely to impact salmon eggs, which are found in streams and intertidal areas. In such a case the source component (an effluent discharge) does not interact with the habitat (streams and intertidal areas), and no impact would be expected (i.e., harm to the salmon eggs).

Impacts can be due to a variety of combinations of stressors and habitats. Integrating these combinations together demonstrates that impact 1 is actually the result of many combinations of sources and habitats (Figure 14.10). It is also apparent that the interactions that lead to impact 1 are different from those that lead to impact 2. In order to fully describe the risk of a single impact occurring, each route needs to be investigated.

This regional approach develops a system of numerical ranks and scalars to address the difficulties encountered when attempting to combine different kinds of risks. Ranks and scalars can be manipulated without regard to the metric of the original measurement. In a complex system

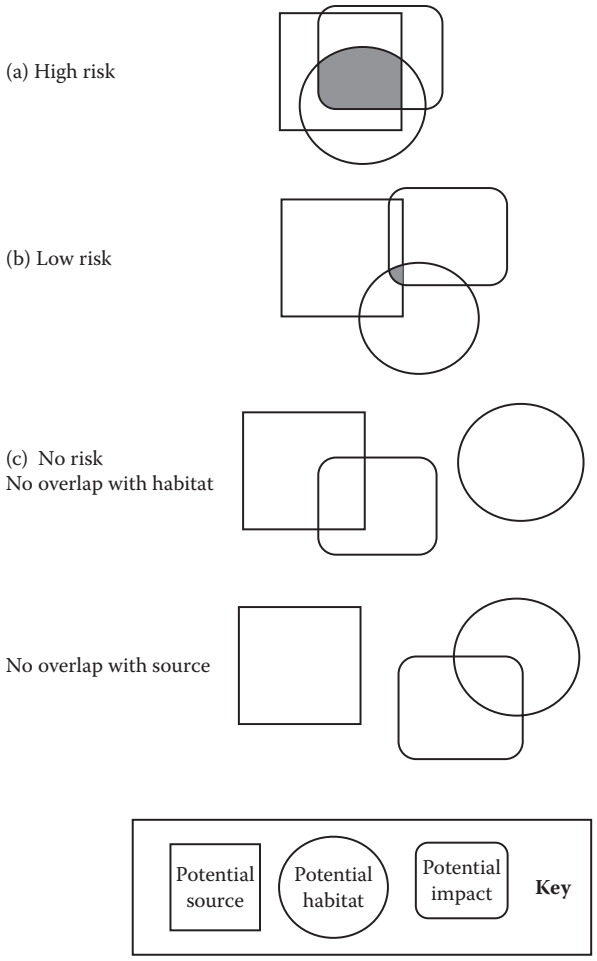

Figure 14.9 The ecological risk resulting from the interaction between sources, habitats, and assessment endpoints. The assumption is that risk is increasingly proportional to the overlap or source, habitat, and impact.

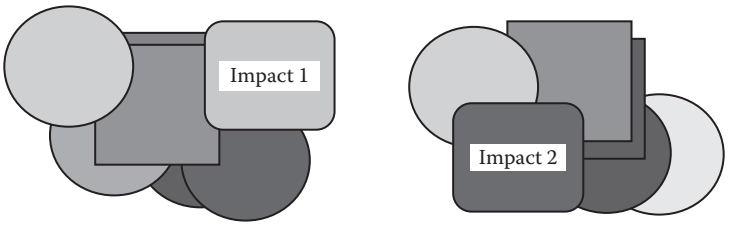

Figure 14.10 Rank integration. Integration (through overlap) of the possible combinations of the two sources and two habitat types that can influence the risk of impacted assessment endpoints (impacts 1 and 2).

with a wide range of dissimilar stressors and effects, there are few measurements that are strictly additive or linear. For example, there is little meaning in adding or multiplying toxicant concentrations to counts of the number of introduced predators in order to determine the total risk in a system. However, it is useful to know that a particular region has both the highest concentrations of a contaminant and the most introduced predators.

14.6.5 A Simple Example

Consider a coastal inlet with a single source type as a concern: wastewater discharges. Two such discharges with effluents of a similar composition exist. Three habitats characterize the region: the subtidal basin, the shoreline, and river deltas. The assessment endpoint of concern is contamination of shellfish harvested by local residents. These shellfish include clams harvested in the shoreline habitat and crabs harvested in the subtidal areas. The relative risks to the endpoint are determined through the following process:

1. *The region is divided into subareas based on source and habitat characteristics.* In this example, three subareas are chosen:
   Subarea A: Contains a small wastewater discharge at the shoreline; several large rivers enter this area.
   Subarea B: Contains a large wastewater discharge in the deep basin.
   Subarea C: Contains an area of the inlet not influenced by either discharge or some of each habitat.
   The shape and size of each subarea incorporate expected transport characteristics of stressors from the source. The edges are chosen to correspond with habitat characteristics.
2. *The sources and habitats are ranked between subareas.* The ranks are chosen to reflect the magnitude of the source and the amount of habitat that could be affected in each subarea.

	Habitats Ranked			
Sources Ranked	*Wastewater*	*Subtidal*	*Shoreline*	*Rivers*
Subarea A	1	0	1	2
Subarea B	2	2	0	0
Subarea C	0	1	2	1

3. *The ranks assigned for each combination of source, habitat, and subarea are multiplied together to form the following matrices:*

	Subarea A	*Subarea B*	*Subarea C*
Subtidal	0	4	0
Shoreline	1	0	0
Rivers	2	0	0
		Wastewater	

4. *The relationships driving possible exposure and effects are determined.* This interaction is represented through a simple binary assignment of 1 (likely to occur) and 0 (less likely to occur). The resulting matrices form exposure and effects filters for the information ranked above.

	Exposures	*Effects to Crabs*	*Effects to Clams*
Subtidal	1	1	0
Shoreline	1	0	1
Rivers	0	0	1
		Wastewater	

5. *Each element of the matrix established in step 3 is multiplied with the appropriate exposure filter and one of the two effects filters in step 4.*

		Subarea A	*Subarea B*	*Subarea C*
Effects to Clams:	Subtidal	0	0	0
	Shoreline	1	0	0
	Rivers	0	0	0

Effects to Clams:	Subtidal	0	4	0
	Shoreline	0	0	0
	Rivers	0	0	0
			Wastewater	

The results for each subarea are summed to determine the relative risks within the region. The risk of shellfish contamination is greatest for crabs in subarea B. Clams are at a lower risk in subarea A.

Impacts to Shellfish	*Subarea A*	*Subarea B*	*Subarea C*
Crabs	0	4	0
Clams	1	0	0

In a simple example such as this, the same conclusions can be reached through spatial examination of the information. However, in a large system with many components, integrating available information can be quite challenging. The above process allows the information to be sorted systematically in order to estimate comparative levels of risk within a region. Field testing these predictions can determine if the model is confirmed in the particular ecological structure. Results that are confirmed can then be traced back to the original components.

14.6.6 Advantages and Dangers of the Ranking Approach

A major advantage of the categorization and ranking approach described above is its intrinsic simplicity. Few assumptions are needed, the most basic being: The more stressors that can affect more habitat result in an increase in the probability of an assessment endpoint being affected. No

reference or control site is necessary, or assumptions about community dynamics, indirect effects, or the linearity of the response. Natural variability can be included as part of the spectrum of stressors. Stressors, for which little research has been conducted, such as the impact of the hatcheries on the population genetics of wild stocks, can be incorporated. A framework for future decision making is constructed by the ranking procedure.

In a properly constructed ranking analysis each assumption has to be documented. A sensitivity analysis can be performed investigating the impacts of ranking decisions upon the final outcome. Uncertainties can also be quantified and data gathered to make the ranking more based upon data-derived rules.

Another advantage of this technique is that it is consistent with methods that rely upon the formation of rules derived from data that may lead to more consistent and accurate rank predictions. In this manner, it is a direct descendant of the nonmetric clustering approach described in Chapter 11.

No technique is without drawbacks. First and foremost is the danger that the ranks will be misinterpreted and abused in a fashion similar to that done for indexing systems such as the index of biological integrity. Ranks and indices are the collapsing of a hypervariate structure into relatively few features. These ranks are not data that can be used in a regression any more than means should be used. In many respects the projection is arbitrary unless it can be based on rules constructed by direct analysis of the ecological structure of interest.

Another drawback is the reliance upon a ranking system without at least some confirmation of the risks projected. The rankings are effectively hypotheses that are testable. There cannot be a substitute for testing the reality of an analysis using a variety of techniques. These methods can include comparison of field concentrations of stressors to benchmark concentrations, analysis of biomonitoring data, and the use of field collections to examine community structure and dynamics.

There has been progress in establishing protocols for confirming risks, or at least the likelihood that a cause–effect relationship may exist. Two of the methods are the use of specific criteria for the establishment of causation and the weight of evidence approach.

14.6.7 Establishing Causation and the Weight of Evidence Approach

The determination of causality is critical to the risk assessment process. Risk assessments require the construction of mechanistic linkages operating at spatial and temporal scales appropriate to the scale of the risk assessment.

First, risk assessments must construct a conceptual model that embeds a series of potential cause-and-effect relationships. In many cases these relationships, such as the concentration-response, are based upon laboratory data. In other instances these relationships have been derived from other field investigations where causality has been established.

Second, risk assessment is a process of hypothesis generation. The uncertainty of a risk assessment can be reduced if at least a part of the hypothesis can be tested and found to be confirmed. A method of accomplishing this is to perform further field research designed to test the causality hypotheses.

To support the processes described above, methods for assigning causality are required that are compatible and consistent with current understandings of the workings of ecological systems. Incorporating scale and dynamics with the inherently open nature of ecological systems is a tremendous challenge.

There have been two parallel approaches to establishing causality. First is the establishment of specific criteria for the identification of mechanisms for observed effects. The second is the weight of evidence approach.

14.6.7.1 Criteria for Causation

There have been a number of efforts to generate frameworks for describing causality. The U.S. Environmental Protection Agency's *Stressor Identification Guidance Document* (U.S. EPA 2000) is a recent example applied to aquatic systems. This type of approach is especially useful if there are clearly identified causative agents in the environment that cause specific abnormalities or symptoms within the organisms or ecological systems. These types of methodologies are based upon explicit rules for assigning the likelihood of causation.

Criteria similar to those listed by Adams (2003) are used to establish causality and are derivatives of Koch's postulates and Hume's criteria. The list includes: (1) strength of association, (2) consistency of association, (3) specificity of association, (4) time order or temporality, (5) biological gradient over space and time, (6) experimental evidence available, and (7) biological plausibility. In many instances, especially at a regional scale and over long periods of time, meeting the requirements for each of these criteria can be difficult.

Items 1 to 3 are dependent upon the coverage of the data across the landscape being sufficient to draw statistical inferences. In some instances there have been monitoring programs at the study site that can provide relevant data. Unfortunately, many monitoring programs are conducted without the questions specific to the risk assessment as a sampling consideration. It may be possible to use data from a variety of sources to establish potential stressor and response relationships, but there will probably still be critical data gaps.

Items 4 and 5 are related. Item 4 is a temporal gradient and requires a data set of sufficient length compared to the dynamics of the effect and the potential causative agents. If a long duration dynamic is involved, such as the Pacific Decadal Oscillation (PDO), then 30 years of data reflects only one cycle. There may also be multiple causative agents acting in a variety of sequences, masking parts of the temporal relationships. Item 5 is a spatial gradient, again requiring sufficient spatial coverage in relationship to the variability of the stressor, and there can be confounding variables in space as well.

