

I n t r o d u c t i o n t o
ENVIRONMENTAL
TOXICOLOGY

Impacts of
Chemicals Upon
Ecological Systems

SECOND EDITION

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Wayne G. Landis
Ming-Ho Yu



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Preface

We have prepared this text because we had no suitable text for teaching courses introducing environmental toxicology and biochemistry. Portions of this book have already been used to teach an introduction to environmental toxicology and biochemical toxicology courses at Western Washington University and changes suggested by these students have been incorporated. In general these students have backgrounds in organic chemistry, ecology, calculus, and often biochemistry. We appreciate any feedback and these suggestions will be incorporated into, hopefully, further editions.

One of the major difficulties in preparing this book has been the rate of change seen in the field. The U.S. Environmental Protection Agency prepared a new Framework for Ecological Risk Assessment, nonlinear dynamics has become a major part of ecological theory, and new methods of examining effects at the level of community and ecosystem have been developed during the writing of this book.

Now it is two years later and we have made major revisions to this edition in order to keep pace with the field of environmental toxicology. Ecological risk assessment has become the operating paradigm and estrogen disruption has taken on a new importance. The field is more sophisticated in the data analysis tools that it uses and multivariate approaches are becoming more common in the literature. Perhaps the most recent development is the awareness that effects and risks must be seen on a regional scale. Multiple natural and anthropogenic stressors occur to a variety of connected habitats. In order to understand the patterns in the environment that result from the introduction of chemicals, we must take a large scale approach. It will be interesting to see what the next several years bring.

The Authors



Wayne G. Landis is the Director of The Institute of Environmental Toxicology and Chemistry of Huxley College, Western Washington University. He received his undergraduate degree from Wake Forest University and his M.A. and Ph.D. in Zoology from Indiana University. With a background in protozoan genetics and ecology, his research has since concentrated on environmental toxicology. In the past several years, he has published over 80 papers and received two patents on microbial degradation. In 1991 he chaired the annual Environ-

mental Toxicology and Risk Assessment Symposium sponsored by the American Society for Testing and Materials held in Atlantic City and served as Organizational Chair for the Annual Meeting of the Society for Environmental Toxicology and Chemistry held in Seattle. During 1992 he served as President of the Pacific Northwest Chapter of the Society for Environmental Toxicology and Chemistry (PNWSETAC). Dr. Landis also has served on the editorial boards of several journals. Since 1989 he has edited three books on aquatic toxicology and risk assessment published by the American Society for Testing and Materials.

Dr. Landis teaches courses in environmental and aquatic toxicology, environmental risk assessment, and population biology. His current research includes developing new methods of evaluating environmental toxicity using birds and marine organisms, establishing interspecies structure activity models, evaluation multispecies toxicity tests, and the description of how ecosystems respond to stressors.

Perhaps the most intriguing avenue of research has been the implementation of chaos and complexity theory to describe the dynamics of ecological systems after toxicant stress. This research has cast a great deal of doubt as to the existence of ecosystem recovery of stability in regards to the dynamics after a stressor event.



Ming-Ho Yu received his B.S. degree in Agricultural Chemistry from National Taiwan University and his M.S. and Ph.D. degrees in Plant Nutrition and Biochemistry from Utah State University. He did his postdoctoral work at the University of Alberta in Edmonton, Canada and Utah State University. Dr. Yu was associated with Huxley College of Environmental Studies, Western Washington University for 27 years, where he taught environmental toxicology and related courses until his retirement in 1997. He spent a year of sabbatical

leave as Visiting Professor at the Department of Public Health and Hygiene, Iwate Medical University in Morioka, Japan. He also spent a summer at the Institute of Whole Body Metabolism in Nauchi, Japan, where he conducted research as a visiting research scientist.

Dr. Yu is a member of the American Association for the Advancement of Science, American Chemical Society, American Society for Nutritional Sciences, International Society for Fluoride Research, New York Academy of Sciences, and the Society of Environmental Toxicology and Chemistry. He is the past president of the International Society for Fluoride Research, and is co-editor of *Environmental Sciences*, an international journal on environmental physiology and toxicology published in Tokyo, Japan.

Acknowledgment

A major part of this book was written based on the notes and other course materials I used in teaching environmental toxicology-related courses at Western Washington University over the past 20 plus years. I want to thank my former students who took those classes from me. Many of them made critical comments on the course materials I used, and their comments inspired me greatly. Special appreciation is due to my wife, Ervena, for her moral support during the course of preparing the manuscript.

M.H.Y.

The students of my environmental toxicology courses during the past eight years at Western Washington University have suffered through the notes, figures, and the first edition, and I thank them for participating in this undertaking. Traci Litwiller compiled the methods summaries, April Markiewicz generated the appendix of methods references, and Lisa Holmquist was instrumental in the editing of the first edition. Kyra Freestar was a great help in the preparation of the second edition. Ruth Noellgen let me modify several of the figures from her thesis for this text. My students and colleagues that have used the first edition have contributed numerous suggestions and we have tried to incorporate them into this edition. Linda S. Landis prepared the study questions and provided her unrelenting support of the project in spite of the evenings alone raising two delightful daughters who place this effort in perspective, Margaret and Eva.

W.G.L.

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APPENDIX A

References for Toxicity Testing and Interpretation

ABBREVIATIONS

ASTM — American Society for Testing and Materials

APHA — American Public Health Association

BCEPD — British Columbia Ministry of the Environment Public Document

USEPA — United States Environmental Protection Agency

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- Appendix F Wilcoxon rank sum test. pp. 225-230
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APPENDIX B

INTRODUCTION

We have decided to include the latest version of the U.S. Environmental Protection Agency's Ecological Risk Assessment Framework. This document is fast becoming the standard conceptual model for ecological risk assessment and the standardized glossary. In order to understand the current literature on ecological risk assessment it is critical to have a working knowledge of the document. In its original form the document has a purple cover, hence it may be referred to as the purple document in transcripts of discussions.

The importance of the document is that it does serve as a framework to organize future research and methods for ecological risk assessment. As with any evolving document, some of the ideas regarding ecosystem resilience and stability mentioned in the framework are dated. However, the inclusion of verification and validation into a risk assessment framework are certainly forward looking. We strongly urge the reader to carefully read this document and to consider the material contained in the preceding chapters. Note inconsistencies and be critical as soon the reader will likely be implementing the framework in a real world situation or be developing improvements.

U.S. EPA Document “A Framework for Ecological Risk Assessment”

*EPA/630/R-92/001, February 1992.
Risk Assessment Forum,
U.S. Environmental Protection Agency,
Washington, D.C. 20460*

DISCLAIMER

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This U.S. Environmental Protection Agency (EPA) report has been developed under the auspices of EPA’s Risk Assessment Forum, a standing committee of EPA scientists charged with developing risk assessment guidance for Agency-wide use. An interoffice work group chaired by Susan Norton (Office of Health and Environmental Assessment), Donald Rodier (Office of Toxic Substances), and Suzanne Marcy (Office of Water) led this effort. Other members of the work group are Michael Brody, David Mauriello, Anne Sergeant, and Molly Whitworth. William van der Schalie and William Wood of the Risk Assessment Forum staff coordinated the project, which included peer review by scientists from EPA, other federal agencies, and the private sector.

FOREWORD

Publication of this report, “Framework for Ecological Risk Assessment” (Framework Report), is a first step in a long-term program to develop risk assessment guidelines for ecological effects. EPA has been developing risk assessment guidelines primarily for human health effects for several years. In 1986, EPA issued five

such guidelines, including cancer, developmental toxicity, and exposure assessment (51 *Federal Register* 33992–34054, 24 September 1986). Although EPA had issued guidance for cancer risk assessment 10 years earlier (41 *Federal Register* 21402, 1976), the 1986 guidelines substantially enlarged the scope of EPA's formal guidance by covering additional health topics and by covering all areas in much greater depth. Each of the guidelines was a product of several years of discussion and review involving scientists and policymakers from EPA, other federal agencies, universities, industry, public interest groups, and the general public.

Preliminary work on comparable guidelines for ecological effects began in 1988. As part of this work, EPA studied existing assessments and identified issues to help develop a basis for articulating guiding principles for the assessment of ecological risks (U.S. EPA, 1991). At the same time, EPA's Science Advisory Board urged EPA to expand its consideration of ecological risk issues to include the broad array of chemical and nonchemical stressors for which research and regulation are authorized in the environmental laws administered by EPA (U.S. EPA, 1990b). As a result, EPA has embarked on a new program to develop guidelines for ecological risk assessment. Like the program for health effects guidance, this activity depends on the expertise of scientists and policymakers from a broad spectrum and draws principles, information, and methods from many sources.

In May 1991, EPA invited experts in ecotoxicology and ecological effects to Rockville, Maryland, to attend a peer review workshop on the draft Framework Report (56 *Federal Register* 20223, 2 May 1991). The workshop draft proposed a framework for ecological risk assessment complemented by preliminary guidance on some of the ecological issues identified in the draft. On the basis of the Rockville workshop recommendations (U.S. EPA, 1991), the revised Framework Report is now limited to discussion of the basic framework (see [Figure 1](#)), complemented by second-order diagrams that give structure and content to each of the major elements in the Framework Report (see [Figures 2](#) through [4](#)). Consistent with peer review recommendations, substantive risk assessment guidance is being reserved for study and development in future guidelines.

The Framework Report is the product of a variety of activities that culminated in the Rockville workshop. Beginning early in 1990, EPA work groups and the National Academy of Sciences' (NAS) Committee on Risk Assessment Methodology began to study the 1983 NAS risk assessment paradigm (NRC, 1983), which provides the organizing principles for EPA's health risk guidelines, as a possible foundation for ecological risk assessment. Early drafts of EPA's Framework Report received preliminary peer comment late in 1990.

In February 1991, NAS sponsored a workshop in Warrenton, Virginia, to discuss whether any single paradigm could accommodate all the diverse kinds of ecological risk assessments. There was a consensus that a single paradigm is feasible but that the 1983 paradigm would require modification to fulfill this role. In April 1991, EPA sponsored a strategic planning workshop in Miami, Florida. The structure and elements of ecological risk assessment were further discussed. Some participants in

both of these earlier meetings also attended the Rockville workshop. EPA then integrated information, concepts, and diagrams from these workshop reviews with EPA practices and needs to propose a working framework for interim use in EPA programs and for continued discussion as a basis for future risk assessment guidelines.

Use of the framework described in this report is not a requirement within EPA, nor is it a regulation of any kind. Rather, it is an interim product that is expected to evolve with use and discussion. EPA is publishing the Framework Report before proposing risk assessment guidelines for public comment to generate discussion within EPA, among government agencies, and with the public to develop concepts, principles, and methods for use in future guidelines. To facilitate such discussion, EPA is presenting the framework at scientific meetings and inviting the public to submit information relevant to use and development of the approaches outlined for ecological risk assessment in the report.

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PREFACE

Increased interest in ecological issues such as global climate change, habitat loss, acid deposition, reduced biological diversity, and the ecological impacts of pesticides and toxic chemicals prompts this Framework Report. This report describes basic elements, or a framework, for evaluating scientific information on the adverse effects of physical and chemical stressors on the environment. The framework offers starting principles and a simple structure as guidance for current ecological risk assessments and as a foundation for future EPA proposals for risk assessment guidelines.

The Framework Report is intended primarily for EPA risk assessors, EPA risk managers, and persons who perform work under EPA contract or sponsorship. The terminology and concepts described in the report may also assist other regulatory agencies, as well as members of the public who are interested in ecological issues.

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This report was prepared by members of the EPA technical panel listed below, with assistance from the staff of EPA's Risk Assessment Forum. Technical review was provided by numerous individuals, including EPA scientists and participants in the May 1991 peer review workshop. Editorial assistance was provided by R.O.W. Sciences, Inc.

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EXECUTIVE SUMMARY

This report, “Framework for Ecological Risk Assessment”, is the first step in a long-term effort to develop risk assessment guidelines for ecological effects. Its primary purpose is to offer a simple, flexible structure for conducting and evaluating ecological risk assessment within EPA. Although the Framework Report will serve as a foundation for development of future subject-specific guidelines, it is neither a procedural guide nor a regulatory requirement within EPA and is expected to evolve with experience. The Framework Report is intended to foster consistent approaches to ecological risk assessment within EPA, identify key issues, and define terms used in these assessments.

Ecological risk assessments evaluate ecological effects caused by human activities such as draining of wetlands or release of chemicals. The term “stressor” is used here to describe any chemical, physical, or biological entity that can induce adverse effects on individuals, populations, communities, or ecosystems. Thus, the ecological risk assessment process must be flexible while providing a logical and scientific structure to accommodate a broad array of stressors.

The framework is conceptually similar to the approach used for human health risk assessment, but it is distinctive in its emphasis in three areas. First, ecological risk

assessment can consider effects beyond those on individuals of a single species and may examine a population, community, or ecosystem. Second, there is no single set of ecological values to be protected that can be generally applied. Rather, these values are selected from a number of possibilities based on both scientific and policy considerations. Finally, there is an increasing awareness of the need for ecological risk assessments to consider nonchemical as well as chemical stressors.

The framework consists of three major phases: (1) problem formulation, (2) analysis, and (3) risk characterization. Problem formulation is a planning and scoping process that establishes the goals, breadth, and focus of the risk assessment. Its end product is a conceptual model that identifies the environmental values to be protected (the assessment endpoints), the data needed, and the analyses to be used.

The analysis phase develops profiles of environmental exposure and the effects of the stressor. The exposure profile characterizes the ecosystems in which the stressor may occur as well as the biota that may be exposed. It also describes the magnitude and spatial and temporal patterns of exposure. The ecological effects profile summarizes data on the effects of the stressor and relates them to the assessment endpoints.

Risk characterization integrates the exposure and effects profiles. Risks can be estimated using a variety of techniques including comparing individual exposure and effects values, comparing the distributions of exposure and effects, or using simulation models. Risk can be expressed as a qualitative or quantitative estimate, depending on available data. In this step, the assessor also:

- describes the risks in terms of the assessment endpoint;
- discusses the ecological significance of the effects;
- summarizes overall confidence in the assessment; and
- discusses the results with the risk manager.

The framework also recognizes several activities that are integral to, but separate from, the risk assessment process as defined in this report. For example, discussions between the risk assessor and risk manager are important. At the initiation of the risk assessment, the risk manager can help ensure that the risk assessment will ultimately provide information that is relevant to making decisions on the issues under consideration, while the risk assessor can ensure that the risk assessment addresses all relevant ecological concerns. Similar discussions of the results of the risk assessment are important to provide the risk manager with a full and complete understanding of the assessment's conclusions, assumptions, and limitations.

Other important companion activities to ecological risk assessment include data acquisition and verification and monitoring studies. New data are frequently required to conduct analyses that are performed during the risk assessment. Data from verification studies can be used to validate the predictions of a specific risk assessment as well as to evaluate the usefulness of the principles set forth in the Framework. Ecological effects or exposure monitoring can aid in the verification process and suggest additional data, methods, or analyses that could improve future risk assessments.

1. INTRODUCTION

Public, private, and government sectors of society are increasingly aware of ecological issues including global climate change, habitat loss, acid deposition, a decrease in biological diversity, and the ecological impacts of xenobiotic compounds such as pesticides and toxic chemicals. To help assess these and other ecological problems, the U.S. Environmental Protection Agency (EPA) has developed this report, "Framework for Ecological Risk Assessment", which describes the basic elements, or framework, of a process for evaluating scientific information on the adverse effects of stressors on the environment. The term "stressor" is defined here as any physical, chemical, or biological entity that can induce an adverse effect (see box¹). Adverse ecological effects encompass a wide range of disturbances ranging from mortality in an individual organism to a loss in ecosystem function.

This introductory section describes the purpose, scope, and intended audience for this report; discusses the definition and application of ecological risk assessment; outlines the basic elements of the proposed framework; and describes the organization of this report.

1.1. PURPOSE AND SCOPE OF THE FRAMEWORK REPORT

An understanding of the finite purpose and scope of this Framework Report is important. EPA, other regulatory agencies, and other organizations need detailed, comprehensive guidance on methods for evaluating ecological risk. However, in discussing tentative plans for developing such guidance with expert consultants (U.S. EPA, 1991; U.S. EPA, in press-a), EPA was advised to first develop a simple framework as a foundation or blueprint for later comprehensive guidance on ecological risk assessment.

With this background, the framework (see Section 1.4) has two simple purposes, one immediate and one longer term. As a broad outline of the assessment process, the framework offers a basic structure and starting principles for EPA's ecological

Physical and Chemical Stressors as a Focus of the Framework

This report does not discuss accidentally or deliberately introduced species, genetically engineered organisms, or organisms used to control horticultural or agricultural pests. While the general principles described in the framework may be helpful in addressing risks associated with these organisms, the capacity of such organisms for reproduction and biological interaction introduces additional considerations that are not addressed in this document.

¹ The boxes used throughout this document serve several purposes. Some boxes provide additional background and rationale for terms, whereas other boxes expand on concepts described in the text. The boxes at the end of each chapter highlight issues that are integral components of the risk assessment process but require more research, analysis, and debate. Further discussion of these issues is reserved for later guidelines.

risk assessments. The process described by the framework provides wide latitude for planning and conducting individual risk assessments in many diverse situations, each based on the common principles discussed in the framework. The process also will help foster a consistent EPA approach for conducting and evaluating ecological risk assessments, identify key issues, and provide operational definitions for terms used in ecological risk assessments.

In addition, the framework offers basic principles around which long-term guidelines for ecological risk assessment can be organized. With this in mind, this report does not provide substantive guidance on factors that are integral to the risk assessment process such as analytical methods, techniques for analyzing and interpreting data, or guidance on factors influencing policy. Rather, on the basis of EPA experience and the recommendations of peer reviewers, EPA has reserved discussion of these important aspects of any risk assessment for future guidelines, which will be based on the process described in this report.

1.2. INTENDED AUDIENCE

The framework is primarily intended for EPA risk assessors, EPA risk managers, and other persons who either perform work under EPA contract or sponsorship or are subject to EPA regulations. The terminology and concepts described here also may be of assistance to other federal, state, and local agencies as well as to members of the general public who are interested in ecological issues.

1.3. DEFINITION AND APPLICATIONS OF ECOLOGICAL RISK ASSESSMENT

Ecological risk assessment is defined as a process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors. A risk does not exist unless (1) the stressor has the inherent ability to cause one or more adverse effects and (2) it co-occurs with or contacts an ecological component (i.e., organisms, populations, communities, or ecosystems) long enough and at a sufficient intensity to elicit the identified adverse effect. Ecological risk assessment may evaluate one or many stressors and ecological components.

Ecological risk may be expressed in a variety of ways. While some ecological risk assessments may provide true probabilistic estimates of both the adverse effect and exposure elements, others may be deterministic or even qualitative in nature. In these cases, the likelihood of adverse effects is expressed through a semiquantitative or qualitative comparison of effects and exposure.

Ecological risk assessments can help identify environmental problems, establish priorities, and provide a scientific basis for regulatory actions. The process can identify existing risks or forecast the risks of stressors not yet present in the environment. However, while ecological risk assessments can play an important role in identifying and resolving environmental problems, risk assessments are not a solution

for addressing all environmental problems, nor are they always a prerequisite for environmental management. Many environmental matters such as the protection of habitats and endangered species are compelling enough that there may not be enough time or data to do a risk assessment. In such cases, professional judgment and the mandates of a particular statute will be the driving forces in making decisions.

1.4. ECOLOGICAL RISK ASSESSMENT FRAMEWORK

The distinctive nature of the framework results primarily from three differences in emphasis relative to previous risk assessment approaches. First, ecological risk assessment can consider effects beyond those on individuals of a single species and may examine population, community, or ecosystem impacts. Second, there is no one set of assessment endpoints (environmental values to be protected) that can be generally applied. Rather, assessment endpoints are selected from a very large number of possibilities based on both scientific and policy considerations. Finally, a comprehensive approach to ecological risk assessment may go beyond the traditional emphasis on chemical effects to consider the possible effects of nonchemical stressors.

The ecological risk assessment framework is shown in [Figure 1](#). The risk assessment process is based on two major elements: characterization of exposure and characterization of ecological effects. Although these two elements are most prominent during the analysis phase, aspects of both exposure and effects also are considered during problem formulation, as illustrated by the arrows in the diagram. The arrows also flow to risk characterization, where the exposure and effects elements are integrated to estimate risk. The framework is conceptually similar to the National Research Council (NRC) paradigm for human health risk assessments (NRC, 1983).

Relationship of the Framework to a Paradigm for Human Health Risk Assessment

In 1983, NRC published a paradigm that has been used in the development of EPA's human health risk assessment guidelines. The paradigm has four phases: hazard identification, dose-response assessment, exposure assessment, and risk characterization (NRC, 1983). Although the framework's problem formulation phase is not explicitly identified in the NRC paradigm, comparable planning issues are addressed in practice at the beginning of all EPA risk assessments. In the framework's analysis phase, characterization of exposure is analogous to exposure assessment, while characterization of ecological effects includes aspects of both hazard identification and dose-response assessment. (The framework uses the term "stressor response" rather than "dose response" because many Agency programs must address stressors other than chemicals, and dose has been used only for chemicals.) Risk characterization is a similar process in both the framework and the NRC paradigm.

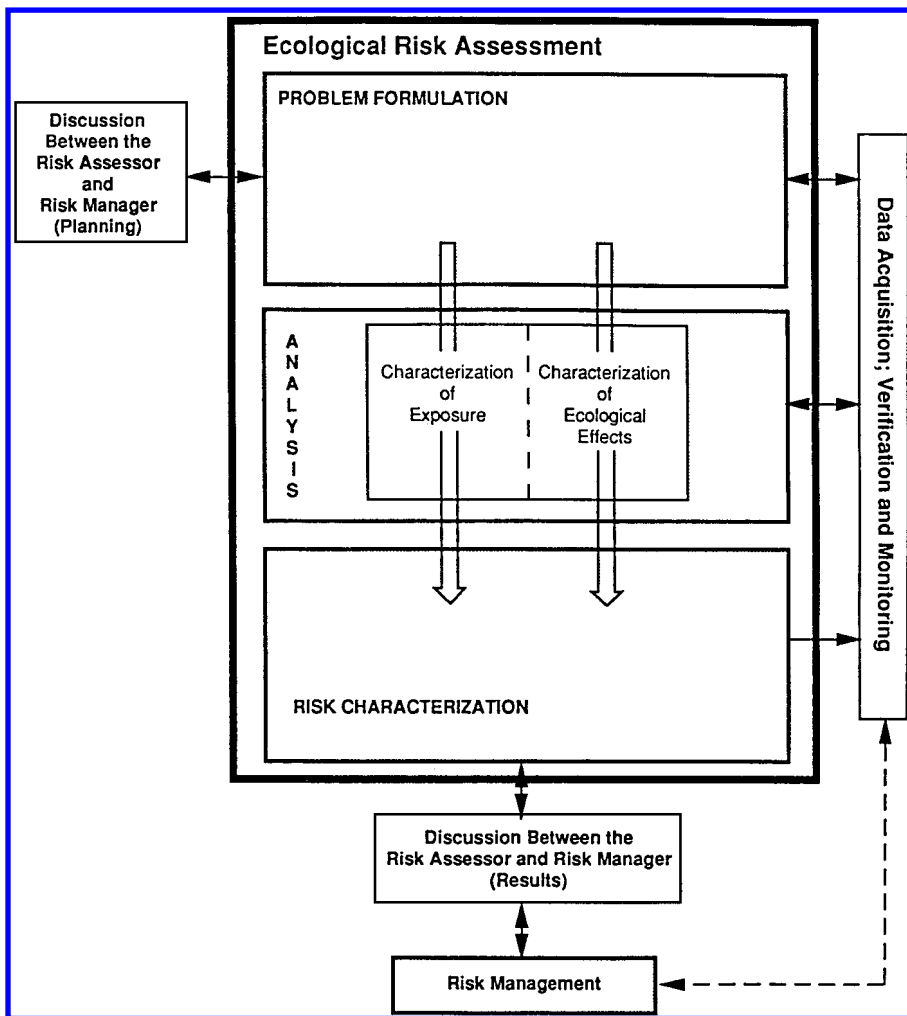


Figure 1. Framework for ecological risk assessment

The first phase of the framework is problem formulation. Problem formulation includes a preliminary characterization of exposure and effects, as well as examination of scientific data and data needs, policy and regulatory issues, and site-specific factors to define the feasibility, scope, and objectives for the ecological risk assessment. The level of detail and the information that will be needed to complete the assessment also are determined. This systematic planning phase is proposed because ecological risk assessments often address the risks of stressors to many species as well as risks to communities and ecosystems. In addition, there may be many ways a stressor can elicit adverse effects (e.g., direct effects on mortality and growth and indirect effects such as decreased food supply). Problem formulation provides an early identification of key factors to be considered, which in turn will produce a more scientifically sound risk assessment.

The second phase of the framework is termed analysis and consists of two activities: characterization of exposure is to predict or measure the spatial and temporal distribution of a stressor and its co-occurrence or contact with the ecological components of concern, while the purpose of characterization of ecological effects is to identify and quantify the adverse effects elicited by a stressor and, to the extent possible, to evaluate cause-and-effect relationships.

The third phase of the framework is risk characterization. Risk characterization uses the results of the exposure and ecological effects analyses to evaluate the likelihood of adverse ecological effects associated with exposure to a stressor. It includes a summary of the assumptions used, the scientific uncertainties, and the strengths and weaknesses of analyses. In addition, the ecological significance of the risks is discussed with consideration of the types and magnitudes of the effects, their spatial and temporal patterns, and the likelihood of recovery. The purpose is to provide a complete picture of the analysis and results.

In addition to showing the three phases of the framework, [Figure 1](#) illustrates the need for discussions between the risk assessor and risk manager. At the initiation of the risk assessment, the risk manager can help ensure that the risk assessment will ultimately provide information that is relevant to making decisions on the issues under consideration, while the risk assessor can ensure that the risk assessment addresses all relevant ecological concerns. Similar discussions of the results of the risk assessment are important to provide the risk manager with a full and complete understanding of the assessment's conclusions, assumptions, and limitations.

Use of the Term “Exposure”

Some reviewers of earlier drafts of this interim framework proposed that the term “exposure” — which, as used in human health risk assessment, generally refers to chemical stressors — not be used for the nonchemical stressors that can affect a variety of ecological components. Other terms, including “characterization of stress”, have been suggested. At this time, EPA prefers exposure, partly because characterization of stress does not convey the important concept of the co-occurrence and interaction of the stressor with an ecological component as well as exposure does.

Characterization of Ecological Effects Used Instead of Hazard Assessment

The framework uses characterization of ecological effects rather than hazard assessment for two reasons. First, the term “hazard” can be ambiguous, because it has been used in the past to mean either evaluating the intrinsic effects of a stressor (U.S. EPA, 1979) or defining a margin of safety or quotient by comparing a toxicological endpoint of interest with an estimate of exposure concentration (SETAC, 1987). Second, many reviewers believed that hazard is more relevant to chemical than to nonchemical stressors.

Figure 1 also indicates a role for verification and monitoring in the framework. Verification can include validation of the ecological risk assessment process as well as confirmation of specific predictions made during a risk assessment. Monitoring can aid in the verification process and may identify additional topics for risk assessment. Verification and monitoring can help determine the overall effectiveness of the framework approach, provide necessary feedback concerning the need for future modifications of the framework, help evaluate the effectiveness and practicality of policy decisions, and point out the need for new or improved scientific techniques (U.S. EPA, in press-a).

The interaction between data acquisition and ecological risk assessment is also shown in Figure 1. In this report, a distinction is made between data acquisition (which is outside of the risk assessment process) and data analysis (which is an integral part of an ecological risk assessment). In the problem formulation and analysis phases, the risk assessor may identify the need for additional data to complete an analysis. At this point, the risk assessment stops until the necessary data are acquired. When a need for additional data is recognized in risk characterization, new information generally is used in the analysis or problem formulation phases. The distinction between data acquisition and analysis generally is maintained in all of EPA's risk assessment guidelines; guidance on data acquisition procedures are provided in documents prepared for specific EPA programs.

The interactions between data acquisition and ecological risk assessment often result in an iterative process. For example, data used during the analysis phase may be collected in tiers of increasing complexity and cost. A decision to advance from one tier to the next is based on decision triggers set at certain levels of effect or exposure. Interactions of the entire risk assessment process also may occur. For example, a screening-level risk assessment may be performed using readily available data and conservative assumptions; depending on the results, more data then may be collected to support a more rigorous assessment.

1.5. THE IMPORTANCE OF PROFESSIONAL JUDGMENT

Ecological risk assessments, like human health risk assessments, are based on scientific data that are frequently difficult and complex, conflicting or ambiguous, or incomplete. Analyses of such data for risk assessment purposes depends on professional judgment based on scientific expertise. Professional judgment is necessary to:

- design and conceptualize the risk assessment;
- evaluate and select methods and models;
- determine the relevance of available data to the risk assessment;
- develop assumptions based on logic and scientific principles to fill data gaps; and
- interpret the ecological significance of predicted or observed effects.

Because professional judgment is so important, specialized knowledge and experience in the various phases of ecological risk assessment is required. Thus, an

interactive multidisciplinary team that includes biologists and ecologists is a prerequisite for a successful ecological risk assessment.

1.6. ORGANIZATION

The next three sections of this report are arranged to follow the framework sequentially. Section 2 describes problem formulation; this section is particularly important for assessors to consider when specific assessment endpoints are not determined *a priori* by statute or other authority. Sections 3 and 4 discuss analysis and risk characterization, respectively. Section 5 defines the terms used in this report. The lists of ecological risk assessment issues at the end of Sections 1 through 4 highlight areas for further discussion and research. EPA believes that these issues will require special attention in developing ecological risk assessment guidelines.

Additional Issues Related to the Framework

- Use of the framework for evaluating risks associated with biological stressors.
- Use of the term exposure (versus characterization of stress) for both chemical and nonchemical stressors.
- Use of the term characterization of ecological effects rather than hazard assessment.

2. PROBLEM FORMULATION

Problem formulation is the first phase of ecological risk assessment and establishes the goals, breadth, and focus of the assessment. It is a systematic planning step that identifies the major factors to be considered in a particular assessment, and it is linked to the regulatory and policy context of the assessment.

Entry into the ecological risk assessment process may be triggered by either an observed ecological effect, such as visible damage to trees in a forest, or by the identification of a stressor or activity of concern, such as the planned filling of a marsh or the manufacture of a new chemical. The problem formulation process (Figure 2) then begins with the initial stages of characterizing exposure and ecological effects, including evaluating the stressor characteristics, the ecosystem potentially at risk, and the ecological effects expected or observed. Next, the assessment and measurement endpoints are identified. (Measurement endpoints are ecological characteristics that can be related to the assessment endpoint.) The outcome of problem formulation is a conceptual model that describes how a given stressor might affect the ecological components in the environment. The conceptual model also describes the relationships among the assessment and measurement endpoints, the data required, and the methodologies that will be used to analyze the data. The conceptual model serves as input to the analysis phase of the assessment.

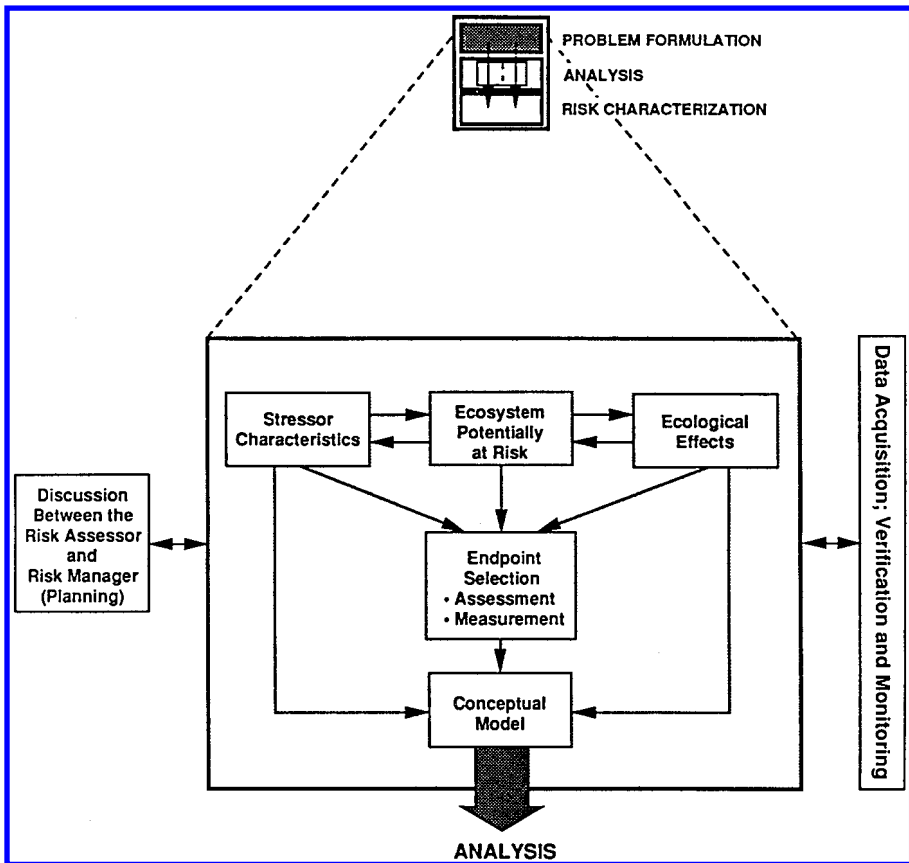


Figure 2. Problem formulation

2.1. DISCUSSION BETWEEN THE RISK ASSESSOR AND RISK MANAGER (PLANNING)

To be meaningful and effective, ecological risk assessments must be relevant to regulatory needs and public concerns as well as scientifically valid. Although risk assessment and risk management are distinct processes, establishing a two-way dialogue between risk assessors and risk managers during the problem formulation phase can be a constructive means of achieving both societal and scientific goals. By bringing the management perspective to the discussion, risk managers charged with protecting societal values can ensure that the risk assessment will provide relevant information to making decisions on the issue under consideration. By bringing scientific knowledge to the discussion, the ecological risk assessor ensures that the assessment addresses all important ecological concerns. Both perspectives are necessary to appropriately utilize resources to produce scientifically sound risk assessments that are relevant to management decisions and public concerns.

2.2. STRESSOR CHARACTERISTICS, ECOSYSTEM POTENTIALLY AT RISK, AND ECOLOGICAL EFFECTS

The initial steps in problem formulation are the identification and preliminary characterization of stressors, the ecosystem potentially at risk, and ecological effects. Performing this analysis is an interactive process that contributes to both the selection of assessment and measurement endpoints and the develop of a conceptual model.

2.2.1. *Stressor Characteristics*

The determination of stressor characteristics begins with the identification of potential chemical or physical stressors. Chemical stressors include a variety of inorganic and organic substances. Some chemicals may result in secondary stressors, as in the case of stratospheric ozone depletion caused by chlorofluorocarbons that could result in increased exposures to ultraviolet radiation. Physical stressors include extremes of natural conditions (e.g., temperature and hydrologic changes) and habitat alteration or destruction. Stressors that may result from management practices, such as harvesting of fishery or forest resources, also may be considered. Example stressor characteristics are summarized in the box below. Gathering information on the characteristics of a stressor helps define the ecosystems potentially at risk from the stressor as well as the ecological effects that may result.

Example Stressor Characteristics

Type — Chemical or physical
Intensity — Concentration or magnitude
Duration — Short or long term
Frequency — Single event, episodic, or continuous
Timing — Occurrence relative to biological cycles
Scale — Spatial heterogeneity and extent

2.2.2. *Ecosystem Potentially at Risk*

The ecosystem within which effects occur provides the ecological context for the assessment. Knowledge of the ecosystem potentially at risk can help identify ecological components that may be affected and stressor-ecosystem interactions relevant to developing exposure scenarios. The approach to identifying the ecosystem potentially at risk from a stressor depends in part on how the risk assessment was initiated. If a stressor first was identified, information on the spatial and temporal distribution patterns of the stressor can be helpful in identifying ecosystems potentially at risk. Similarly, if the risk assessment is initiated by observing effects, these effects can directly indicate ecosystems or ecological components that may be considered in the assessment.