Experimental evidence such as toxicity tests or the induction of disease under controlled conditions (item 6) can also be coupled with field observations to establish cause–effect mechanisms. Experimental evidence is critical for testing specific predictions made by hypotheses designed to predict large-scale relationships, and should be included whenever practical.

Item 7 is a composite of items 1 to 6, but also is related to the sensitivity of the observer to accept uncertainty in what defines plausible. Clearly, a mechanism that has been confirmed experimentally and by field observations is ideal. The paradigm in which the risk assessment group is working also bounds the expectations of plausibility. In working at a landscape level over a period of decades where multiple causes are likely present, this criterion becomes less attainable.

14.6.7.2 Weight of Evidence

A parallel effort has been the assignment of cause or risk using a weight of evidence (WoE) approach. Menzie et al. (1996) provide one of the earliest and clearest descriptions of the WoE approach to assigning causality. The usefulness of the WoE approach has been extensively discussed in a series of recent papers (Burton et al. 2002a, 2002b; Chapman et al. 2002; Forbes and

Calow 2002). Clearly this approach has proven useful; however, it requires improvement in order to address the needs of regional risk assessment.

The WoE approach (Chapman et al. 2002) combines lines of evidence (LoE), including the presence of a proposed stressor, the ability of the stressor to cause an effect, and the observed effect in the field to establish causation. This is a powerful approach, especially for systems that are limited in spatial and temporal scales, have clearly characterized stressors, and have extensive effects data sets.

To illustrate, we will apply the WoE approach to the evaluation of toxicity causing or being a risk factor in the alteration of a benthic community structure in a waterway (Figure 14.11). Extensive data on chemical concentrations in sediments are obtained at the site under investigation (Figure 14.11a). Data on the chemical contaminants are matched with laboratory tests of sediment toxicity to the chemicals (Figure 14.1b). A comparison of the chemical concentrations to the toxicity data indicates that the materials are toxic under laboratory conditions (Figure 14.11c). A hypothesis is then generated that identifies the sediment under consideration as likely to be toxic. Sediment bioassays of the sediment can confirm the hypothesis (Figure 14.11d). Since the assessment endpoint is the preservation of benthos, measurements are made of the benthic community structure in the region (Figure 14.11e). Chemical concentrations and toxicity results are also compared to measures of benthic community structure. Chemicals that are positively associated with toxicity observed in the laboratory and effects seen in the field can be identified as one of the risk factors (Figure 14.11f). There can also be conflicting lines of evidence. In our example, nonchemical habitat alteration (dredging, piers, etc.) also corresponds to the observed impacts (Figure 14.11g) and can also be identified as a risk factor. Differentiating between the two will require a new set of investigations.

A probabilistic approach may be used to differentiate the two. This approach is particularly useful in ruling out potential risk factors with low probabilities of occurrence. It is important for the risk assessors to observe the impacts, list the potential stressors, identify exposure pathways, and review the evidence that a particular stressor can cause the observed effect. The causality

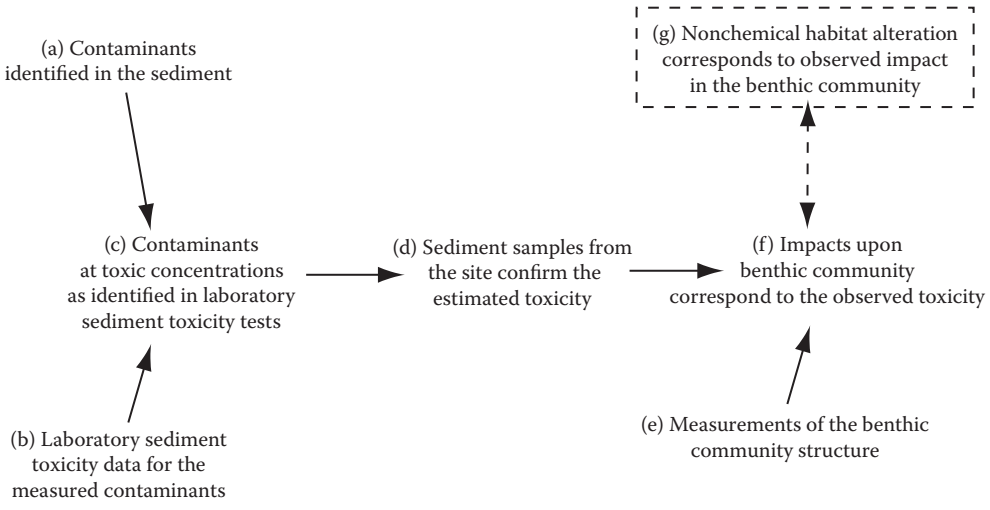

Figure 14.11 Illustration of the use of a WoE approach in order to establish risk due to contaminants to a benthic community.

criteria set in the previous section can be useful in this process; the more criteria that are met, the more likely the causation. The output is the probability of a particular stressor and its source being the causative agent for the observed or predicted impact. Multiple stressors might have similar probabilities due to uncertainties from the understanding of the exposure-effects link.

The appropriateness of each line of evidence and the criteria used to establish a linkage approach should be considered in the problem formulation and the conceptual model development. In this manner, the rules for accepting a potential stressor as a cause can be set before the analysis begins. It is critical that these rules be established and not altered unless there is compelling evidence to do so. This process prevents a *post hoc* WoE approach and the introduction of investigator bias.

Post hoc approaches to WoE should be avoided unless there is a clear revisiting of the problem formulation–conceptual model development to ensure that the *post hoc* analysis meets the decision-making criteria of the risk assessment.

Both the criteria for causality and the WoE approaches improve the transparency of the process. The criteria for establishing suggested links between causes and effects are clearly presented before the initial analysis. This process also improves communication between each of the communities involved in the process. Each stakeholder group can see the clear connections between the studies being conducted, the effectiveness of the studies' results in reducing the uncertainties, and the progress toward the final risk assessment.

14.6.8 A General Model for Regional Risk Assessment: The 10 Steps

The previous reviews of the application of the relative risk model (RRM) have led to the formulation of 10 procedural steps that formalize the process. The process can also generate three specific outputs useful in decision making.

The procedural steps are:

1. List the important management goals for the region. What do you care about and where?
2. Make a map. Include potential sources and habitats relevant to the management goals.
3. Break the map into regions based upon a combination of management goals, sources, and habitats.
4. Make a conceptual model that links sources of stressors to the receptors and to the assessment endpoints.
5. Decide on a scheme to allow the calculation of relative risk to the assessment endpoints.
6. Calculate the relative risks.
7. Evaluate uncertainty and sensitivity analysis of the relative rankings.
8. Generate testable hypotheses for future field and laboratory investigation to reduce uncertainties and to confirm the risk rankings.
9. Test the hypotheses listed in step 8.
10. Communicate the results in a fashion that portrays the relative risks and uncertainty in a response to the management goals.

These 10 steps correspond to the portions of the ecological risk assessment framework depicted in Figure 14.2. The first four steps of the RRM correspond to the initial segments of the framework, especially problem formulation. These initial steps largely determine the success of the risk assessment. Steps 4 to 6 are closely related steps and do not fit cleanly into a conventional framework. The conceptual model is based upon knowledge of source-stressor-habitat-effects linkages. Determination of the ranking scheme incorporates large amounts of data generated on the

amounts of stressors, habitats, and what knowledge is available on potential outcomes. Once the conceptual model and ranking scheme are established, the actual calculation is straightforward. Analysis of uncertainty and sensitivity and generation of testable hypotheses are the more difficult steps that most closely correspond to risk characterization. Testing the hypotheses corresponds to the verification step, and should be incorporated whenever possible. Step 10 corresponds to risk communication and is a critical step. The next paragraphs briefly describe each of the 10 steps and the 3 outputs.

The first four steps are critical to performing a regional ecological risk assessment and are the foundation of a useful risk assessment that can be applied to the decision-making process and to long-term environmental management. These steps should involve a close interaction with all of the interested parties. The parties include the regulators, the regulated community, the stakeholders, including private citizens and nongovernmental organizations, and the risk assessors. There are likely to be environmental managers in the first three groups that will be involved in the decision-making process. The risk assessors need to clearly understand the decision-making needs of each of the other groups, communicate the strengths and limitations of the risk assessment process, and attempt to translate management goals stated in nonscientific terminology to features that can be quantified and evaluated. In this interaction the role of the risk assessor is clearly not decision making, but as scientific and technical support. At times the decision makers may need to be informed that a particular goal is not part of ecological reality, or that the field of science is not sufficiently advanced to provide predictive measures. However, the interaction is critical if a successful risk assessment is to occur.

1. *List the important management goals for the region. What do you care about and where?* The management goals are the key to rest of the risk assessment. EPA states, "Ecological risk assessment is a process used to systematically evaluate and organize data, information, assumptions, and uncertainties to help understand and predict the relationship between stressors and ecological effects in a way that is useful for environmental decision making" (U.S. EPA 1998). Likewise, regional risk assessments are most effective when they target the decision-making needs and goals of environmental managers. It is important to identify difficult or even conflicting goals, and decisions must be identified early in the process. Without identifying, discussing, and resolving these issues, the assessment results will not appear to be useful to managers, and in fact may not be usable for the decisions at hand.

   There are four sets of interactions among the regulated community, the regulators, and the interested stakeholders in the decision-making process. Interaction among these three groups is expected in three forms. First, each will interact with the other two parties in a bipartite fashion. Second, all three parties must interact at the same time to clearly define the management and decision options in order to answer basic questions about the future management of the area. Third, there are also interactions between the three groups and the risk assessment team.

   The role of the risk assessment team is critical. In some instances the desired uncertainty reduction is not possible due to resource limitations (Suter 1993), and some management goals are also unattainable as well. While a goal may be to restore the balance of nature or to return the system to a pristine state, given our current understanding of ecological systems, neither of these goals is attainable. However, stakeholders envision the restoration of certain ecological resources to within usable limits, and these goals can be quantified and engineered.

   As this process is completed, the management goals are then placed into a spatial context with the appropriate sources and habitats.

2. *Make a map. Include potential sources and habitats relevant to the management goals.* First, the potential sources within the study area are located, characterized, and placed on a map that includes the critical topological features of the system. The boundaries are set by the management goals of the decision makers, but also taking into account the life history of the various endpoints. Habitat information is also plotted for the endpoints under consideration. Maps can be produced in a variety of ways; the Port Valdez study utilized conventional maps scanned into a computer, and the additional information was added in a graphics program. Subsequent studies have made extensive use of geographical information systems (GIS), which have distinct advantages and disadvantages. The advantages are clearly the ability to display and analyze geographical information in a variety of formats. Unfortunately, not all spatial data are in digital form, digital data can often be expensive when they do exist, and digital data are kept in a variety of projections, which take time to combine. Uncertainty related to geographical information is also an issue, which will be discussed in step 7.

   The next step is to combine management objectives, source information, and habitat data into geographically explicit portions that can be analyzed in a relative manner.
3. *Break the map into regions based upon a combination of management goals, sources, and habitats.* The next step is the creation of risk regions that delineate the boundaries of the areas for which risks will be calculated. This map is the basis of the rest of the analysis because risks are all relative based upon the delineated regions. The map is also based upon possible pathways of exposure in a spatial sense to the locations where habitat can be found for the assessment endpoints. In this regard it may be very important to follow fate of the water, groundwater, soil, and air within the landscape to ensure that appropriate sources, stressors, and habitats are incorporated into a risk region.
4. *Make a conceptual model that ties the stressors to the receptors and to the assessment endpoints.* The conceptual model delineates the potential connections between sources, stressors, habitat, and endpoints that will be used in each risk region. The conceptual model is an extension of the basic framework for a regional risk assessment with sources providing stressors into particular habitats. In this instance the habitats are broadly defined as terrestrial and aquatic to capture the exposure pathways and location within the region of our endpoints. In this instance there are numerous interconnected endpoints both to show the valued ecosystem components and to illustrate the interdependence and potential indirect effects.