A wide range of ecosystem properties may be considered during problem formulation. These properties include aspects of the abiotic environment (such as climatic conditions and soil or sediment properties), ecosystem structure (including the types and abundances of different species and their trophic level relationships), and ecosystem function (such as the ecosystem energy source, pathways of energy utilization, and nutrient processing) (U.S. EPA, in press-b). In addition, knowledge of the types and patterns of historical disturbances may be helpful in predicting ecological responses to stressors.

The need to evaluate spatial and temporal distribution and variation is inherent in many of these example characteristics. Such information is especially useful for determining potential exposure, that is, where there is co-occurrence of or contact between the stressor and ecological components.

2.2.3. *Ecological Effects*

Ecological effects data may come from a variety of sources. Relevant sources of information include field observations (e.g., fish or bird kills, changes in aquatic community structure), field tests (e.g., microcosm or mesocosm tests), laboratory tests (e.g., single species or microcosm tests), and chemical structure-activity relationships. Available information on ecological effects can help focus the assessment on specific stressors and on ecological components that should be evaluated.

Many factors can influence the utility of available ecological effects data for problem formulation. For example, the applicability of laboratory-based tests may be affected by any extrapolations required to specific field situations, while the interpretation of field observations may be influenced by factors such as natural variability or the possible presence of stressors other than the ones that are the primary focus of the risk assessment.

2.3. ENDPOINT SELECTION

Information compiled in the first stage of problem formulation is used to help select ecologically based endpoints that are relevant to decisions made about protecting the environment. An endpoint is a characteristic of an ecological component (e.g., increased mortality in fish) that may be affected by exposure to a stressor (Suter, 1990a). Two types of endpoints are distinguished in this report. Assessment endpoints are explicit expressions of the actual environmental value that is to be protected. Measurement endpoints are measurable responses to a stressor that are related to the valued characteristics chosen as the assessment endpoints (Suter, 1990a).

Assessment endpoints are the ultimate focus in risk characterization and link the measurement endpoints to the risk management process (e.g., policy goals). When an assessment endpoint can be directly measured, the measurement and assessment endpoints are the same. In most cases, however, the assessment endpoint cannot be directly measured, so a measurement endpoint (or a suite of measurement endpoints)

is selected that can be related, either qualitatively or quantitatively, to the assessment endpoint. For example, a decline in a sport fish population (the assessment endpoint) may be evaluated using laboratory studies on the mortality of surrogate species, such as the fathead minnow (the measurement endpoint). Sound professional judgment is necessary for proper assessment and measurement endpoint selection, and it is important that both the selection rationale and the linkages between measurement endpoints, assessment endpoints, and policy goals be clearly stated.

Assessment and measurement endpoints may involve ecological components from any level of biological organization, ranging from individual organisms to the ecosystem itself. In general, the use of a suite of assessment and measurement endpoints at different organizational levels can build greater confidence in the conclusions of the risk assessment and ensure that all important endpoints are evaluated. In some situations, measurement endpoints at one level of organization may be related to an assessment endpoint at a higher level. For example, measurement endpoints at the individual level (e.g., mortality, reproduction, and growth) could be used in a model to predict effects on an assessment endpoint at the population level (e.g., viability of a trout population in a stream).

General considerations for selecting assessment and measurement endpoints are detailed in the following boxes. More detailed discussions of endpoints and selection criteria can be found in Suter (1989, 1990a), Kelly and Harwell (1990), U.S. Department of the Interior (1987), and U.S. EPA (1990a).

Endpoint Terminology

Several reviewers have suggested using the term “indicator” in place of “measurement endpoint”. At this time, measurement endpoint is preferred because it has a specific meaning (a characteristic of an ecological system that can be related to an assessment endpoint), whereas indicator can have several different meanings. For example, indicator has been used at EPA to mean (1) measures of administrative accomplishments (e.g., number of permits issued), (2) measures of exposure (e.g., chemical levels in sediments), or (3) measures of ecosystem integrity. These indicators cannot always be related to an assessment endpoint.

2.4. THE CONCEPTUAL MODEL

The major focus of the conceptual model (Figure 2) is the development of working hypotheses regarding how the stressor might affect ecological components of the natural environment (NRC, 1986). The conceptual model also includes descriptions of the ecosystem potentially at risk and the relationship between measurement and assessment endpoints.

During conceptual model development, a preliminary analysis of the ecosystem, stressor characteristics, and ecological effects is used to define possible exposure

Considerations in Selecting Assessment Endpoints

Ecological Relevance

Ecologically relevant endpoints reflect important characteristics of the system and are functionally related to other endpoints. Selection of ecologically relevant endpoints requires some understanding of the structure and function of the ecosystem potentially at risk. For example, an assessment endpoint could focus on changes in a species known to have a controlling influence on the abundance and distribution of many other species in its community. Changes at higher levels of organization may be significant because of their potential for causing major effects at lower organizational levels.

Policy Goals and Societal Values

Good communication between the risk assessor and risk manager is important to ensure that ecologically relevant assessment endpoints reflect policy goals and societal values. Societal concerns can range from protection of endangered or commercially or recreationally important species to preservation of ecosystem attributes for functional reasons (e.g., flood water retention by wetlands) or aesthetic reasons (e.g., visibility in the Grand Canyon).

Susceptibility to the Stressor

Ideally, an assessment endpoint would be likely to be both affected by exposure to a stressor and sensitive to the specific type of effects caused by the stressor. For example, if a chemical is known to bioaccumulate and is suspected of causing eggshell thinning, an appropriate assessment endpoint might be raptor population viability.

scenarios. Exposure scenarios consist of a qualitative description of how the various ecological components co-occur with or contact the stressor. Each scenario is defined in terms of the stressor, the type of biological system and principal ecological components, how the stressor will contact or interact with the system, and the spatial and temporal scales.

For chemical stressors, the exposure scenario usually involves consideration of sources, environmental transport, partitioning of the chemical among various environmental media, chemical/biological transformation or speciation processes, and identification of potential routes of exposure (e.g., ingestion). For nonchemical stressors such as water level or temperature changes or physical disturbance, the exposure scenario describes the ecological components exposed and the general temporal and spatial patterns of their co-occurrence with the stressor. For example, for habitat alterations, the exposure scenario may describe the extent and distributional pattern of disturbance, the populations residing within or using the disturbed areas, and the spatial relationship of the disturbed area to undisturbed areas.

Considerations in Selecting Measurement Endpoints

Relevance to an Assessment Endpoint

When an assessment endpoint cannot be directly measured, measurement endpoints are identified that are correlated with or can be used to infer or predict changes in the assessment endpoint.

Consideration of Indirect Effects

Indirect effects occur when a stressor acts on elements of the ecosystem that are required by the ecological component of concern. For example, if the assessment endpoint is the population viability of trout, measurement endpoints could evaluate possible stressor effects on trout prey species or habitat requirements.

Sensitivity and Response Time

Rapidly responding measurement endpoints may be useful in providing early warnings of ecological effects, and measurement endpoints also may be selected because they are sensitive surrogates of the assessment endpoint. In many cases, measurement endpoints at lower levels of biological organization may be more sensitive than those at higher levels. However, because of compensatory mechanisms and other factors, a change in a measurement endpoint at a lower organizational level (e.g., a biochemical alteration) may not necessarily be reflected in changes at a higher level (e.g., population effects).

Signal-to-Noise Ratio

If a measurement endpoint is highly variable, the possibility of detecting stressor-related effects may be greatly reduced even if the endpoint is sensitive to the stressor.

Consistency with Assessment Endpoint Exposure Scenarios

The ecological component of the measurement endpoint should be exposed by similar routes and at similar or greater stressor levels as the ecological component of the assessment endpoint.

Diagnostic Ability

Measurement endpoints that are unique or specific responses to a stressor may be very useful in diagnosing the presence or effects of a stressor. For example, measurement of acetylcholinesterase inhibition may be useful for demonstrating responses to certain types of pesticides.

Practicality Issues

Ideal measurement endpoints are cost effective and easily measured. The availability of a large database for a measurement endpoint is desirable to facilitate comparisons and develop models.

Although many hypotheses may be generated during problem formulation, only those that are considered most likely to contribute to risk are selected for further evaluation in the analysis phase. For these hypotheses, the conceptual model describes the approach that will be used for the analysis phase and the types of data and analytical tools that will be needed. It is important that hypotheses that are not carried forward in the assessment because of data gaps be acknowledged when uncertainty is addressed in risk characterization. Professional judgment is needed to select the most appropriate risk hypotheses, and it is important to document the selection rationale.

Additional Issues in Problem Formulation

- Role of risk management concerns in establishing assessment endpoints. Although it is important to consider risk management concerns when assessment endpoints are selected, there is still uncertainty as to how these inputs should influence the goals of the risk assessment, the ecological components to be protected, and the level of protection required.
- Identifying specific assessment and measurement endpoints for different stressors and ecosystems.

3. ANALYSIS PHASE

The analysis phase of ecological risk assessment (Figure 3) consists of the technical evaluation of data on the potential effects and exposure of the stressor. The analysis phase is based on the conceptual model developed during problem formulation. Although this phase consists of characterization of ecological effects and characterization of exposure, the dotted line in Figure 3 illustrates that the two are performed interactively. An interaction between the two elements will ensure that the ecological effects characterized are compatible with the biota and exposure pathways identified in the exposure characterization. The output of ecological effects characterization and exposure characterization are summary profiles that are used in the risk characterization phase (Section 4). Discussion of uncertainty analysis, which is an important part of the analysis phase, may be found in Section 4.1.2.

Characterization of exposure and ecological effects often requires the application of statistical methods. While the discussion of specific statistical methods is beyond the scope of this document, selection of an appropriate statistical method involves both method assumptions (e.g., independence of errors, normality, equality of variances) and data set characteristics (e.g., distribution, presence of outliers or influential data). It should be noted that statistical significance does not always reflect biological significance, and profound biological changes may not be detected by statistical tests. Professional judgment often is required to evaluate the relationship between statistical and biological significance.

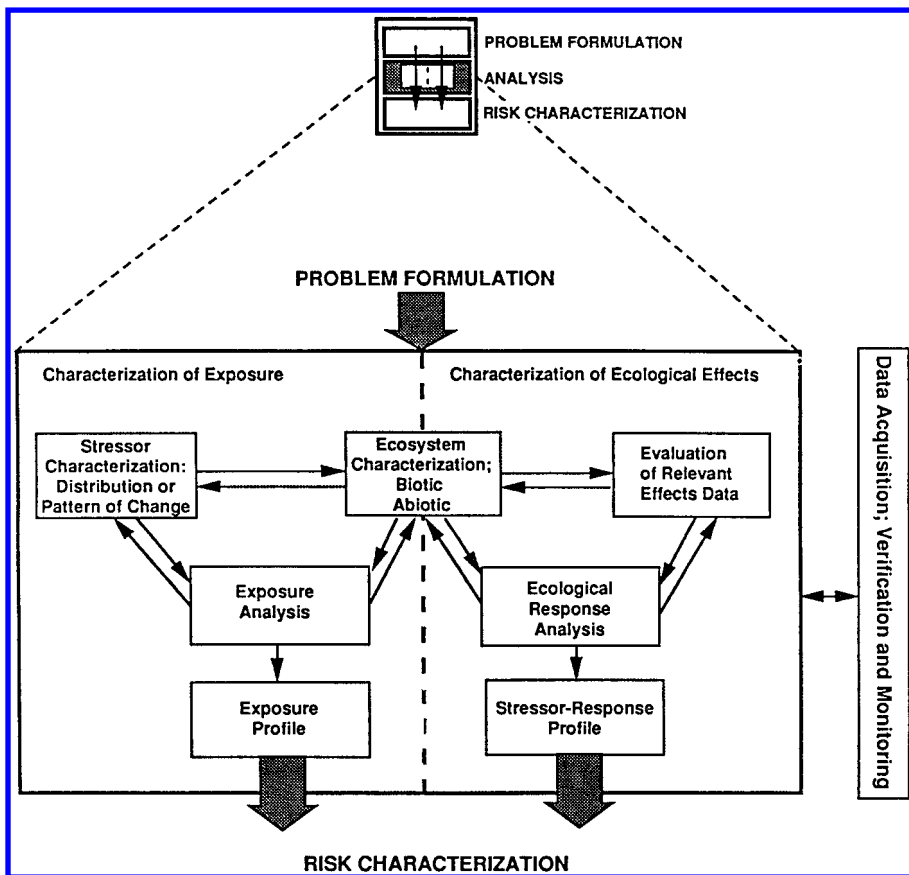


Figure 3. Analysis

3.1. CHARACTERIZATION OF EXPOSURE

Characterization of exposure (half of the analysis phase shown in Figure 3) evaluates the interaction of the stressor with the ecological component. Exposure can be expressed as co-occurrence or contact depending on the stressor and the ecological component involved. An exposure profile is developed that quantifies the magnitude and spatial and temporal distributions of exposure for the scenarios developed during problem formulation and serves as input to the risk characterization.

3.1.1. Stressor Characterization: Distribution or Pattern of Change

Stressor characterization involves determining the stressor's distribution or pattern of change. Many techniques can be applied to assist in this stressor characterization process. For chemical stressors, a combination of modeling and monitoring

data often is used. Available monitoring data may include measures of releases into the environment and media concentrations over space and time. Fate and transport models often are used that rely on physical and chemical characteristics of the chemical coupled with the characteristics of the ecosystem. For nonchemical stressors such as physical alterations or harvesting, the pattern of change may depend on resource management or land-use practices. Depending on the scale of the disturbance, the data for stressor characterization can be provided by a variety of techniques, including ground reconnaissance, aerial photographs, or satellite imagery.

During stressor characterization, one considers not only the primary stressor but also secondary stressors that can arise as a result of various processes. For example, removal of riparian (stream-side) vegetation not only alters habitat structure directly, but can have additional ramifications such as increased siltation and temperature rise. For chemicals, secondary stressors can be produced by a range of environmental fate processes.

The timing of the stressor's interaction with the biological system is another important consideration. If the stressor is episodic in nature, different species and life stages may be affected. In addition, the ultimate distribution of a stressor is rarely homogeneous; it is important to quantify such heterogeneity whenever possible.

3.1.2. *Ecosystem Characterization*

During ecosystem characterization, the ecological context of the assessment is further analyzed. In particular, the spatial and temporal distributions of the ecological component are characterized, and the ecosystem attributes that influence the distribution and nature of the stressor are considered.

Characteristics of the ecosystem can greatly modify the ultimate nature and distribution of the stressor. Chemical stressors can be modified through biotransformation by microbial communities or through other environmental fate processes, such as photolysis, hydrolysis, and sorption. The bioavailability of chemical stressors also can be affected by the environment, which in turn influences the exposure of ecological components.

Physical stressors can be modified by the ecosystem as well. For example, siltation in streams depends not only on sediment volume, but on flow regime and physical stream characteristics. Similarly, nearby wetlands and levees influence water behavior during flood events.

The spatial and temporal distributions of ecological components also are considered in ecosystem characterization. Characteristics of ecological components that influence their exposure to the stressor are evaluated, including habitat needs, food preferences, reproductive cycles, and seasonal activities such as migration and selective use of resources. Spatial and temporal variations in the distribution of the ecological component (e.g., sediment invertebrate distribution) may complicate evaluations of exposure. When available, species-specific information about activity patterns, abundance, and life histories can be very useful in evaluating spatial and temporal distributions.

Another important consideration is how exposure to a stressor may alter natural behavior, thereby affecting further exposure. In some cases, this may lead to enhanced exposure (e.g., increased preening by birds after aerial pesticide spraying), while in other situations initial exposure may lead to avoidance of contaminated locations or food sources (e.g., avoidance of certain waste effluents or physically altered spawning beds by some fish species).

3.1.3. Exposure Analyses

The next step is to combine the spatial and temporal distributions of both the ecological component and the stressor to evaluate exposure. In the case of physical alterations of communities and ecosystems, exposure can be expressed broadly as co-occurrence. Exposure analyses of individuals often focus on actual contact with the stressor, because organisms may not contact all of the stressors present in an area. For chemical stressors, the analyses may focus further on the amount of chemical that is bioavailable, that is, available for uptake by the organism. Some chemical exposure analyses also follow the chemical within the organism's body and estimate the amount that reaches the target organ. The focus of the analyses will depend on the stressors being evaluated and the assessment and measurement endpoints.

The temporal and spatial scales used to evaluate the stressor need to be compatible with the characteristics of the ecological component of interest. A temporal scale may encompass the lifespan of a species, a particular life stage, or a particular cycle, for example, the long-term succession of a forest community. A spatial scale may encompass a forest, a lake, a watershed, or an entire region. Stressor timing relative to organism life stage and activity patterns can greatly influence the occurrence of adverse effects. Even short-term events may be significant if they coincide with critical life stages. Periods of reproductive activity may be especially important, because early life stages often are more sensitive to stressors, and adults also may be more vulnerable at this time.

The most common approach to exposure analysis is to measure concentrations or amounts of a stressor and combine them with assumptions about co-occurrence, contact, or uptake. For example, exposure of aquatic organisms to chemicals often is expressed simply as concentration in the water column; aquatic organisms are assumed to contact the chemical. Similarly, exposures of organisms to habitat alteration often are expressed as hectares of habitat altered; organisms that utilize the habitat are assumed to co-occur with the alteration. Stressor measurements can also be combined with quantitative parameters describing the frequency and magnitude of contact. For example, concentrations of chemicals in food items can be combined with ingestion rates to estimate dietary exposure of organisms.

In some situations, the stressor can be measured at the actual point of contact while exposure occurs. An example is the use of food collected from the mouths of nestling birds to evaluate exposure to pesticides through contaminated food (Kendall, 1991). Although such point-of-contact measurements can be difficult to obtain, they reduce the need for assumptions about the frequency and magnitude of contact.

Patterns of exposure can be described using models that combine abiotic ecosystem attributes, stressor properties, and ecological component characteristics. Model selection is based on the model's suitability for the ecosystem or component of interest, the availability of the requisite data, and the study objectives. Model choices range from simple, screening-level procedures that require a minimum of data to more sophisticated methods that describe processes in more detail but require a considerable amount of data.

Another approach to evaluating exposure uses chemical, biochemical, or physiological evidence (e.g., biomarkers) of a previous exposure. This approach has been used primarily for assessing chemical exposures and is particularly useful when a residue or biomarker is diagnostic of exposure to a particular chemical. These types of measurements are most useful for exposure characterization when they can be quantitatively linked to the amount of stressor originally contacted by the organism. Pharmacokinetic models are sometimes used to provide this linkage.

3.1.4. Exposure Profile

Using information obtained from the exposure analysis, the exposure profile quantifies the magnitude and spatial and temporal patterns of exposure for the scenarios developed during problem formulation and serves as input to risk characterization. The exposure profile is only effective when its results are compatible with the stressor-response profile. For example, appraisals of potential acute effects of chemical exposure may be averaged over short time periods to account for short-term pulsed stressor events. It is important that characterizations for chronic stressors account for both long-term, low-level exposure and possible shorter term, higher level contact that may elicit similar adverse chronic effects.

Exposure profiles can be expressed using a variety of units. For chemical stressors operating at the organism level, the usual metric is expressed in dose units (e.g., mg/kg body weight/day). For higher levels of organization (e.g., an entire ecosystem), exposure may be expressed in units of concentration/unit area/time. For physical disturbance, the exposure profile may be expressed in other terms (e.g., percentage of habitat removed or the extent of flooding/year).

An uncertainty assessment is an integral part of the characterizations of exposure. In the majority of assessments, data will not be available for all aspects of the characterization of exposure, and those data that are available may be of questionable or unknown quality. Typically, the assessor will have to rely on a number of assumptions with varying degrees of uncertainty associated with each. These assumptions will be based on a combination of professional judgment, inferences based on analogy with similar chemicals and conditions and estimation techniques, all of which contribute to the overall uncertainty. It is important that the assessor characterize each of the various sources of uncertainty and carry them forward to the risk characterization so that they may be combined with a similar analysis conducted as part of the characterization of ecological effects.

3.2. CHARACTERIZATION OF ECOLOGICAL EFFECTS

The relationship between the stressor and the assessment and measurement endpoints identified during problem formulation is analyzed in characterization of ecological effects (Figure 3). The evaluation begins with the evaluation of effects data that are relevant to the stressor. During ecological response analysis, the relationship between the stressor and the ecological effects elicited is quantified, and cause-and-effect relationships are evaluated. In addition, extrapolations from measurement endpoints to assessment endpoints are conducted during this phase. The product is a stressor-response profile that quantifies and summarizes the relationship of the stressor to the assessment endpoint. The stressor-response profile is then used as input to risk characterization.

3.2.1. *Evaluation of Relevant Effects Data*

The type of effects data that are evaluated depends largely on the nature of the stressor and the ecological component under evaluation. Effects elicited by a stressor may range from mortality and reproductive impairment in individuals and populations to disruptions in community and ecosystem function such as primary productivity. The evaluation process relies on professional judgment, especially when few data are available or when choices among several sources of data are required. If available data are inadequate, new data may be needed before the assessment can be completed.

Data are evaluated by considering their relevance to the measurement and assessment endpoints selected during problem formulation. The analysis techniques that will be used also are considered; data that minimize the need for extrapolation are desirable. Data quality (e.g., sufficiency of replications, adherence to good laboratory practices) is another important consideration. Finally, characteristics of the ecosystem potentially at risk will influence what data will be used. Ideally, the test system reflects the physical attributes of the ecosystem and will include the ecological components and life stages examined in the risk assessment.

Data from both field observations and experiments in controlled settings can be used to evaluate ecological effects. In some cases, such as for chemicals that have yet to be manufactured, test data for the specific stressor are not available. Quantitative structure-activity relationships (QSARs) are useful in these situations (Auer et al., 1990; Clements et al., 1988; McKim et al., 1987).

Controlled laboratory and field tests (e.g., mesocosms) can provide strong causal evidence linking a stressor with a response and can also help discriminate between multiple stressors. Data from laboratory studies tend to be less variable than those from field studies, but because environmental factors are controlled, responses may differ from those in the natural environment.

Observational field studies (e.g., comparison with reference sites) provide environmental realism that laboratory studies lack, although the presence of multiple

stressors and other confounding factors (e.g., habitat quality) in the natural environment can make it difficult to attribute observed effects to specific stressors. Confidence in causal relationships can be improved by carefully selecting comparable reference sites or by evaluating changes along a stressor gradient where differences in other environmental factors are minimized. It is important to consider potential confounding factors during the analysis.

3.2.2. *Ecological Response Analyses*

The data used in characterization of ecological effects are analyzed to quantify the stressor-response relationship and to evaluate the evidence for causality. A variety of techniques may be used, including statistical methods and mathematical modeling. In some cases, additional analyses to relate the measurement endpoint to the assessment endpoint may be necessary.

Stressor-Response Analyses

The stressor-response analysis describes the relationship between the magnitude, frequency, or duration of the stressor in an observational or experimental setting and the magnitude of response. The stressor-response analysis may focus on different aspects of the stressor-response relationship, depending on the assessment objectives, the conceptual model, and the type of data used for the analysis. Stressor-response analyses, such as those used for toxicity tests, often portray the magnitude of the stressor with respect to the magnitude of response. Other important aspects to consider include the temporal (e.g., frequency, duration, and timing) and spatial distributions of the stressor in the experimental or observational setting. For physical stressors, specific attributes of the environment after disturbance (e.g., reduce forest stand age) can be related to the response (e.g., decreased use by spotted owls) (Thomas et al., 1990).

Analyses Relating Measurement and Assessment Endpoints

Ideally, the stressor-response evaluation quantifies the relationship between the stressor and the assessment endpoint. When the assessment endpoint can be measured, this analysis is straightforward. When it cannot be measured, the relationship between the stressor and measurement endpoint is established first, then additional extrapolations, analyses, and assumptions are used to predict or infer changes in the assessment endpoint. The need for analyses relating measurement and assessment endpoints also may be identified during risk characterization, after an initial evaluation of risk.

Measurement endpoints are related to assessment endpoints using the logical structure present in the conceptual model. In some cases, quantitative methods and models are available, but often the relationship can be described only qualitatively. Because of the lack of standard methods for many of these analyses, professional

Extrapolations and Other Analyses Relating Measurement and Assessment Endpoints

Extrapolation between Taxa

example: from bluegill sunfish mortality to rainbow trout mortality

Extrapolation between Responses

example: from bobwhite quail LC_{50} to bobwhite quail NOEL (no observed effect level)

Extrapolation from Laboratory to Field

example: from mouse mortality under laboratory conditions to mouse mortality in the field

Extrapolation from Field to Field

example: from reduced invertebrate community diversity in one stream to another stream

Analysis of Indirect Effects

example: relating removal of long-leaf pine to reduced populations of red-cockaded woodpecker

Analysis of Higher Organizational Levels

example: relating reduced individual fecundity to reduced population size

Analysis of Spatial and Temporal Scales

example: evaluation of the loss of a specific wetland used by migratory birds in relation to the larger scale habitat requirements of the species

Analysis of Recovery

example: relating short-term mortality to long-term depauperation

judgment is an essential component of the evaluation. It is important to clearly explain the rationale for any analyses and assumptions.

Extrapolations commonly used include those between species, between responses, from laboratory to field, and from field to field. Differences in responses among taxa depend on many factors, including physiology, metabolism, resource utilization, and life history strategy. The relationship between responses also depends on many factors, including the mechanism of action and internal distribution of the stressor within the organism. When extrapolating between different laboratory and field settings, important considerations include differences in the physical environment and organism behavior that will alter exposure, interactions with other stressors, and interactions with other ecological components.

In addition to these extrapolations, an evaluation of indirect effects, other levels of organization, other temporal and spatial scales, and recovery potential may be necessary. Whether these analyses are required in a particular risk assessment will depend on the assessment endpoints identified during problem formulation.

Important factors to consider when evaluating indirect effects include interspecies interactions (e.g., competition, disease), trophic-level relationships (e.g., predation),

and resource utilization. Effects on higher (or lower) organizational levels depend on the severity of the effect, the number and life stage of organisms affected, the role of those organisms in the community or ecosystem, and ecological compensatory mechanisms.

The implications of adverse effects at spatial scales beyond the immediate area of concern may be evaluated by considering ecological characteristics such as community structure and energy and nutrient dynamics. In addition, information from the characterization of exposure on the stressor's spatial distribution may be useful. Extrapolations between different temporal scales (e.g., from short-term impacts to long-term effects) may consider the stressors' distribution through time (intensity, duration, and frequency) relative to ecological dynamics (e.g., seasonal cycles, life cycle patterns).

In some cases, evaluation of long-term impacts will require consideration of ecological recovery. Ecological recovery is difficult to predict and depends on the existence of a nearby source of organisms, life history and dispersal strategies of the ecological components, and the chemical-physical environmental quality following exposure to the stressor (Cairns, 1990; Poff and Ward, 1990; Kelly and Harwell, 1990). In addition, there is some evidence to suggest that the types and frequency of natural disturbances can influence the ability of communities to recover (Schlosser, 1990).

Evaluation of Causal Evidence

Another important aspect of the ecological response analysis is to evaluate the strength of the causal association between the stressor and the measurement and assessment endpoints. This information supports and complements the stressor-response assessment and is of particular importance when the stressor-response relationship is based on field observations. Although proof of causality is not a requirement for risk assessment, an evaluation of causal evidence augments the risk assessment. Many of the concepts applied in human epidemiology can be useful for evaluating causality in observational field studies. For example, Hill (1965) suggested nine evaluation criteria for causal associations. An example of ecological causality analysis was provided by Woodman and Cowling (1987), who evaluated the causal association between air pollutants and injury to forests.

3.2.3. Stressor-Response Profile

The results of the characterization of ecological effects are summarized in a stressor-response profile that describes the stressor-response relationship, any extrapolations and additional analyses conducted, and evidence of causality (e.g., field effects data).

Ideally, the stressor-response relationship will relate the magnitude, duration, frequency, and timing of exposure in the study setting to the magnitude of effects.

Hill's Criteria for Evaluating Causal Associations (Hill, 1965)

1. **Strength:** A high magnitude of effect is associated with exposure to the stressor.
2. **Consistency:** The association is repeatedly observed under different circumstances.
3. **Specificity:** The effect is diagnostic of a stressor.
4. **Temporality:** The stressor precedes the effect in time.
5. **Presence of a biological gradient:** A positive correlation between the stressor and response.
6. **A plausible mechanism of action.**
7. **Coherence:** The hypothesis does not conflict with knowledge of natural history and biology.
8. **Experimental evidence.**
9. **Analogy:** Similar stressors cause similar responses.

Not all of these criteria must be satisfied, but each incrementally reinforces the argument for causality. Negative evidence does not rule out a causal association but may indicate incomplete knowledge of the relationship (Rothman, 1986).

For practical reasons, the results of stressor-response curves are often Summarized as one reference point, for instance, a 48-h LC_{50} . Although useful, such values provide no information about the slope or shape of the stressor-response curve. When the entire curve is used, or when points on the curve are identified, the difference in magnitude of effect at different exposure levels can be reflected in risk characterization.

It is important to clearly describe and quantitatively estimate the assumptions and uncertainties involved in the evaluation, where possible. Examples include natural variability in ecological characteristics and responses and uncertainties in the test system and extrapolations. The description and analysis of uncertainty in characterization of ecological effects are combined with uncertainty analyses for the other ecological risk assessment elements during risk characterization.

Additional Issues Related to the Analysis Phase

- Quantifying cumulative impacts and stress-response relationships for multiple stressors.
- Improving the prediction of ecosystem recovery.
- Improving the quantification of indirect effects.
- Describing stressor-response relationships for physical perturbations.
- Distinguishing ecosystem changes due to natural processes from those caused by man.

4. RISK CHARACTERIZATION

Risk characterization (Figure 4) is the final phase of risk assessment. During this phase, the likelihood of adverse effects occurring as a result of exposure to a stressor are evaluated. Risk characterization contains two major steps: risk estimation and risk description. The stressor-response profile and the exposure profile from the analysis phase serve as input to risk estimation. The uncertainties identified during all phases of the risk assessment also are analyzed and summarized. The estimated risks are discussed by considering the types and magnitude of effects anticipated, the spatial and temporal extent of the effects, and recovery potential. Supporting information in the form of a weight-of-evidence discussion also is presented during this step. The results of the risk assessment, including the relevance of the identified risks to the original goals of the risk assessment, then are discussed with the risk manager.

4.1. RISK ESTIMATION

Risk estimation consists of comparing the exposure and stressor-response profiles as well as estimating and summarizing the associated uncertainties.

4.1.1. *Integration of Stressor-Response and Exposure Profiles*

Three general approaches are discussed to illustrate the integration of the stressor-response and exposure profiles: (1) comparing single effect and exposure values; (2) comparing distributions of effects and exposure; and (3) conducting simulation modeling. Because these are areas of active research, particularly in the assessment of community- and landscape-level perturbations, additional integration approaches are likely to be available in the future. The final choice as to which approach will be selected depends on the original purpose of the assessment as well as time and data constraints.

Comparing Single Effect and Exposure Values

Many risk assessments compare single effect values with predicted or measured levels of the stressor. The effect values from the stressor-response profile may be used as is, or more commonly, uncertainty or safety factors may be used to adjust the value. The ratio or quotient of the exposure value to the effect value provides the risk estimate. If the quotient is one or more, an adverse effect is considered likely to occur. This approach, known as the quotient method (Barnthouse et al., 1986), has been used extensively to evaluate the risks of chemical stressors (Nabholz 1991; Urban and Cook, 1986). Although the quotient method is commonly used and accepted, it is the least probabilistic of the approaches described here. Also, correct usage of the quotient method is highly dependent on professional judgment, particularly in instances when the quotient approaches one. Greater insight into the magnitude

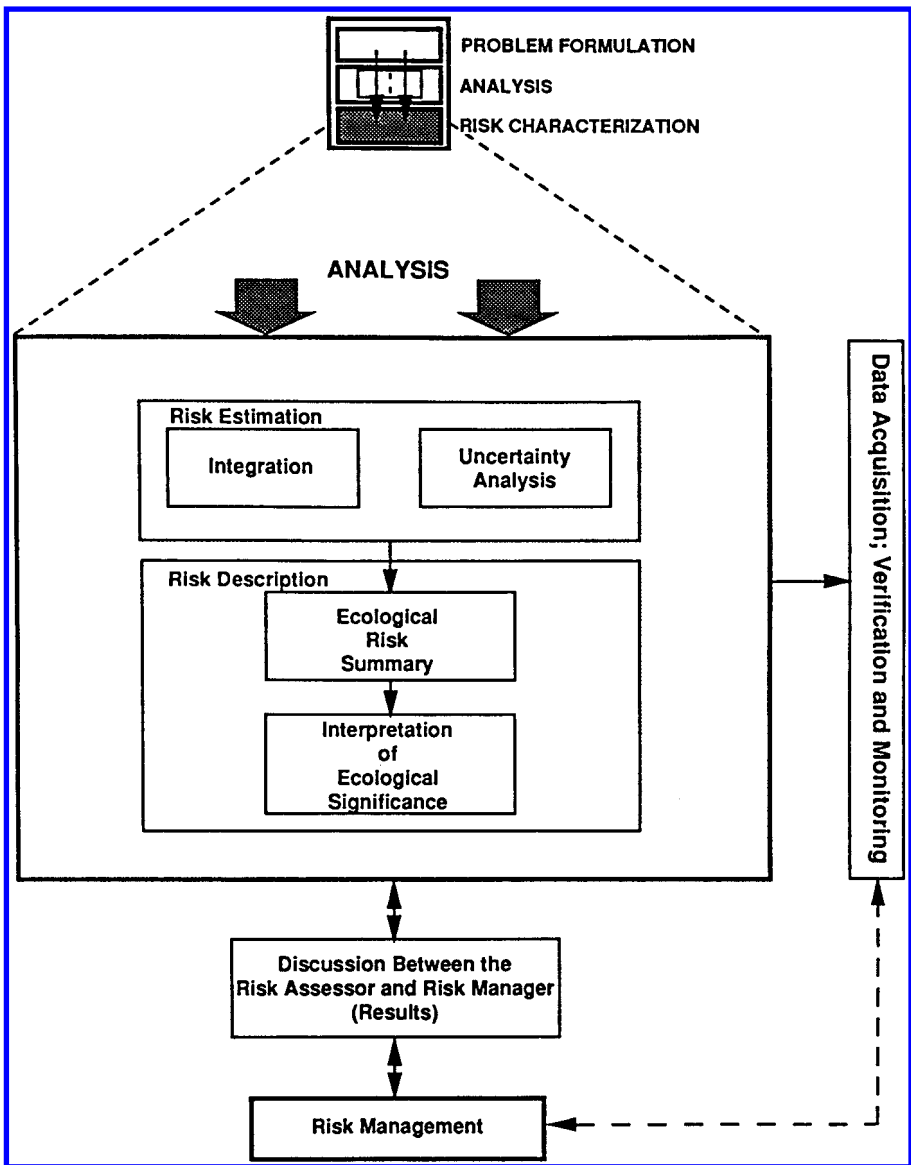


Figure 4. Risk characterization

of the effects expected at various levels of exposure can be obtained by evaluating the full stressor-response curve instead of a single point and by considering the frequency, timing, and duration of the exposure.

Comparing Distributions of Effects and Exposure

This approach uses distributions of effects and exposure (as opposed to single values) and thus makes probabilistic risk estimates easier to develop. Risk is quantified by the degree of overlap between the two distributions; the more overlap, the greater the risk. An example of this approach, analysis of extrapolation error, is given in Barnthouse et al. (1986). To construct valid distributions, it is important that sufficient data amenable to statistical treatment are available.

Conducting Simulation Modeling

Simulation models that can integrate both the stressor-response profile and exposure profile are useful for obtaining probabilistic estimates of risk. Two categories of simulation models are used for ecological risk assessment: single-species population models are used to predict direct effects on a single population of concern using measurement endpoints at the individual level, while multispecies models include aquatic food web models and terrestrial plant succession models and are useful for evaluating both direct and indirect effects.

When selecting a model, it is important to determine the appropriateness of the model for a particular application. For example, if indirect effects are of concern, a model of community-level interactions will be needed. Direct effects to a particular population of concern may be better addressed with population models. The validation status and use history of a model also are important considerations in model selection. Although simulation models are not commonly used for ecological risk assessment at the present time, this is an area of active research, and the use of simulation models is likely to increase.

In addition to providing estimates of risks, simulation models also can be useful in discussing the results of the risk characterization to the risk manager. This dialogue is particularly effective when the relationship between risks to certain measurement endpoints and the assessment endpoint are not readily apparent (e.g., certain indirect effects and large-scale ecosystem-level disturbances).

4.1.2. Uncertainty

The uncertainty analysis identifies, and, to the extent possible, quantifies the uncertainty in problem formulation, analysis, and risk characterization. The uncertainties from each of these phases of the process are carried through as part of the total uncertainty in the risk assessment. The output from the uncertainty analysis is an evaluation of the impact of the uncertainties on the overall assessment and, when feasible, a description of the ways in which uncertainty could be reduced.

A complete discussion of uncertainty is beyond the scope of this report, and the reader is referred to the works of Finkel (1990), Holling (1978), and Suter (1990b). However, a brief discussion of the major sources of uncertainty in ecological risk assessment is appropriate. For illustrative purposes, four major areas of uncertainty are presented below. These are not discrete categories, and overlap does exist among them. Any specific risk assessment may have uncertainties in one or all of these categories.

Conceptual Model Formulation

As noted earlier, the conceptual model is the product of the problem formulation phase, which, in turn, provides the foundation for the analysis phase and the development of the exposure and stressor-response profiles. If incorrect assumptions are made during conceptual model development regarding the potential effects of a stressor, the environments impacted, or the species residing within those systems, then the final risk assessment will be flawed. These types of uncertainties are perhaps the most difficult to identify, quantify, and reduce.

Information and Data

Another important contributor of uncertainty is the incompleteness of the data or information upon which the risk assessment is based. In some instances, the risk assessment may be halted temporarily until additional information is obtained. In other cases, certain basic information such as life history data may be unobtainable with the resources available to the risk assessment. In yet other cases, fundamental understanding of some natural processes with an ecosystem may be lacking. In instances where additional information cannot be obtained, the role of professional judgment and judicious use of assumptions are critical for the completion of the assessment.

Stochasticity (Natural Variability)

Natural variability is a basic characteristic of stressors and ecological components as well as the factors that influence their distribution (e.g., weather patterns, nutrient availability). As noted by Suter (1990b), of all the contributions to uncertainty, stochasticity is the only one that can be acknowledged and described but not reduced. Natural variability is amenable to quantitative analyses, including Monte Carlo simulation and statistical uncertainty analysis (O'Neill and Gardner, 1979; O'Neill et al., 1982).

Error

Errors can be introduced through experimental design or the procedures used for measurement and sampling. Such errors can be reduced by adherence to good

laboratory practices and adherence to established experimental protocols. Errors also can be introduced during simulation model development. Uncertainty in the development and use of models can be reduced through sensitivity analyses, comparison with similar models, and field validation.

In summary, uncertainty analyses provide the risk manager with an insight into the strengths and weaknesses of an assessment. The uncertainty analysis also can serve as a basis for making rational decisions regarding alternative actions as well as for obtaining additional information to reduce uncertainty in the risk estimates.

4.2. RISK DESCRIPTION

Risk description has two primary elements. The first is the ecological risk summary, which summarizes the results of the risk estimation and uncertainty analysis and assesses confidence in the risk estimates through a discussion of the weight of evidence. The second element is interpretation of ecological significance, which describes the magnitude of the identified risks to the assessment endpoint.

4.2.1. *Ecological Risk Summary*

The ecological risk summary summarizes the results of the risk estimation and discusses the uncertainties associated with problem formulation, analysis, and risk characterization. Next, the confidence in the risk estimates is expressed through a weight-of-evidence discussion. The ecological risk summary may conclude with an identification of additional analyses or data that might reduce the uncertainty in the risk estimates. These three aspects of the ecological risk summary are discussed in the following sections.

Summary of Risk Estimation and Uncertainty

Ideally, the conclusions of the risk estimation are described as some type of quantitative statement (e.g., there is a 20% change of 50% mortality). However, in most instances, likelihood is expressed in a qualitative statement (e.g., there is a high likelihood of mortality occurring). The uncertainties identified during the risk assessment are summarized either quantitatively or qualitatively, and the relative contribution of the various uncertainties to the risk estimates are discussed whenever possible.

Weight of Evidence

The weight-of-evidence discussion provides the risk manager with insight about the confidence of the conclusions reached in the risk assessment by comparing the positive and negative aspects of the data, including uncertainties identified throughout the process. The considerations listed below are useful in a weight-of-evidence discussion:

- The sufficiency and quality of the data. A risk assessment conducted with studies that completely characterize both the effects and exposure of the stressor has more credibility and support than an assessment that contains data gaps. It is important to state if the data at hand were sufficient to support the findings of the assessment. In addition, data validity (e.g., adherence to protocols, having sufficient replications) is an important facet of the weight-of-evidence analysis.
- Corroborative information. Here the assessor incorporates supplementary information that is relevant to the conclusions reached in the assessment. Examples include reported incidences of effects elicited by the stressor (or similar stressor) and studies demonstrating agreement between model predictions and observed effects.
- Evidence of causality. The degree of correlation between the presence of a stressor and some adverse effect is an important consideration for many ecological risk assessments. This correlation is particularly true when an assessor is attempting to establish a link between certain observed field effects and the cause of those effects. Further discussions of the evaluation of causal relationships may be found in the section on characterization of ecological effects (Section 3.2.2.).

Identification of Additional Analyses

The need for certain analyses may not be identified until after the risk estimation step. For example, the need to analyze the risks to a fish population (an assessment endpoint) due to an indirect effect such as zooplankton mortality (a measurement endpoint) may not be established until after the risk to zooplankton has been characterized. In such cases, another iteration through analysis or even problem formulation may be necessary.

4.2.2. Interpretation of Ecological Significance

The interpretation of ecological significance places risk estimates in the context of the types and extent of anticipated effects. It provides a critical link between the estimation of risks and the communication of assessment results. The interpretation step relies on professional judgment and may emphasize different aspects depending on the assessment. Several aspects of ecological significance that may be considered include the nature and magnitude of the effects, the spatial and temporal patterns of the effects, and the potential for recovery once a stressor is removed.

Nature and Magnitude of the Effects

The relative significance of different effects may require further interpretation, especially when changes in several assessment or measurement endpoints are observed or predicted. For example, if a risk assessment is concerned with the effects of stressors on several ecosystems in an area (such as a forest, stream, and wetland), it is important to discuss the types of effects associated with each ecosystem and where the greatest impact is likely to occur.

The magnitude of an effect will depend on its ecological context. For example, a reduction in the reproductive rate may have little effect on a population that reproduces rapidly, but it may dramatically reduce the numbers of a population that reproduces slowly. Population-dependent and -independent factors in the ecosystem also may influence the expression of the effect.

Finally, it is important to consider the effects in the context of both magnitude and the likelihood of the effect occurring. In some cases, the likelihood of exposure to a stressor may be low, but the effect resulting from the exposure would be devastating. For example, large oil spills may not be common, but they can cause severe and extensive effects in ecologically sensitive areas.

Spatial and Temporal Patterns of the Effects

The spatial and temporal distributions of the effect provide another perspective important to interpreting ecological significance. The extent of the area where the stressor is likely to occur is a primary consideration when evaluating the spatial pattern of effects. Clearly, a stressor distributed over a larger area has a greater potential to affect more organisms than one confined to a small area. However, a stressor that adversely affects small areas can have devastating effects if those areas provide critical resources for certain species. In addition, adverse effects to a resource that is small in scale (e.g., acidic bogs) may have a small spatial effect but may represent a significant degradation of the resource because of its overall scarcity.

The duration of any effect is dependent on the persistence of the stressor as well as how often the stressor is likely to occur in the environment. It is important to remember that even short-term effects can be devastating if such exposure occurs during critical life stages of organisms.

Recovery Potential

A discussion of the recovery potential may be an integral part of risk description, although the need for such an evaluation will depend on the objective of the assessment and the assessment endpoints. An evaluation of the recovery potential may require additional analyses, as discussed in Section 3.1., and will depend on the nature, duration, and extent of the stressor.

Depending on the assessment objectives, all of the above factors may be used to place the risks into the broader ecological context. This discussion may consider the ramifications of the effects on other ecological components that were not specifically addressed in the assessment. For example, an assessment that focused on the decline of alligator populations may include a discussion of the broader ecological role of the alligator, such as the construction of wallows that act as water reservoirs during droughts. In this way, the potential effects on the community that depends on the alligator wallows can be brought out in risk characterization.

4.3. DISCUSSION BETWEEN THE RISK ASSESSOR AND RISK MANAGER (RESULTS)

Risk characterization concludes the risk assessment process and provides the basis for discussions between the risk assessor and risk manager that pave the way for regulatory decision-making. The purpose of these discussions is to ensure that the results of the risk assessment are clearly and fully presented and to provide an opportunity for the risk manager to ask for any necessary clarification. Proper presentation of the risk assessment is essential to reduce the chance of over- or under-interpretation of the results. To permit the risk manager to evaluate the full range of possibilities contained in the risk assessment, it is important that the risk assessor provide the following types of information:

- the goal of the risk assessment;
- the connection between the measurement and assessment endpoints;
- the magnitude and extent of the effect, including spatial and temporal considerations and, if possible, recovery potential;
- the assumptions used and the uncertainties encountered during the risk assessment;
- a summary profile of the degrees of risk as well as a weight-of-evidence analysis; and
- the incremental risk from stressors other than those already under consideration (if possible).

The results of the risk assessment serve as input to the risk management process, where they are used along with other inputs defined in EPA statutes, such as social and economic concerns, to evaluate risk management options.

In addition, based on the discussions between the risk assessor and risk manager, follow-up activities to the risk assessment may be identified, including monitoring, studies to verify the predictions of the risk assessment, or the collection of additional data to reduce the uncertainties in the risk assessment. While a detailed discussion of the risk management process is beyond the scope of this report, consideration of the basic principles of ecological risk assessment described here will contribute to a final product that is both credible and germane to the needs of the risk manager.

Additional Issues Related to the Risk Characterization Phase

- Predicting the time required for an ecological component to recover from a stressor.
- Combining chemical and nonchemical stressors in risk characterization.
- Incorporating critical effect levels into risk characterization.
- Better quantification of uncertainty.
- Developing alternative techniques for expressing uncertainty in risk characterization.

5. KEY TERMS

- Assessment endpoint** — An explicit expression of the environmental value that is to be protected.
- Characterization of ecological effects** — A portion of the analysis phase of ecological risk assessment that evaluates the ability of a stressor to cause adverse effects under a particular set of circumstances.
- Characterization of exposure** — A portion of the analysis phase of ecological risk assessment that evaluates the interaction of the stressor with one or more ecological components. Exposure can be expressed as co-occurrence, or contact depending on the stressor and ecological component involved.
- Community** — An assemblage of populations of different species within a specified location in space and time.
- Conceptual model** — The conceptual model describes a series of working hypotheses of how the stressor might affect ecological components. The conceptual model also describes the ecosystem potentially at risk, the relationship between measurement and assessment endpoints, and exposure scenarios.
- Direct effect** — An effect where the stressor acts on the ecological component of interest itself, not through effects on other components of the ecosystem (compare with definition for indirect effect).
- Ecological component** — Any part of an ecological system, including individuals, populations, communities, and the ecosystem itself.
- Ecological risk assessment** — The process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to more or more stressors.
- Ecosystem** — The biotic community and abiotic environment within a specified location in space and time.
- Exposure** — Co-occurrence of or contact between a stressor and an ecological component.
- Exposure profile** — The product of characterization of exposure in the analysis phase of ecological risk assessment. The exposure profile summarizes the magnitude and spatial and temporal patterns of exposure for the scenarios described in the conceptual model.
- Exposure scenario** — A set of assumptions concerning how an exposure may take place, including assumptions about the exposure setting, stressor characteristics, and activities that may lead to exposure.
- Indirect effect** — An effect where the stressor acts on supporting components of the ecosystem, which in turn have an effect on the ecological component of interest.
- Measurement endpoint** — A measurable ecological characteristic that is related to the valued characteristic chosen as the assessment endpoint. Measurement endpoints are often expressed as the statistical or arithmetic summaries of the observations that comprise the measurement.
- Median lethal concentration (LC₅₀)** — A statistically or graphically estimated concentration that is expected to be lethal to 50% of a group of organisms under specified conditions (ASTM, 1990).
- No observed effect level (NOEL)** — The highest level of a stressor evaluated in a test that does not cause statistically significant differences from the controls.
- Population** — An aggregate of individuals of a species within a specified location in space and time.
- Recovery** — The partial or full return of a population or community to a condition that existed before the introduction of the stressor.

Risk characterization — A phase of ecological risk assessment that integrates the results of the exposure and ecological effects analyses to evaluate the likelihood of adverse ecological effects associated with exposure to a stressor. The ecological significance of the adverse effects is discussed, including consideration of the types and magnitudes of the effects, their spatial and temporal patterns, and the likelihood of recovery.

Stressor — Any physical, chemical, or biological entity that can induce an adverse response.

Stressor-response profile — The product of characterization of ecological effects in the analysis phase of ecological risk assessment. The stressor-response profile summarizes the data on the effects of a stressor and the relationship of the data to the assessment endpoint.

Trophic levels — A functional classification of taxa within a community that is based on feeding relationships (e.g., aquatic and terrestrial green plants comprise the first trophic level and herbivores comprise the second).

Xenobiotic — A chemical or other stressor that does not occur naturally in the environment. Xenobiotics occur as a result of anthropogenic activities such as the application of pesticides and the discharge of industrial chemicals to air, land, or water.

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CHAPTER 1

Introduction to Environmental Toxicology

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 the ecosystem. The broad scope of environmental toxicology requires a multidisciplinary approach of a variety of specialists.

ENVIRONMENTAL TOXICOLOGY AS AN INTERDISCIPLINARY SCIENCE

Environmental toxicology takes and assimilates from a variety of disciplines. Terrestrial and aquatic ecologists, chemists, molecular biologists, geneticists, and mathematicians are all 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 also can 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 may 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.

Environmental Toxicology and Some of its Components

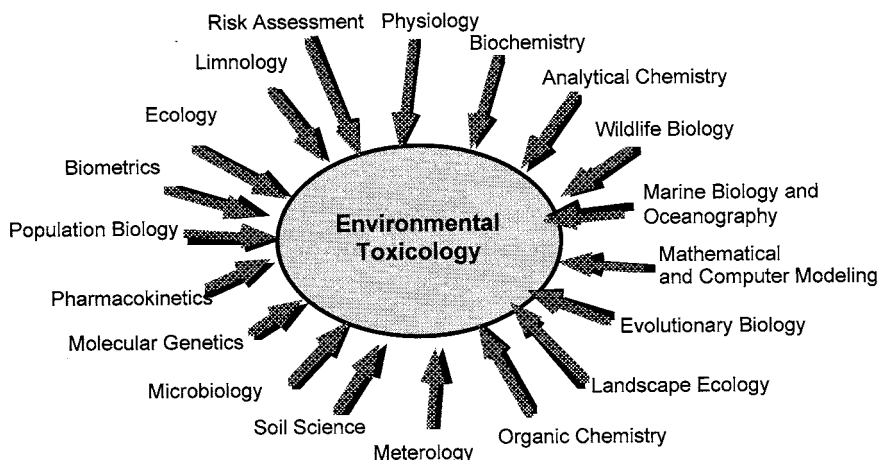


Figure 1.1 The components of environmental toxicology. Environmental toxicology borrows heavily from a variety of scientific disciplines. The very nature of the field is multidisciplinary, making a basic knowledge of the basics of biology, chemistry, mathematics, and physics essential.

Of increasing importance to the field is 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 cross many biological and physical scales. New tools will lead to new insights into the interaction of chemicals with ecological structures.

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

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 is necessary. Simulation models are generally detailed requiring complex computations and not mathematically tractable for simplification.

Strategic Models are usually simple and mathematically tractable and designed to explore basic principals, but not designed to mimic a particular population or environment. Such models include the logistic equation, simple growth models, and competition equations. Although the models may be simple, complex dynamics yielding intense discussion can result.

Testable Models of laboratory or field data are the final broad class of models. They involve the derivation of specific testable predictions. Good theory should lead to models of this type. Throughout this book it should be recognized that many of the topics covered are tactical, strategic, or testable models of reality.

A BRIEF HISTORY AND ORGANIZATIONS IN ENVIRONMENTAL TOXICOLOGY

As a discipline, environmental toxicology is relatively new. In 1991, the 15th Annual Symposium sponsored by the American Society for Testing and Materials and the 12th Annual Meeting sponsored by the Society of Environmental Toxicology and Chemistry on environmental toxicology were held. Of a rapidly evolving field, this text is only a snapshot of the directions and research of the late 1980s and early 1990s. The science evolved from the efficacy testing of pesticides in the 1940s to the cleanup of burning rivers, polluted lakes, and wildlife kills of 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 worldwide. The Organization for Economic Cooperation and Development serves a similar role in Europe. In 1979 the Society for Environmental Toxicology and Chemistry (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, 2230 scientists and policymakers attended in Seattle, and 3000 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 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.

LEGISLATION

Unlike basic research, environmental toxicology often has been 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 and amended in 1976 (33 USC Sections 1251 to 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 is 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, commonly referred to as 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 into 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 manmade 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 (1976, 42 USC Sections 2601 to 2629), referred to as TSCA, 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 but 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 manufacturer. The toxicity tests typically involve a single-species approach.

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 the 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.

Although the federal 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.

INTRODUCTION TO THE TEXTBOOK

The purpose of this volume is to provide the background knowledge so that the short- and long-term effects of chemical pollution can be evaluated and the risks understood. There are 10 more chapters, each with a specific building block towards the understanding of the status of the field of environmental toxicology.

The next chapter, A Framework 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, its effect upon the site of action, and finally the impacts upon an ecosystem. Many of the terms used throughout the 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 called 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 are 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. Particular attention is paid to sediment tests since these are currently the focus of a

great deal of attention. 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 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 understanding 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. Estrogen mimics and the possible modes of action of these materials are discussed with particular emphasis on dioxins and the polychlorinated biphenyls (PCBs). 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 (QSAR), 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 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 6 (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.

Many of the examples used in the preceding chapters emphasize organic pollutants. However, inorganic materials comprise an important class of contaminants. Chapter 7 (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 8 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 run-off, 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 9 (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 also may yield strategies for the reduction or elimination of xenobiotics.

One of the major sections of the textbook is the chapter dealing with the response of various ecological systems to the stress of toxicants. Chapter 10 (Measurement and Interpretation of the Ecological Effects of Toxicants) deals with broad categories of responses to toxicants, as well as specific examples. Biomonitoring and biomonitoring strategies also are discussed. As this text is written, several new, exciting, and controversial ideas about the nature of complex systems, chaos, and the interactions with communities 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. Also introduced in this chapter is the background of metapopulation dynamics which may provide a theory for dealing with contamination in heterogeneous environments.

The discipline that ties together environmental toxicology is that of risk assessment. Chapter 11 (Ecological Risk Assessment) provides a framework for the integration of the classical toxicology at the molecular and organismal levels and the prediction of events at the level of the community and ecosystem. Exciting research is currently underway examining the importance of indirect effects, landscape and global changes, and management of these risks. In Chapter 10 we review the current paradigm of the United States Environmental Protection Agency. Particularly interesting is a review of approaches for dealing with regional risk assessments and their potential for the management of ecological structures.

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

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A Framework for Environmental Toxicology

Environmental toxicology can be simplified to the understanding of only three functions. These functions are presented in [Figure 2.1](#). First, there is the interaction of the introduced chemical, 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 a suitable understanding of these functions. The remainder of the chapter introduces the critical factors for each of these functions. Unfortunately, we do not clearly understand how the impacts seen at the population and community levels are propagated from molecular interactions.

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 levels of biological interaction with a xenobiotic:

Chemical Physical-Chemical Characteristics
Bioaccumulation/Biotransformation/Biodegradation
Site of Action

Biochemical Monitoring
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 2.2. Examples of these factors at each level of biological organization are given below.

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 quantitative structure-activity relationships (QSAR). QSAR has the potential of enabling 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 byproduct 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.

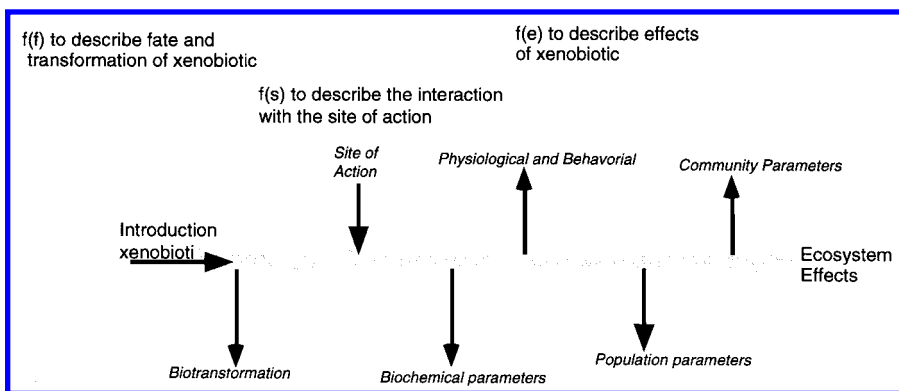


Figure 2.1 The three functions of environmental toxicology. Only three basic functions need to be described after the introduction of a xenobiotic into the environment. The first describes the fate and distribution of the material in the biosphere and the organism after the initial release to the environment ($f(f)$). 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 ($f(e)$).

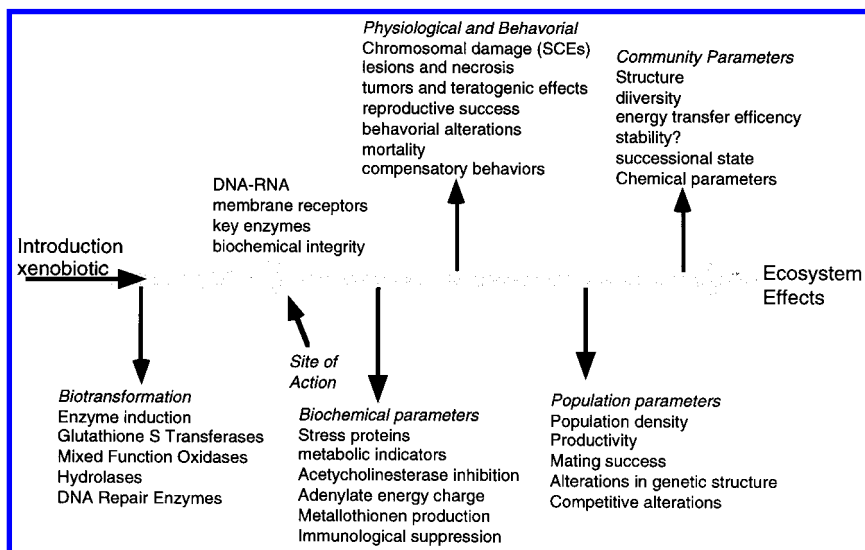


Figure 2.2 Parameters and indications of the interaction of a xenobiotic with the ecosystem. The examples listed are only a selection of the parameters that need to be understood for the explanation of the effects of a xenobiotic upon an ecosystem. However, biological systems appear to be organized within a hierarchy and that is how environmental toxicology must frame its outlook upon environmental problems.

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.

Receptor 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.

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 trisomys 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 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 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 in assessing the health of an ecosystem at the population level.

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 are those that are tumorigenic. Tumors in fish, especially flatfish, have been extensively studied as indicators of oncogenic materials in marine sediments. Oncogenesis also has been extensively studied in Medaka and trout as 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 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 monitor continually the water quality as perceived by the test organisms with a desktop computer analysis system at a relatively low cost.

Population Parameters

A variety of endpoints have been used, including number and structure of a population, to indicate stress. Population numbers or density have 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, i.e., 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 *Drosophilla*. 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. Although with 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 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.

Community Effects

The structure of biological communities has always been a commonly used indicator of stress in a biological 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 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 indexes 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 indexes, 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. May's work in the early 1970s 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 are based upon human constructs. The members of the marine benthic invertebrate community interact with many other types of organisms, microorganisms, vertebrates, and protists that in many ways determine the diversity and persistence of an organism. Communities also can be defined as functional groups, such as the intertidal community or alpine forest community, that may more accurately describe functional groupings of organisms.

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.

AN ALTERNATIVE FRAMEWORK INCORPORATING COMPLEXITY THEORY

The framework presented above 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 recently 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 risk assessment ([Figure 2.3](#)).

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

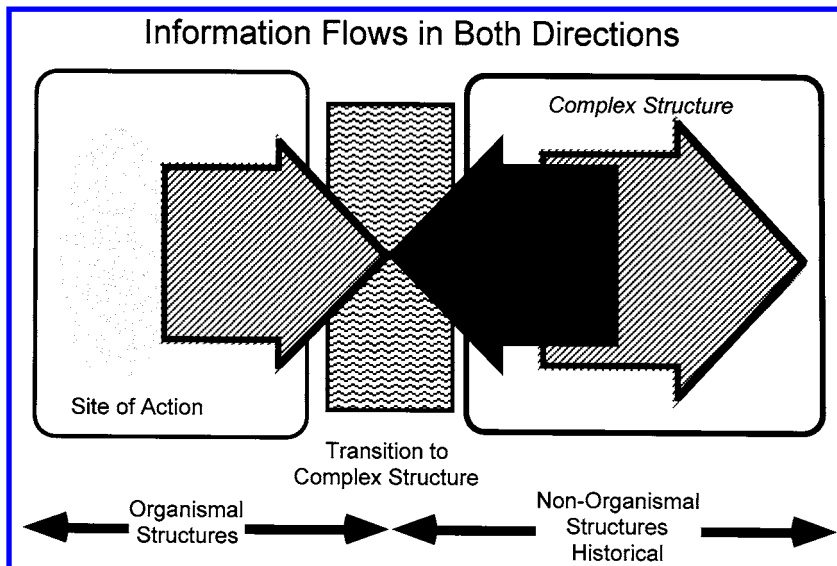


Figure 2.3 Organismal and nonorganismal framework. As the information is passed on to the complex structure, it becomes part of the history of the ecosystem.

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 means that the history of past events is written into the structure and dynamics. The many nonlinear dynamics and historical nature of ecosystems confer upon the system the property of complexity.

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

1. Complex structures are neither completely deterministic or 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.

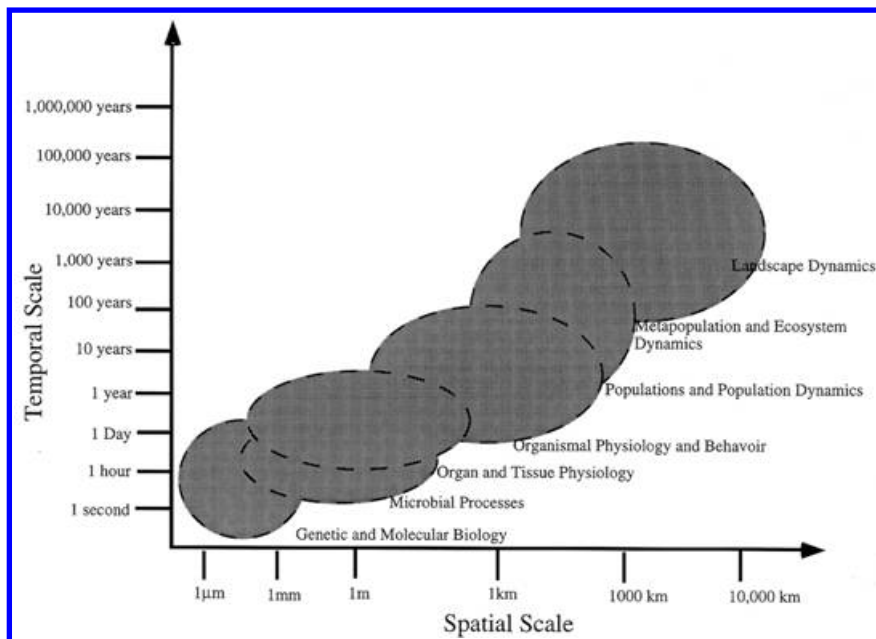


Figure 2.4 The overlap of spatial and temporal scales in environmental toxicology. Not only are there scales in organization, but scales over space and time exist. Many molecular activities exist over short periods and volumes. Populations can exist over relatively small areas, even a few square meters for microorganisms, thousands of square kilometers for many bird and mammal populations. Although often diagrammed as discrete, each of these levels are intimately connected and phase one into another along both the space and time scales.

SPATIAL AND TEMPORAL SCALES

Not only are there scales in organization, but 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 range from the few angstroms of molecular interactions to the hundreds of thousands of square kilometers affected by large-scale events. Figure 2.4 presents some of the organizational aspects of ecological systems with their corresponding temporal and spatial scale. 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.

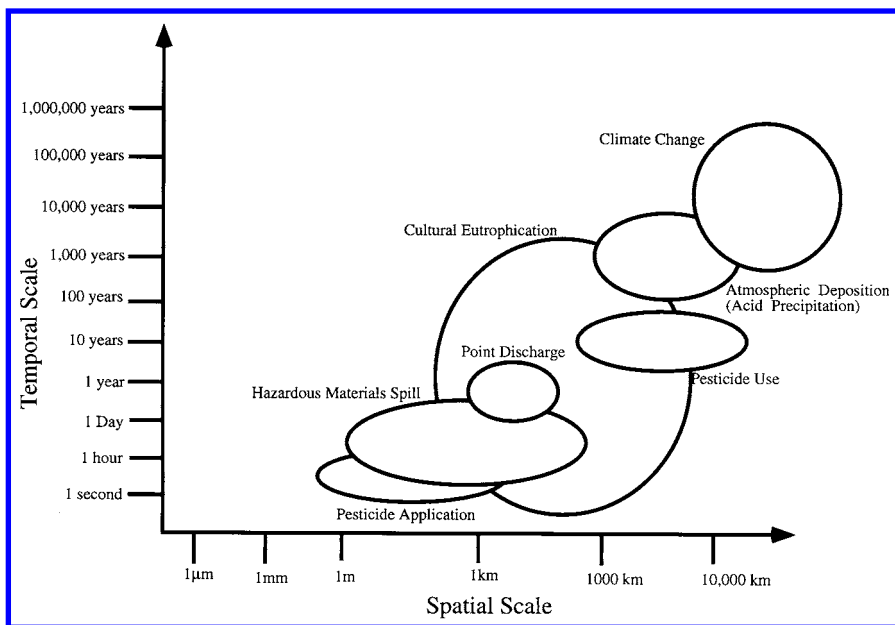


Figure 2.5 The overlap of spatial and temporal scales in chemical contamination. Just as there are scales of ecological processes, contamination events also range in scale. Pesticide applications can range from small-scale household use to large-scale agricultural applications. The addition of surplus nutrients and other materials due to agriculture or human habitation is generally large scale and long lived. Acid precipitation generated by the tall stacks in the midwestern United States is a fairly recent phenomena, but the effects will likely be long term. However, each of these events has molecular scale interactions.

Perhaps the most important example of a new biochemical pathway generating a global impact was the development of photosynthesis. The atmosphere of Earth originally 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.

Effects at the community and ecosystem level conversely 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 even increase due to its rescue from a neighboring population. If the structure of the ecological landscape provides few opportunities for rescue, localized extinctions would be more likely.

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 2.5). 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.

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STUDY QUESTIONS

1. Define the three functions to be understood to simplify environmental toxicology.
2. Define QSAR.
3. Define bioaccumulation, biotransformation, and biodegradation.
4. What is “site of action”?
5. Describe limits to the use of DNA alteration as an indicator of genotoxic materials.
6. Describe immunological suppression.
7. Name three major physiological indicators of impact by a xenobiotic on a population.
8. Describe a problem with using population parameters to indicate xenobiotic challenge.
9. Name two means by which a xenobiotic can alter competitive abilities of organisms.
10. What are the most dramatic impacts observable on ecosystems by xenobiotics?
11. Is the arrow describing the interactions of the ecological system with a chemical pollutant unidirectional?
12. In what ways are organisms simple structures?
13. What are the characteristics of complex structures?
14. If ecosystems are complex structures, can they be in equilibrium?
15. What are the disadvantages and advantages to the organismal/nonorganismal model compared to the conventional model?
16. Characterize ecological functions and processes by temporal and spatial scale.
17. What are the interactions between the scale of a chemical contamination and that of the affected ecological system?

An Introduction to Toxicity Testing

Toxicity is the property or properties of a material that produces 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. Anthropogenically derived compounds can be produced in the millions of pounds 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 run-offs, contaminated soils and aquatic sediments, atmospheric deposition, and urban run-off 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 rates and the 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 are the pesticides. Pesticide can refer to any compound that exhibits toxicity to an undesirable organism. Since the biochemistry and physiology of all organisms are linked by the stochastic processes of evolution, 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 also are released into 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 normally

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 dose and the 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, characteristics of the organisms 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.

THE DOSE-RESPONSE 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 that of a normal distribution or a bell-shaped curve (Figure 3.2). This distribution is not surprising. 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 normal distribution.

The distribution of mortality vs. 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

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
Compound 1									
Cumulative toxicity	0.0	2.0	7.0	23.0	78.0	92.0	97.0	100.0	100.0
Percent 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 endpoints at a concentration of 2 mg/kg, then the percentage responding would be 7%.

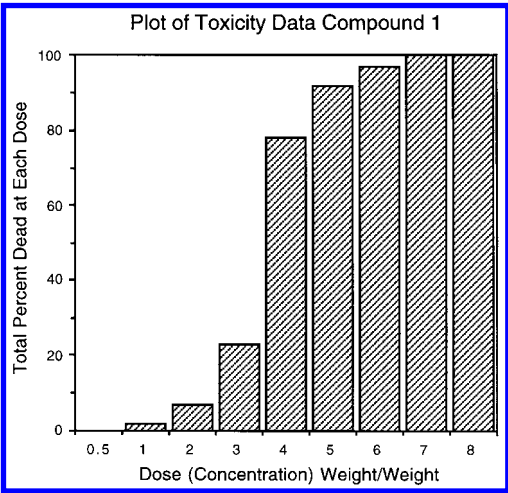


Figure 3.1 Plot of cumulative mortality vs. environmental concentration or dose. The data are plotted as cumulative number of dead by each dose using the data presented in [Table 3.1](#). The x-axis is in units of weight to volume (concentration) or weight of toxicant per unit weight of animal (dose).

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

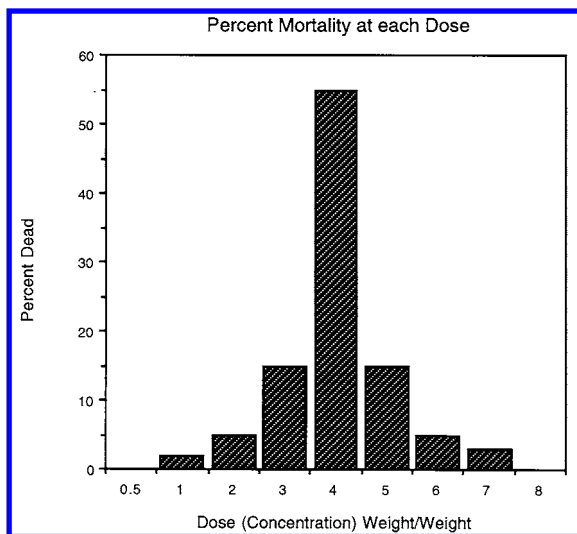


Figure 3.2 Plot of mortality vs. environmental concentration or dose. Not surprisingly, the distribution that results is that of a normal distribution or a bell-shaped curve. This distribution is not surprising. Responses or traits from organisms that are controlled by numerous sets of genes follow bell-shaped curves. Length, coat color, and fecundity are examples of multigenic traits whose distribution result 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).