   In cases, such as this illustration, where metals can be assumed as the principal contaminant, it is important to incorporate all of the confounding stressors as well. The shaded boxes highlight the conceptual model if only metals are being considered. However, other stressors are also impacting all of the endpoints. A metals-only assessment would take the endpoints and the metals out of context.
5. *Decide on an evaluation scheme for each source, stressor, and habitat to allow the calculation of risk to the assessment endpoints.* There has to be a scheme for evaluating sources, stressors, and habitats and translating this into a risk calculation. There are many methods, typically using quotients between an observed concentration and a concentration deemed as a threshold above which an unacceptable effect will occur. As previously discussed, this quotient method has drawbacks. Ranking methods are also available, as previously discussed.

   The critical issue is that the evaluation scheme be decided before the collection of field data or the initiation of toxicity tests. Some types of evaluation schemes may require very specific sampling in order to produce the required statistical power. At a regional scale, a sampling scale that ensures an efficient use of resources is critical to prevent the depletion of resources.

6. *Calculate the risks.* Calculate the risks using the scheme planned in step 5. Examples of such methods are described elsewhere in this chapter.
7. *Evaluate uncertainty and sensitivity analysis of the relative rankings.* Uncertainty needs to be accounted for and tracked in the risk assessment process. At times it may be an accounting process, listing factors that introduced uncertainty into the assessment process. At other times the uncertainty can be represented by a distribution and a Monte Carlo process employed to provide a range of values.
8. *Generate testable hypotheses for future field and laboratory investigation to reduce uncertainties and to confirm the risk rankings.* A risk assessment should be able to provide predictions that can be tested using a variety of methods. It may not be possible to perform landscape-scale experimental manipulations, but it is clearly possible to make predictions about patterns that should already exist. The hypothesis to be tested may be a subhypothesis of the overall risk estimation that is clearly testable. Being able to test and confirm at least part of the hypotheses generated by the risk assessment should increase the confidence of the risk assessors, stakeholders, and decision makers in using the results for environmental management.
9. *Test the hypotheses listed in step 8.* Hypotheses can be tested using a variety of field, mesocosm, or laboratory test methods. In an ideal situation it should be possible to make predictions based upon known concentrations and then sample that field site in order to confirm effect or no effect. It may be necessary to rework the risk assessment in order to reduce uncertainty, or a stressor-habitat-effect linkage may be incorrect. Testing the risk predictions allows feedback into the assessment process, improving future predictions.
10. *Communicate the results in a fashion that portrays the relative risks and uncertainty in a response to the management goals.* The risk assessment process, no matter how scientifically valid, is still not useful unless the results are clearly communicated to the stakeholders and decision makers that commissioned the study. A variety of tools can be used. A variety of publications can be placed upon the Internet or made available in libraries. These publications can range from very technical to plain English, depending upon the audience. Public meetings can also be conducted to provide assessment results, but also to receive comments from the interested parties. Communication to the decision maker is equally vital. This communication needs to be tailored to the decision-making criteria and clear.

    The three outputs that can be incorporated into step 10 are:

    a. Maps of the risk regions with the associated sources, land uses, habitats, and the spatial distribution of the assessment endpoints.
    b. A regional comparison of the relative risks, their causes, the patterns of impacts to the assessment endpoints, and the associated uncertainty. These regional comparisons and estimates of the contribution of each source and stressor create a spatially explicit risk hypothesis.
    c. A model of source-habitat-impact that can be used to ask *what if* questions about different scenarios that are potential options in environmental management.

    These outputs effectively summarize the data, provide risk assessments, and provide a tool for the examination of different risk scenarios. These outputs facilitate communication and decision making for the environmental managers.

The Cherry Point Case Study

The Cherry Point relative risk assessment (Hart Hayes and Landis 2004, 2005) is an example of the use of the relative risk model for regional scale risk assessment. This section takes each of the 10 steps and describes how they were applied in this particular case study.

Figure 14.12 Aerial photograph of the shoreline and landscape that are part of the Cherry Point study area. (See color insert following page 268.)

The primary objective of the study was to analyze cumulative impacts from multiple sources of stress to assess risk to multiple biological endpoints that utilize the region. An aerial photograph of the region (Figure 14.12) portrays the mixed marine-terrestrial nature of the area.

The first two steps are essentially simultaneous in the case of Cherry Point. In order to answer the *where* question, a map is going to be necessary.

1. *List the important management goals for the region. What do you care about and where?*

The Washington Department of Natural Resources (WDNR) manages the aquatic lands of Washington State "for current and future citizens of the state to sustain long-term ecosystem and economic viability." This joint mission to both protect natural resources and generate income from them creates a framework in which difficult management decisions must be made. The purpose of this regional risk assessment was to provide estimates of the relative contributions of risk from anthropogenic sources to biological endpoints in the Cherry Point region to aid WDNR in their management decisions.

The Cherry Point Technical Working Group, organized by WDNR Aquatic Resources Division, represented stakeholders for the endpoint selection process. The working group included representatives from WDNR, Washington Department of Fish and Wildlife (WDFW), Washington State Department of Ecology (Ecology), the Lummi Nation Indian tribe, citizens' groups, and the three major industries in the region (British Petroleum, Alcoa Intalco Works, and Phillips 66). This stakeholder group generated a list of species based on accepted criteria for the selection of assessment endpoints. We then shortened the list to six biological endpoints that included representative components of the Cherry Point reach, paying special attention to select endpoints that are susceptible to site-specific stressors in the region. The selected assessment endpoints included three fish, two macroinvertebrates, and one bird (great blue heron).

Coho salmon are known to utilize nearshore and stream habitats in the study area and are culturally valued by stakeholders. Coho salmon are connected to Pacific herring in the marine food web via predator-prey and competition for food.

Juvenile English sole are known to use the nearshore region at Cherry Point. Because the juvenile life stage is benthic, they are likely to be exposed to and exhibit effects from contaminated

sediments. If contaminated sediments were present in the study region in a high enough concentration, English sole would likely exhibit a response.

Pacific surf smelt embryos have been documented, and the species is known to spawn year-round on beaches within the Cherry Point study area. The association of surf smelt embryos with sediments makes them vulnerable to potential stressors in the region. Surf smelt also support both commercial and recreational fisheries in Washington State.

The juvenile life-stage of Dungeness crab is known to inhabit nearshore waters in the Cherry Point study area. Like English sole, their close association with sediments makes them vulnerable to potential stressors in the region, including sediment changes and contaminants. The crab has a commercial and recreational value, making them relevant to stakeholders.

Littleneck clams are sediment dwellers and have a high probability of exposure to sediment-bound contaminants. Large numbers are known to occur in the study area and are heavily harvested by both recreational and commercial clam diggers. Because adult clams are sedentary, any response they exhibit is likely to be due to local stressors, providing a good indication of the local condition of the Cherry Point region.

Great blue heron use both intertidal and terrestrial habitats, providing a link between the aquatic and terrestrial components of the study area. Two large nesting colonies consisting of about 300 nesting pairs each are located within the study area at Point Roberts and Birch Bay. Because great blue heron are predators and potentially prey on Pacific herring, English sole, and shellfish, they are likely to be exposed to and bioaccumulate persistent chemicals that may occur in the study area. Because the local subspecies is nonmigratory, Great blue heron provide an indication of the local condition of the Cherry Point region, reducing the probability of observing effects caused by stressors outside the region.

2. *Make a map. Include potential sources and habitats relevant to the management goals.*

The Cherry Point study area is located in Whatcom County, Washington, and consists of the coastline from Point Roberts and the U.S. border in the north to the southern boundary of Lummi Bay in the south (Figure 14.13). The area incorporates approximately 715 km^2 and includes the nearshore watersheds that drain into Semiahmoo Bay, Birch Bay, Lummi Bay, and the Strait of Georgia, as well as the inter- and subtidal regions in these water bodies.

3. *Break the map into regions based upon a combination of management goals, sources, and habitats.*

Using a variety of sources of spatial data and ArcView® GIS software, we defined the boundaries of the study area and divided it into six subregions (Figure 14.14) based on watershed and bathymetric boundaries, the location of the recently established WNDR aquatic reserve, land use, and locations of likely sources of stressors. Upland, the study area ends at the boundaries of watersheds draining directly into coastal waters. Nearshore, the study area was limited to waters within the 60 m contour, where assessment endpoint species are most likely to be found. The six subregions were:

1. Point Roberts subregion, consisting of Point Roberts proper, a peninsula protruding into the northern boundary of the study area immediately south of the U.S.–Canadian border plus the adjacent waters to 60 m depth
2. Drayton Harbor subregion, comprising Drayton Harbor itself and the watersheds that drain into this water body, including the city of Blaine, California and Dakota Creeks, Semiahmoo Spit, and adjacent waters
3. Birch Bay subregion, containing the bay and Birch Bay State Park, Terrell Lake, Terrell Creek, and the remaining upland watershed
4. Cherry Point subregion, which includes the newly designated Cherry Point aquatic reserve, three large industrial piers and much of the upland industrial complexes, the site of a proposed pier and shipping facility, as well as several small unnamed creeks
5. Lummi Bay subregion, consisting of Lummi River, part of the city of Ferndale, a large portion of the southern oil refinery complex, the Lummi Nation Indian Reservation, and Lummi Bay itself

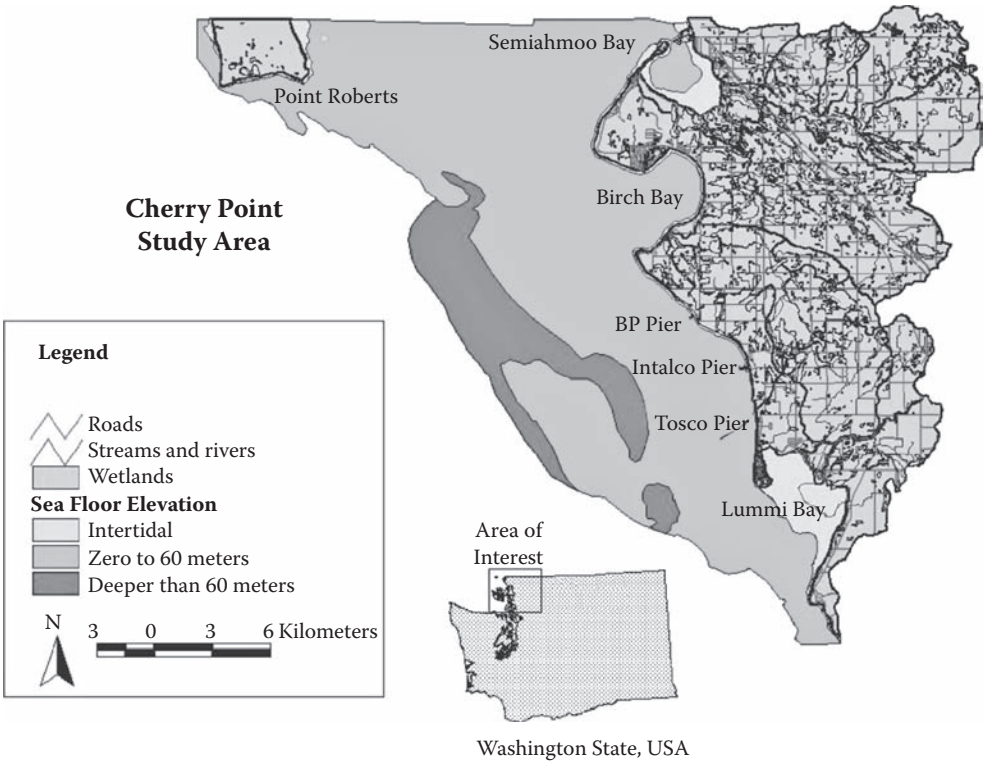

Figure 14.13 The Cherry Point study area in northern Whatcom County, Washington. BP (British Petroleum) Oil Company, Alcoa Intalco Works Aluminum, and Tosco Oil Company maintain shipping piers on the coast. (See color insert.)