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 apparent that the midpoint for compound 2 will likely be higher than that of 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 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 reported 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 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

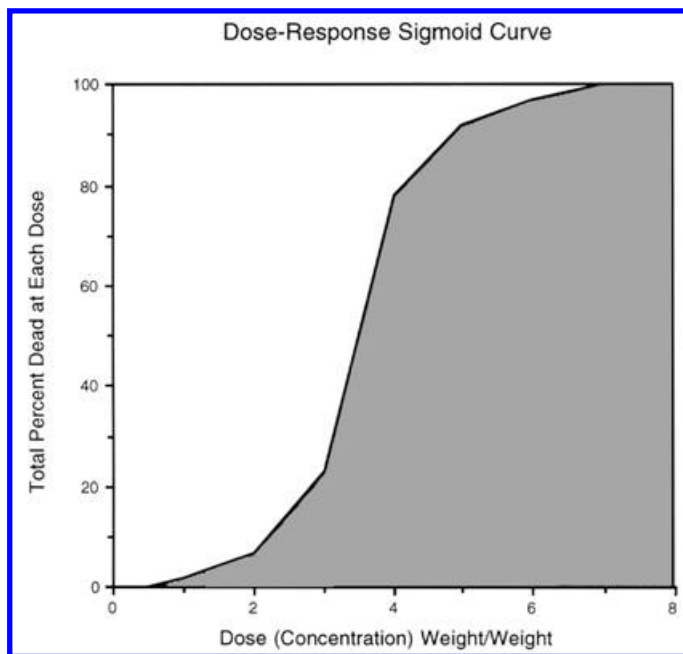


Figure 3.3 The sigmoid dose-response curve. Converted from the discontinuous bar graph of Figure 3.2 to a line graph. If mortality is a continuous function of the toxicant, the result is the typical sigmoid dose-response curve. The x-axis is in units of weight to volume (concentration) or weight of toxicant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

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 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 additional deaths at each concentration	0.0	5.0	10.0	15.0	40.0	15.0	10.0	5.0	0.0

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-h test simply because the concentration in the tissue has not reached toxic levels within the specified testing time. L. McCarty has written extensively on this topic

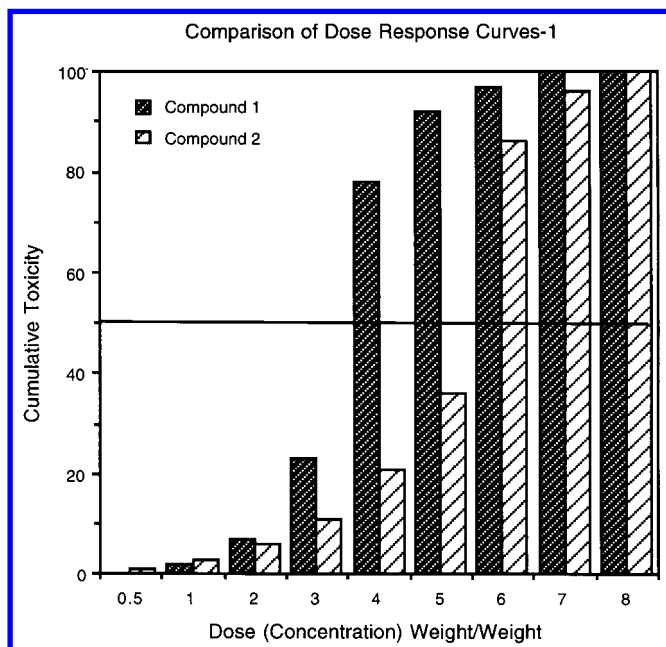


Figure 3.4 Comparison of dose-response curves-1. One of the primary goals of toxicity testing is the comparison or ranking of toxicity. The cumulative plots comparing compound 1 and compound 2 demonstrate the distinct nature of the two different toxicity curves.

and suggests that a “Lethal Body Burden” or some other measurement be used to reflect tissue concentrations. These ideas are discussed in a later chapter.

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 graphical or statistical methods.

NOEL — No observed effects level determined by graphical or statistical methods. This parameter is reported as a dose.

NOAEC — No observed adverse effects concentration determined by graphical or statistical methods. The effect is usually chosen for its impact upon the species tested.

NOAEL — No observed adverse effects level determined by graphical or statistical methods.

LOEC — Lowest observed effects concentration determined by graphical or statistical methods.

LOEL — Lowest observed effects level determined by graphical or statistical methods.

MTC — Minimum threshold concentration determined by graphical or statistical methods.

MATC — Maximum allowable toxicant concentration determined by graphical or statistical methods.

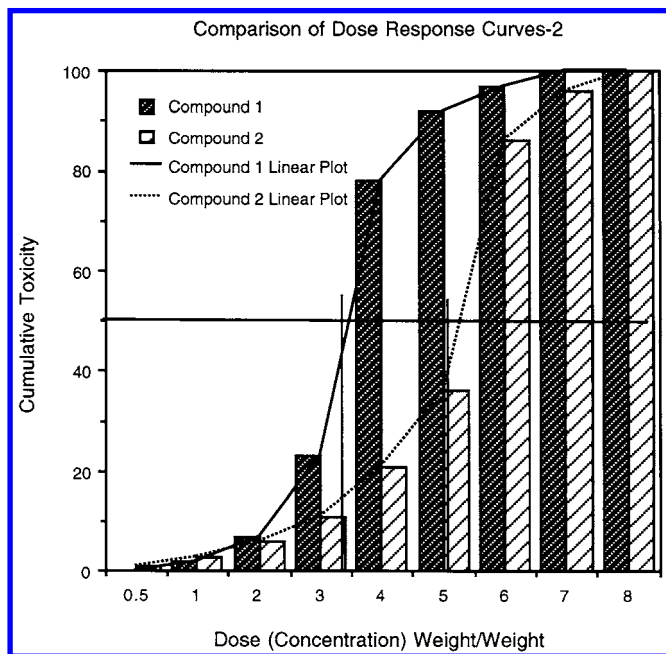


Figure 3.5 Comparison of dose-response curves-2. Plotting the dose-response curve demonstrates that the concentrations that cause mortality in 50% of the population are distinctly different. However, the slopes of the two curves appear to be the same. In many cases this may indicate that the compounds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

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.

Given the difficulty of determining these endpoints, great caution should be taken when using these parameters. An implicit assumption of these endpoints is that there is a threshold concentration or dose. That is, 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 (Figure 3.8). In some cases biological effects occur at succeeding lower concentrations until the chemical is removed from the environment. There is much debate about which model of dose vs. effects is more accurate and useful.

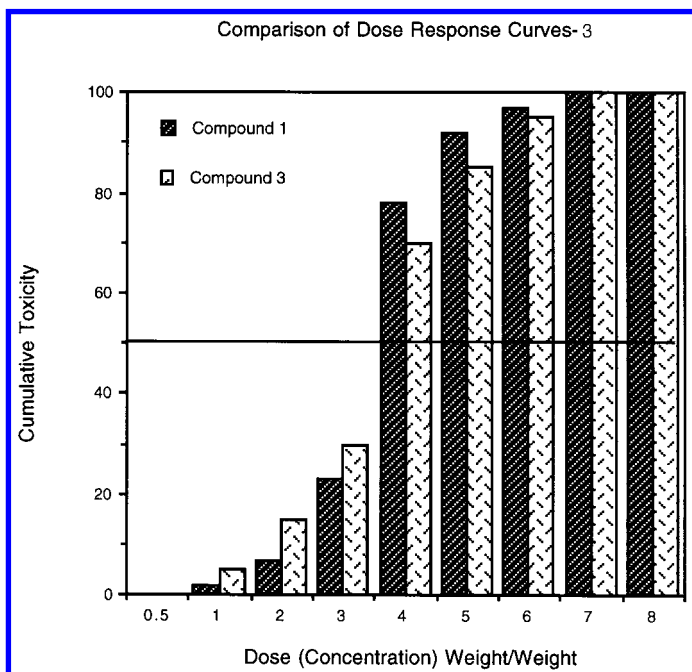


Figure 3.6 Comparison of dose-response curves-3. Cumulative toxicity plots for compounds 1 and 3. Notice that the plots intersect at roughly 50% mortality.

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 and Development (OECD), the National Toxicology Program (NTP), and are available as United States Environmental Protection Agency publications, the *Federal Register*, and often from the researchers that developed the standard methodology.

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, such as:

- Uniformity and comparability of test results.

- Allows replication of the result by other laboratories.

- Provides criteria as to the suitability of the test data for decisionmaking.

- Logistics are simplified, little or no developmental work.

- Data can be compiled with that of other laboratories for use when large data sets are required. Examples are quantitative structure activity research and risk assessment.

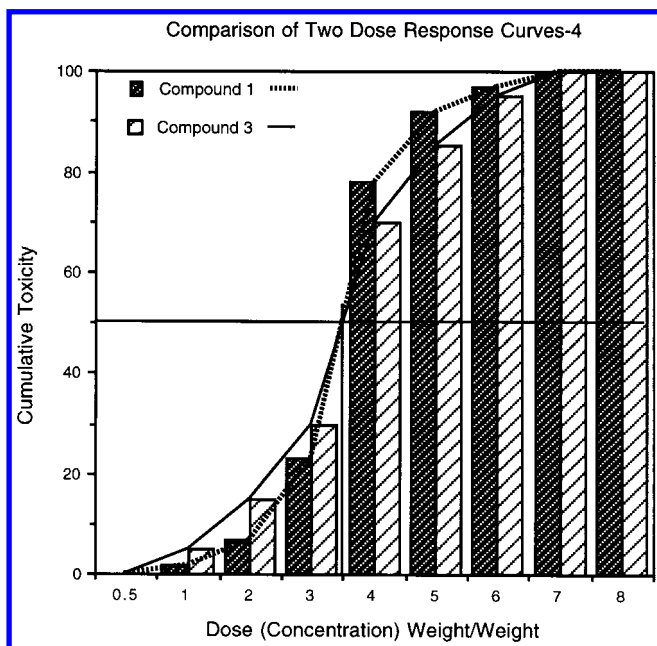


Figure 3.7 Comparison of dose-response curves-4. Although the mid-points of the curves for compounds 1 and 3 are the same, compound 3 is more toxic at low concentrations more typical of exposure in the environment.

The method establishes a defined baseline from which modifications can be made to answer specific research questions.

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:

The scope of the method or guide is identified.

Reference documents, terminology specific to the standards organization, a summary, and the utility of the methodology are listed and discussed.

Hazards and recommended safeguards are now routinely listed.

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.

Specifications for the material undergoing testing are provided.

Test organisms are listed along with criteria for health, size, and sources.

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.

Analytical methodologies for making the measurements during the experiment are often given a separate listing.

Acceptability criteria are listed by which to judge the reliability of the toxicity test. Methods for the calculation of results are listed. Often several methods of determining the EC_{50} , LD_{50} or NOEL are referenced.

Specifications are listed for the documentation of the results.

Appendixes are often added to provide specifics for particular species or strains of animals and the alterations to the basic protocol to accommodate these organisms.

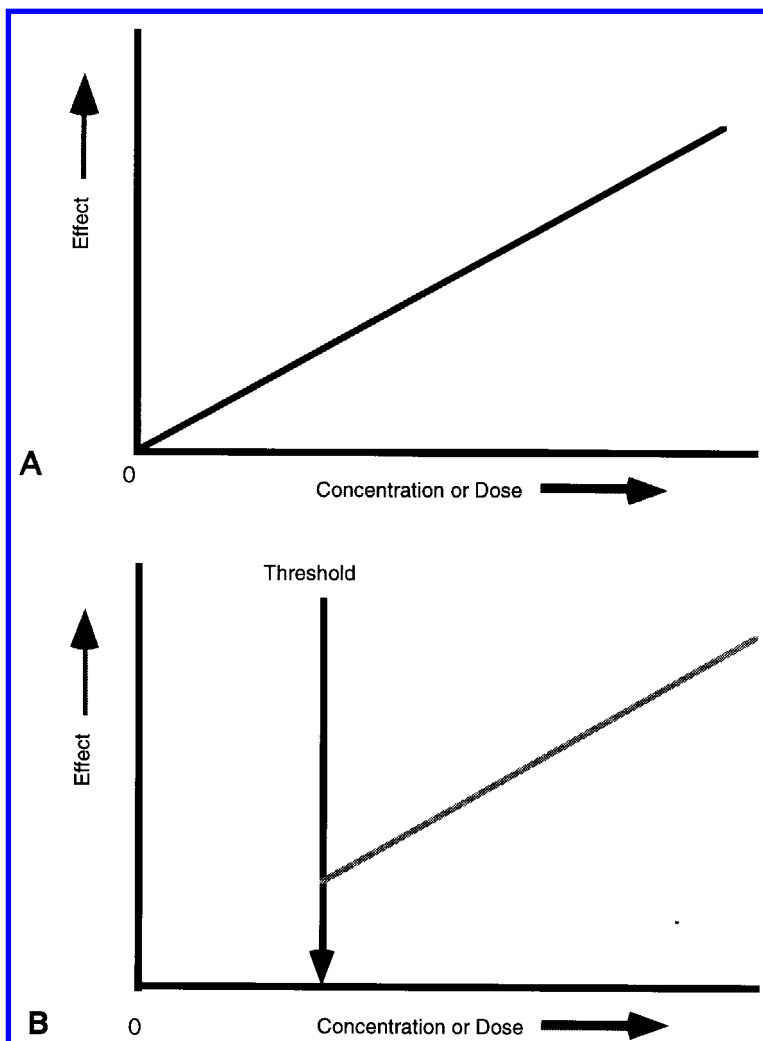


Figure 3.8 Threshold concentration. There are two prevailing ideas on the toxicity of compounds at low concentrations. Often it is presumed that a compound has a toxic effect as long as any amount of the compound is available to the organism (A). Only at zero concentration will the effect disappear. The other prevailing idea is that a threshold dose exists below which the compound is present but no effects can be discerned (B). There is a great deal of debate about which model is accurate.

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

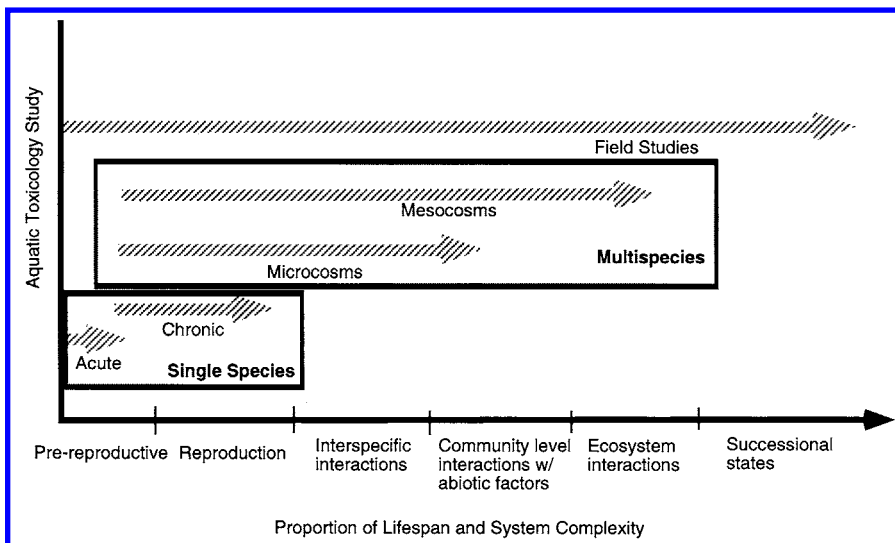


Figure 3.9 Classification of toxicity tests in environmental toxicology. Generally the two parameters involved are the length of the test relative to the test organism and the species composition of the test system.

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.

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, daphnia, rats, and birds, periods of 24 to 48 h have been used. Even in the case of the short-lived *Daphnia magna*, a 48-h 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 toxicity tests of similar periods of time using bacteria, protists, and algae also constitute acute toxicity tests. Many bacteria can divide in less than 1 h under optimal conditions. Most protists and algae are capable of undergoing binary fission in less than a 24-h period. A 24-h 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) for males a significant portion of the time for spermatogenesis. 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 aspects of population dynamics such as predator-prey interactions and competition are a goal of these tests. Usually these tests are called microcosm or small cosmos toxicity tests. There is no clear definition of what volume, acreage, or other measure of size constitute a microcosm. A larger microcosm is a mesocosm. Mesocosms usually, but not always, have more trophic levels and generally 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 enable a look at structural and functional dynamics that are generally thought of as ecosystem level. Unfortunately, one man's mesocosm is another person's microcosm, making classification difficult. The types of multispecies comparisons 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 also are affected by the temporal, spatial, and evolutionary heterogeneities 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.

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 advantages of being simpler and 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 test. 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 to ensuring a 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 diluters used to ensure accurate delivery of various toxicant concentrations to the test chambers: the venturi and the 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 vacuum 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 advantage of being easy to set up and transport and often they are 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.

Exposure Scenarios

In aquatic test systems, exposure is usually a whole-body exposure. That means that the toxicant can enter the organism through the skin, cell wall, respiratory system (gills, stomata), and digestive system. 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 into 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 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 a food or water to ensure toxicant delivery.

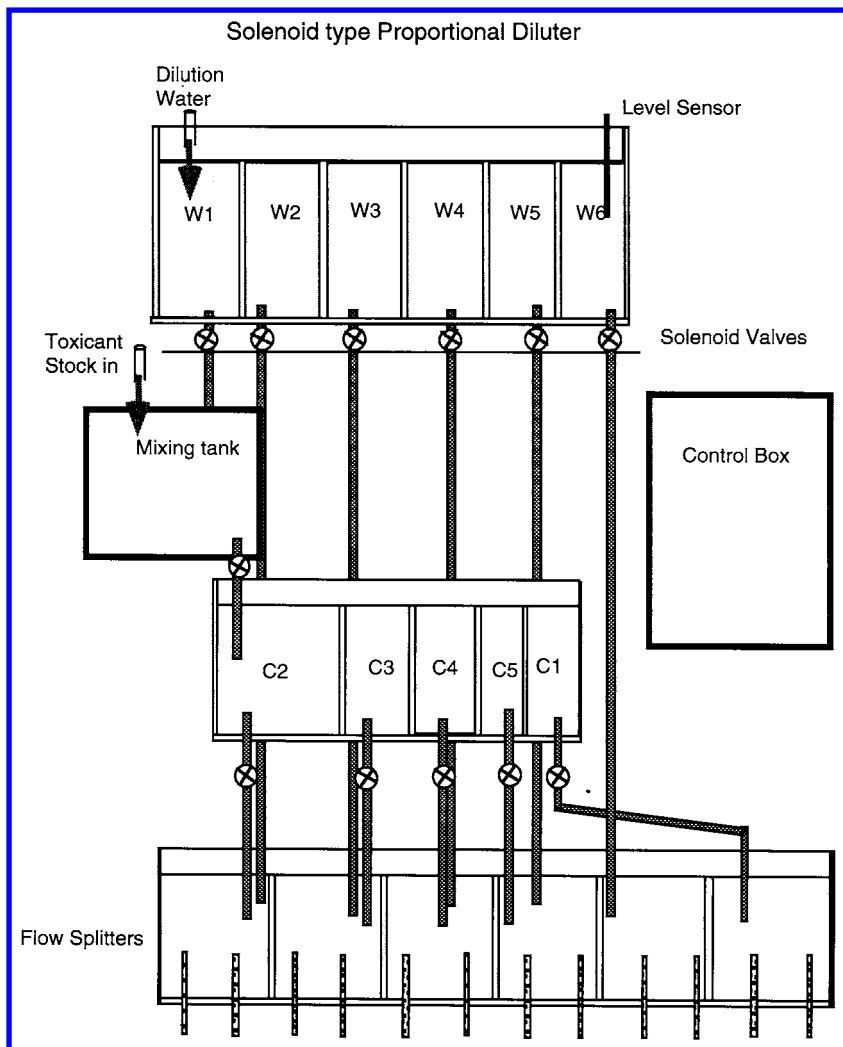


Figure 3.10 Schematic of a proportional diluter with flow controlled by solenoid valves. This mechanism ensures that an accurate concentration of the test material is reliably introduced to the test organisms at a specified rate.

Unfortunately, many compounds are not palatable and the test organisms quickly cease to eat.

Other routes of exposure include inhalation exposure for atmospherically 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 airtight 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.

Plant-, 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 to 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 performed with contaminated sediments or with a material added to a 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 bare 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 particulates 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.

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, phyla.

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 strains of 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, the cultures often originated from collections and the genetic relationships to the organisms used by other laboratories is 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 over the past 10 years, yet fewer than a thousand compounds have been examined. In contrast, the acute toxicity of over 2000 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 Suter (1993) in a recent review. 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. 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.

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 are teleost

fish. However, the *Daphnia* also fit the pattern. The exceptions about 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 10. 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. Though, 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.

Statistical Design Parameters

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(s) 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 also is 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 work load. 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 differentiate accurately differences in populations. The usual case in toxicity testing is the comparison of a treatment group to 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 number of test vessels or field samples required. Upon full consideration it may be necessary to rephrase the question or use another test methodology.

OVERVIEW OF AVAILABLE STATISTICAL METHODS FOR THE EVALUATION OF SINGLE SPECIES TOXICITY TESTS

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.

Commonly Used Methods for the Calculation of Endpoints

As reviewed by 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 essentially is the plotting of the dose-response curve and reading the concentration that corresponds to the LC_{50} or the 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, illuminating 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.

The probit method is perhaps the most widely used method for calculating toxicity vs. concentration. As its name implies, the method used a probit transformation of the data. 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, provide 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 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-Kärber 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

Table 3.3 Estimates of LD₅₀ 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)

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.

**Comparison of Calculations of Several Programs
for Calculating Probit Analysis**

Each of the methods for the estimation of an LC₅₀ 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 that 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 dataset was constructed using a normal distribution with a mean (LD₅₀) 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 equal to 8 and a standard deviation equal to 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. First, that the response was normal with regard to the dosage. Second, 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 LC₅₀. SAS log 10 and the ASTM-PROBIT were consistently identical in the calculated values of the LD₅₀s 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 is based on the log 10 of the dose, the SAS default overestimated the LD₅₀ 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 conclude that these methods are not robust to departures from the underlying assumptions about the response distributions.

Roberts made a comparison between several commonly available programs used to calculate probit estimates of LD₅₀s. These programs were

DULUTH-TOX, written by C. Stephan of the Duluth Environmental Protection Agency's Environmental Research Laboratory, was used to calculate toxicity end-points.

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

UG-PROBIT was developed by the Department of Mathematics and Statistics and the University of Guelph, Canada.

SPSSx-PROBIT is a part of the SPSSx statistical program available commercially and on many mainframes of universities and industry.

SAS-PROBIT is analogous to the SPSSx-PROBIT in that it is part of a widely available SAS statistical package.

After an extensive analysis, Roberts concluded that most of the programs provided useful and comparable LC₅₀ 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-PROBIT incorporated statistical tests to examine the data to assure that the assumptions of the probit calculations were met.

Data Analysis for Chronic Toxicity Tests

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. Transformation of the data.
2. Testing for equivalence of the control or not dosed treatment with the carrier control.
3. Analysis of variance performed on the treatment groups.
4. 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 a 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 comparisons with a statistically significant result will appear 5 times out of 100 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 (LOEC). The concentration that demonstrates no difference from the control is the no observed effects concentration (NOEC). 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 are 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 the derived MATC. The closer the spacing and the more concentrations used, the more accurate are these derived parameters.

Gelber et al. suggest that a major improvement can be made in the analysis of chronic toxicity tests. They suggest that Williams' 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,

Regression after a Log Transformation Test Example Number 3

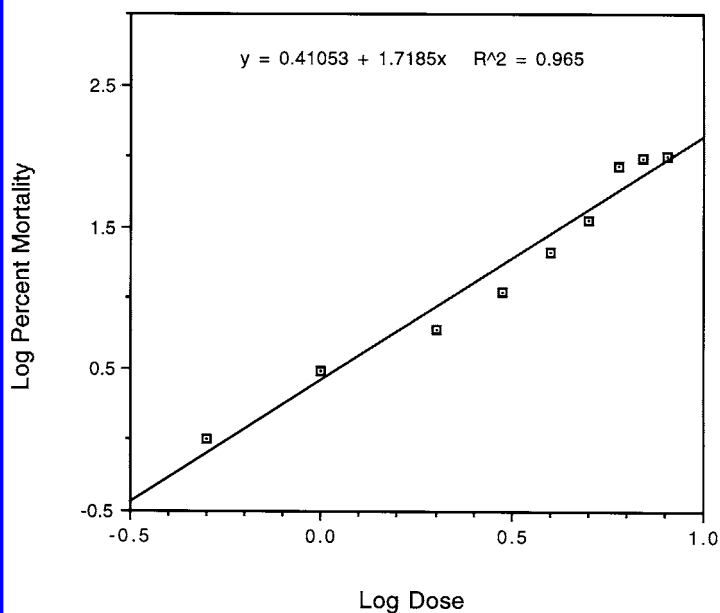


Figure 3.11 Plot of a log-log regression for toxicity data set 3.

since performing both the ANOVA and the multiple comparison tests both have a 5% error rate. They suggest performing multiple Williams' tests to arrive at the concentration that is not significantly different from the control set.

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. When ranking compounds in 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_1 s 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 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) leaves the β value unconstrained and skews the importance of reporting the toxic result. In other words, the typical test will be conservative on the side of saying there is no 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.

Given these disadvantages, hypothesis testing is still the prevalent method of determining NOELs and is the preferred method stated in such publications as *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (Weber et al. 1989). However, there is an alternative.

Moore and Caux (1997) advocate the use of the EC_x approach. This approach reconstructs the concentration-response curve from a data set and uses a previously specified effects concentration as the endpoint. This approach is very similar to that advocated by Stephan and offers a variety of advantages. A program for the reconstructions has been prepared (Caux and Moore 1997) relieving the computational burden. There is now no reason not to use a regression-type approach.

THE DESIGN OF MULTISPECIES TOXICITY TESTS

Over the past 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 8400 l, and 18 outdoor freshwater microcosms ranging from 8 to 18,000,000 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, Matthews, and Matthews 1997) and the major points are summarized below.

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 trophic structure, although 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 being incorporated. 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 naturally synthesized 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 when 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 are one key to species richness, as in "The Paradox of the Plankton" (Hutchinson 1961). Environmental heterogeneity is key to the establishment of metapopulations, a key 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.

Data Analysis and Interpretation of Multispecies Toxicity Tests

A large number of data analysis methods have been used to examine the dynamics of these structures. 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 able to perform hypothesis testing on those patterns.

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 also are critical considering the large size and complexity of multispecies tests. However tempting, it is inappropriate to take several samples from the same microcosm sample and label them 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.

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 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 are 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 NOECs, 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.

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 10 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 also can 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 used 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 (DPC) uses 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 recently fashionable technique derived from a principal components approach is the coupling of PCA with redundancy analysis (RDA) (van der Brink et al. 1996, Van Breukelen et al. 1993). 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 ground work 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 a part of the 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 to conduct a sorting based on a predetermined set of rules. There are a variety of available techniques. The groupings then can 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 dataset 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.

Recently 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, 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 use of nonmetric clustering and other methods have been compared in a number of field and laboratory tests (Matthews and Hearne 1991; Matthews, Matthews, and Hachmoller 1991; Landis et al. 1993).

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 assets of NCAA is that it is nondimensional, nonmetric, and that 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 that it is computationally intensive and there is no assurance that a global maxima of clustering has been obtained. Furthermore, NCAA is not available as part of packaged statistical programs.

Visualization

Methods of visualization that are useful in interpreting the dynamics of ecological structures also are available. In the past numerous graphs of each variable were plotted over the course of the experiment 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 de Brink et al. 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 10.

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 as well as 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 because 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 are 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.

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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 of a dose-response curve which determine the 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 for the use of a standard method in 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 six criteria for choosing a test species for use in a toxicity test.
17. Discuss the natural source vs. laboratory-derived composition of species in multi-species 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 are the critical design considerations for multispecies toxicity tests?
21. Why are univariate toxicity tests not always appropriated for microcosm studies?
22. List the characteristics that microcosms have with naturally occurring ecosystems.
23. Why is spatial and temporal heterogeneity reduced in microcosm test systems?

Survey and Review of Typical Toxicity Test Methods

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 an EC_{50} or an NOEL 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. A more complete listing of toxicity tests and references for the methods are presented in Appendix A.

The following survey starts with single species toxicity tests and concludes 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 to some length the various types of toxicity (acute, chronic, partial life cycle, etc.), it is in many ways logical to list 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.

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 (K_{oc}) for an Organic Chemical in Soil and Sediments
Inhibition of Respiration in Microbial Cultures in the Activated Sludge Process
Algal Growth Potential Testing with <i>Selenastrum capricornutum</i>
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 <i>Daphnia magna</i>
Conducting Sediment Toxicity Tests with Freshwater Invertebrates
Conducting 10-Day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods
Conducting Static 96-h Toxicity Tests with Microalgae
Conducting Static Acute Aquatic Toxicity Screening Tests with the Mosquito, <i>Wyeomyia smithii</i> (Coquillett)
Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Mollusks
Conducting Static Toxicity Tests with the <i>Lemna gibba</i> G3
Conducting a Terrestrial Soil-Core Microcosm Test
Conducting Three-Brood, Renewal Toxicity Tests With <i>Ceriodaphnia dubia</i>
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

SINGLE SPECIES TOXICITY TESTS

Daphnia 48-H Acute Toxicity Test

This test along with the fish 96-h acute toxicity test is one of the standbys in aquatic toxicology. *Daphnia magna* and *D. pulex* are the common test species. *D. magna* require a relatively hard water for their culture. *D. magna* are large, commonly available, and easy to culture. *D. pulex* are not quite as large as *D. magna* and tolerate 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 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 daphnia and the quality of the water and test methodology.

In general, 10 neonates that are less than 24 h 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 daphnia is used as the endpoint. An organism is considered immobile (nonmotile) if it does not resume swimming after prodding with a pipet or glass rod. Measurements are made at 24-h intervals. No feeding occurs during the course of this toxicity test.

The daphnia 48-h 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 short time frame, 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 daphnia through the production of three broods with generally 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 reinhardtii*, and *Selenastrum capricornutum* as the food source.

This toxicity test is usually run as a static renewal but some researchers have used a continuous flow set up 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

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

Test type	Chronic (partial life cycle)	Acute 48 h
Organisms	<i>D. magna</i>	<i>D. magna</i>
Age of test organisms	≤24-h old	≤24-h 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 chow, yeast, alfalfa, green algae, and diatoms given in excess	Do not feed
Test duration	21 days	48 h
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 to 1080 lux
Photoperiod	16 h light and 8 h dark (with 15- to 30-min transition)	16 h light and 8 h dark
pH range	7.0–8.6	7.0–8.6
DO concentration	40–100%	60–100%
Aeration	Not necessary	None
Endpoint	Survival, growth, and reproduction	Immobilization

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 reproducible reproduction of the *C. dubia* during the course of the toxicity tests. A combination of trout chow, yeast, rye grass powder, and algae have been used. Nonetheless, the *C. dubia* three-brood toxicity test has been proven to be useful and replicable.

Clonal variability in sensitivity to toxicants does exist. Soares, Baird, and Calow (1992) examined the relative sensitivities of nine clones of *D. magna* of sodium bromide and 3,4-dichloroaniline (3,4-DCA) using chronic NOECs and LOECs as endpoints. All tests were conducted in the same laboratory. There was as much as a 15 fold difference in the LOECs in the tests using sodium bromide. A two- to

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

Test type	Static renewal/chronic
Organisms	<i>Ceriodaphnia dubia</i>
Age of test organisms	<12 h 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 <i>C. dubia</i> 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: <i>Ankistrodesmus convolutus</i> , <i>A. falcatus</i> , <i>Chlamydomonas reinhardtii</i> , and <i>Selenastrum capricornutum</i>
Test duration	7 days
Physical and chemical parameters	
Temperature	25° ± 1°C
Test solution pH	Not specified
DO concentration	40–100%
Endpoint	Reproduction

four-fold difference in LOECs was observed for the 3,4-DCA toxicity tests. Interclonal variation was therefore substantial. These results demonstrated the importance of identifying the specific clone used in toxicity testing when attempting to compare results.

Algal 96-H 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 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

Freshwater:

Green Algae: *Selenastrum capricornutum*, *Scenedesmus subspicatus*, *Chlorella vulgaris*

Blue-green algae (bacteria): *Microcystus aeruginosa*, *Anabena flos-aquae*

Diatom: *Navicula pelliculosa*

Saltwater:

Diatom: *Skeletonema costatum*, *Thalassiosira pseudonana*

Flagellate: *Dunaliella tertiolecta*

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

Test type	Static
Organisms	Fresh water species: <i>Selenastrum capricornutum</i> , <i>Scenedesmus subspicatus</i> , <i>Chlorella vulgaris</i> , <i>Microcystis aeruginosa</i> <i>Anabaena flos-aquae</i> , <i>Navicula pelliculosa</i> ; Salt water species: <i>Skeletonema costatum</i> , <i>Thalassiosira pseudonana</i> , and <i>Dunaliella tertiolecta</i>
Number of organisms per chamber ($\pm 10\%$)	<i>Selenastrum capricornutum</i> and other freshwater green algae 2×10^4 cells/ml <i>Navicula pelliculosa</i> 2×10^4 cells/ml <i>Microcystis aeruginosa</i> 5×10^4 cells/ml <i>Anabaena flos-aquae</i> 2×10^4 cells/ml Saltwater Species 2×10^4 cells/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 h
Physical and chemical parameters	
Water temperature	$24 \pm 2^\circ\text{C}$ for freshwater green and blue-green alga $20 \pm 2^\circ\text{C}$ for <i>Navicula pelliculosa</i> and other saltwater alga
Light quality	Continuous "cool-white" fluorescent
Light intensity	Should not vary by more than $\pm 15\%$ $60 \mu\text{E m}^{-2}/\text{s}^{-1}$ ($4300 \text{ lm}/\text{m}^2$) for freshwater diatoms and green algae $30 \mu\text{E m}^{-2}/\text{s}^{-1}$ ($2150 \text{ lm}/\text{m}^2$) for freshwater blue-green algae $82\text{--}90 \mu\text{E m}^{-2}/\text{s}^{-1}$ (5900 to $6500 \text{ lm}/\text{m}^2$) for <i>Thalassiosira</i> $60 \mu\text{E m}^{-2}/\text{s}^{-1}$ ($4300 \text{ lm}/\text{m}^2$) for <i>Skeletonema</i>
Photoperiod	14 h light/10 h dark for <i>Skeletonema</i>
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

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 a 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 volatilization can produce a concentration different from that of the expected or nominal concentration.

Good microbiological sterilization 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 as well as nutrients contained in effluents. It is crucial that the data be appropriately plotted and analyzed.