6. Alden Bank subregion, an offshore area with no terrestrial component and centered around a shallow bank that rises from deeper waters closer to shore

Identification of Habitats

Habitats were identified according to the classification system defined by WDNR's Nearshore Habitat Program, U.S. EPA Region 10 Estuarine Habitat Assessment Protocol, and the published literature about the habitat requirements of the listed assessment endpoints The 10 habitats represent different vegetation and substrate types in the upland, intertidal, and subtidal areas in the study area. The 10 types of habitats are (1) gravel-cobble intertidal, (2) sandy intertidal, (3) nearshore soft-bottom subtidal, (4) intertidal mudflats, (5) inter- or subtidal eelgrass, (6) inter- or subtidal macroalgae, (7) water column, (8) stream, (9) wetlands, and (10) forest.

Identification of Sources of Stressors

The nearshore lands in the Cherry Point region are dominated by agriculture interspersed with residential, industrial, forested, and undeveloped lands. Large shipping vessels travel to and from three deep water shipping piers, and hundreds of recreational and fishing vessels have moorage in private and public marinas in the area (WDNR 1997a). Beaches are popular for clam digging, crabbing, and other recreational uses. To portray this mixture of multiple human uses, we partitioned anthropogenic sources of stressors into eight categories for use in the RRM: (1) accidental and chemical spills, (2) agricultural land use, (3) ballast water, (4) piers, (5) point sources of pollution, (6) recreational activities, (7) urban land use, and (8) vessel traffic. Natural sources of stressors

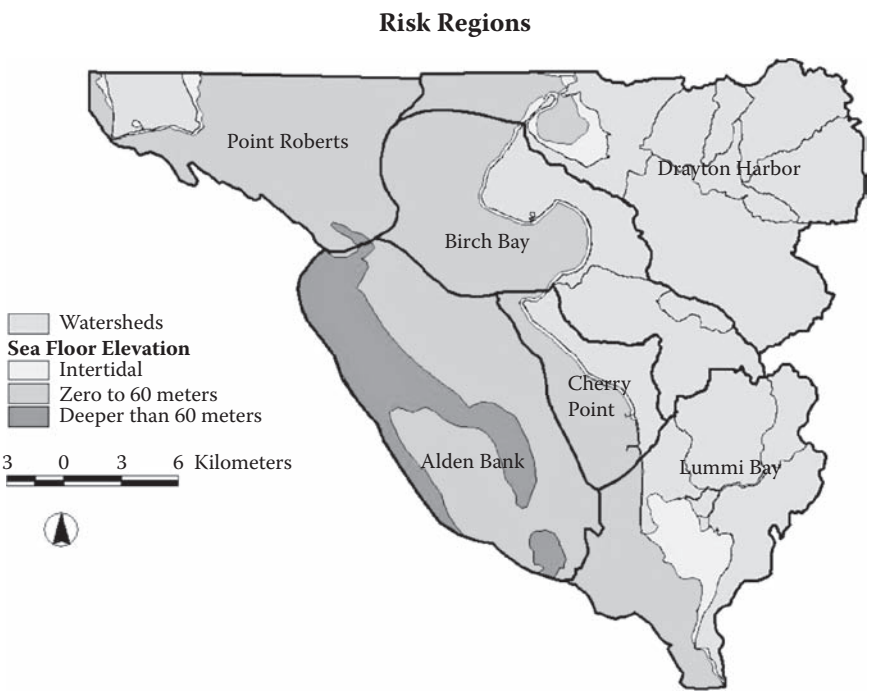

Figure 14.14 The study area divided into six subregions based on watershed and bathymetric boundaries. (See color insert.)

were eliminated from this study due to a lack of site-specific data and in order to limit the study to sources relevant to the regional land use, nearshore, and coastal management decisions facing local managers.

4. *Make a conceptual model that ties the stressors to the receptors and to the assessment endpoints.*

We constructed a conceptual model (Figure 14.15) to depict the interconnections between sources, stressors, habitats, and endpoints based on information in the published and unpublished literature. In most of our case studies straightforward box and line diagrams could depict the cause–effect relationships. However, in this case a different graphical presentation was used. The conceptual model depicts preliminary exposure and effects filters for each source-stressor-habitat-endpoint combination. A complete exposure pathway met the following criteria based on a review of published and unpublished literature: The source releases or causes the stressor; the stressor will occur and persist in the habitat; the endpoint uses the habitat type; the stressor can negatively affect the assessment endpoint.

The conceptual model diagram is read differently than the box and line conceptual models describe earlier in this chapter and in previous publications. In this instance the sources are listed along the upper-left hand column, with the stressors along the top of the chart. The sources and their associated stressors can be found by looking at the intersection of the two factors. The endpoints are listed in the middle of the chart and the intersection between all three can be read directly by examining the shaded areas. The kind of disturbance is keyed to the symbols in each cell.

The problem formulation process resulted in maps and a conceptual model that later became the foundation of the analysis and risk characterization phases of the assessment.

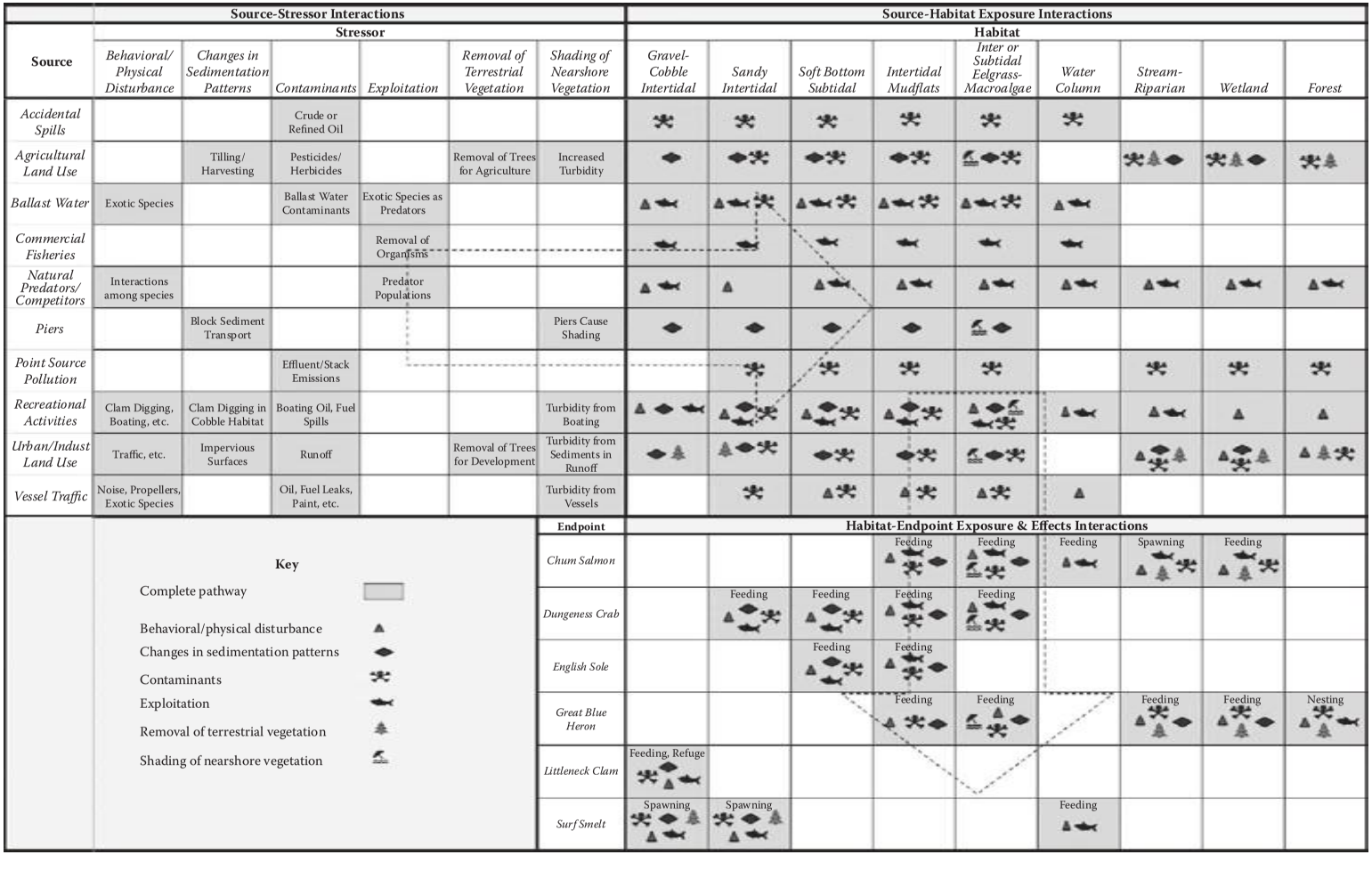

Figure 14.15 Conceptual model depicting potential exposure and effects pathways from source to stressor to habitat to endpoint. (See color insert.)

Table 14.2 Examples of Geographical Information Used in the Cherry Point RRM

Name	*Data Description (Sources of the Data Are in Parentheses)*
Accidental spills	Locations and volumes of spills ranging from 1 pint to hundreds of gallons (Ecology)
Land use	Land use as designated by the Whatcom County 2000 tax assessment codes (Whatcom County)
Ballast water releases	Locations, dates, and volumes of ballast water releases from 1999 to 2001 (WDFW)
Piers	Locations of piers and docks on Washington coasts (WDNR)
Point sources of pollution	Locations of National Pollution Discharge Elimination System (NPDES) permit holders, toxic release inventory sites, and solid and hazardous waste sites (U.S. EPA)
Recreational clam diggers and crab buoy locations	Locations of Washington Department of Fish and Wildlife aerial observations of recreational shellfish harvesters and crabbers, 2001 (WDFW)
Vessel traffic	Locations of boat slips for both recreational and commercial vessels; Washington Department of Natural Resources Shorezone Inventory (WDNR)
Intertidal substrates and vegetation	Locations of nearshore habitat types (WDNR)
Bathymetry	Sea floor depths as measured by National Ocean Service (National Oceanographic Survey)
Streams	Streams and rivers (USGS)
Wetlands	Location and area of wetlands (Whatcom County)
Forest	Land parcels designated as forest based on Whatcom County 2000 tax assessment codes (Whatcom County)

5. *Decide on an evaluation scheme for each source, stressor, and habitat to allow the calculation of risk to the assessment endpoints.*

GIS and other site-specific data (Table 14.2) were used to rank (1) sources of stressors (e.g., human land use, point sources of pollution, vessel traffic) and (2) habitats (e.g., cobble-gravel intertidal habitat, wetlands) for subregions within the study area. We then assigned exposure and effects filters for each source-stressor-habitat-endpoint combination based on the conceptual model as well as geographic data. Ranks and filters were integrated to derive risk estimates for subregions, sources, and endpoints in the study area.

Sources and Habitat Ranks

Geographical data sets were used to assign source and habitat ranks to the six subregions in the study area. Using Jenk's optimization, a technique for finding clusters of similar characteristics, data sets were broken into four categories to assign ranks of 0, 2, 4, and 6. Table 14.3 provides samples of the criteria for the source and habitat-ranking schemes. Table 14.4 contains the risk ranks assigned for each habitat and Table 14.5 presents the source ranks for the subregions.

Table 14.3 Examples of Criteria for Ranking Sources of Stressors and Habitats

Source	*Ranking Criterion*	*Range (Divided by Natural Breaks)*	*Rank*	*Drayton Harbor Example*
Sources				
Accidental spills	Volume of spills (gallons) per year per square kilometer in subregions	0 0.001–0.023 0.024–35.395 35.396–280.677	0 (Zero) 2 (Low) 4 (Medium) 6 (High)	255.8 gallons = rank of 6
Agricultural land use	Percent agricultural land	0 0.69–16.94 16.95–42.02 42.03–50.41	0 (Zero) 2 (Low) 4 (Medium) 6 (High)	42.02% agriculture = rank of 4
Point sources of pollution	Number of point sources of pollution in region per square kilometer land	0 0.001–0.04 0.050–0.18 0.19–0.020	0 (Zero) 2 (Low) 4 (Medium) 6 (High)	0.18 point sources per square kilometer land = rank of 4
Habitats				
Gravel-cobble intertidal	Area (km^2)	0 0.062–0.271 0.272–0.636 0.636–2.316	0 (Zero) 2 (Low) 4 (Medium) 6 (High)	2.316 km^2 = rank of 6
Sandy intertidal	Area (km^2)	0 0.001–0.852 0.853–1.894 1.895–8.914	0 (Zero) 2 (Low) 4 (Medium) 6 (High)	1.783 km^2 = rank of 4
Eelgrass	Area (km^2)	0 0.245–1.367 1.368–3.755 6.491–6.922	0 (Zero) 2 (Low) 4 (Medium) 6 (High)	6.493 km^2= rank of 6

Table 14.4 Example of Habitat Ranks for Subregions

Habitat		*Risk Region*					
		Point Roberts	*Drayton Harbor*	*Birch Bay*	*Cherry Point*	*Lummi Bay*	*Alden Bank*
Gravel-cobble intertidal	RRM rank (km^2)	4 (0.636)	6 (2.316)	2 (0.062)	4 (0.436)	4 (0.272)	0 0.000
Sandy intertidal	RRM rank (km^2)	2 (0.609)	4 (1.783)	4 (1.894)	2 (0.853)	6 (8.914)	0 0.000
Mudflats	RRM rank (km^2)	2 (0.009)	0 (0.000)	0 (0.000)	0 (0.000)	6 (0.347)	0 (0.000)
Eelgrass	RRM rank (km^2)	4 (2.368)	6 (6.491)	4 (3.755)	2 (0.245)	6 (6.922)	0 (0.000)

Table 14.5 Source Ranks for Subregions (Data Used to Make the Assigned Ranks Are in Parentheses)

Source		*Risk Region*					
		Point Roberts	*Drayton Harbor*	*Birch Bay*	*Cherry Point*	*Lummi Bay*	*Alden Bank*
Accidental spills	RRM rank (gallons/km^2)	2 (0)	6 (255.84)	2 (0.02)	4 (35.39)	6 (280.68)	0 (0)
Agricultural land use	RRM rank (% land use)	2 (1.39)	4 (42.02)	4 (35.44)	2 (16.94)	6 (50.41)	0 (0)
Ballast water	RRM rank (yes/no)	0 (no)	0 (no)	0 (no)	6 (yes)	0 (no)	0 (no)
Piers	RRM rank (no./km shoreline)	4 (0.07)	2 (0.03)	0 0.00	6 (0.24)	2 (0.03)	0 (0)
Point source pollution	RRM rank (no./km^2)	0 0.00	4 (0.18)	2 (0.04)	6 (0.20)	4 (0.16)	0 (0)
Recreational activities	RRM rank (no. ind./km shoreline)	0 (0)	2 (7.98)	6 (156.13)	4 (21.76)	2 (8.73)	0 (0)
Urban and industrial land use	RRM rank (% land use)	4 (31.17)	4 (28.30)	4 (29.04)	6 (41.34)	2 (21.00)	0 (0)
Vessel traffic	RRM rank (no. slips/km shoreline)	6 (51.03)	4 (29.88)	2 (11.64)	6 (0.081)	6 (5.694)	4 (0)

Exposure and Effects Filters

Exposure and effects filters of 0, 0.5, or 1 were assigned to reflect low, medium, or high probability of exposure or effects for each source-endpoint combination. These filters were based primarily on linkages described in the conceptual model.

Exposure filters received a score of 1 if the conceptual model pathway between source and habitat was complete, and a 0 if the pathway was not complete. A score of 1 was reduced to 0.5 if site-specific data indicated that the stressor occurred in small amounts, thus reducing the probability of exposure to endpoints.

Likewise, an effects filter received a score of 1 if the conceptual model pathway from habitat to endpoint was complete, and a score of 0 for an incomplete pathway. A score of 1 was reduced to 0.5 if site-specific data indicated the endpoint uses the habitat only marginally, reducing the probability of exposure, and therefore effects. If no site-specific data were available, the score was left as 1.

Effects filters were also assigned according to the conceptual model and site-specific data. Site-specific data were available for great blue heron, surf smelt embryos, and juvenile Dungeness crab.

6. *Calculate the risks.*

We combined source and habitat ranks with exposure and effects filters to determine the relative risk estimates. Risk estimates were derived by first multiplying the source and habitat ranks by the exposure and effects filters for each subregion. The sum of the products of each source-habitat-filter combination determined the final estimate of risk. These risk estimates were compared among subregions, sources, habitats, and endpoints to reveal:

1. The subregions where most risk occurs
2. The sources contributing the most risk
3. The habitats where most risk occurs
4. The endpoints most at risk in the Cherry Point area

The calculations (Table 14.6) revealed (1) the subregions and (2) habitats where most of the risk occurs, (3) which sources contribute the most risk, and (4) the endpoints most likely to be affected by anthropogenic stressors in the Cherry Point region. The risk predictions resulting from this assessment are estimates about the relative risk to endpoints in the region. These patterns of risk form hypotheses that can be tested by field-based observations. It is often useful to present the results in both tables and graphs. The next discussion uses maps and bar graphs to present the results of the risk assessment.

The RRM predicted the highest risk in Lummi Bay and Drayton Harbor, medium risk in Cherry Point, Birch Bay, and Point Roberts, and low risk in Alden Bank (Figure 14.16a). Habitats where most of the risk occurs are eelgrass, sandy intertidal, and macroalgae. The major contributors of risk in the region are commercial and recreational vessel traffic, upland urban and agricultural land use, and shoreline recreational activities (Figures 14.17 and 14.18). The biological endpoints most likely to be at risk are great blue heron and juvenile Dungeness crab.

Vessel traffic was identified as a major contributor of risk in Point Roberts, Drayton Harbor, Lummi Bay, and Alden Bank subregions. Urban land use was important in Drayton Harbor and Cherry Point. The model predicted agricultural land use contributed much of the risk in Drayton Harbor and Lummi Bay. Recreational activities were important in Birch Bay. Ballast water was the most important source for Cherry Point. All other sources ranked low.

7. *Evaluate uncertainty and sensitivity analysis of the relative rankings.*

Uncertainty analysis differed from previous RRM assessments with the addition of an alternative habitat-ranking scheme to analyze the effects of model uncertainty and Monte Carlo techniques to quantitatively describe parameter uncertainty in risk predictions. The risk predictions produced in the RRM are point estimates based on ranks and filters derived from imperfect data. To communicate the uncertainty associated with these point estimates, Monte Carlo

Table 14.6 Risk Scores for the Risk Regions by Habitat, Source, and Endpoint (the Shaded Areas Bring Attention to the Areas and Endpoints at Highest Risk)

	Risk Region						
	Point Roberts	*Drayton Harbor*	*Birch Bay*	*Cherry Point*	*Lummi Bay*	*Alden Bank*	*Total*
Habitat							
Gravel-cobble intertidal	192	300	168	528	220	0	1,408
Sandy intertidal	125	440	420	400	612	0	1,997
Mudflats	125	0	0	0	756	0	881
Eelgrass	282	744	376	212	696	0	2,310
Macroalgae	282	248	564	424	232	0	1,750
Soft-bottom subtidal	279	368	124	246	172	180	1,369
Water column	96	36	128	128	64	48	500
Stream	0	504	304	144	480	0	1,432
Wetland	40	240	192	88	152	0	712
Forest	28	192	96	36	180	0	532
Total	1,449	3,072	2,372	2,206	3,564	228	**12,891**
Source							
Accidental spills	35	348	54	80	318	0	835
Agricultural land use	157	660	484	126	840	0	2,267
Ballast water	0	0	0	444	0	0	444
Piers	282	198	0	276	264	0	1,020
Point source pollution	0	232	54	120	300	0	706
Recreational activities	0	362	984	368	454	0	2,168
Urban land use	330	756	556	414	428	0	2,484
Vessel traffic	645	516	240	378	960	228	2,967
Total	1,449	3,072	2,372	2,206	3,564	228	**12,891**
Assessment Endpoint							
Coho salmon	112	396	272	336	360	48	1,524
Juvenile Dungeness crab	331	856	672	480	796	36	3,171
Juvenile English sole	236	184	62	164	392	72	1,110
Great blue heron	494	1180	910	490	1420	72	4,566
Littleneck clam	114	296	196	336	376	0	1,318
Surf smelt embryos	162	160	260	400	220	0	1,202
Total	1,449	3,072	2,372	2,206	3,564	228	**12,891**