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. Animals collected from the wild have some advantages and some 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 dilution. At the least, the tap water should be

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

Test type	Static, renewal, flow-through
Organisms	Fresh water Vertebrates Frog (<i>Rana</i> sp.), toad (<i>Bufo</i> sp.), coho salmon (<i>Oncorhynchus kisutch</i>), rainbow trout (<i>Oncorhynchus mykiss</i>), brook trout (<i>Salvelinus fontinalis</i>), goldfish (<i>Carassius auratus</i>), fathead minnow (<i>Pimephales promelas</i>), channel catfish (<i>Ictalurus punctatus</i>), bluegill (<i>Lepomis macrochirus</i>), green sunfish (<i>Lepomis cyanellus</i>) Invertebrates Daphnids (<i>Daphnia magna</i>, <i>D. pulex</i>, <i>D. pulicaria</i>), amphipods (<i>Gammarus lacustris</i>, <i>G. fasciatus</i>, <i>G. pseudolimnaeus</i>), crayfish (<i>Orconectes</i> sp., <i>Combarus</i> sp., <i>Procambarus</i> sp., <i>Pacifastacus leniusculus</i>), stoneflies (<i>Pteronarcys</i> sp.), mayflies (<i>Baetis</i> sp., <i>Ephemerella</i> sp., <i>Hexagenia limbata</i>, <i>H. bilineata</i>), midges (<i>Chironomus</i> sp.), snails (<i>Physa integra</i>, <i>P. heterostropha</i>, <i>Amnicola limosa</i>), planaria (<i>Dugesia tigrina</i>) Saltwater Vertebrates Sheepshead minnow (<i>Cyprinodon variegatus</i>), mummichog (<i>Fundulus heteroclitus</i>), longnose killifish (<i>Fundulus similis</i>), silverside (<i>Menidia</i> sp.), threespine stickleback (<i>Gasterosteus aculeatus</i>), pinfish (<i>Lagodon rhomboides</i>), spot (<i>Leiostomus xanthurus</i>), shiner perch (<i>Cymatogaster aggregata</i>), tidepool sculpin (<i>Oligocottus maculosus</i>), sanddab (<i>Citharichthys stigmaeus</i>), flounder (<i>Paralichthys dentatus</i>, <i>P. lethostigma</i>), starry flounder (<i>Platichthys stellatus</i>), English sole (<i>Parophrys vetulus</i>), herring (<i>Clupea harengus</i>) Invertebrates Copepods (<i>Acartia clausi</i>, <i>Acartia tonsa</i>), shrimp (<i>Penaeus setiferus</i>, <i>P. duorarum</i>, <i>P. aztecus</i>), grass shrimp (<i>Palaemonetes pugio</i>, <i>P. intermedius</i>, <i>P. vulgaris</i>), sand shrimp (<i>Crangon septemspinosa</i>), shrimp (<i>Pandalus jordani</i>, <i>P. danae</i>), bay shrimp (<i>Crangon nigricauda</i>), mysid (<i>Mysidopsis bahia</i>, <i>M. bigelowi</i>, <i>M. almyra</i>), blue crab (<i>Callinectes sapidus</i>), shore crab (<i>Hemigrapsus</i> sp., <i>Pachygrapsus</i> sp.), green crab (<i>Carcinus maenas</i>), fiddler crab (<i>Uca</i> sp.), oyster (<i>Crassostrea virginica</i>, <i>C. gigas</i>), polychaete (<i>Capitella capitata</i>)
Age and size of test organisms	All organisms should be as uniform as possible in age and size. Fish: juvenile; weight between 0.1–5.0 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 h old; Amphipods, mayflies, and stone flies: early instar Midges: second or third instar Saltwater mysids: less than 24 h post-release from the brood sac Do not use ovigerous decapod crustaceans or polychaetes with visible developing eggs in coelom Amphibians: use young larvae whenever possible

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

Test type	Static, renewal, flow-through
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 h All other species: 96 h in static tests, at least 96 h in renewal and flow-through test
Physical and chemical parameters	
Water temperature (°C)	Fresh water Vertebrates Frog, <i>Rana</i> sp. (22) Toad, <i>Bufo</i> sp. (22) Coho salmon, <i>Oncorhynchus kisutch</i> (12) Rainbow trout, <i>Oncorhynchus mykiss</i> (12) Brook trout, <i>Salvelinus fontinalis</i> (12) Goldfish, <i>Carassius auratus</i> (17, 22) Fathead minnow, <i>Pimephales promelas</i> (25) Channel catfish, <i>Ictalurus punctatus</i> (17, 22) Bluegill, <i>Lepomis macrochirus</i> (17, 22) Green sunfish, <i>Lepomis cyanellus</i> (17, 22) Invertebrates Daphnids, <i>Daphnia magna</i>, <i>D. pulex</i>, <i>D. pulicaria</i> (20) Amphipods, <i>Gammarus lacustris</i>, <i>G. fasciatus</i>, <i>G. pseudolimnaeus</i> (17) Crayfish, <i>Orconectes</i> sp., <i>Combarus</i> sp., <i>Procambarus</i> sp., (17, 22) <i>Pacifastacus leniusculus</i> (17) Stoneflies, <i>Pteronarcys</i> sp. (12) Mayflies, <i>Baetis</i> sp., <i>Ephemerella</i> sp. (17) Mayflies, <i>Hexagenia limbata</i>, <i>H. bilineata</i> (22) Midges, <i>Chironomus</i> sp. (22) Snails, <i>Physa integra</i>, <i>P. heterostrophia</i>, <i>Amnicola limosa</i> (22) Planaria, <i>Dugesia tigrina</i> (22) Saltwater Vertebrates Sheepshead minnow, <i>Cyprinodon variegatus</i> (22) Mummichog, <i>Fundulus heteroclitus</i> (22) Longnose killifish, <i>Fundulus similis</i> (22) Silverside, <i>Menidia</i> sp. (22) Threespine stickleback, <i>Gasterosteus aculeatus</i> (17) Pinfish, <i>Lagodon rhomboides</i> (22) Spot, <i>Leiostomus xanthurus</i> (22) Shiner perch, <i>Cymatogaster aggregata</i> (12) Tidepool sculpin, <i>Oligocottus maculosus</i> (12) Sanddab, <i>Citharichthys stigmaeus</i> (12) Flounder, <i>Paralichthys dentatus</i>, <i>lethostigma</i> (22) Starry flounder, <i>Platichthys stellatus</i> (12)

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

Test type	Static, renewal, flow-through
	English sole, <i>Parophrys vetulus</i> (12) Herring, <i>Clupea harengus</i> (12) Invertebrates Copepods, <i>Acartia clausi</i> (12) <i>Acartia tonsa</i> (22) Shrimp, <i>Penaeus setiferus</i> , <i>P. duorarum</i> , <i>P. aztecus</i> (22) Grass shrimp, <i>Palaemonetes pugio</i> , <i>P. intermedius</i> , <i>P. vulgaris</i> (22) Sand shrimp, <i>Crangon septemspinosa</i> (17) Shrimp, <i>Pandalus jordani</i> , <i>P. danae</i> (12) Bay shrimp, <i>Crangon</i> (17) Mysid, <i>Mysidopsis bahia</i> , <i>M. bigelowi</i> , <i>M. almyra</i> (27) Blue crab, <i>Callinectes sapidus</i> (22) Shore crab, <i>Hemigrapsus</i> sp., <i>Pachygrapsus</i> sp. (12) Green crab, <i>Carcinus maenas</i> (22) Fiddler crab, <i>Uca</i> sp. (22) Oyster, <i>Crassostrea virginica</i> , <i>C. gigas</i> (22) Polychaete, <i>Capitella capitata</i> (22)
Light quality	Not specified
Light intensity	Not specified
Photoperiod	16 h light/8 h dark with a 15 to 30 min 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 h 40–100% for static test after 48 h 60–100% for renewal and flow-through tests (all times)
Endpoint	Death, immobilization

doubly distilled and filtered before being used to make culture media. Systems that use distilled water supplied by a central system, filtered through 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 tests 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 LC₅₀ 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.

The fish species used in these tests can be far ranging, although the most popular are the fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), the channel catfish (*Ictalurus punctatus*), and the 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.).

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 $\pm 20\%$ 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 h/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 $\pm$ 2°C	
Humidity	Ideally 40–60%	
Oxygen content	19%	
Dynamic airflow	12 to 15 air changes per hour	
Endpoint	Death	

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

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 of this developmental process is that a great deal of toxicity data does occur for a variety of compounds, both in their structure and their mode of action. Often the only toxicity data available for a compound is a rat or mouse toxicity endpoint. An enormous amount of physiological and behavioral data are 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

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 $\pm 20\%$ 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)
Test chamber type	Height should be at least 17.8 cm (7 in.) 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 by gavage; if by gavage, a 5-day/week dosing regimen is acceptable	
Test duration	90 days	
Clinical examinations	Urinalysis, hematology, blood chemistry, and necropsy	
Physical and chemical parameters		
Temperature	22 $\pm$ 2°C	
Endpoint	Death	

genetically with some having extensive pedigrees available. The drawback to environmental toxicology, however, is that the focus has traditionally been the extrapolation of the toxicity data to primates and not towards 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 organisms caught in the wild, 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 both acceptable. [Table 4.10](#)

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 (<i>Colinus virginianus</i>), Japanese quail (<i>Coturnix japonica</i>), mallard (<i>Anas platyrhynchos</i>), and ring-necked pheasant (<i>Phasianus colchicus</i>)
Age of organism	14, 14, 5, and 10 days, respectively
Experimental design	
Test chamber	Construction materials in contact with birds should not be toxic nor be 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 <i>ad libitum</i>
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 approx. 38°C to approx. 22°C should be established in brooders
Photoperiod	Minimum of 14 h of light
Humidity	45–70% (higher relative humidities may be appropriate for waterfowl)
Ventilation	Sufficient to supply 10 to 15 air changes per hour
Endpoint	Mortality

compares two methods, one being the ASTM consensus method and the other from the U.S. EPA. The ASTM method is broader and includes species that the U.S. EPA method does not. This does allow 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, while 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 the potential differences when comparing toxicity results.

ANIMAL CARE AND USE CONSIDERATIONS

Since the care and well-being of terrestrial vertebrates has 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

Table 4.9 Summary for Conducting Reproductive Studies with Avian Species

Test type	Avian reproduction
Organisms	Ring-necked pheasant (<i>Phasianus colchicus</i>), bobwhite (<i>Colinus virginianus</i>), Japanese quail (<i>Coturnix japonica</i>), chicken (<i>Tympanuchus cupido</i>), mallard (<i>Anas platyrhynchos</i>), black duck (<i>Anas rubripes</i>), screech owl (<i>Otus asio</i>), American kestrel, ring dove (<i>Streptopelia risoria</i>), gray partridge, crowned guinea-fowl
Age of organism	Should be within $\pm 10\%$ of the mean age of the group
Feeding	Feed and water should be available <i>ad libitum</i> ; 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% (1000 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)
Light quality	Should emit a spectrum simulating daylight
Light intensity	65 lux (6 fc)
Photoperiod	For adults: 8 h light/16 h dark prior to photostimulation; 17 h light/7 h dark from onset of photostimulation For hatchlings: at least 14 h of light for precocial species
Endpoint	Reproduction

of Health. These guidelines were not welcomed by many toxicologists during their implementation. The net effect, however, has been in the improvement of research. Now almost all research is reviewed by animal use committees and strict protocols exist that help to ensure the health of the test organisms. In addition, these rules help to ensure a more efficient utilization of laboratory animals.

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

Test type	ASTM	EPA
	Avian subacute dietary	Avian subacute dietary
Organisms	Northern bobwhite (<i>Colinus virginianus</i>), Japanese quail (<i>Coturnix japonica</i>), mallard (<i>Anas platyrhynchos</i>), ring-necked pheasant (<i>Phasianus colchicus</i>)	Northern bobwhite (<i>Colinus virginianus</i>), mallard (<i>Anas platyrhynchos</i>)
Age of organism	14, 14, 5, and 10 days, respectively	10 to 14 and 5 to 10 days, respectively
Experimental design		
Test chamber	Construction materials in contact with birds should not be toxic nor be 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 Mallards: 70 × 100 × 24 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 <i>ad libitum</i>	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 days observation, clean diet for both groups
Clinical examinations	Body weight (record at beginning and end) and feed consumption	Body weight and feed consumption

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

Test type	ASTM	EPA
	Avian subacute dietary	Avian subacute dietary
Physical and chemical parameters		
Temperature	A temperature gradient from approx. 38°C to approx. 22°C should be established in brooders	22 to 27°C outside, about 35°C inside brooder
Photoperiod	Minimum of 14 h of light	Diurnal recommended, 24 h lighting acceptable
Humidity	45 to 70% (higher relative humidities may be appropriate for waterfowl)	30 to 80%
Ventilation	Sufficient to supply 10 to 15 air changes per hour	Adequate supply should be maintained
Endpoint	Mortality	Mortality

Facilities and animal husbandry are a major consideration with the avian or any other test using a terrestrial vertebrate. Guidelines exist and are promulgated by the United States Department of Agriculture and the National Institutes of Health to ensure that test animals are maintained to an acceptable standard.

An additional consideration when using mammals and birds 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. Animal use committees are set up to counsel investigators as to the wise use of animals in research.

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 also can 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.

Also it is 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. Frequently 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 as 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.

Although useful, and 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.

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 past 10 years. The methodology has been performed in a number of laboratories with repeatable results. This toxicity test has a number of uses. FETAX has been touted as an alternative to performing mammalian teratogenicity test and its correlation with known mammalian teratogens is very good. Teratogenicity of run-off, water collected from lakes and streams, and even elutriates from soil samples have 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 which use mammals. Validation studies using compounds with known mammalian and/or human developmental toxicity suggest the predictive accuracy rate compares favorably with other currently available “*in vitro* teratogenesis screening assays” (Bantle 1992). It is important to measure developmental toxicity because embryo mortality, malformation, and growth inhibition often can 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 of other vertebrates. *Xenopus* is, of course, not native to the Americas, but it is a typical amphibian and its comparability in teratogenic response to mammalian species has already been documented. *Xenopus* are

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

Test type	96 h static renewal
Organism	<i>Xenopus laevis</i>
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: three feedings per week of ground beef liver; liquid multiple vitamins should be added to the liver in concentrations from 0.05–0.075 cc/5 g liver
Experimental design	
Test vessel type and size	Adults: large aquarium or fiberglass 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 fiberglass 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 h
Number of concentrations	5
Number of replicate per sample	2
Number of organism per chamber	Adults: 4–6 per 1800 cm ² of water surface area Breeding Adults: 2 Embryos: 25
Test duration	96 h
Physical and chemical parameters	
Temperature	Adult: 23 ± 3°C Embryos: 24 ± 2°C
Photoperiod	12 h light/12 h dark
pH range	6.5 to 9
TOC	10 mg/l
Alkalinity and hardness	Between 16 and 400 mg/l as CaCO ₃
Endpoint	Acute (mortality) and subacute (teratogenesis)

also widely available and the basic methodology also can 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 a rapid acceptance as a teratogenicity screen in environmental toxicology.

**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

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.

The overriding characteristic of a multispecies toxicity test is that it consists 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 all 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 lead to the selection of one of the current methods with slight modification as to which is 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 a 55-gal aquarium. Larger test systems also can be used outside the laboratory. A recently proposed outdoor aquatic microcosm proposal 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 past 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 chlorpyrifos. 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, that 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 particular scientific question.

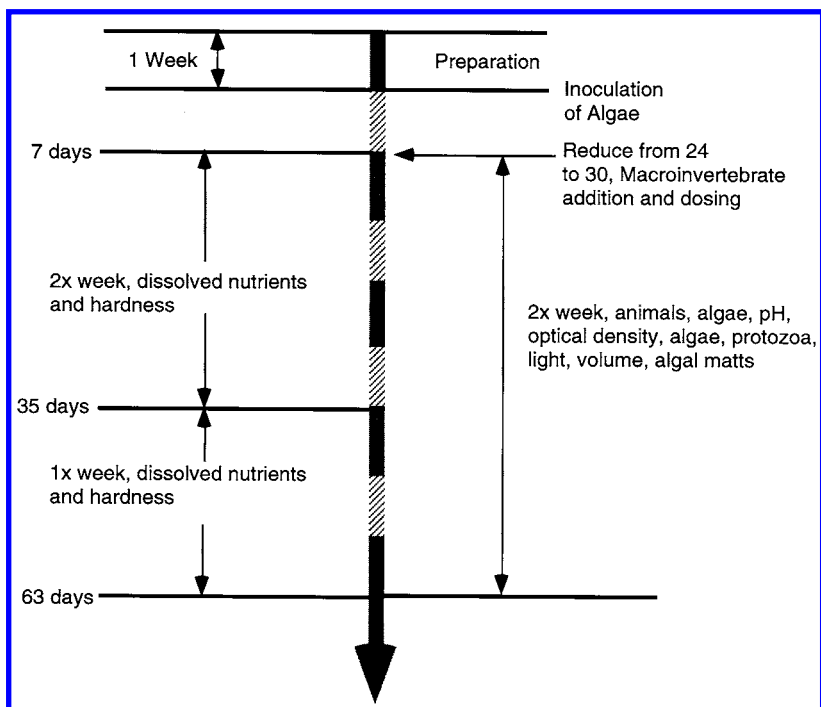


Figure 4.1 Timeline for the standardized aquatic microcosm. The 63-day toxicity test is specific in its sampling requirements, acclimation times, and dosing.

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. An autoclaved sediment consisting of 200 g silica sand and 0.5 g of ground chitin are autoclaved separately and then added to the already autoclaved jar and media.

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 $20^{\circ} \pm 2^{\circ}\text{C}$. Illumination is set at $79.2 \mu\text{E m}^{-2} \text{sec}^{-1}$ 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 dimensions with 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.

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

Test type	Multi-species
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) <i>Anabaena cylindrica</i> <i>Ankistrodesmus</i> sp. <i>Chlamydomonas reinhardi</i> 90 <i>Chlorella vulgaris</i> <i>Lyngbya</i> sp. <i>Nitzschia kutzigiana</i> (Diatom 216) <i>Scenedesmus obliquus</i> <i>Selenastrum capricornutum</i> <i>Stigeoclonium</i> sp. <i>Ulothrix</i> sp. Animals (added on day 4 at the initial numbers indicated in parentheses) <i>Daphnia magna</i> (16/microcosm) <i>Hyalella azteca</i> (12/microcosm) <i>Cypridopsis</i> sp. or <i>Cyprinotus</i> sp. (ostracod) (6/microcosm) Hypotrichs [protozoa] (0.1/ml) (optional) <i>Philodina</i> sp. (rotifer) (0.03/ml)
Experimental design	
Test vessel type and size	1-gal (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 (approx. 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 20 to 25°C with minimal dimensions of $2.6 \times 0.85 \times 0.8$ 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
Light intensity	$80 \mu\text{E m}^{-2}$ photosynthetically active radiation s^{-1} (850–1000 fc)
Photoperiod	12 h light/12 h dark
Microcosm medium	Medium T82MV
Sediment	Composed of silica sand (200 g), ground, crude chitin (0.5g), 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

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 also is computed for each sampling day.

Mixed Flask Culture

The 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. Over a 6-month period repeated inocula are made in 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 are 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 prokaryotic 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” inocula 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.

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 as invertebrate predators. Proposed in late 1991 was a microcosm/mesocosm blend that is substantially larger than the MFC or the SAM experimental units. The

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	a) Two species of single-celled green algae or diatoms b) One species of filamentous green alga c) One species of nitrogen-fixing blue-green alga (bacteria) d) One grazing macroinvertebrate e) One benthic, detrital-feeding macroinvertebrate f) 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 # 82 medium [20], 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 to 18 weeks Allow to mature 6 weeks prior to treatment; follow 6 to 12 weeks after exposure	
Physical and chemical parameters		
Temperature	20°C	
Photoperiod	12 h light/12 h dark	
Endpoint	Oxygen content, algal densities, microbial activity, respiratory activity, biomass, protozoan population	

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 m³ 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 a variety of emergent invertebrates. A diagrammatic representation of one system for the examination of the effects of a model herbicide is presented in Figure 4.2.

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

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 (<i>Lepomis macrochirus</i>), fathead minnow (<i>Pimephales promelas</i>), channel catfish (<i>Ictalurus punctatus</i>), 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 to 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 $\pm 10\%$ throughout study; if water level falls more than 10%, add pond water, fresh well water, or rain water; 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

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 have been established. In some locations it also may 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 also are 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

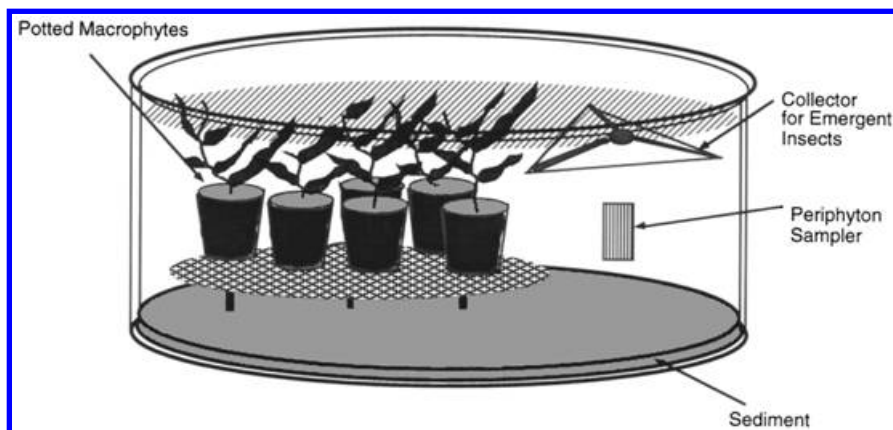


Figure 4.2 FIFRA microcosm experimental unit. An example of a microcosm experimental unit designed to test the effects of a herbicide on an aquatic environment. This particular setup does not include fish since the predatory effects would tend to hide lower trophic level effects upon the invertebrate populations. Typically, a FIFRA microcosm experiment includes fish species, particularly when acetylcholinesterase inhibitors or other toxicants particularly effective against animal species are tested.

are controlled by the local environmental conditions. On the other hand, the sensitivity to local conditions also can 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. A recent publication, *Aquatic Mesocosm Studies in Ecological Risk Assessment* (Graney et al. 1994), does review and discuss the system typically used for the purposes of pesticide registration.

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 its 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.

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 non-plasticized 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 to 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

SUMMARY

This chapter reviewed a wide variety of toxicity tests, yet only a small fraction of the toxicity tests that are currently performed or that 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 dealing with the topic of toxicity testing.

First, there is a tendency to over extrapolate 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 either because of overzealous salesmanship, undo 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 onto 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.

APPENDIX: 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 in. (457 to 610 mm) in length and 8 to 12 lb in weight as marine adults and 10.8 to 25.8 in. (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 fresh water in North America from Monterey Bay, CA (in the sea infrequently to Baja California) to Point Hope, AK. In Asia, it occurs from the Anadyr River, Russia, 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; segregation into summer and autumn, or autumn and winter runs is more apparent in Asia than in North America; spawning takes place in swifter water of shallow, gravelly 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 troutlike, elongate, average length is 12 to 18 in. (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 darker, colors more intense; lake residents lighter, brighter, more silvery.

Systematic notes: Populations in different watersheds were long called by different scientific names and still by different regional common names in the South.

Distribution: Native range was eastern Pacific Ocean and fresh water, mainly west of the Rocky Mountains, from northwestern Mexico (including extreme northern Baja California) to the Kuskokwim River, AK; 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, temp. being 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 in. (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.

Biology: 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, thickset, average total length about 5 to 10 in. (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, 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 in. (51 mm), thickset, 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 sub-specifically 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 in. (356 to 533 mm), weight is 2 to 4 lb.

Color: Individuals less than 12 to 14 in. (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 is now recognized 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 fresh waters, 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 in. (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 fresh waters of eastern and central North America; has been introduced throughout the U.S., into Africa, and possibly other areas of 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 in. (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 fresh waters of east-central North America.

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

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 in 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 also are 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 fish, especially young fish, 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, *Pontooporeia 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 (*Aquatic Entomology*, 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 is a 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 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) are 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 burrow or under stones and debris. They retreat rapidly backwards when disturbed. Depending on the species, crayfish may be herbivores, carnivores, detritivores, or omnivores; their very robust first pair of legs (chela) are used to cut or crush food. These chela also are used as defensive weapons. Prawns and river shrimps are generally swimmers (*Aquatic Entomology*, 390 to 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 (*Aquatic Entomology*, 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 and/or herbivores and are themselves a preferred food of many freshwater carnivores, including other insects and fish, 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 proved 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 also may 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 (*Aquatic Entomology*, 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 conelike. 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 fish 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 flat-worms, many of which are parasitic or marine. Most of the free-living, freshwater forms are planarians, and 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. Cephalothorax is covered by a carapace. 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 Lernaepodoida) are parasitic on fish and are highly modified for this type of existence. The vast majority of copepods are free-living. One genus (*Cyclopoida*) is parasitic on fish.

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 fish.

Algae

Chlamydomonas reinhardtii

Unicellular, green alga which possesses one nucleus, one chloroplast, and several mitochondria. It is facultatively photosynthetic and can grow in the dark with acetate as its 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.**

Filamentous member of the Chlorophyta, a multicellular algae which is immobile in the mature state. Reproduction frequently involves the formation and the 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 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 the 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)

A small, ruddy, chicken-like bird, about 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, southern Minnesota, southern 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 with 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 for toxicity testing as the Northern bobwhite.

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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 a algal 96-h 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.

Routes of Exposure and Modes of Action

THE DAMAGE PROCESS

Given sufficiently high concentrations, environmental toxicants can cause adverse effects on the physiological processes of a living organism. In order for a pollutant to exert its toxicity on an organism exposed to it, the pollutant 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, a few commonalities that are shared by different pollutants are summarized here to provide a general background. The damage processes in plants and in animals are considered separately below.

ATMOSPHERIC POLLUTANTS AND PLANTS

An atmospheric pollutant-induced plant injury may follow a pathway that includes exposure, uptake, transport, storage, metabolism, and excretion ([Figure 5.1](#)). To cause injury to any vegetation, an air pollutant must first be taken up by 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 characteristics, and air movement across the leaf are important determinants affecting the initial flux of gases to the leaf surface. More pollutant would enter a leaf when there is some air movement.

Stomatal resistance is a critical factor affecting pollutant uptake. The resistance is determined by stomatal number, size, anatomical characteristics, and the size of the stomatal aperture. Little or no uptake occurs when the stoma is closed. Stomatal opening is regulated by internal CO₂ content, temperature, humidity, light, water availability, and nutrient status, particularly, potassium ions. It should be mentioned

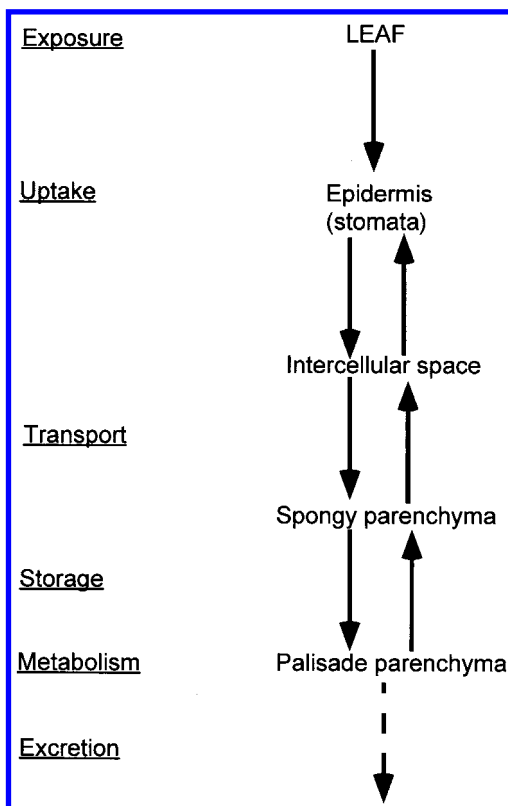


Figure 5.1 Schematic pathway of plant injury induced by atmospheric pollutants.

that, although stomatal resistance is an important factor influencing pollutant uptake, genetic sensitivity of individual species and cultivars are the overriding factors determining plant injury. It should be noted also that the level of a pollutant within the leaf is most critical to plant health.

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 into 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 parent compound. The formation of free radicals following the initial reaction in the cell is an example. 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 cell metabolism and causing plant injury.

Changes in ultrastructure of various organelles such as chloroplasts and mitochondria can impair photosynthesis and energy metabolism of the plant cell.

As a pollutant moves in the liquid phase from the substomatal regions to the cellular target sites, 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 can adversely affect the cell.

Certain enzymes in the cell may be inhibited when exposed to an air pollutant. For instance, Pb and Cd may inhibit the activity of an enzyme by disrupting its active site containing a sulfhydryl (SH) group. Likewise, sulfur dioxide may oxidize and break apart the sulfur bonds in critical enzymes of the membrane, impairing cellular function.

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

Vertebrates

A pollutant may get into an animal through a series of pathways similar to those shown previously (see [Figure 5.1](#)). The routes may include exposure, uptake, transport, storage, metabolism, and excretion. [Figure 5.2](#) shows the pathways through which a pollutant may pass during its presence in a terrestrial animal or fish.

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, through inhalation, or through ingestion.

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.

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 (a) filtration through spaces or pores in membranes; (b) passive diffusion through the spaces or pores, or by dissolving in the lipid material of the membrane; and (c) facilitated transport, whereby specialized transport systems carry water soluble substances across the membrane by a lipid

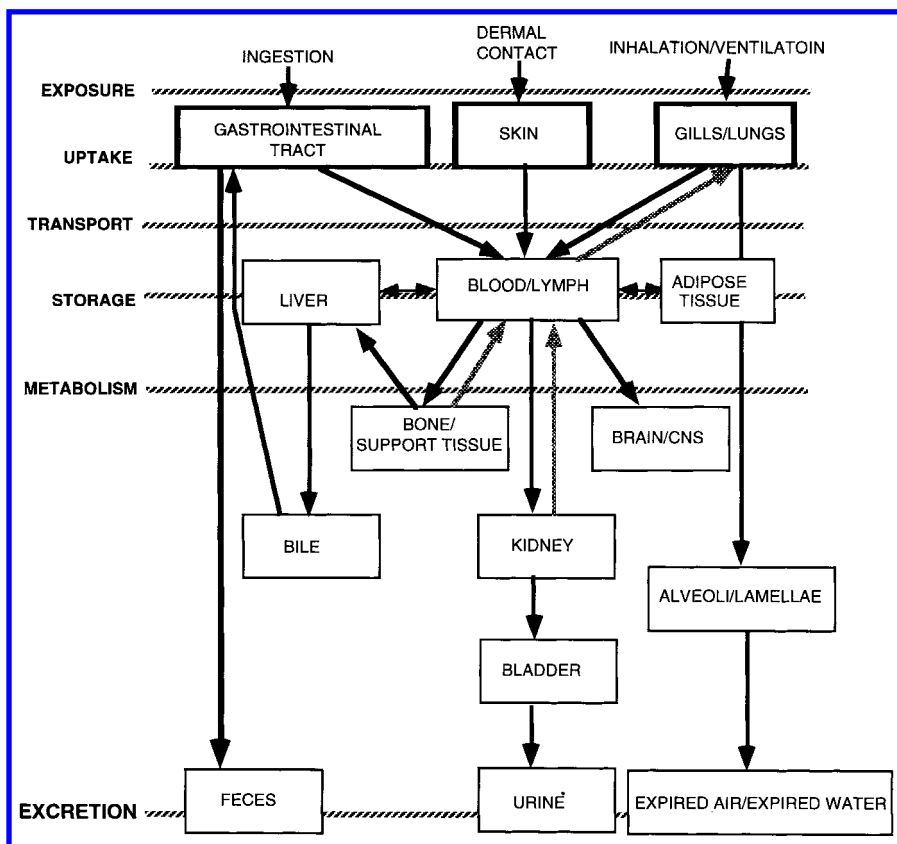


Figure 5.2 Routes of absorption, translocation, and excretion of toxicants in a vertebrate.

soluble “carrier” molecule, which complexes with the chemical; and (d) active transport which requires both a carrier molecule and energy. It can be seen then that, as far as the chemical properties are concerned, **lipophilicity** is the most important factor affecting absorption.

Transport

Once absorbed, a rapid transport of the toxicant throughout the body takes place. A pollutant or a toxicant may be transported via the lymphatic system or blood stream and distributed to various body tissues, including those of storage depots and sites of metabolism or biotransformation.

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 toxicant may be transported to a storage depot or to sites 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.

Metabolism

The metabolism of toxicants may be carried out at 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 enables the liver to metabolize a broad spectrum of organic substances. The reactions involved in the metabolism of these substances 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, on the other hand, involves conjugation reactions in which an endogenous substance combines with the primary metabolite, forming a complex secondary metabolite. An important feature of these reactions is the conversion of lipophilic compounds to more water-soluble and thus more excretable metabolites. While many toxicants are detoxified through these reactions, others may be activated as well (see Chapter 9).

Excretion

The final step involved in the action of a pollutant is excretion from the body. Excretion may take place through the kidneys, lungs, or intestinal tract. A toxicant may be excreted in its original form or as its metabolite(s), depending upon its chemical properties. Excretion is the most permanent means by which toxic substances are removed from the body.

MECHANISMS OF ACTION

The toxic action of chemical agents involves compounds with intrinsic toxicity or activated metabolites. These interact with cellular components at their site of action to initiate toxic effects that 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 toxicant or a pollutant may be terminated by metabolic transformation, storage, 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 (a) disruption or destruction of cellular structure, (b) direct chemical combination with

a cell constituent, (c) its influence on enzymes, and (d) initiation of a secondary action. These are examined below.

Disruption or Destruction of Cellular Structure

A toxicant or a pollutant may exert its injurious effect on a specific 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 certain concentrations can be structurally damaged leading to 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, disrupting 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 ozone may lead 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 lead to disruption of the lung tissue.

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) forming carboxyhemoglobin (COHb) as shown below:



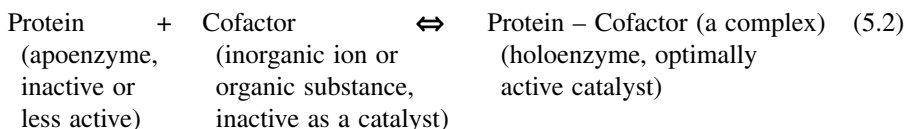
Since hemoglobin in the body is essential in the carbon dioxide-oxygen exchange system between the lungs and the tissues, interference with the functioning of hemoglobin as a result of COHb formation can be detrimental.

Another example is cadmium, a highly toxic heavy metal. Once absorbed, cadmium in the body is mainly bound to the protein metallothionein. This protein is involved in the transport and selective storage of cadmium. A rather selective accumulation of cadmium occurs in the kidneys, leading to eventual tubular dysfunction with proteinuria (Friberg et al. 1974).

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

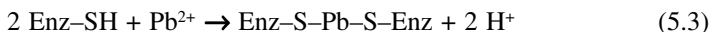
holoenzyme and exhibits maximal catalytic activity. The protein component without its cofactor is termed an apoenzyme and exhibits very low activity, or none at all.



There are two categories of cofactors: organic and inorganic. The organic cofactors include several 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, vitamin K after ingestion is unchanged and used directly as vitamin K. The vitamin niacin after being ingested, however, is converted to either of two cofactors — nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). On the other hand, the inorganic cofactors include several simple inorganic ions such as Mg^{2+} , Mn^{2+} , Zn^{2+} , Ca^{2+} , Fe^{2+} , Cu^{2+} , K^{+} , and Na^{+} ions.

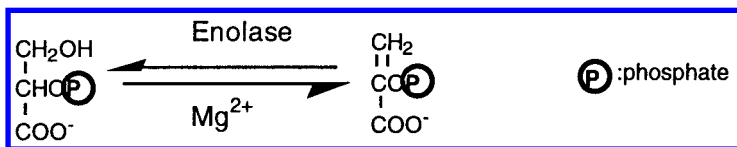
Several ways in which environmental pollutants may inactivate an enzyme system are described below:

1. A pollutant may combine with the active site or sites of an enzyme thus inactivating it. For example, a heavy metal such as mercury, lead, or cadmium can attach itself to the thiol or 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 happens to be the active site of the enzyme. Transaminases and δ -aminolevulinate dehydratase are susceptible to inhibition by lead because they contain the SH group at their active sites.



Additionally, the conformation of an enzyme protein may be altered through disruption of the hydrogen bonds.

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 requires Mg^{2+} ions for its activity. In the presence of phosphate, fluoride inactivates the Mg^{2+} cofactor, presumably by causing the formation of a magnesium fluorophosphate complex.



2-Phosphoglycerate

Phosphoenol
pyruvate

(5.4)

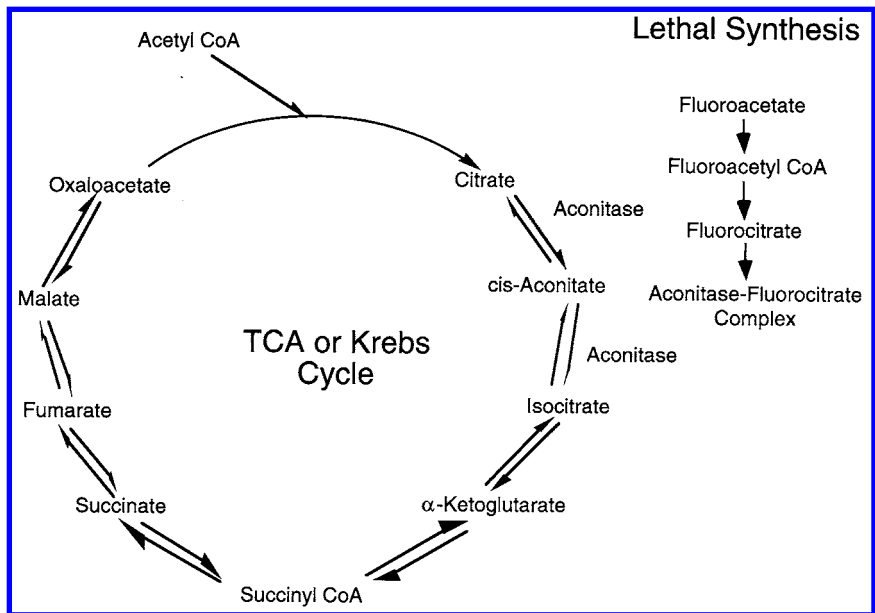


Figure 5.3 Synthesis of fluorocitrate from fluoroacetate through “lethal synthesis”. Inhibition of aconitase shuts down TCA cycle.

3. A pollutant may exert its toxicity through competing with the cofactor for the active site, thus inactivating the enzyme. For example, Be (beryllium) competes with Mg and Mn, and 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 but to a metabolic conversion product, fluorocitrate, formed through a reaction commonly known as “lethal synthesis”, as shown in [Figure 5.3](#). 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 Krebs cycle. Inhibition of aconitase results in citrate accumulation. The cycle stops for lack of metabolites, impairing energy metabolism.

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 which are injurious 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 5.4](#)). 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

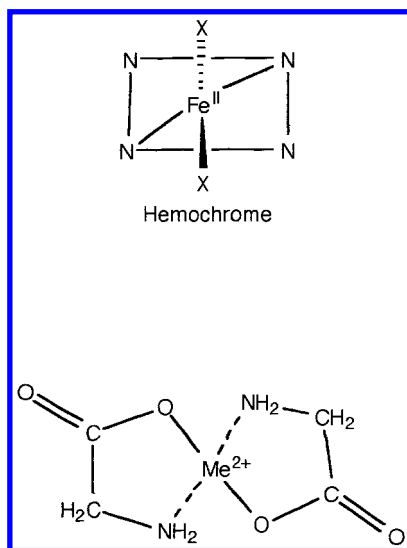


Figure 5.5 Examples of chelation.

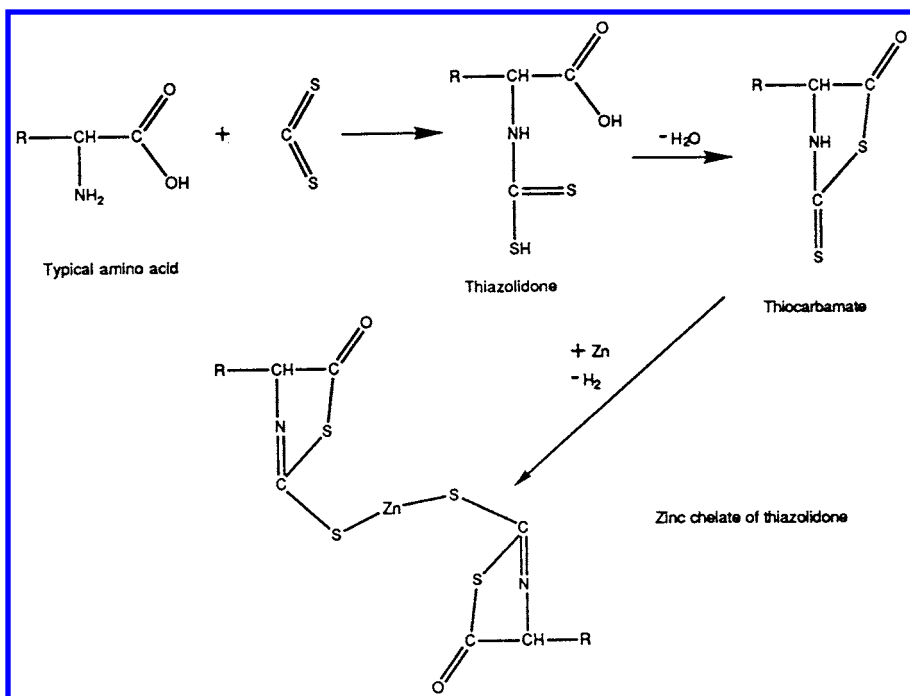


Figure 5.6 Reactions of CS_2 with proteins and amino acids.

It has been suggested that metal chelation may be one of the mechanisms involved in carcinogenesis. Many carcinogens possess structures or can be metabolized to structures capable of metal-binding. This in turn will aid the entrance of metals into cells. Once inside the cells, interaction between normal metals and abnormal metals can occur thus altering cell metabolism. Certain anticancer agents may function through metal binding, i.e., they may inactivate abnormal metals more than the normal metals within the cells. There are, moreover, numerous toxic environmental chemicals that have either chelate structures or can become so through the usual metabolic processes.

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 toxicant. 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 to move into the liver and spleen. When vanadium concentrations were at 250 ppm or above, however, iron moved out of the liver and spleen. As a result, the iron level in the spleen was decreased to one half to one third of the normal content, while the iron level in the liver decreased to one third of the normal content (Furst 1960). These results indicate that treatment with vanadium will lead to depletion of iron in these tissues.

The phenomenon of metal shift in rats exposed to fluoride has recently been reported (Yoshida et al. 1991). Administration of fluoride to rats increased serum Zn levels, whereas the levels of Se and Al in the whiskers were decreased.

A similar phenomenon has been observed with rats exposed to ozone. When the rats were exposed to this pulmonary irritant for 4 h, the animals showed increased levels of copper, molybdenum, and zinc in the lungs, while these metals were decreased in the liver. This would indicate an altered hemodynamics and changes in cellular permeability in a secondary affected organ.

COMMON MODES OF ACTION IN DETAIL

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 5.7 is a diagram of a typical cellular membrane with the lipid bilayer and its associated proteins. Three site of actions 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

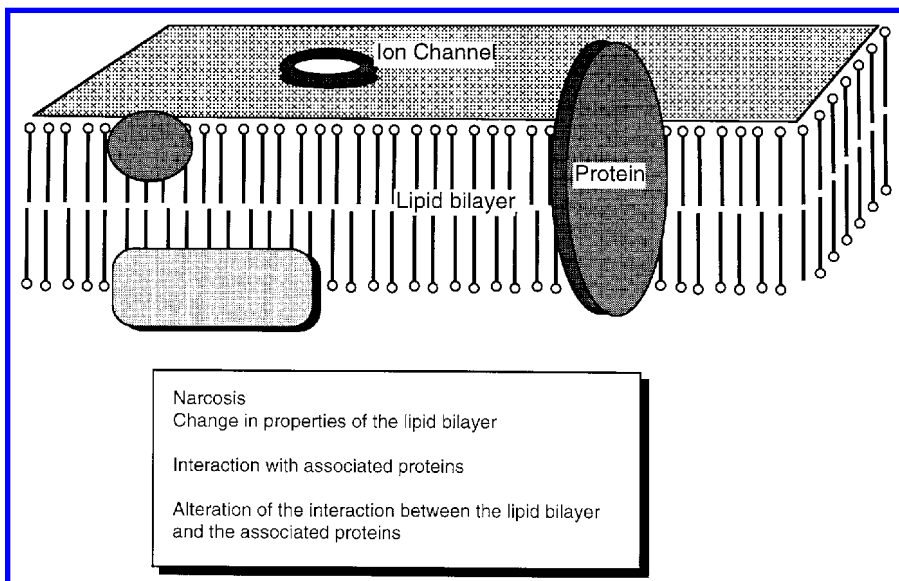


Figure 5.7 Schematic of cell membrane with associated proteins.

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 compounds 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 5.8](#). Apparently at higher values of log P, the nonpolar compounds demonstrate a lesser slope. Perhaps two different mechanisms are at play.

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 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 5.9](#). The more toxic compounds generally have short phosphonate side groups with fluoride or a cyano leaving group. The metabolic replacement of

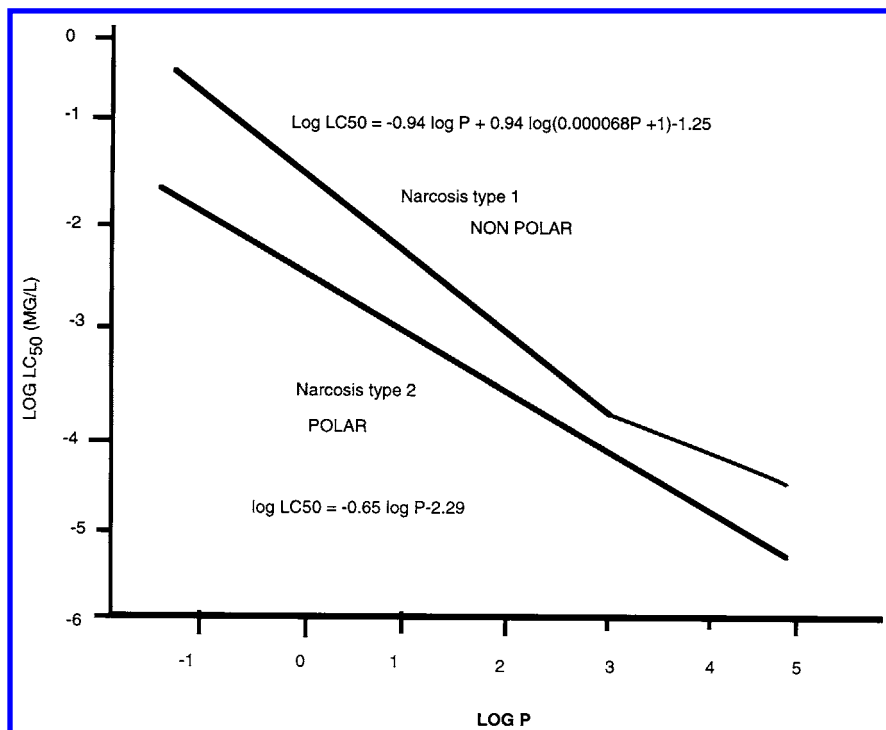


Figure 5.8 Comparison of the relationship between polar and nonpolar compounds, toxicity, and octanol/water partition coefficient. Note that the slopes are similar for both groups of compounds until higher log P values are reached.

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 5.10). 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 serine with a proton donated by the amino acid. This process is graphically demonstrated in Figure 5.11. 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.

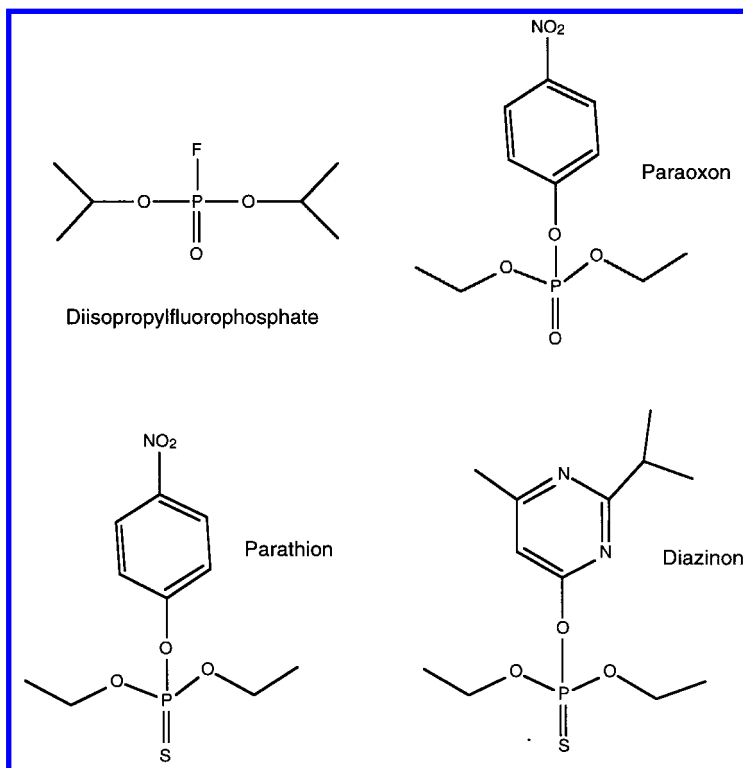


Figure 5.9 Typical organophosphates and related compound structures.

Organophosphates are able to participate in part of the reaction depicted above. However, as shown in the accompanying figure ([Figure 5.12](#)), all does not work as if the organophosphate was acetylcholinesterase. The typical organophosphate is able to enter at the active site and the initial 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. The 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 fact that the binding of an organophosphate to an enzyme such as 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 recently been shown that organophosphates bind to a variety of liver proteins and these protein act, accidentally perhaps, as sinks protecting enzymes of the central nervous system (CNS) from exposure. Of course, a second

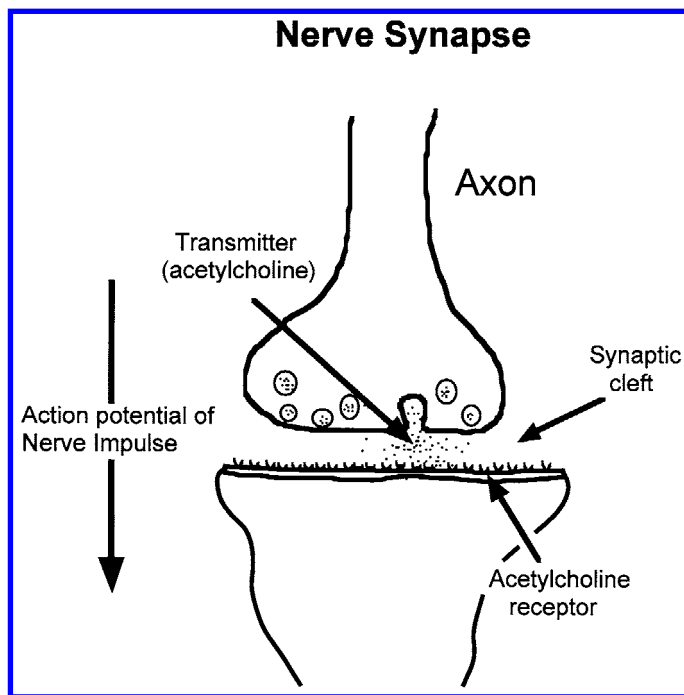


Figure 5.10 Schematic of the synapse. Acetylcholine is an important neurotransmitter and the intervention of acetylcholinesterase prevents subsequent firing of the adjacent neuron.

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.

Monohaloacetic Acids

Monohaloacetic 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 is manufactured in large amounts.

Comparing the toxicity of the chloroacetate, fluoroacetate, and iodoacetate in rats, the 24-h 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} for chloroacetate, fluoroacetate, and iodoacetate was 130, 310, and 480 min, respectively. Based upon this comparative study, fluoroacetate was the most toxic, iodoacetate the intermediate, and chloroacetic acid was 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 the halogen cause very different physiological effects.

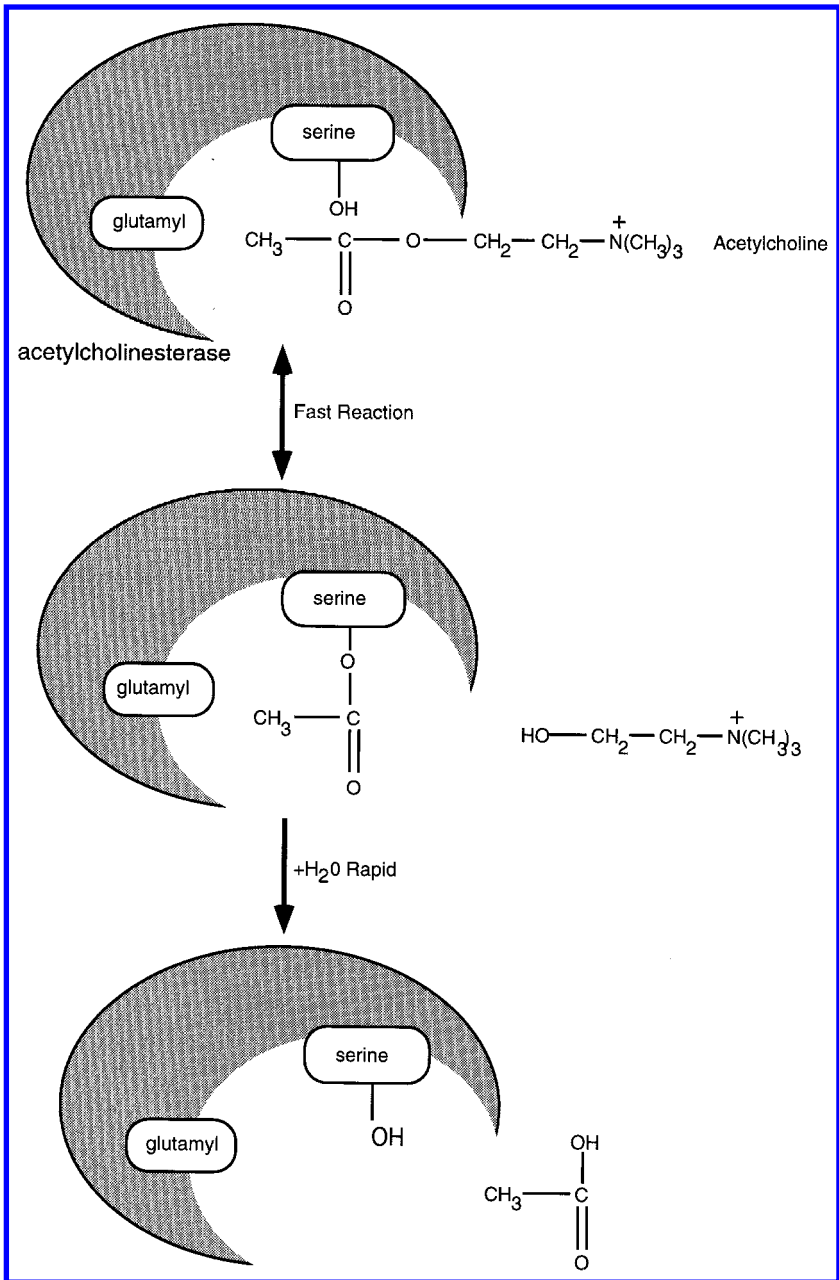


Figure 5.11 Normal hydrolysis of acetylcholinesterase. The amino acid serine is important in the donation of a proton used in the catalytic process. The proton is regenerated during the reaction.

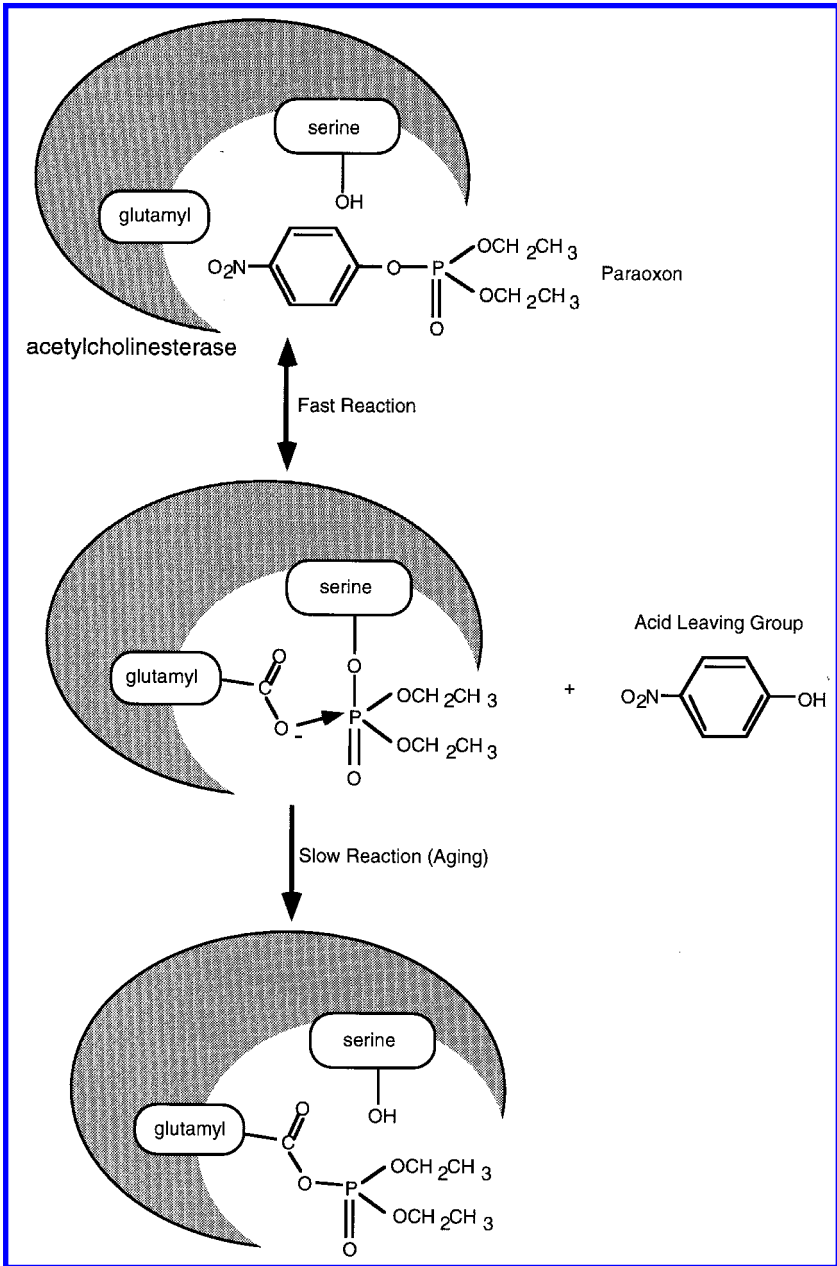


Figure 5.12 Inhibition of acetylcholinesterase by an organophosphate. The initial binding of the organophosphate to the active site prevents the normal substrate from entering the active site. The aging process subsequently binds the organophosphate to the active site permanently, inactivating the enzyme.

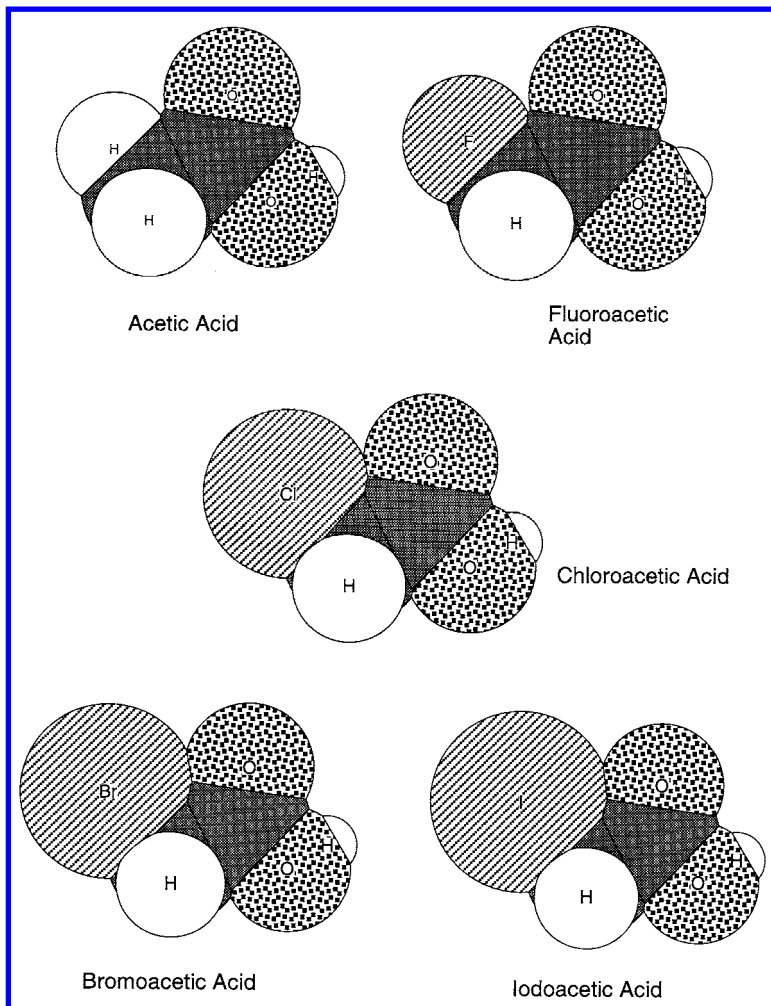


Figure 5.13 Relative configurations of the monohaloacetic acids and acetic acid.

Figure 5.13 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 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 $-\text{CH}_2\text{COOH}$. 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 $-\text{SH}$ 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 $-\text{SH}$ groups.

INTRODUCTION TO QSAR

Quantitative structure-activity relationships (QSAR) 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, rat, mouse, daphnia and fathead minnow acute toxicity, and at establishing toxicological relationships across species boundaries.

Toxicity data is generally of two types. First, most toxicity data are continuous; that is they may have virtually any numerical value. LD_{50} , NOEC, EC_{50} , EC_{10} data 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 5.14](#). 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:

$$y = mx + b \quad (5.5)$$

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 5.1](#)

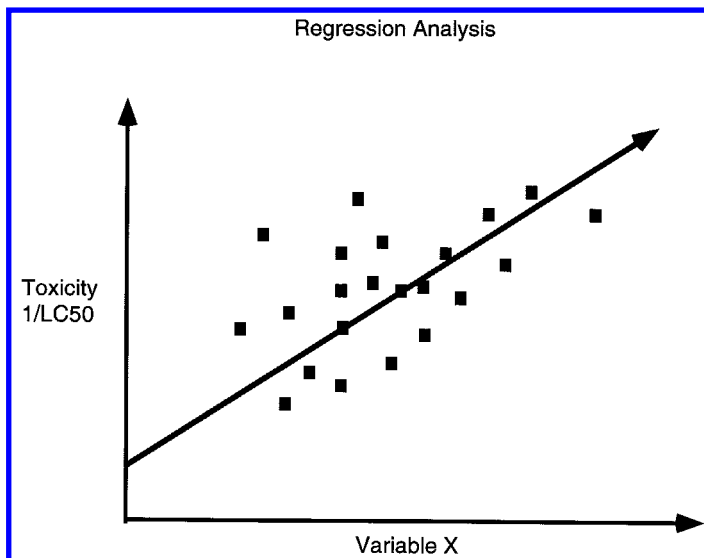


Figure 5.14 Linear regression model for continuous data in QSAR analysis. The model is a simple linear regression with toxicity plotted against the physical or structural variable being used for the estimate.

Table 5.1 *Daphnia* EC₅₀ Equation for Model Incorporating Molecular Connectivity Indices and Substructural Keys

Key	Coefficient
Primary amine bound to aromatic ring atom	1.0167
Primay 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 (nonbeta phynyl)	0.8091
1,1-Divinyl chloride (nonbeta 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

After Enslein et al. 1989.

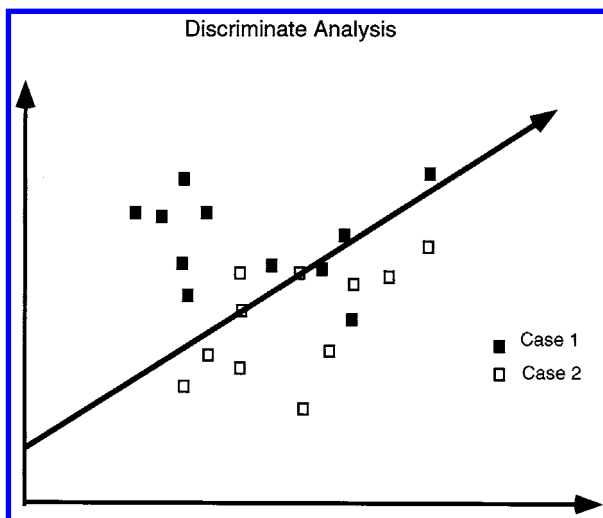


Figure 5.15 Discriminant analysis. In this case the goal is to differentiate data that are in two categories, Case 1 and Case 2. Case 1 could be mutagenic and Case 2 not mutagenic. Many toxicological measurements are categorical in nature.

portrays such an equation in tabular form. 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 5.15). 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.

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. Models also are often produced that attempt to describe a particular subset of compounds unique in their composition or mode of action. And, 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 broadly map 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 LD₅₀, *Daphnia* EC₅₀, and fathead minnow, to name a few. In addition to modeling

continuous endpoints, this approach also has 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. An example of such compounds are the acetylcholinesterase inhibitors. These compounds are very specific in their inhibition of serine enzymes. In the instance of predicting *Daphnia* EC₅₀, 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 are 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 LD₅₀ to *Daphnia magna* EC₅₀. 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 available 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.

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 5.16. 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 this 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 are 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 a mg/kg, it is generally necessary and desirable to transform the LD₅₀ and LC₅₀ values into molar form as follows:

$$\log 1/C = \log (\text{mol w} \times 1000/\text{LD}_{50} \text{ or } \text{LC}_{50}) \quad (5.6)$$

where C is the molar concentration.

Procedure for Constructing Qsar Models

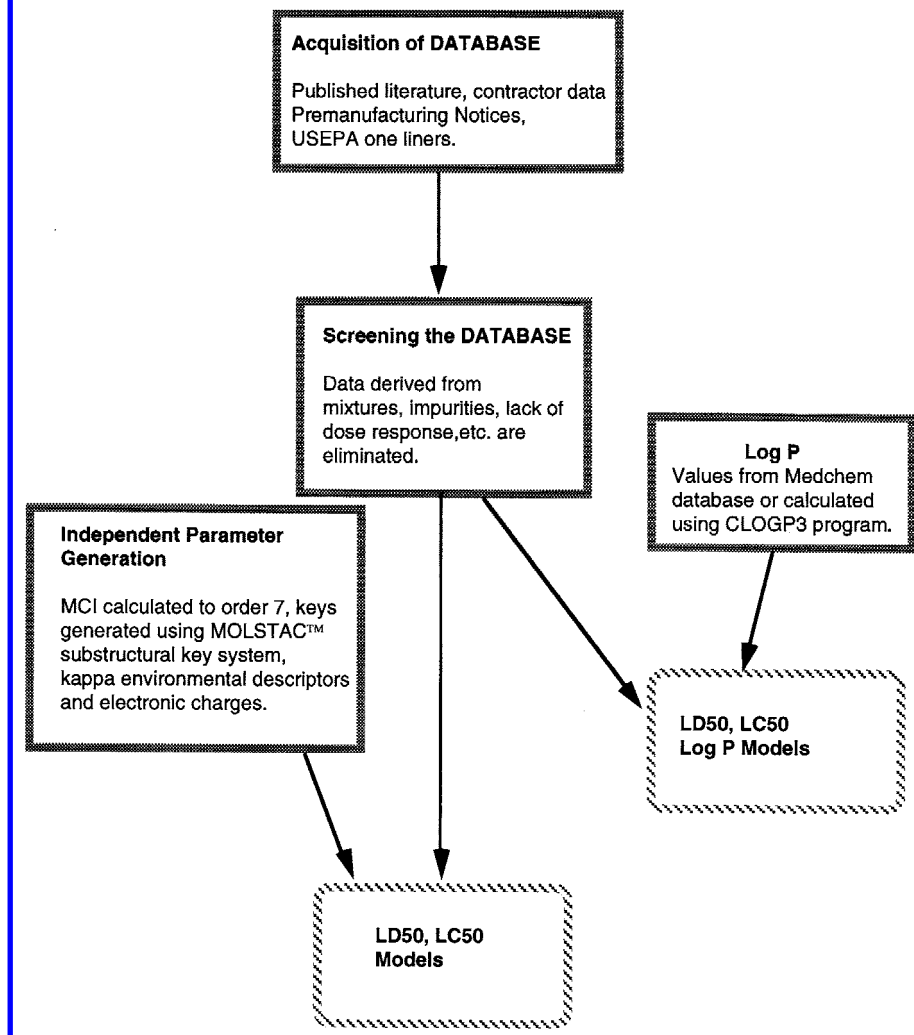


Figure 5.16 The developmental process for the construction of a structure-activity model.

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 LD₅₀ or LC₅₀ value is incorporated as a typical parameter. Molecular connectivity indexes, electronic charge distributions, and kappa environmental descriptors have been proven recently 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:

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 5.17](#) 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 into the final equation. Care is taken to avoid spurious correlations or collinearity difficulties.

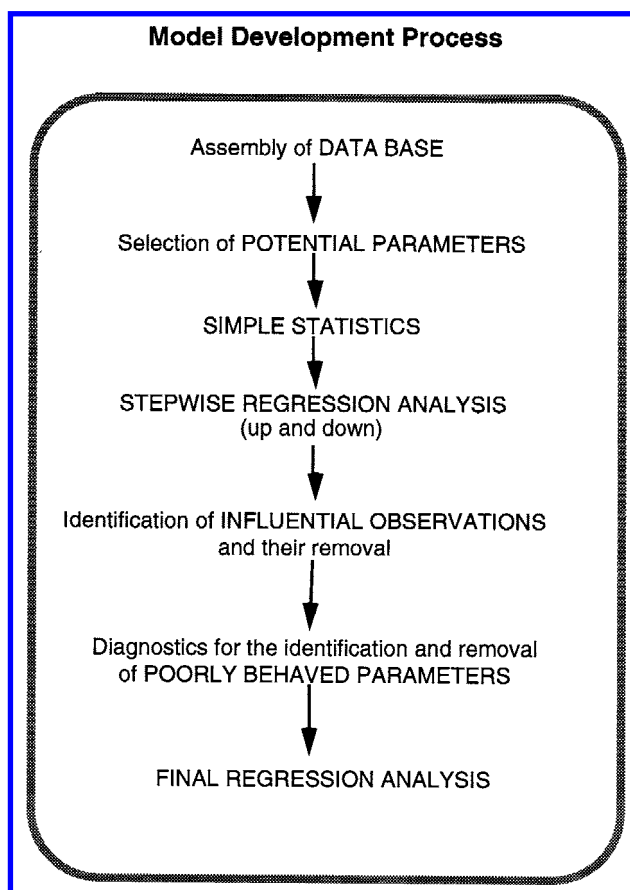


Figure 5.17 The statistical processes of QSAR model development using regression.

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 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.

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 LD₅₀ 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

Key	Cross Product
Primary amine (noncyclic) r-NH ₂ (R = alkyl)	0.961
Valence adjusted path MCI order 1	0.437
Constant term	2.287
Total	3.685

Estimate of EC₅₀ as $\log(1000/\text{Molar}) = 3.685$ 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
Allyamine	110.0	14.1
Cyclohexylamine	80.0	6.9
<i>n</i> -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 h *D. magna* EC₅₀ 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 in 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 a 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.

RECEPTOR-MEDIATED TOXICITY, ENDOCRINE DISRUPTION, AND A MECHANISTIC SAR ANALYSIS OF PCB TOXICITY

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 are 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 5.18](#) diagrams 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, which 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 genes 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 androstenedione and initiate male sexual development. Estrogens include estradiol, estrone, and estriol and are important in the development of female sexual characteristics and in regulating female receptiveness and reproduction.

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 receptors. Toxicants that are endocrine disruptors work in two basic ways ([Figure 5.19](#)). 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 not only occupies the active site but also 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

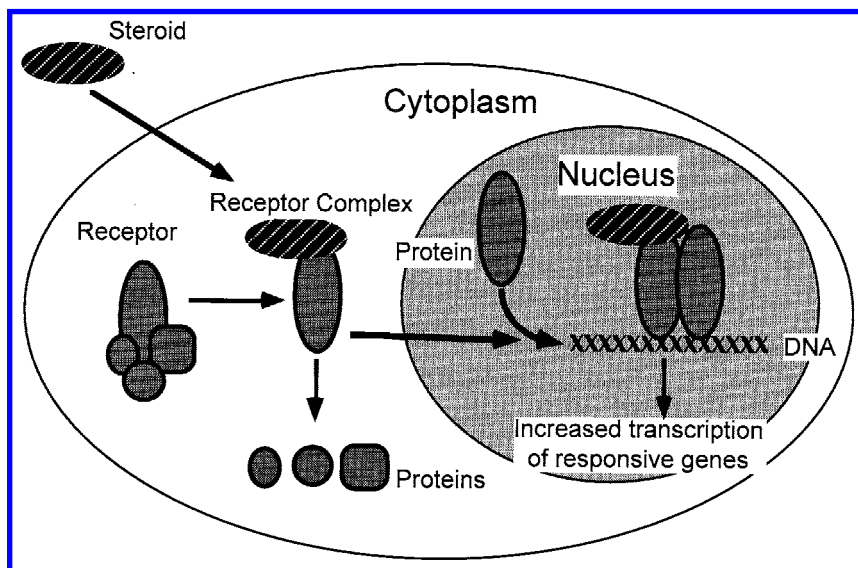


Figure 5.18 Regulatory role of steroidal hormones.

the instance of a xenobiotic blocking an estrogen receptor, masculinization of females can occur.