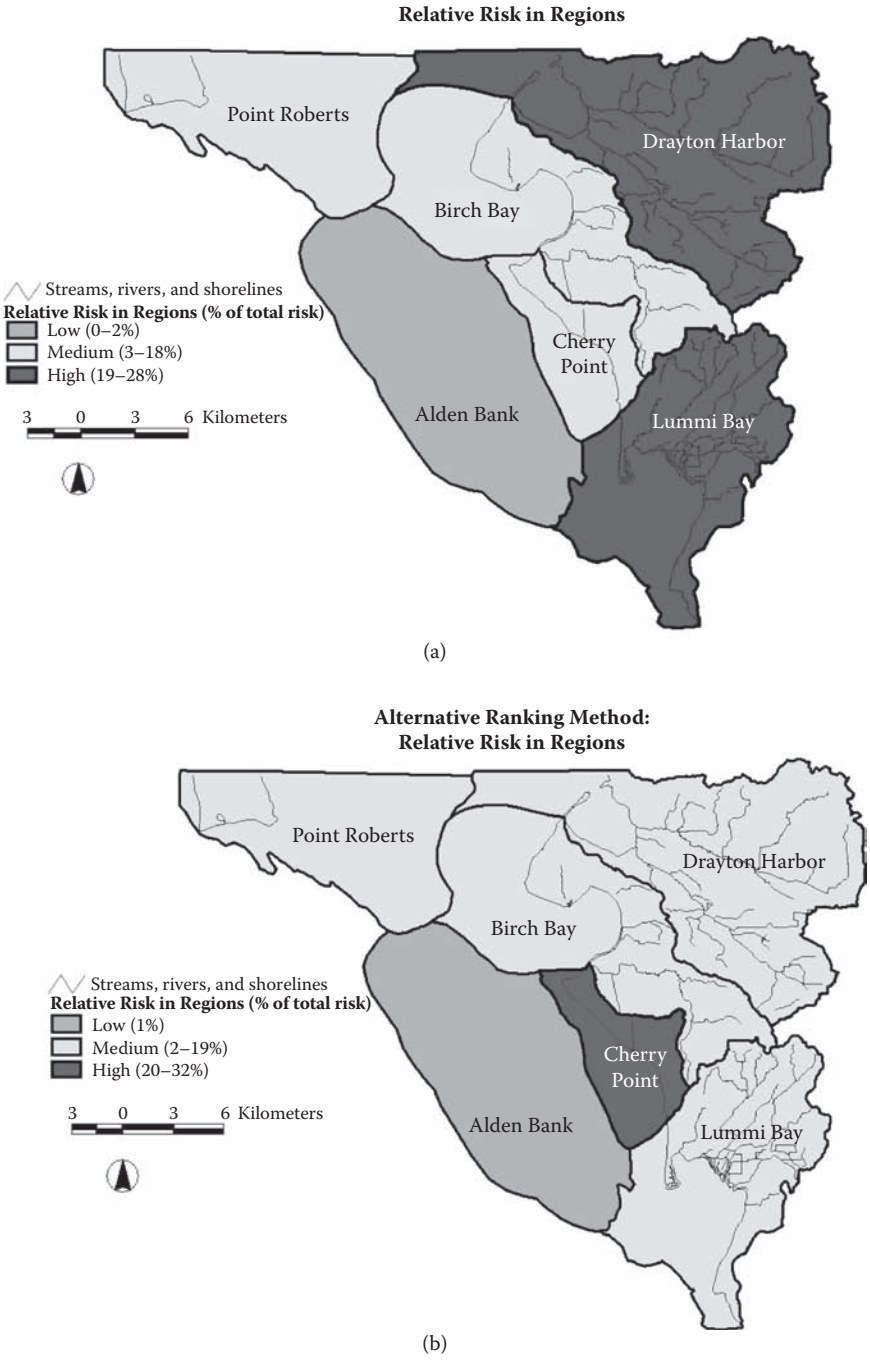

Figure 14.16 Comparisons of the relative risks depending upon assumptions about the sensitivity of the habitat versus area. (a) The distribution of relative risk under the usual assumptions. (b) The distribution when smaller habitat size corresponds to a higher risk probability. (See color insert.)

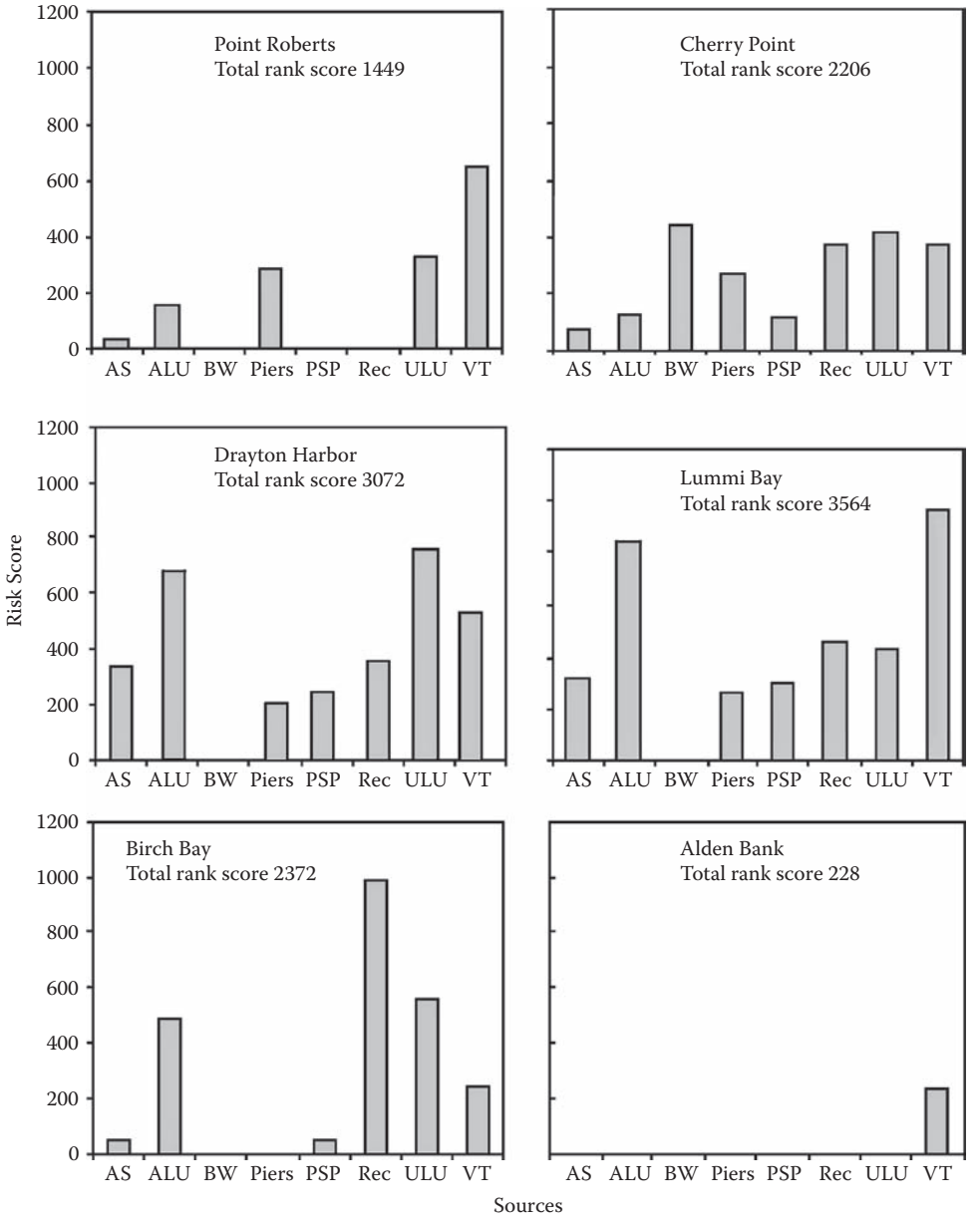

Figure 14.17 Relative contribution to risk from sources in subregions. Y axis is the relative risk score. X axis from left to right: AS = accidental spills, ALU = agricultural land use, BW = ballast water, Piers = piers, PSP = point source pollution, Rec = recreational activities, ULU = urban land use, and VT = vessel traffic.

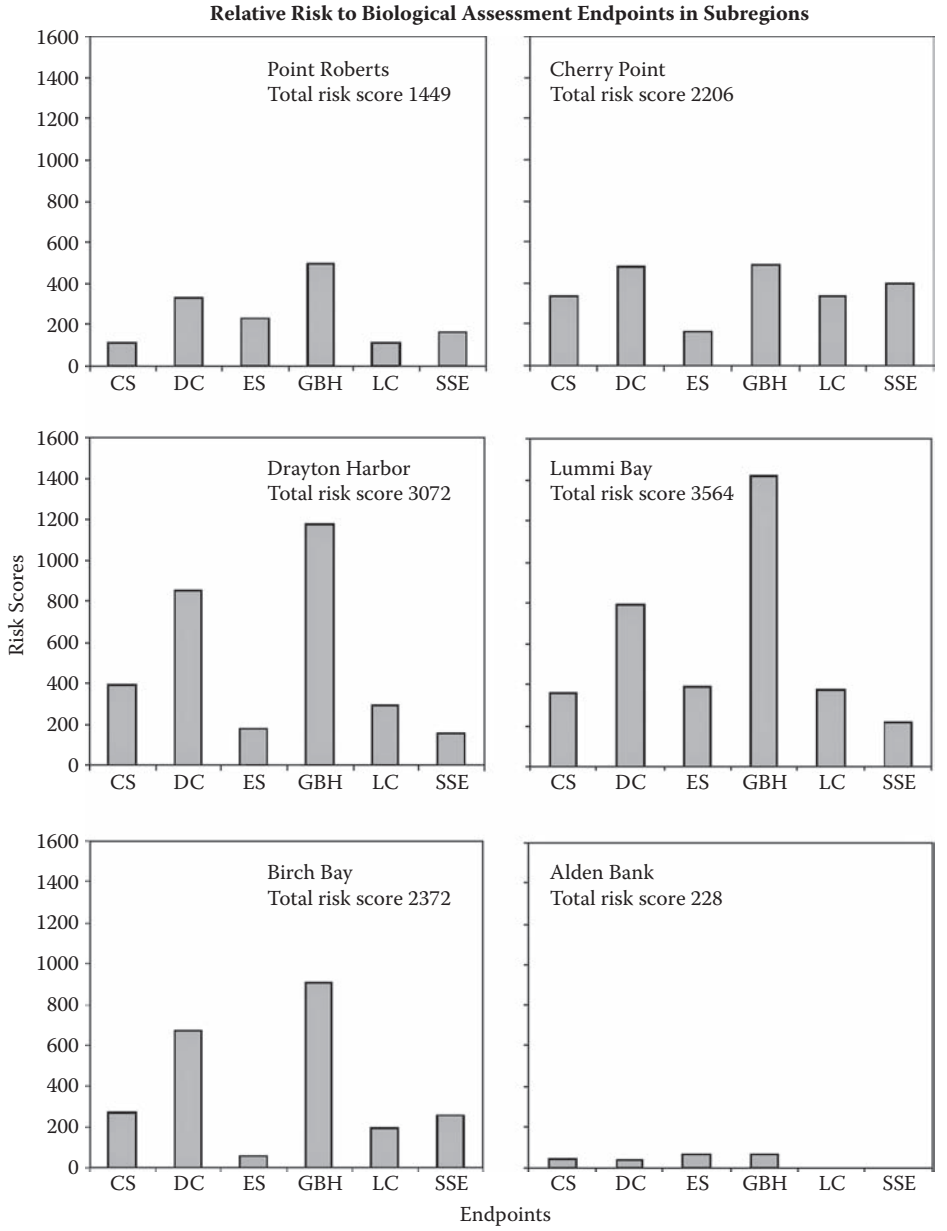

Figure 14.18 Relative risk to biological assessment endpoints in subregions. Y axis is the relative risk score. X axis from left to right: CS = Coho salmon, DC = juvenile Dungeness crab, ES = juvenile English sole, GBH = great blue heron, LC = native littleneck clam, SSE = surf smelt embryos.

analysis was used to generate distributions of probable predictions for each risk component. In addition to using Monte Carlo analysis to describe parameter uncertainty in the assessment, we also applied an alternative habitat-ranking scheme to the RRM to investigate uncertainty in the model and the effects of habitat-ranking assumptions on the risk estimates.

Monte Carlo Analysis

The first phase of uncertainty analysis applied Monte Carlo techniques to analyze parameter uncertainty in the risk predictions. In risk assessment, Monte Carlo uncertainty analysis combines assigned probability distributions of input variables to estimate a probability distribution for output variables. In the case of the Cherry Point regional risk assessment, the input variables are the ranks and filters with medium or high uncertainty, and the output variables are the risk estimates.

For the Monte Carlo uncertainty analysis, we first assigned designations of low, medium, or high uncertainty to each source and habitat rank and exposure and effects filter based on data quality and availability. We assigned discrete probability distributions to ranks and filters with medium and high uncertainty according to the criteria in Table 14.7. We did not assign distributions to ranks and filters with low uncertainty but left them simply as the original point estimate.

We assigned high uncertainty to accidental spills ranks for all subregions because the Ecology (2001) spills data set was incomplete and lacked the spill volume for many of the records, and locations of several records in the database were undeterminable. This poor data quality resulted in high uncertainty in the accidental spills ranks.

The recreational activities rank for Point Roberts was assigned high uncertainty because the WDFW data set (2001a) on which ranks were based did not survey the Point Roberts region. We assigned gravel-cobble, sandy intertidal, and mudflats habitat ranks for the Point Roberts subregion medium uncertainty because no habitat area data were available for this region.