The different affinities for the estrogen receptor has been demonstrated by Vonier et al. (1996) investigating the binding of a variety of xenobiotics to alligator estrogen receptor (ER) (Table 5.2). Inhibition of a tritiated 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 [³H]17B-estradiol binding at low concentrations. The compounds *o,p'*-DDD and *o,p'*-DDT were particularly potent, while the related compounds, differing in only the substitution pattern *p,p'*-DDD and *p,p'*-DDT, 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.

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 and the 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 PCBs.

Dioxin and PCBs are hypothesized to be toxic because of three modes of action (McKinney and Walker 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

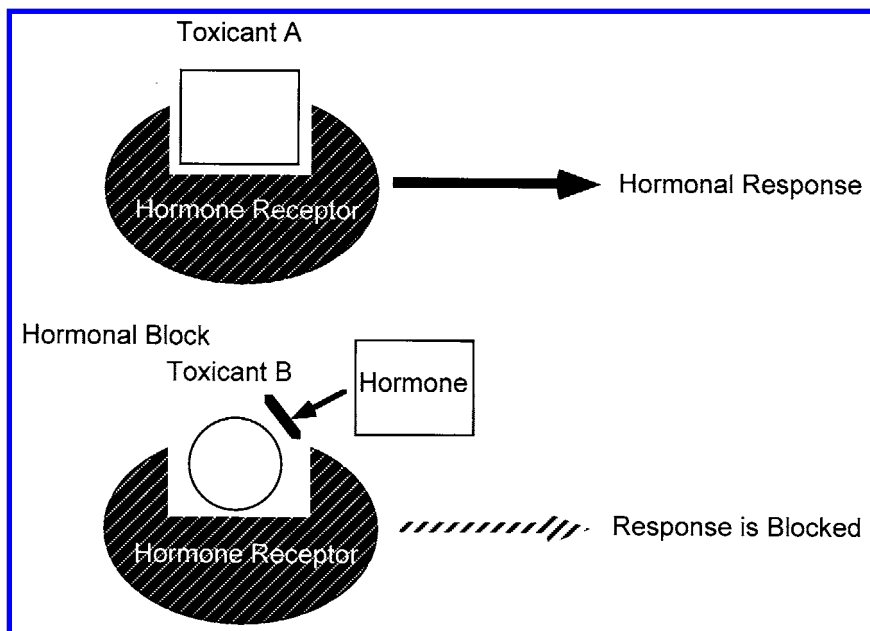


Figure 5.19 Mechanisms for the xenobiotic disruption of hormonal activity. In certain cases the xenobiotic may interact with the hormone receptor in such a manner that a hormonal response is generated. In some instances this hormonal response is inappropriate for the sex or normal breeding state of the 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 thus the response is blocked.

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 5.3 presents data for the toxicity of TCDD to a variety of 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 had little or no effect. Conversely, Table 5.4 presents data for several vertebrate species. 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, that 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. Given that vertebrates and invertebrates have diverged more than 500 million years ago, it is ironic that an evolutionary event of the Cambrian period determines the pattern of toxicity to compounds not formed until the 20th century.

Table 5.2 IC₅₀ for the Inhibition of [³H]17B-Estradiol Binding to Alligator Estrogen Receptor of Several Environmental Chemicals. Estradiol, DDD and Related Compounds Were Strong Inhibitors of Radioactive Estradiol to the Receptor.

Chemical	Alligator ER-binding IC ₅₀ (M)
17B-estradiol	0.0078
<i>o,p'</i> -DDD	2.26
<i>o,p'</i> -DDT	9.1
DDDH	11.1
<i>o,p'</i> -DDE	37.25
Dicofol	45.6
<i>p,p'</i> -DDT	>50 ^a
<i>p,p'</i> -DDD	>50 ^a
<i>p,p'</i> -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

^a Compounds inhibited binding but were insoluble at concentrations necessary for an IC₅₀. NS, not significant. 2,4-D, 2,4-(dichlorophenoxy) acetic acid.

After Vonier et al. 1996.

This summary of the structure activity relationship to the modes of action of dioxin and PCBs is based on the review of McKinney and Walker (1994). 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 5.20). This specific configuration apparently allows for very specific modes of action. One of the important dioxin modes of action is its ability to stack when reacting with a variety of ring structures in proteins (Figure 5.21). 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 5.22). This thyroxin-type or cleft-type model is another specific mode of toxicity (Figure 5.23).

In a review of structure and nomenclature (Figure 5.24), PCBs are two biphenyl rings linked by a single carbon bond and 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

Table 5.3 Toxicity of TCDD to a Variety of Invertebrates

Test Species	Water Conc. (ng/l) ^a	Organism Conc. (pg/g) ^b	Duration of Exposure	Effects
Algae				
<i>Oedogonium cardiacum</i>	1330	2,295,000	33 d	No toxic effect
Vascular plant				
Duckweed	1300		33 d	No toxic effect
<i>Lemna minor</i>				
Duckweed	7.13	30,700	33 d	No toxic effect
<i>Lemna minor</i>				
Annelid				
Worm	200 ^c		55 d	No decrease in reproductive success
<i>Paranais</i> sp.				
Mollusk				
Snail (adult)	1330	502,000	33 d	No toxic effect
<i>Physa</i> sp.				
Arthropod				
Cladoceran (adult)	1330	1,570,000	33 d	No toxic effect
<i>Daphnia magna</i>				

^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 5.4 Toxicity of TCDD to a Variety of Vertebrates

Test Species	Water Conc. (ng/l) ^a	Organism Conc. (pg/g) ^b	Duration of Exposure	Effects
Fish				
Coho salmon				
<i>Oncorhynchus kisutch</i>				
Juvenile (3.5 g)	5.60		96 h	50% mortality
Mink				
<i>Mustela vison</i>				
Newborn		1000 ^c	Daily for 12 d	100% mortality after 14 d

^a Measured TCDD concentration in water.

^b Measured TCDD concentration in organism (wet weight).

^c Unmeasured TCDD concentraton in water or organism (wet weight).

mode of action resembles that of dioxin; in other cases the PCB may act as an estrogen analogue.

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' hexachlorobiphenyl (HCB) does resemble 2,3,7,8 TCDD when in the coplanar configuration (Figure 5.25). In contrast, this structure is not available to 2,2',4,4',6,6' HCB due to the steric hindrance to the chlorine atoms. It is hypothesized that the coplanar configuration of the PCB allows these

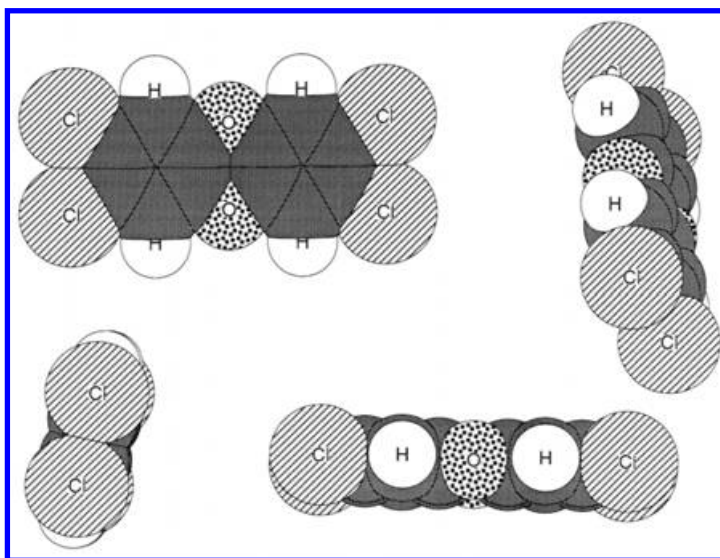


Figure 5.20 The three-dimensional structure of 2,3,7,8 TCDD.

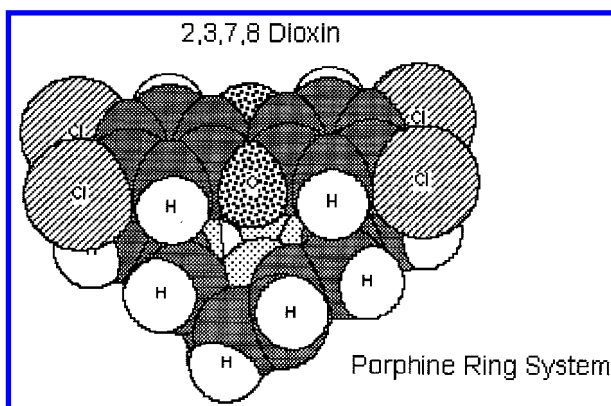


Figure 5.21 Stacking model for dioxin toxicity.

types of compounds to share the stacking and cleft-type modes of action hypothesized for dioxin. In [Figure 5.26](#), 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 5.27](#) 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. Although the ortho-substituted PCBs are less dioxin-like compared to other PCBs, upon hydroxylation they become more potent estrogenic compounds.

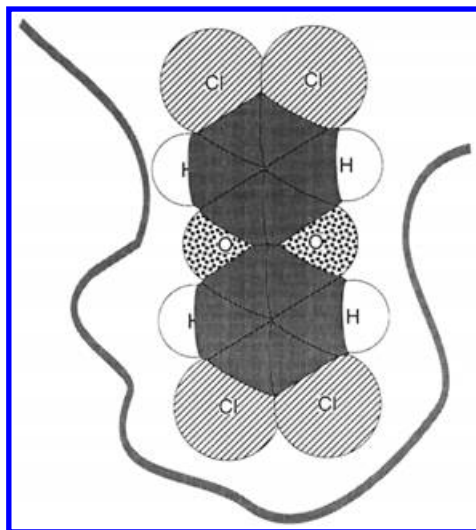


Figure 5.22 Cleft-type model for dioxin toxicity.

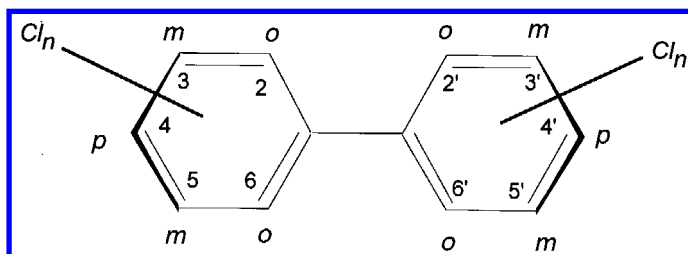
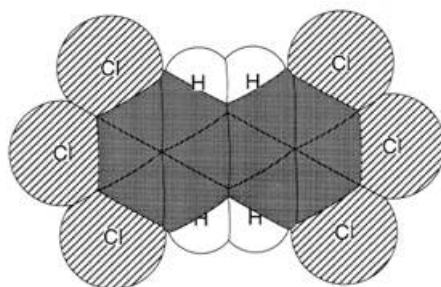


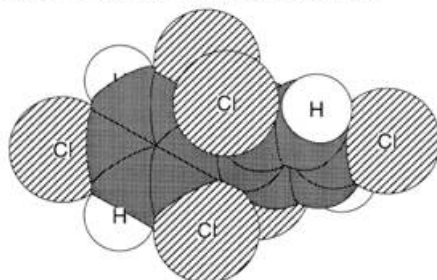
Figure 5.23 Structure and nomenclature for PCBs. A number of compounds exist with this same general structure, with varying numbers of chlorine atoms and positions along the two aromatic rings. The positions of these substituted chlorines are denoted by the number of carbons in each ring. Relative positions also are denoted by the *o*, *m*, and *p*, which are shorthand for ortho, meta, and para.

Coplanar conformation



3,3', 4,4',5,5' Hexachlorobiphenyl

Co-planar confirmation not available



2,2', 4,4',6,6' Hexachlorobiphenyl

Figure 5.24 Conformations of coplanar and nonplanar PCBs.

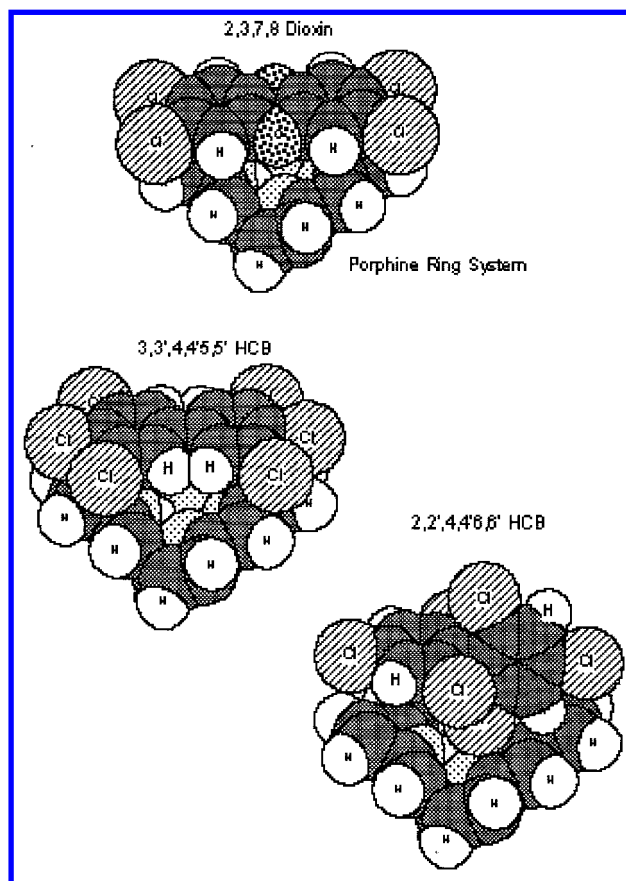


Figure 5.25 Stacking by 2,3,7,8 dioxin and PCBs.

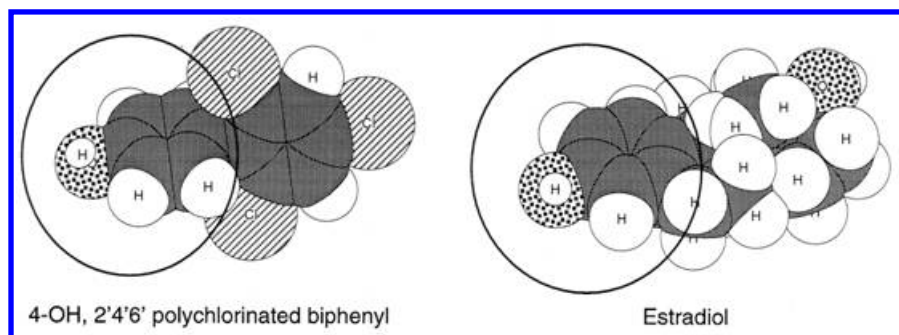


Figure 5.26 Similarity of a substituted PCB with estradiol.

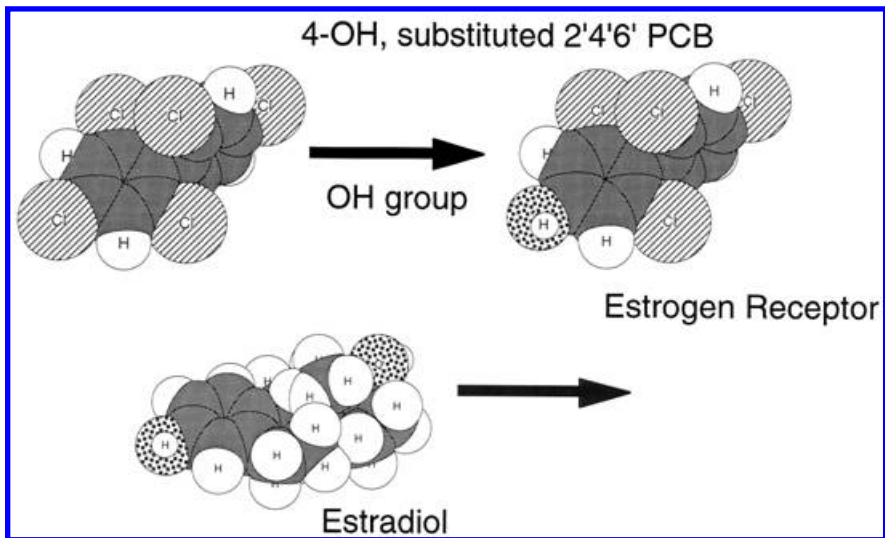


Figure 5.27 Suggested mode of action of a PCB with a substituted -OH group.

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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 which 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 are monohaloacetic acids? Describe the mode of action of fluoroacetic acid, iodoacetic and bromoacetic acids, and chloroacetic acid.
17. What are the potential modes of action for 2,3,7,8 TCDD?
18. Explain the potential modes of action for coplanar PCBs.
19. Why is the term "coplanar PCB" really a misnomer?
20. Diagram the stacking and cleft models that describe PCB and dioxin toxicity.
21. What mode of action do PCBs without coplanarity share with synthetic estrogens?
22. Explain the difference in toxicity of TCDD between vertebrates and invertebrates.
23. What are QSAR?
24. What are the two general types of toxicity data? How are they modeled?
25. Describe the three sets of traditional models for toxicity using regression and discriminate analysis.
26. Describe the developmental process for the construction of a structure-activity model. What is the importance of the reliability of the database?
27. What is MOLSTAC™ and how is it used?
28. Describe the statistical processes of the QSAR model.
29. Explain cross-validation of the QSAR model.
30. Describe the TOPKAT system.
31. 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?

CHAPTER 6

Factors Modifying the Activity of Toxicants

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, exposure time, environmental factors, interaction, biological factors, and nutritional factors. The parameters that modify the toxic action of a compound are examined in this chapter.

PHYSICOCHEMICAL PROPERTIES OF POLLUTANTS

Characteristics such as whether a pollutant is solid, liquid, or gas; whether the pollutant is soluble in water or in lipid; 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 non-ionized 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.

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 recover homeostatic balance during an “off” phase of intermittent exposure than if they are exposed to the same level of toxicant continuously. In addition, organisms may be able to develop tolerance after an intermittent dose.

ENVIRONMENTAL FACTORS

Environmental factors such as temperature, humidity, and light intensity also influence the toxicity of pollutants.

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 with power plants. Thermal pollution is the release of effluent that is at a higher temperature than the body of water it is released in. Vast amounts of water are used for cooling purposes by steam-electric power plants. Cooling water is often discharged at an elevated temperature causing river water temperatures to be raised to such an extent that the water may be incompatible for fish life.

Temperature changes in a volume of water affect the amount of dissolved oxygen (DO). The amount of DO present at saturation in water decreases with increasing temperature. On the other hand, the rate at which most chemical reactions occur increases with increased temperatures. Many enzymes have a peak temperature range. Above and below that range they are much more inefficient at catalyzing reactions. An elevated temperature leads to faster assimilation of waste and therefore faster depletion of oxygen. This depletion also adversely affects the ability of fish and other animals to survive in these heated waters. Additionally, 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.

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 gladioli to be more sensitive to fluoride as relative humidity increased from 50 to 80%.

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 4000 foot-candles (fc) (Dunning and Heck 1973). In contrast, the sensitivity of pinto bean to PAN (peroxyacetyl nitrate), a gaseous pollutant, increased with increasing light intensity. 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 1000 fc, light intensity must have an effect on plant response beyond its effect on stomatal opening.

INTERACTION OF POLLUTANTS

Seldom are living organisms exposed to a single pollutant. Instead, they are exposed to combinations of pollutants simultaneously. In addition, the effect of pollutants is dependent on many factors including portals of entry, action mode, metabolism, and others previously described above. Exposure to combinations of pollutants may 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.

Synergism and Potentiation

These terms have been variously used and defined 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 synergistic effect, resulting in increased lung cancer incidence. The presence of particulate matter such as sodium chloride (NaCl) and sulfur dioxide (SO₂), or SO₂ and sulfuric acid mist simultaneously, would have potentiative or synergistic effects on animals.

Similarly, exposure of plants to both O₃ and SO₂ simultaneously is more injurious than exposure to either of these gases alone. For example, laboratory work indicated that a single 2-h or 4-h exposure to O₃ at 0.03 ppm and to SO₂ at 0.24 ppm did not injure tobacco plants. Exposure for 2 h to a mixture of 0.031 ppm of O₃ and 0.24 ppm of SO₂, however, produced moderate (38%) injury to the older leaves of Tobacco Bel W3 (Menser and Heggstad 1966) ([Table 6.1](#)).

Table 6.1 Synergistic Effect of Ozone and Sulfur Dioxide on Tobacco Bel W3 Plants

Duration, h	Toxicants, ppm		Leaf damage, %
	O ₃	SO ₂	
2	0.03	—	0
2	—	0.24	0
2	0.031	+0.24	38

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.

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 when administered separately. Antagonism may be due to chemical or physical characteristics of the xenobiotics, or it may be due to the biological actions of the chemicals 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.

Physical means of antagonism can also exist. For example, oil mists have been shown to decrease the toxic effects of O₃ and NO₂ 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.

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).

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). Calculations are based on knowing the “site of dosage”, “site of action”, and the “physiological system” which are well documented in the pharmacological literature. Additionally, numerous models exist for predicting mixture toxicity but require prior knowledge of pair-wise 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 the Multiple Toxicity Indices (Konemann 1981). These models, working in combination, will be most useful for the amount of data that is 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:

$$= \frac{P_s}{P_{T_{50}}} + \frac{Q_s}{Q_{T_{50}}} \quad (6.1)$$

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 devised a more refined system to determine toxicity based on the formula:

$$\frac{A_m}{A_i} + \frac{B_m}{B_i} = S \quad (6.2)$$

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 than 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 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 that 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 value 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 6.1). A summary of this procedure is as follows:

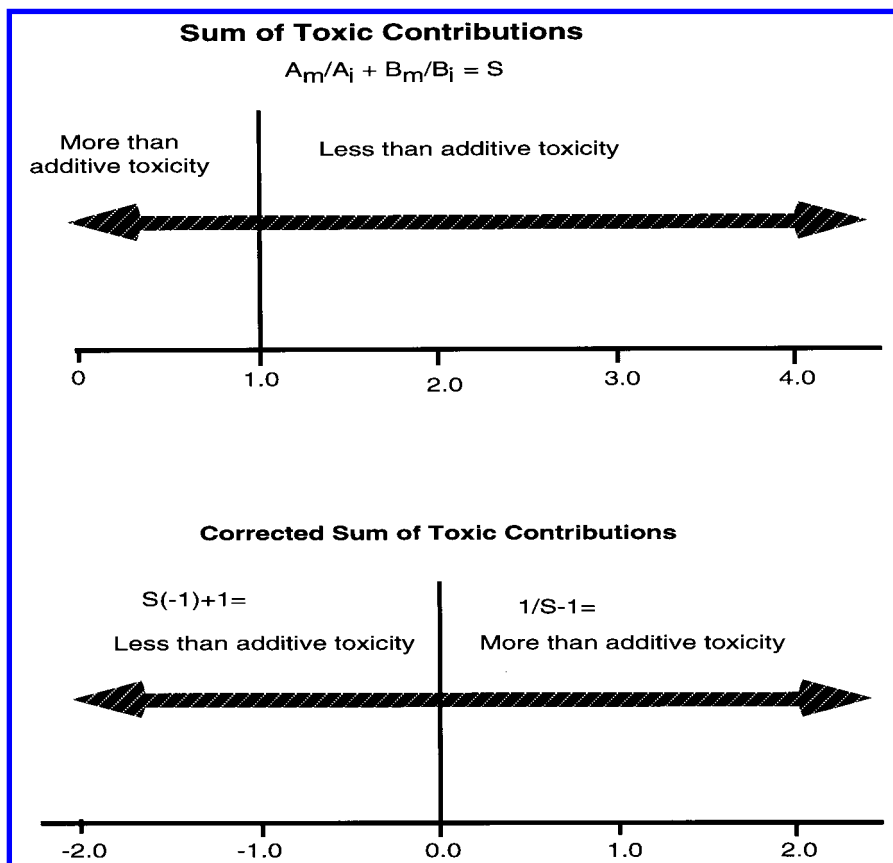


Figure 6.1 Graphical representation of the sum of toxic contributions. In the top of the figure the sum of toxic contributions is counterintuitive, the more than additive toxicity has a ratio of less than one and the proportions are nonlinear. With the corrections in the corrected sum of toxic contributions, the less than additive toxicity is less than one with the more than additive toxicity greater than one.

$$\frac{A_m}{A_i} + \frac{B_m}{B_i} = S, \text{ the sum of biological effects} \quad (6.3)$$

$$\text{Additive Index} = 1/S - 1.0 \text{ for } S \leq 1.0 \text{ and} \quad (6.4)$$

$$\text{Additive Index} = S(-1) + 1.0 \text{ for } S \geq 1.0 \quad (6.5)$$

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 H. Konemann introduced a Multiple Toxicity Index (MTI):

$$MTI = 1 - \frac{\log M}{\log m_0} \quad (6.6)$$

where $m_0 = M/f_{\max}$; $f_{\max}$ = 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); $M = \sum_{i=1}^n z_i/Z_i$ = sum of toxic units giving the desired response; 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:

$$MTI = 1 - \frac{\log M}{\log n} \quad (6.7)$$

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 to interpret.

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 disadvantage to the implementation is that these equations are not set up for easy use and the lack of environmental toxicity data. A combination of implementation of the selected methodology into a computer program coupled to a large database and 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, CAS numbers for the compounds with an unknown toxicity and the toxicity of the mixture, if known. Concentrations of each material also are input.
- A system for searching the appropriate databases for toxicity data or SAR models for estimating the desired parameter. The 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 the difficulty of 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

$$A_c/A_i + B_i/B_i + C_i/C_i + = MT \quad (6.8)$$

where A_c = environmental concentration of compound A, A_i = concentration resulting in the endpoint selected, for example a EC_{50} or LC_{10} , and MT is the mixture toxicity as a fraction with 1 equal to the mixture having the 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.

ESTIMATING THE TOXICITY 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) and predicts the sediment toxicity of polynuclear aromatic hydrocarbons (PAH). 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 ΣPAH 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 also was 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 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.

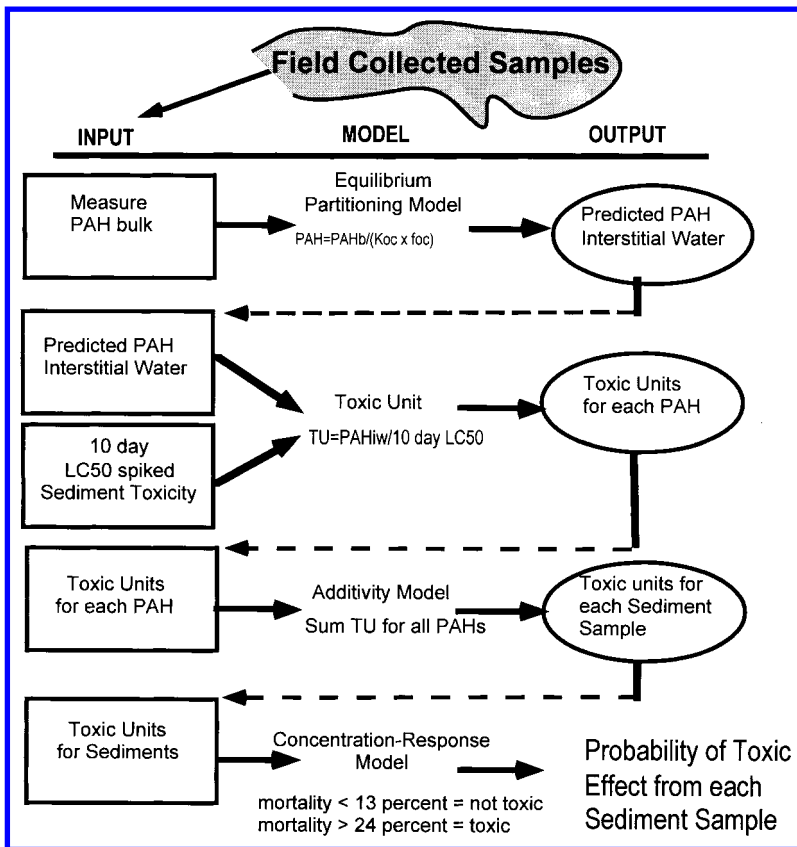


Figure 6.2 The steps in calculating the toxicity of PAHs to amphipods.

The estimates of toxicity are expressed as nontoxic, 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 nontoxic. Between 13 and 24% mortality, the toxicity prediction is considered uncertain. Above a prediction of 24% mortality the sediment is considered toxic.

A flow chart for estimating sediment toxicity is presented in Figure 6.2. 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 using the 10-day amphipod LC_{50} as the toxicity benchmark. The toxic units 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

**Table 6.2 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
W. Port	0.00001 ± 0.00002
E. Port	0.00001 ± 0.00001

Note: The mean sum of the toxic units with the standard deviations are listed. In this instance the probability of toxicity was low at each sampling site.

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%.

Wiegiers et al. (1997) also have applied the model to the concentrations of 10 PAHs (data for all 13 PAHs were not consistently available) for samples collected throughout Port Valdez, AK. 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 the Solomon Gulch Hatchery also were collected. All of the acute toxicity levels predicted in Port Valdez occurred 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 6.2](#) as a comparison between samples collected from the identified sub-areas.

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 ΣPAH 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 using set criteria for specific compounds, the ΣPAH offers a distinct advantage to the accurate prediction of toxicity.

BIOLOGICAL FACTORS AFFECTING TOXICITY

Plants

In plants, the most widely studied and probably the most important factor affecting 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 also have been observed. Plants show differences in their susceptibility to different pollutants. For instance, some plants may be sensitive to O_3 but relatively resistant to SO_2 , while in others the opposite may be true.

The sensitivity of plants to atmospheric pollutants such as O_3 is known to be related, in part, to stomatal opening and closure.

Leaf maturity also affects the sensitivity of plants to air pollutants. Generally, young tissues are more sensitive to PAN and hydrogen sulfide, and maturing leaves are most sensitive to the other airborne pollutants.

Animals

Genetic, developmental, health status, sex variation, and behavior are among the important factors affecting the response of animals and humans to pollutant toxicity (Hodgson 1980).

Genetic Factors

Similar to the situation in plants previously discussed, different species of animals respond differently 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 genetically induced malabsorption can contribute to differences in their response to environmental stresses. Individuals with malabsorption syndrome, for example, may suffer nutritional deficiencies, which in turn may lead to an increased susceptibility to environmental chemicals.

Developmental Factors

Immature immune system, aging, pregnancy, immature detoxication systems, and circadian rhythms are included in this category. For example, lack of γ -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 immature hepatic MFO system in the young; are all contributing factors to varying responses exhibited by the individuals to xenobiotics.

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. Diseases in any of these organs would lead to impaired functioning and decreased ability to cope with xenobiotics. For instance, cardiovascular and respiratory diseases of other origins decrease the individual's ability to withstand superimposed stresses. An impaired renal function will certainly affect the kidney's ability

Table 6.3 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

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. Liver disorders, therefore, will seriously impair detoxication processes.

Lifestyle

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 thus may 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.

Sex Variation

The rate of metabolism of foreign compounds varies with the difference in sex of both humans and animals. For example, response to chloroform (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 is affected by different strains as well (Table 6.3).

NUTRITIONAL FACTORS

The importance of nutrition as a major factor affecting the toxicity of chemicals has been recognized in the recent years. Results obtained from human epidemiological and animal experimental studies strongly support the relationship between nutrition and pollutant toxicity. For example, laboratory animals fed low protein diets have been reported to be more susceptible to the toxicity of chemicals under test. The interaction between nutrition and environmental pollutants is complex, and understanding its nature is a great challenge in the study of both toxicology and

nutritional biochemistry. It may be mentioned that a new area of study called **nutritional toxicology** has emerged in the recent years.

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 circulating level of those chemicals. It can cause changes in body composition leading to altered tissue distribution of chemicals. Dietary factors also can influence renal function and pH of body fluids, resulting in altered toxicity of chemicals. In addition, responsiveness of the target organ may be modified as a result of changing nutrition.

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. Animal studies also showed that glucuronide conjugation was decreased under starvation.

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 effect 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 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 6.4). On the other hand, rats treated under the same conditions

Table 6.4 Effect of Protein on Pesticide Toxicity

Compounds	Casein content of diet	
	3.5%	26%
Acetylcholinesterase inhibitors	LD ₅₀ , mg	
Parathion	4.86	37.1
Diazinon	215	466
Malathion	759	1401
Carbaryl	89	575
Chlorinated hydrocarbons		
DDT	45	481
Chlordane	137	217
Toxaphene	80	293
Endrin	6.69	16.6
Herbicide and fungicides		
Diuron	437	2390
Captan	480	12,600

Note: Male rats fed for 28 days from weaning on diets of varying casein contents, then given an oral dose of pesticides.

may show a decreased mortality with respect to heptachlor, CCl₄, and aflatoxin. It is known that, in the liver, heptachlor is metabolized to the epoxide, which is more toxic than heptachlor itself, while CCl₄ is metabolized to CCl₃, a highly reactive free radical. As for aflatoxin, the decreased mortality is due to reduced binding of its metabolites to DNA.

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 also may lead to a greater vulnerability of fasted animals to xenobiotics such as acetaminophen, whose metabolism is associated with depletion of the glutathione (GSH) component of the hepatic antioxidant defense system.

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 lipophilic 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 (Parke and Loannides 1981). A high-fat diet may favor more oxidation to occur, as it may contribute to more incorporation of membrane material.

Types of lipids also can 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 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 Augustinem 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 body burden of fluoride. Dietary fat does not increase 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.

Vitamin A

Interest in vitamin A and its synthetic analogues 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 8000 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. Experimental studies show that rats exposed to PCB, DDT, and dieldrin caused a marked reduction in liver vitamin A store suggesting that the metabolism of vitamin A may be affected by exposure to these organisms. 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 detoxification, however, depends on several factors such as substrate, tissue, and animal species.

Vitamin D

The role that vitamin D plays in the prevention of rickets and osteomalacia is widely known. Studies have shown that the mechanism 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-D₃. This is then converted in the kidney to 1,25-dihydroxy-D₃, the “hormone-like” substance that is the active form of the vitamin. The 25-hydroxylation of cholecalciferol requires NADPH, O₂, and an enzyme whose properties are similar to those of microsomal MFO (Bjorkhelm et al. 1979). In addition, 25-hydroxy-D₃ has been shown to competitively inhibit some cytochrome P-450 reactions *in vitro*.

Vitamin E

Vitamin E (α -tocopherol), a potent membrane-protecting antioxidant, protects against toxicants causing membrane damage through peroxidation. Male rats supplemented with daily doses of 100 mg tocopheryl acetate and exposed to 1.0 ppm O₃ have been shown to survive longer than vitamin E-deficient rats. The action of O₃ is attributed at least 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 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; 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.

Vitamin C

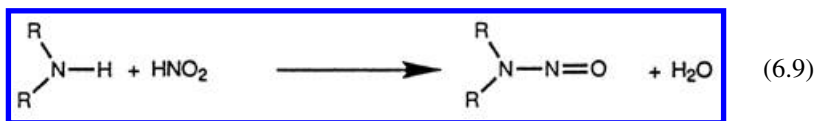
Vitamin C (ascorbic acid) is found in varying amounts in almost all of our body tissues. In particular, high contents are found in adrenal and pituitary glands, eye lens, and various soft tissues (Table 6.5). Ascorbic acid is a potent antioxidant and participates in a large number of cellular oxidation-reduction reactions. Thus, vitamin C protects against superoxide formation in the cytosol. Its relationship to drug

Table 6.5 Ascorbic Acid Content of Adult Human Tissues

Tissue	Ascorbic acid (mg/100 g wet tissue)
Pituitary glands	40–50
Leucocytes	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

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:



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 RDA (Recommended Dietary Allowances) 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, 4.1 mg nitrite/day, in addition to exposure to ambient air containing CO, O₃, Pb, cigarette smoke, and others (Calabrese 1980). Recommendations for increasing the RDA for vitamin C to meet such additional needs, however, has not received general support. Moreover, it is known that a dietary excess of vitamin C can produce various adverse effects, based on nutritional and clinical point of view.