Eelgrass and macroalgae habitat ranks for the Alden Bank subregion were assigned high uncertainty because no vegetation data were available this far offshore. These habitat ranks received ranks of 0; however, because the sea floor depth becomes quite shallow again at Alden Bank (NOS 2001), some vegetation is most likely present (the amount is undetermined).

We assigned medium uncertainty to subtidal soft-bottom habitat ranks for all subregions because subtidal substrate data were unavailable for the entire study area. We derived areas for this habitat on which ranks were based using GIS analysis and bathymetry data (NOS 2001), assuming the majority of subtidal substrate to be soft bottom (vs. vegetation or rocky substrate). While this assumption may overestimate the amount of soft substrate on the sea floor bottom, it overestimates this habitat in all subregions evenly. While the area values are not precise, the final ranks most likely represent the relative amount of this habitat type in each subregion, and are therefore appropriately assigned, albeit with a degree of uncertainty.

Inconsistent land use data quality resulted in medium uncertainty in the Lummi Bay forest habitat rank. Because a large portion of the Lummi Bay subregion falls within the boundaries of the Lummi Nation Indian reservation, the Whatcom County assessor's tax parcel data set did not accurately cover these areas. Instead, land use areas in this subregion were assigned according to another data set developed by the Whatcom County Public Utility District. Inconsistency of the data sets warranted assigning medium uncertainty to the forest habitat rank in the Lummi Bay subregion.

Vessel traffic ranks for Lummi Bay, Cherry Point, and Alden Bank were assigned medium uncertainty because the data set on which we based ranks for the other subregions (number of slips per kilometer of shoreline) did not accurately portray the amount of vessel traffic occurring as a result of the industrial piers and ferry terminal in these subregions. Taking this into account, but lacking a data set that characterized both recreational and commercial vessel traffic, we instead assigned ranks of the next higher category in these subregions.

All other ranks were assigned low uncertainty. Filter similarly received designations of high, medium, or low uncertainty. A lack of understanding of the fate and transport of stressors, deficient site-specific information about the locations and amounts of stressors, and variance in the quantity of a stressor that sources may release were all grounds for assigning medium and high uncertainties to filters.

Table 14.7 Uncertainty Analysis Monte Carlo Input Distributions

Assigned Rank Value	*Uncertainty*	*Assigned Probability (%) for Ranks*			
		0	*2*	*4*	*6*
Ranks					
0	High	60	20	20	0
0	Medium	80	10	10	0
2	High	0	60	20	20
2	Medium	0	80	10	10
4	High	0	20	60	20
4	Medium	0	10	80	10
6	High	0	20	20	60
6	Medium	0	10	10	80
		Assigned Probability (%) for Ranks			
		0	*0.5*	*1*	
Filters					
0	High	60	20	20	
0	Medium	80	10	10	
0.5	High	0	60	40	
0.5	Medium	0	80	20	
1	High	0	40	60	
1	Medium	0	20	80	

The Monte Carlo simulations were run for 1,000 iterations and output distributions for each subregion, source, habitat, and endpoint risk prediction were derived. These distributions show a range of probable risk estimates associated with each point estimate.

During the second phase of the uncertainty analysis, we applied an alternative habitat-ranking method to investigate the effects of the underlying assumptions of the habitat-ranking scheme on the final risk estimates. The original RRM method assumes that a large amount of habitat in a subregion increases the probability that an organism utilizing that habitat will come into contact with a stressor, thus increasing the probability of exposure, and therefore risk. Accordingly, subregions with a larger amount of habitat receive a high rank, signifying a high probability of impact to endpoints. This ranking method can become difficult when analyzing risk at a population, rather than an individual or organism, scale because the effects of stressors differ between individuals and populations.

The alternative method for ranking habitat in subregions has an entirely different set of assumptions. This second method assigns high ranks to subregions with a small amount of habitat and assumes:

1. A small habitat size supports a small population of organisms. This small population would theoretically be more susceptible to the effects of stressors in the environment and is at greater risk of becoming extinct than a larger, more resilient population.
2. Stressor concentration is greater in small habitats, thus increasing the likelihood of both exposure and effects.

We investigated the uncertainty associated with these assumptions by applying the alternative habitat-ranking scheme to the RRM and compared these results with the original RRM risk predictions. We also performed Monte Carlo uncertainty analysis on the alternative habitat-ranking results. Although the scores were not affected greatly, the relative rankings of the areas changed. The Cherry Point region is now at high risk, and the rest of the study area is now at medium risk, except for Alden Bank (Figure 14.16b).

8. *Generate testable hypotheses for future field and laboratory investigation to reduce uncertainties and to confirm the risk rankings.*

The distribution of risks and the contribution of the various sources and stressors constitute a set of hypotheses. Risk calculations (Table 14.6) revealed (1) the subregions and (2) habitats where most of the risk occurs, (3) which sources contribute the most risk, and (4) the endpoints most likely to be affected by anthropogenic stressors in the Cherry Point region. The risk predictions resulting from this assessment are estimates about the relative risk to endpoints in the region. These patterns of risk form hypotheses that can be tested.

The RRM predicted the highest risk in Lummi Bay and Drayton Harbor, medium risk in Cherry Point, Birch Bay, and Point Roberts, and low risk in Alden Bank (Figure 14.16a). Habitats where most of the risk occurs are eelgrass, sandy intertidal, and macroalgae (Table 14.6). The major contributors of risk in the region are commercial and recreational vessel traffic, upland urban and agricultural land use, and shoreline recreational activities. The biological endpoints most likely to be at risk are great blue heron and juvenile Dungeness crab (Table 14.6).

Vessel traffic was identified as a major contributor of risk in Point Roberts, Drayton Harbor, Lummi Bay, and Alden Bank subregions. Urban land use was important in Drayton Harbor and Cherry Point. The model predicted that agricultural land use contributed much of the risk in Drayton Harbor and Lummi Bay. Recreational activities were important in Birch Bay. Ballast water was the most important source for Cherry Point. All other sources ranked comparatively low.

Results of the Uncertainty Analysis

The model developed for this risk assessment was based on a combination of site-specific data and general knowledge about interconnections between risk components. Uncertainty in the assessment arose from both flaws in input data and imperfections in the model, and includes a lack of site-specific data in some or all subregions within the study area, poor data quality, misunderstanding of the fate and transport of stressors in the Cherry Point environment, omission of contributing sources and stressors, a failure to identify and incorporate temporal and spatial patterns, and incorrect assumptions in the model.

To quantify the effects of parameter uncertainty on the risk predictions, Monte Carlo analysis was applied to the RRM to derive probability distributions of possible risk estimates. The Monte Carlo analysis resulted in probability distributions of risk predictions for each subregion, habitat, source, and assessment endpoint (Figure 14.19).

During the second component of uncertainty analysis, we applied an alternative habitat-ranking scheme to investigate the assumptions about habitat use by biological assessment endpoints and how those assumptions affect the predicted risk values.

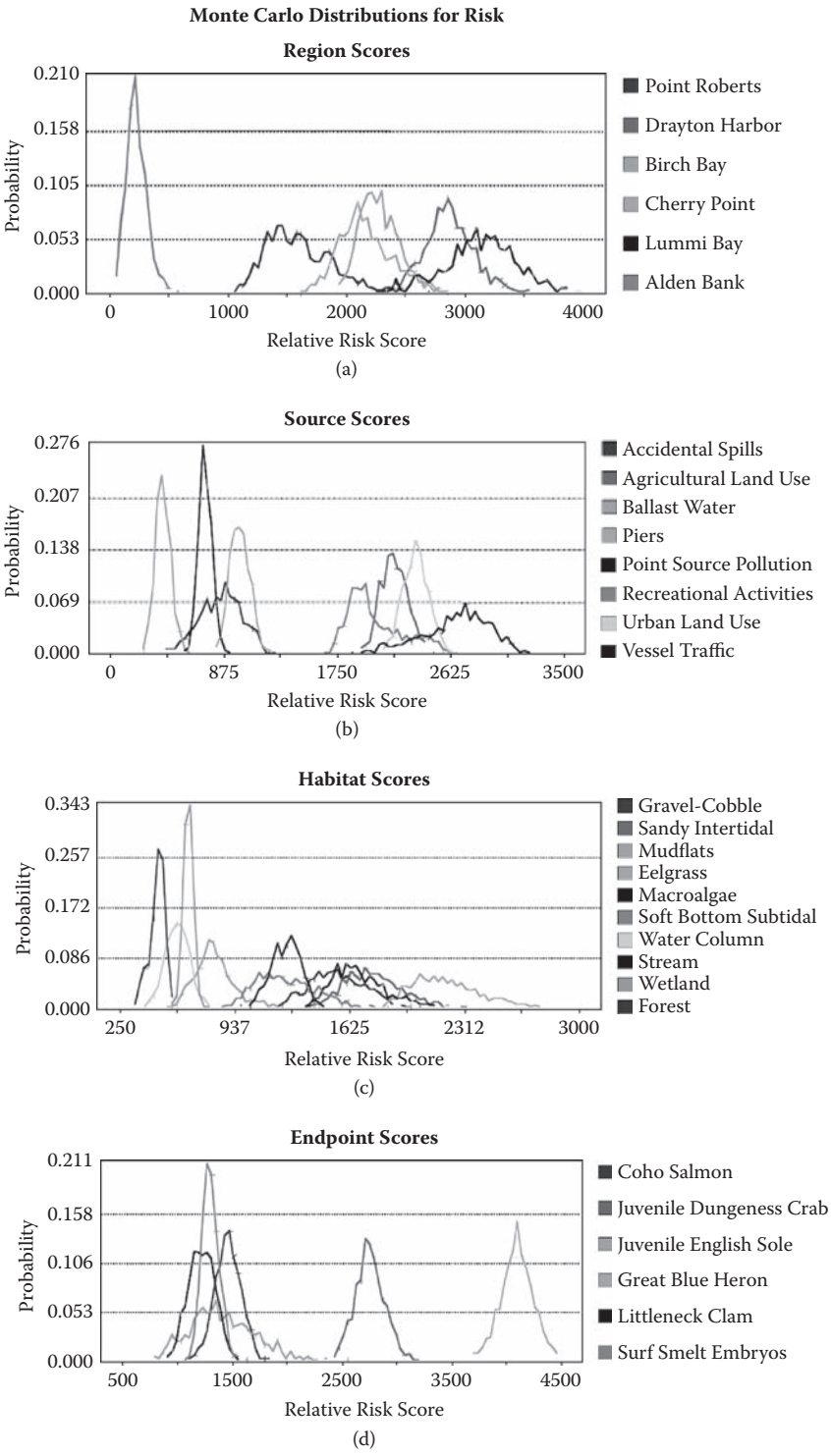

Figure 14.19 Monte Carlo results of the RRM calculation. (See color insert.)

The Monte Carlo analysis (Figure 14.19) produced distributions with means close to the predicted risk values for most risk predictions, suggesting low uncertainty in RRM predictions for most risk components. The Alden Bank Monte Carlo distribution was the narrowest of all the distributions for subregions, suggesting the most confidence in the risk prediction. Point Roberts, Lummi Bay, and Cherry Point had the widest distributions, indicating more relative uncertainty in these predictions. Point Roberts and Alden Bank distributions were right skewed, suggesting the possibility that, despite the mean and risk prediction being similar, the models may have underestimated risk in these subregions. The Lummi Bay distribution was left skewed.

For source contribution to risk, the RRM prediction for vessel traffic was on the upper end of the Monte Carlo distribution of risk values. The distribution was also left skewed. The distribution for recreational activities was right skewed, suggesting a possible underestimation of risk from this source. The ranges of the distributions for agricultural land use, ballast water, piers, point sources of pollution, and urban land use were relatively narrow, demonstrating high confidence in their risk predictions. Relatively, the ranges of the Monte Carlo probability distributions for accidental spills, recreational activities, and vessel traffic were wider, demonstrating less confidence in their predictions. These results are consistent with the relatively poor data quality and higher uncertainty in the initial ranks for accidental spills, recreational activities, and vessel traffic.

Monte Carlo distributions for habitats also revealed components with high and low uncertainty. The ranges of the distributions for mudflats, streams, and wetlands were narrower than those for the other habitats, suggesting higher confidence in habitat risk predictions for these three habitats than for the remaining seven. Distributions for mudflats and streams were left skewed, however, suggesting a possible overestimation of risk. Eelgrass, macroalgae, and soft-bottom subtidal distributions were right skewed, indicating the model may have underestimated risk in these habitats.

The right-skewed probability distributions for biological endpoints indicated an overestimation of risk in the predictions for juvenile Dungeness crab and surf smelt embryos, as evidenced by their right-skewed probability distributions. All other biological assessment endpoints had approximately normal distributions and low uncertainty.

Alternative Habitat-Ranking Scheme

One of the unknowns in risk assessment is the relationship between habitat size and risk. However, the alternative habitat-ranking scheme did not change risk predictions for assessment endpoints, habitats, and sources, suggesting the RRM is fairly robust to changes in habitat ranks. However, the spatial distribution of relative risk changed when the alternative habitat-ranking scheme was applied. Risk in Lummi Bay and Drayton Harbor moved from high risk in the original assessment to medium risk using the alternative ranking scheme. Cherry Point region moved from its original medium risk to high risk (Figure 14.6b). The Monte Carlo uncertainty results from the alternative habitat-ranking scheme calculations were similar to the original Cherry Point RRM Monte Carlo results.

9. *Test the hypotheses listed in step 8.*

To date there has not been an effort to independently measure the risks to endpoints at Cherry Point. Several types of studies would be helpful in confirming the risk results:

1. Studies confirming the habitat types, land use, and other information contained in the GIS data files. It has been noted by our research team that some of the areas identified as mudflats are in fact dark sands. Land use can change rapidly. Areas marked as agriculture can range from intensive use of fertilizer and the growth of crops to low-intensity management and perhaps an occasional cut of hay.
2. Studies confirming the location of endpoints within the region. In some cases the endpoints are known to exist but specific information on the duration of habitat use can be limited.

On the other hand, the nests for the great blue heron have been located and the aquatic and terrestrial range of the adults has been documented.

3. Studies examining the changes in the status of the endpoints, habitats, and stressors entering the Cherry Point region. The areas at highest risk would be expected to see the effects upon the appropriate endpoints. The dynamics of the rates of habitat change, land use, amount of stressor input, and endpoints are generally unknown. At the Cherry Point region the Pacific herring is the only marine species that is regularly monitored, and the great blue heron is closely followed.

10. *Communicate the results in a fashion that portrays the relative risks and uncertainty in a response to the management goals.*

The outcome of this risk assessment was presented to the Cherry Point Technical Working Group, the Washington Department of Natural Resources, and in the peer-reviewed literature. This example is a summary of those reports.

In 2007 WDNR initiated a collaborative process to aid in the development of a management plan for the Cherry Point Marine Reserve and the surrounding area. The stakeholder group included the resource management agencies, industries, nongovernmental agencies, and the tribes. We have participated in that process, making available the research described above, along with work on the risks to the Cherry Point Pacific herring and refinements to the original risk assessment described above. The group also incorporated numerous other sources of information into the process.

The draft management plan and process has been extensively documented (http://www.dnr.wa.gov/ResearchScience/Topics/AquaticHabitats/Pages/aqr_rsve_cherry_point.aspx, accessed November 28, 2009). Many of the endpoints selected for the original Cherry Point RRM are now included in the management plan. There are also plans to monitor the endpoints along with the potential sources and stressors within the area. New additions also added to the document are invasive species and climate change, topics once considered outside the domain of risk assessment.

The risk assessment for Cherry Point was the first study to employ Monte Carlo techniques to describe the uncertainty in a relative risk type of assessment. One of the outcomes of the assessment was also the inclusion of numerous endpoints and stressors that formed some of the basis of the current management plan.

An unforeseen outcome of the risk assessment was that the extensive work done in order to perform the risk assessment has created a risk assessment laboratory. The development of the relative risk model for invasive species was aided by having the Cherry Point data in hand. Colnar and Landis (2007) published a risk assessment scheme for invasive species using the European green crab at Cherry Point as the example.

14.6.9 Life Cycle Assessment

Life cycle assessment (LCA) is an additional assessment approach for making environmental decisions. LCA can be defined as an inventory of all the steps in the development, manufacture, use, and disposal of a product or a commodity with a determination of the environmental consequences (Todd and Curran 1999). The purpose of a LCA is to provide information to a decision maker so that choices can be made in the design of a manufacturing process to minimize environmental impacts or risks.

The basic components of the LCA process are illustrated in Figure 14.20. In the manufacturing process there are segments that are upstream of the final process. These upstream segments can include the manufacture of subcomponents, packaging materials, solvents and paints to be used in final assembly, pallets for transportation, and so forth. The downstream aspects are likely to include transportation of the product, use of the product, and eventually disposal. The inputs are materials or substances that are incorporated into that step. If one of the upstream segments

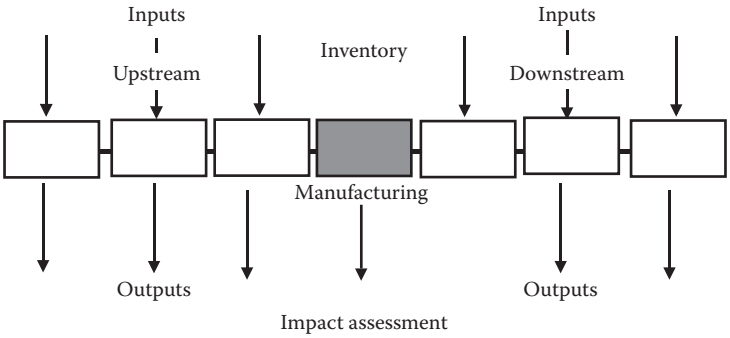

Figure 14.20 The manufacturing process and LCA.

is the manufacture of a plastic, then oil or coal would be a likely input. If a car is manufactured, then one of its downstream inputs to being driven would be gasoline, tires, etc. The outputs are the materials released from each part of the process. In the case of plastics production, it would be the effluents, air emissions, and waste materials from the process. The outputs from operating a car would be the air emissions, disposal of used oil and lubricants, disposal of tires, and other consumable parts.

Keeping track of the manufacturing process, the inputs and outputs comprise the inventory aspect of the LCA process. The inventory should include each aspect of the process, the amounts, and final disposition, and the eventual use and disposal of the manufactured material.

The impact segment of the LCA process is an attempt to understand the potential effects that each segment will have upon the environment. The impacts should include not just toxicology, but physical alterations of habitat, water use, land use, and other factors. Factors that can be taken into account during the impact analysis can also include recycling compatibility, energy use, and product reuse.

The LCA process begins with a phase similar to ecological risk assessment by defining the goals and scope of the assessment. The initial steps are (Todd and Curran 1999):

1. Develop a detailed understanding of the decisions to be made.
2. Design and direct the study based on the organization's principles.
3. Discern how LCA can assist these decisions, i.e., the degree to which both inventory and impact assessment can provide the information needed.
4. Tailor the LCA study to these decisions.
5. Undertake the process of making value decisions and information limitations explicit to the study users and audiences.

Todd and Curran (1999) also list a variety of reasons for conducting an LCA. These reasons can be broken down into three sectors: government, manufacture, and consumer.

A government agency would choose to perform an LCA to evaluate:

- The environmental performance to make a purchasing decision
- Environmental regulations to ensure that management goals are being met
- Policies in regards to sustainability

A manufacturer would choose an LCA process to:

- Determine the environmental impact of a product
- Acquire an overview of a manufacturing process to identify significant impacts
- Evaluate the impacts due to a change in process, and to compare source or supply alternatives

A consumer or consumer group would choose an LCA to:

- Compare total environmental impacts of products or activities to guide purchase decisions
- Assess the effects of lifestyle changes on the environment
- Evaluate public and corporate policies as to supporting sustainability to guide purchasing and voting selections

A detailed step-by-step analysis of the process is beyond the scope of this chapter but can be found in Barnthouse et al. (1997).

Similar to risk assessment, LCA is deeply involved in the decision-making process. Impact assessment will likely be replaced in the LCA process by a probabilistic risk assessment, and tools will be developed.

Study Questions

1. What are ecological risk assessment, stressor, hazard, and exposure?
2. Define *problem formulation*, *hazard assessment*, *exposure assessment*, and *risk characterization*, as in Figure 14.1.
3. Which aspect of the ecological risk assessment framework defines the question under consideration? What are subunits to this formulation?
4. Stresses can be of what three categories? What five characteristics can stressors have that are derived in part from use patterns?
5. What are some interactions between the stressor and the ecological system?
6. What is an endpoint? An assessment endpoint? A measurement endpoint?
7. How can the variance-to-mean relationship classify the type of sampling distribution?
8. What scenario is the most relevant and diagnostic?
9. What factors make risk assessment a scientific process?
10. What two components have been incorporated into the analysis component in the new framework for ecological risk assessment (as opposed to their separation in traditional risk assessment)?
11. What is the goal of the exposure analysis?
12. What are several ways to determine exposure?
13. What is the most critical aspect of the risk assessment process?
14. What are the criteria used to judge the importance of data when characterizing ecological effects?
15. Describe the stressor-response profile.
16. Describe the eight EPA framework-listed relationships between assessment and measurement endpoints.
17. What is one of the difficulties in evaluating the stressor-response relationship?

18. Describe risk characterization.
19. What is the quotient method of estimating risk? Discuss a difficulty with this analysis.
20. Discuss possible erroneous conclusions that may be drawn if secondary results are deduced or extrapolated from multispecies tests and field studies.
21. List the three general aspects of the analysis for the ecological risk summary and describe each.
22. What is a good question to be examined concerning the interpretation of ecological significance?
23. List the factors of crucial importance in the report to the risk manager.
24. Describe alternate methods to the simple quotient method for evaluating the spatial component of risk.
25. What are the problems particular to the performance of an ecological risk assessment for a large geographical area?
26. Why is a ranking method used when there are several distinct types of stressors, environments, and receptors in an environment?
27. How were the steps of the RRM applied to the Cherry Point region?
28. How are the connections between sources, stressors, habitats, and impacts portrayed?
29. How did the Monte Carlo analysis contribute to the understanding of the uncertainty in the Cherry Point risk analysis?
30. How can the predictions of the Cherry Point risk assessment be tested?
31. What are the general principles for life cycle assessment?

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Appendix A: References for Toxicity Testing and Interpretation

Compiled by April J. Markiewicz

This appendix is a source of methods and guidance to be used in environmental toxicology and risk assessment. Methods are periodically updated and the latest version should be used. Many of the methods are now available online from ASTM, U.S. EPA, and other sources. The list was compiled in October 2009.

Abbreviations

APHA	American Public Health Association
ASTM	American Society for Testing and Materials International
AWWA	American Water Works Association
BCME	British Columbia Ministry of Environment
BCMELP	British Columbia Ministry of Environment, Lands and Parks
BCMWLAP	British Columbia Ministry of Water, Land and Air Protection
OECD	Organization for Economic Cooperation and Development
U.S. EPA	U.S. Environmental Protection Agency
WEF	Water Environment Federation

General Information

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