Minerals

About 20 mineral elements are considered to be essential in human nutrition, and seven of these, including calcium, phosphorus, sodium, potassium, magnesium, sulfur, and chlorine are called macrominerals, while the rest are often referred to as trace elements. 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 complexes 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 H₂O₂ and organic hydroperoxides before they can cause membrane disruption.

As previously mentioned, increasing evidence suggests that oxygen radicals play a major role in the pathophysiology of many diseases including cancer. Reference was also made that antioxidants play a vital role in counteracting these radicals. Like other antioxidants such as vitamins C and E and β-carotene, selenium is considered by many to exhibit anticarcinogenic action. It should be noted that only relatively small amounts of selenium are needed to meet known physiological functions and, like other essential nutrients, selenium is toxic when consumed in excess.

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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 ΣPAH model for estimating the sediment toxicity of mixtures of PAHs.
13. What are plants' most important factor affecting response to air pollutants? What is another factor for plant sensitivity?
14. Name five important factors affecting the response of animals to pollutant toxicity.
15. What effects can nutritional modulation have on response to pollutant toxicity?
16. What effect does a high-carbohydrate diet have on detoxification? What effect do dietary lipids have?
17. What are several possibilities of mechanisms involved in vitamin A action in relation to carcinogenesis?
18. Discuss the relationships of vitamin E and vitamin C with nitrosamine.

CHAPTER 7

Inorganic Gaseous Pollutants

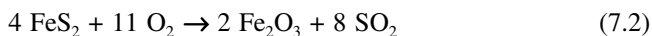
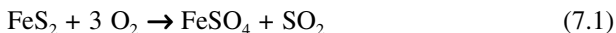
In this Chapter, five of the major gaseous air pollutants are considered, i.e., sulfur oxides (SO_x), nitrogen oxides (NO_x), ozone (O₃), carbon monoxide (CO), and fluoride (F). Although much of the fluoride emitted into the atmosphere from various sources is in the particulate form, fluoride is included in our discussion here with other inorganic gaseous pollutants mainly because gaseous fluoride causes the most damage to living organisms, especially plants.

SULFUR OXIDES

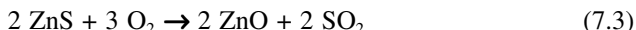
Sulfur oxides include both sulfur dioxide (SO₂) and sulfur trioxide (SO₃), of which SO₂ is more important as an air pollutant. Sulfur trioxide may be formed in the furnace by reaction between sulfur and O₂ or SO₂ and O₂. Sulfur dioxide is probably the most dangerous of all gaseous pollutants on the basis of amounts emitted.

Sources of SO₂

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 (FeS₂) or pyrite. When heated at high temperatures, pyrite undergoes the following reactions:



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 SO₂ as shown below:



In the U.S., SO₂ emission from stationary sources and industry accounts for about 95% of all SO₂ emission.

Characteristics of SO₂

SO₂ is highly soluble in water, with a solubility of 11.3g/100 ml. Once emitted into the atmosphere, SO₂ may undergo oxidation in the gaseous phase, forming H₂SO₄ aerosol. Gaseous SO₂ also may become dissolved in water droplets and, following oxidation, form H₂SO₄ aerosol droplets. Both forms of H₂SO₄ thus produced may be removed by deposition to the Earth's surface (Figure 7.1).

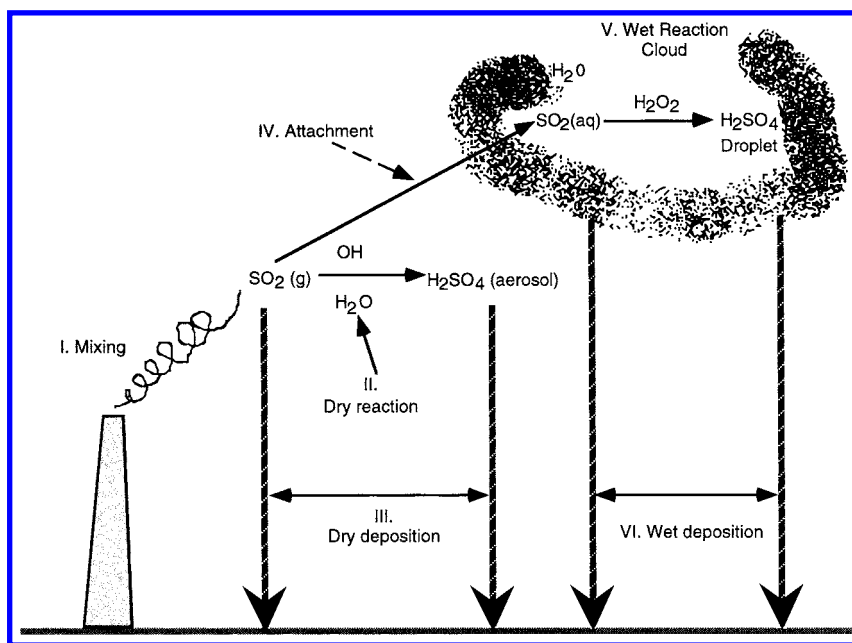


Figure 7.1 SO₂ transport, transformation, and deposition processes. Initially SO₂ is mixed into the atmosphere (I). Gaseous SO₂ may undergo oxidation in the gaseous phase with subsequent formation of H₂SO₄ aerosol (II). Both gaseous SO₂ and H₂SO₄ aerosol may be deposited at the Earth's surface (III). Gaseous SO₂ may become dissolved in a water droplet (IV). The dissolved SO₂ can be oxidized in solution to form H₂SO₄ aerosol droplets (V). The H₂SO₄ aerosol and the H₂SO₄ droplet may be removed to the Earth's surface by wet deposition (VI). (From Fox, D.L. 1986. The transformation of pollutants. In *Air Pollution*, 3rd ed., Vol. VI, A.C. Stern, Ed., Academic Press, New York, pp. 86-87. With permission.)

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 7.2.

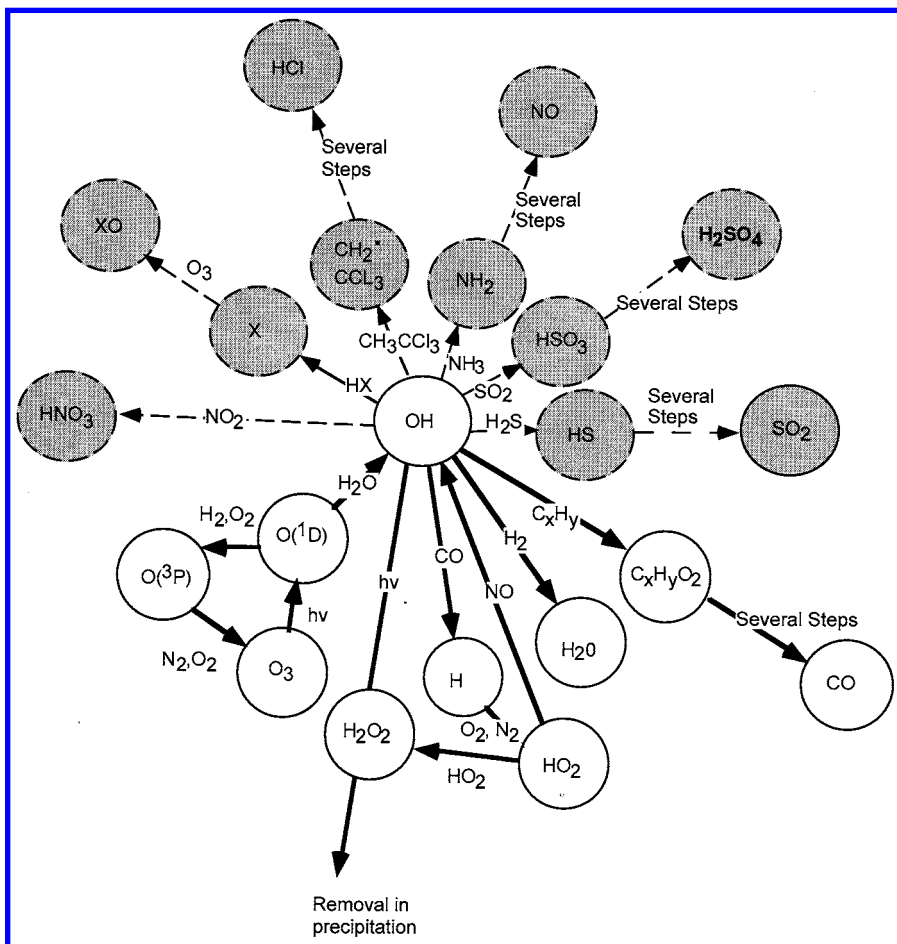


Figure 7.2 Photochemistry of the OH radical controls the trace gas concentration. The photochemistry of the free hydroxyl radical controls the rate at which many trace gases are oxidized and removed from the atmosphere. Processes that are of primary importance in controlling the concentration of OH in 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 broken lines. 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. $\text{HX} = \text{HCl}, \text{HBr}, \text{HI}$ or HF . C_xH_y denotes hydrocarbons. (From W.L. Chameides and D.D. Davis, *Chem. Eng. News*, 60 (40): 38-52, 1982. American Chemical Society. With permission.)

Effects 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 also are 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. 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 nonenzyme mechanisms. Several factors, including cellular enzymes such as peroxidase and cytochrome oxidase, metals, ultraviolet light, and O_2^- stimulate the oxidation of SO_2 . In the presence of SO_3^{2-} and HSO_3^- , more O_2^- 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- biphosphatase, and ribulose-5-phosphate kinase. Enzyme activity may be increased or decreased by exposure to SO_2 at different concentrations. As mentioned previously, there are differences in sensitivity of plant species to SO_2 under similar biophysical conditions. This suggests that delicate biochemical and physiological differences operating in different plant species could affect the sensitivity of a particular plant to SO_2 .

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 the ambient values produced little distress (Alarie et al. 1970; Alarie et al. 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 h 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 gains, loss of hair, nephrosis in kidneys, myocardial degeneration, and accelerated aging.

Many studies have demonstrated 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.

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 exhibited 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 perception 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 recommended 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 mouthpiece, while the increase was less with nasal breathing (Frank et al. 1962). Exercise augments responses to the pollutants.

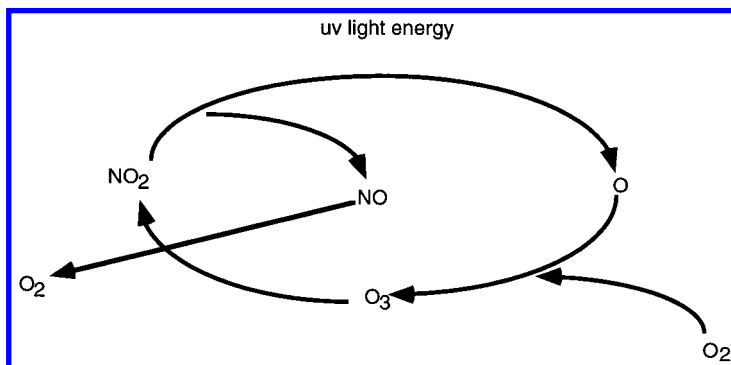


Figure 7.3 The photolytic cycle of NO_2 .

NITROGEN OXIDES

Forms and Formation of Nitrogen Oxides

Six forms of nitrogen oxides are present, i.e., 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 toxicities and air pollution significance. Basic chemical reactions involved in the formation of NO_2 are shown below:

1210°C



The NO formed in Equation 7.4 persists when temperature is rapidly lowered, as is the case in ambient air. The reaction shown in Equation 7.5 is one of the few reactions which are slowed down with increase in temperature.

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 7.3). It is clear that, as far as the cycle is concerned, there is no net loss or gain of chemical substances.

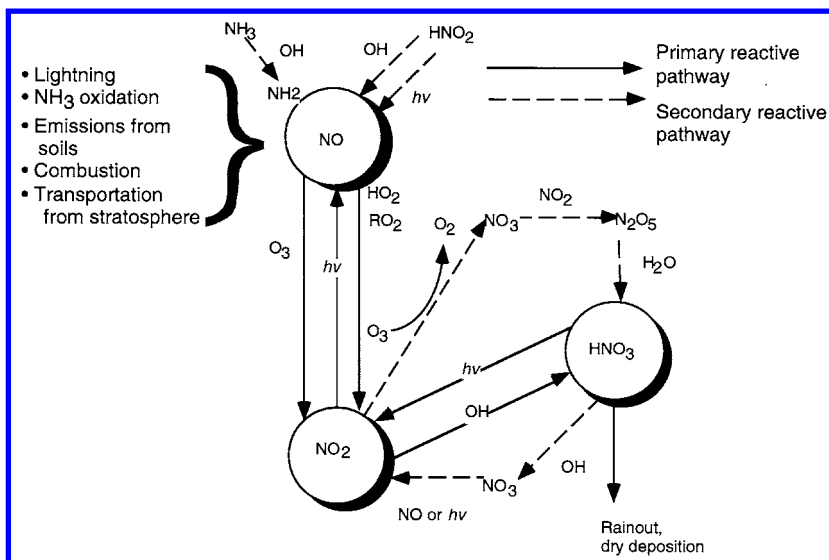


Figure 7.4 Major reactive N species in troposphere. (From W.L. Chameides and D.D. Davis, *Chem. Eng. News*, 60(40): 38–52, 1982. American Chemical Society. With permission.)

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 , nitric acid also is an important N compound in the troposphere. It is formed mainly from NO_2 and OH radicals. Nitric acid also is 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 7.4.

Effects on Plants

Plants absorb gaseous NO_x through stomata. NO_2 is more rapidly absorbed than NO, mainly because NO_2 reacts readily 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 (Zeevart 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 NO_x 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 injury may be caused by ammonia produced

from NO_3^- , if it is not utilized soon after its formation. Plants can metabolize the dissolved NO_x through their NO_2 assimilation pathway:



Other biochemical pathways affected by NO_x include inhibition of lipid biosynthesis, oxidation of unsaturated fatty acids *in vivo*, and stimulation of peroxidase activity. It should be noted that the general sense of the plant scientists is that, at current ambient concentrations, nitrogen oxides by themselves play little to no role in observed plant damages.

Effects on Humans and Animals

Studies on the pathological and physiological effects of NO_2 on animals have been conducted 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 h resulted in bits of fibrin in the airway, increased number of macrophages, and altered appearance of the cells in the distal airway and adjacent pulmonary alveoli. Terminal bronchioles showed hyperplasia and hypertrophy, loss of cilia, and disturbed ciliogenesis. Large crystalloid 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).

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+} ions and is thus unable to reversibly combine with molecular oxygen. As mentioned previously, although almost all the studies were conducted by using much higher concentrations of NO_2 than are found in ambient air; a few recent papers did deal with low NO_2 concentrations. Orehek et al. (1976) showed that in asthmatic subjects exposure to 0.1 ppm of NO_2 significantly aggravated the hyperreactivity in the airway. While at the prevailing concentrations of NO_2 the 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.

Biochemical Effects

Extracts of lung lipids from rats exposed to NO_2 have been shown to involve oxidation. Lipid peroxidation was more severe in animals fed a diet deficient in vitamin E (Roehm et al. 1971). In contrast to O_3 , 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 (1965, 1967a, 1967b) have demonstrated that exposure for 10 weeks or longer at 10 ppm or for 2 h at 50 ppm increased both tissue oxygen consumption and LDH and aldolase activity. Stimulation of glycolysis has also been reported.

OZONE

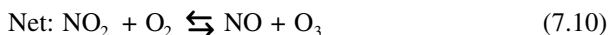
Sources

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 resultant atomic oxygen reacting with another oxygen molecule to form ozone:

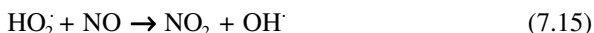
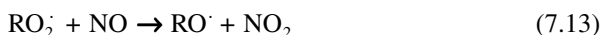


Ozone in the lower atmosphere also is produced as a result of modern technology. Equipment that produces sparks, arcs, or static discharge; ultraviolet and other ionizing radiation; commercial applications, such as air purifiers and deodorizers in homes, hospitals, and offices; and closed environmental 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 the previous section on nitrogen oxides, disruption of the photolytic cycle of NO_2 (Figure 7.3 and Equations 7.8 through 7.10) by atmospheric hydrocarbons is the principal cause of photochemical smog.



In the above equations, theoretically, the 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, $O^{\cdot}D$; 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:

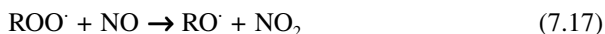


It is noticeable that the process starts with an $OH^\cdot$ radical. After one pass through the cycle, two molecules of NO are oxidized to NO_2 . The $OH^\cdot$ radical formed in the last step (Equation 7.15) can start the cycle again. On the other hand, O_3 also can be formed from O_2 reacting with hydrocarbon free radicals:

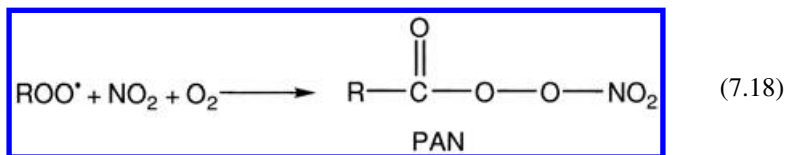


Photochemical Smog

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,



The free radical $RO_2^\cdot$ can also react with O_2 and NO_2 to produce peroxyacyl nitrate (PAN):



Peroxyacyl nitrate also can be formed from a reaction involving $\text{RO}_3^\cdot$ and NO_2 :



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 photochemical 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%), NO_x (mainly NO_2 , about 10%), PAN (0.6%), free radical oxygen forms, and other organic compounds such as aldehydes, ketones, and alkyl nitrates.

Effects 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 (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); (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; Izuta et al. 1991); (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 h in cucumber, bean, and monkey flower (Dugger and Ting 1970), suggesting an inhibition of amylase or phosphorylase. While decrease in glyceraldehyde-3-phosphate dehydrogenase activity suggests inhibition of glycolysis, 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. Recent studies showed that mung bean seedlings exposed to 250 ppb of O_3 for 2 h caused a marked decrease in invertase activity (Yu 1994). 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.

Effects on Humans and Animals

Ozone and other oxidants cause respiratory and eye irritation. The TLV (threshold limit value) for O_3 in industry is 0.1 ppm. Exposure to 0.6 to 0.8 ppm O_3 for 60 min 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 (Bates 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 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 increase the 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 that observed in community pollution in Los Angeles, CA.

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**.

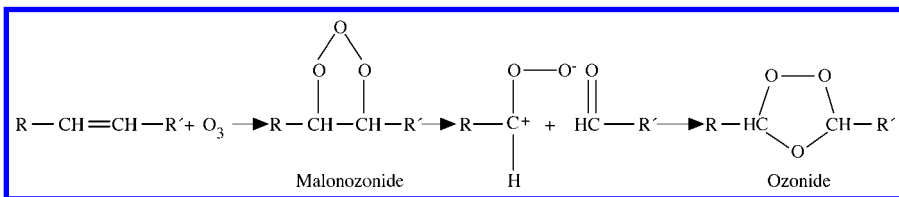
Biochemical Effects

Research on the biochemical effects of O_3 has been extensive. Among the many postulated mechanisms that have been advanced concerning the toxicity of O_3 , the following is 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.

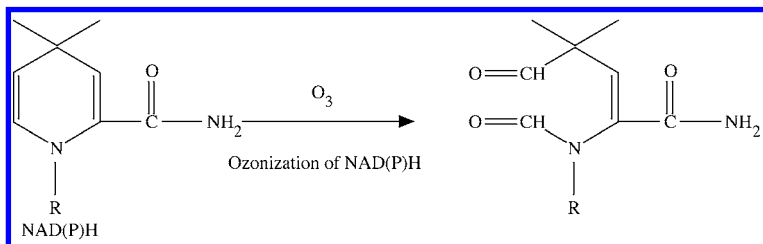
Ozone interacts with proteins and some amino acids causing alteration. For instance, the lysozyme in tears of individuals exposed to smog has been reported to be 60% less than the normal. Concentrations of protein sulfhydryl and nonprotein sulfhydryl in the lungs of rats exposed to 2 ppm O_3 for 4 to 8 h have been shown to be decreased. Mudd et al. (1969) showed that aqueous solutions of amino acids such as tyrosine, histidine, 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 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 h, and increase in glucose-6-phosphate dehydrogenase, 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 as 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).

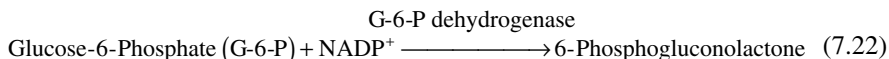
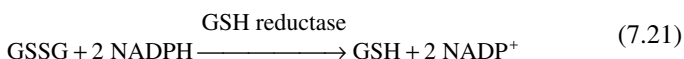
Another chemical pathway leading to O_3 -dependent unsaturated fatty acid oxidation is through incorporation of O_3 into the fatty acid double bond, resulting in ozonide formation. This process is generally known as ozonolysis:



Ozone also is 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. NADPH oxidation increases the activity of the pentose phosphate pathway. Such 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 O₃ exposure.



CARBON MONOXIDE

Carbon monoxide is an odorless, colorless, and tasteless gas that is often found in high concentrations in urban atmosphere. 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 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 fire fighters, traffic police, toll booth attendants, coal miners, coke oven and smelter workers, and transportation mechanics.

Formation of CO

Formation of CO occurs usually 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. Reactions between CO₂ and carbon-containing materials at high temperature. This is common in many industrial devices such as blast furnaces.



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 Fe₂O₃ ores as shown below. Some CO may escape into the atmosphere, however.



3. Dissociation of CO₂ at high temperature. Carbon dioxide dissociates into CO and O at high temperature as follows:

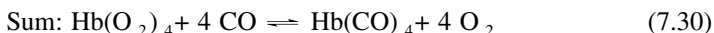
high temp.



High temperatures favor the dissociation of CO₂. For example, at 1745°C the dissociation is 1%, while at 1940°C, it is 5%.

Toxicological Effects

The most widely known physiological effect of CO is interference with O₂ transfer. This interference is brought about by the combination of CO gas with hemoglobin (Hb) leading to the formation of carboxyhemoglobin, HbCO or COHb:



The dissociation constant, K, for Equation 7.30 is 210 at 37°C. In other words, CO has a more than 200-times greater affinity for combination with Hb than O₂ does. A binding site on a Hb molecule cannot be occupied by both CO and O₂. Although increases in oxygen concentrations can shift the equilibrium in Equation 7.30 to the left, recovery of Hb is slow, while the asphyxiating effect of putting Hb out of business is rapid. Normal or background level of blood carboxyhemoglobin (HbCO or COHb) 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 COHb 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:

$$\text{Percent COHb in blood} = 0.16 \times (\text{CO conc. in the air in ppm}) + 0.5 \quad (7.31)$$

Table 7.1 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, of manual dexterity, of learning, and impairment of 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), vomiting, occasionally
30–35	Clouding of mental alertness occurs with increasing weakness
35–45	Collapse and coma
>50	Death (in young people)

Based on COHb levels, various health effects may be expected to occur. [Table 7.1](#) summarizes demonstrated health effects associated with COHb levels.

Carbon monoxide also inhibits the function of alveolar macrophages. This can lead to weakened tissue defenses against airborne bacterial infection. Maternal CO poisoning during pregnancy has been shown to cause fetal death due to lack of O₂ in the fetal circulatory system. Carbon monoxide poisoning causing unconsciousness for 30 min to 5 h 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 h at rest in room air. It is shortened to 60 to 90 min if 100% oxygen is given using a face mask. Since more than 2 h at 100% oxygen can cause pulmonary oxygen toxicity, the oxygen concentration should be reduced to 60% at 2 h.

Mechanism of Action

As mentioned previously, CO competes with O₂ 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 O₂ to the mitochondria, shifts the oxyhemoglobin dissociation curve to the left. Alteration of oxyhemoglobin dissociation curve by COHb occurs in such a manner that O₂ is released to tissues with great difficulty and at a lower O₂ tension.

Human Exposure to CO

Exposure to CO comes mainly from three sources: (1) CO in the surrounding ambient environment particularly 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, garages connected to living quarters, and space heaters in campers; (2) occupational exposure such as experienced by fire fighters (>10,000 ppm CO), traffic police, coal miners, coke oven and smelter workers, toll booth attendants, and

Table 7.2 Blood COHb Levels of Smokers

Category of smokers	Median equilibrium blood COHb (%)
Never smoked	1.3
Ex-smoker	1.4
Pipe and/or cigar smokers only	1.7
Light cigarette smoker (<1/2 pack/day; noninhaler)	2.3
(<1/2 pack/day; inhaler)	3.8
Moderate smoker (1/2–2 packs/day; inhaler)	5.9
Heavy smoker (>2 packs/day; inhaler)	6.9

transportation mechanics; and (3) cigarette smoking. Smokers have higher COHb levels than nonsmokers (Table 7.2). Nonsmokers may be subjected to inhalation of CO from cigarette smoke in confined places.

FLUORIDE

Environmental Sources and Forms of Fluoride

Fluorine is the lightest element in Group VII of the periodic table, with atomic number 9 and an atomic weight of 18.998. It has a single isotope and its valence in all naturally occurring compounds is 1. Fluoride (F) is ubiquitous: it occurs in minerals and soils, air, natural waters, and foods.

- **Minerals and soils.** — The fluoride content of rocks is 0.06 to 0.09% by weight of upper layer of lithosphere. The chief F-containing minerals are: fluorspar (CaF_2), cryolite (Na_3AlF_6), and fluorapatite ($\text{Ca}_{10}\text{F}_2(\text{PO}_4)_6$).
- **Natural waters** — The fluoride content of natural waters in the northeastern part of the U.S. ranges from 0.02 to 0.1 ppm, while it is about 0.2 ppm in the West and Midwest. River waters contain 0.0 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. Seawater contains about 1.2 ppm F.
- **Foods** — Virtually all foods contain trace amounts of fluoride. Fluoride-containing foods and beverages are, therefore, the most important sources of F intake. In the U.S., an adult takes in about 0.2 to 0.3 mg/day from foods, whereas the intake from drinking water ranges from 0.1 to 0.5 mg. In communities where the water supply is fluoridated, fluoride intake may amount to 1 to 2 mg/day. Plants can absorb F from soil, water, or air. Most plants contain 0.1 to 10 ppm F (on dry basis); forage plants generally contain 5 to 10 ppm (on dry basis). Fluoride contents in plants vary with plant species. Several species of plants are F accumulators. For instance, tea leaves may contain as high as 760 ppm F; camellia, 620 ppm; and elderberry, 3600 ppm (all on dry basis). Tea beverage, however, may contain less than 0.5 mg F per cup. The F contents of some foods are shown in Table 7.3.
- **Air** — The air in U.S. residential and/or rural communities contains less than 0.04 to 1.2 ppb (0.03 to 0.90 $\mu\text{g F/m}^3$).

Table 7.3 Fluoride Content of Selected Foods

Food	Fluoride content (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)	av. 60

Adapted from Committee on Biologic Effects of Atmospheric Pollutants, 1971.

- **Industrial sources of fluoride in the environment** — The major sources of fluoride emissions into the atmosphere from industrial sources include steel, phosphate fertilizer, ceramics (brick, tile, glass, etc.), and aluminum industries, combustion of coal (F content of coal: 0.001 to 0.048% in U.S., average 0.008%), nonferrous metal foundries; and welding operations.

Fluoride Pollution

Fluoride emitted into the atmosphere from volcanism, industries, and other sources includes both gaseous and particulate forms. They also contribute F to surface waters. The forms of F emitted from various industrial processes include hydrogen fluoride, cryolite, calcium fluoride, and silicon tetrafluoride (SiF_4). Some heavy discharges of F into the atmosphere and waters have occurred in connection with the phosphate and aluminum industries. Combustion of coal in power plants can emit considerable amounts of F into the atmosphere. In addition to deposition into surface waters, airborne F may eventually be deposited onto the ground and taken up by soils, plants, and animals. [Figure 7.5](#) shows environmental transfer of F.

Effects on Plants

Fluoride is by far the most important of the source-specific pollutants. Fluoride-induced effects in plants may be viewed based on four levels of biologic organization including ecosystem, organism, tissue or organ, and cellular levels. Fluoride ions accumulate in plant leaves mainly as a result of diffusion through the stomata from the atmosphere. Very little fluoride enters the plant system from the soil unless the soil is very acidic. In contrast to other major air pollutants such as SO_2 , NO_x , O_3 , PAN, etc. discussed in the previous sections, F accumulates in the leaf tips and margins of many species as a result of translocation in the transpiration stream. 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

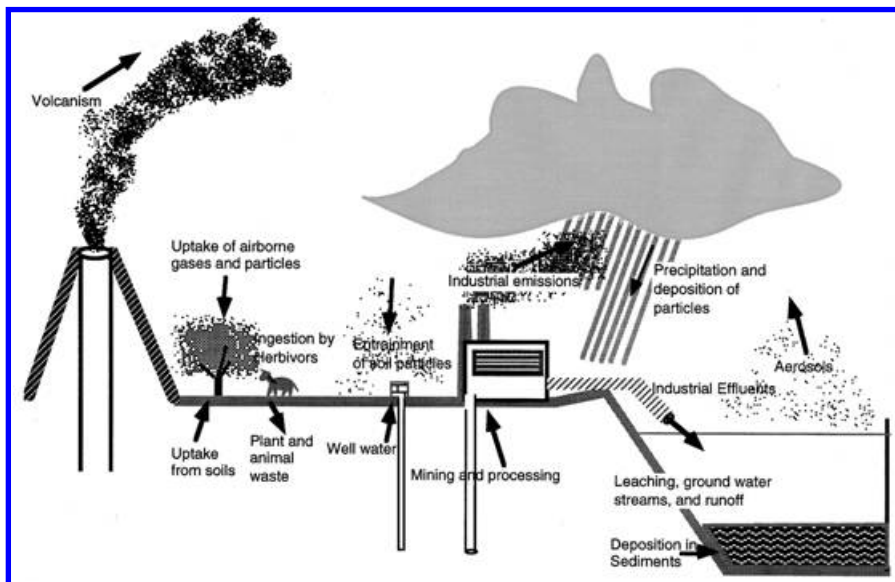


Figure 7.5 Environmental transfer of fluoride and other elemental pollutants.

than are particulate fluorides. Although plants differ widely in their susceptibility to F injury, accumulation of high levels of F in leaves normally leads to chlorosis or necrosis, which in turn can lead to reduced growth and yield.

Fluoride causes both structural and functional changes in plant cells. These include altered cellular and subcellular membranes with subsequent injuries. Many metabolic processes such as glycolysis, Krebs cycle reactions, photosynthesis, protein synthesis, lipid metabolism, and others have been shown to be affected by fluoride. Much of the action of fluoride on these processes can be attributed to fluoride-dependent inhibition of enzymes. A large number of enzymes have been shown to be affected by fluoride. Some examples include enolase, phosphoglucomutase, phosphatase, hexokinase, PEP carboxylase, pyruvate kinase, succinic dehydrogenase, malic dehydrogenase, pyrophosphatase, phytase, nitrate reductase, mitochondrial ATPase, urease (Miller et al. 1983), lipase (Yu et al. 1987), and amylase (Yu et al. 1988).

Inhibition of enzymes such as these may cause compositional changes in leaf tissues, such as changes in soluble sugar concentrations, organic acids, and amino acids.

Effect on Animals

Excessive intakes of F can injure domestic animals leading to acute or chronic effects depending on F concentrations.

- **Acute effects** — Fluoride has caused serious injurious effects on livestock in the U.S. and other industrialized 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

Table 7.4 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	—

From Committee on Biologic Effects of Atmospheric Pollutants. 1971. *Fluorides*. National Academy of Sciences, Washington, D.C., p. 295.

range from 30 to 50 mg/kg for cattle and from 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, collapse, and respiratory and cardiac failure are observed, leading to eventual death of the animals.

- **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 (a thin enamel). An affected tooth also is subject to more rapid wear and to an erosion of the enamel away from the dentine. It is noteworthy that dental lesions will not be seen in animals brought into endemic fluorosis areas after their permanent teeth have erupted (Shupe and Olson 1983). In skeletal fluorosis, the affected bones lose their normal, hard, smooth luster and appear rough, porous, and chalky white. Lameness or stiffness is an intermittent sign of fluoride toxicity. The clinical basis for the lameness is not well understood. Appetite is normally impaired and this may result in decreased weight gain, cachexia, and lowered milk yield. The animals may become more susceptible to other environmental stresses and suffer a decrease in longevity.

A number of factors influence the manifestation of dental and skeletal fluorosis. Some examples are the amount and the bioavailability of F ingested, duration of ingestion, species of animals involved (Table 7.4), age at time of excessive F ingestion, nutritional and general health status of animals, mode of F exposure (i.e., continuous or intermittent), presence of synergistic or antagonistic substances, presence of other stress factors and individual biologic response.

Several nutrients such as proteins, Ca, and vitamin C influence the severity of F toxicity. Adverse effects of F are alleviated by these nutrients. For example, both vitamin C and Ca have been shown to decrease the toxicity in guinea pigs (Hodge and Smith 1965). Laboratory experiments showed that mice fed a low-protein (4%) diet deposited 500% more fluoride in the tibia than control animals fed a regular

diet containing 27% protein, and that supplemental vitamin C greatly reduced the F deposition in the bone (Yu and Hwang 1986). It should be mentioned that mice produce vitamin C as well.

Effects on Human Health

- **Daily intake** — Daily intake of F from food is about 0.2 to 0.3 mg, from water 0.1 to 0.5 mg (1 to 2 mg if water is fluoridated), and varying quantities from beverages (F content of wine, 0 to 6.3 ppm; beer, 0.15 to 0.86 ppm; milk, 0.04 to 0.55 ppm). The amount of F inhaled from air is about 0.05 mg/day.
- **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. 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, rats, and some other species of animals has been recognized. With humans, water supplies containing 1 ppm F have been reported 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.
- **Acute intoxication** — The lethal dose of inorganic F has been 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 LD₅₀ 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 digestive tract, and heart failure.
- **Chronic effects** — Fluoride accumulates in the skeleton during prolonged, high-level exposures. Radiological evidence of hypermineralization (osteofluorosis) is shown when bone F concentrations reach about 5000 ppm (Hodge and Smith 1965). 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. Increases of serum and urinary F levels usually occur. In some parts of the world, such as India and China where the water supply (from wells) in many villages and towns contain high levels of F, osteofluorosis is common. In China alone, it is estimated that about 20 million people may be suffering chronic F poisoning (Yu and Tsunoda 1988).

Biochemical Effects

While it is clear that the action of F on living organisms is complex and involves a variety of enzymes, the mode of action of F ions on the enzymes is not so clear. The principal mechanisms that have been suggested include (1) formation of complex with metalloenzymes, (2) removal of a metal cofactor from the enzyme system, and (3) binding to the free enzyme or to the enzyme substrate complex (Miller et al. 1983). Using a model system, Edwards and coworkers (Edwards et al. 1984) indicated that fluoride could disrupt the hydrogen bonding of protein molecules. Because hydrogen bonding is important in the maintenance of the tertiary structure of protein molecules, such disruption would result in enzyme inhibition.

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STUDY QUESTIONS

1. What is the most dangerous gaseous pollutant and why?
2. How does SO₂ affect a plant's structure and function? What affects SO₂ uptake by a plant? How is plant metabolism affected?
3. At what levels does SO₂ affect experimental animals? What does it affect?
4. What condition of SO₂ might have a greater health significance than the air concentration of SO₂?
5. What effect does SO₂ have on humans?
6. Which form of nitrogen oxide is the major toxicant and why?
7. How do gaseous NO₂ affect plants?
8. How does NO₂ affect animals?
9. What is the most important source of O₃ which contributes to environmental pollution? What causes this source?
10. How do photochemical oxidants affect plant enzyme activity and lipid synthesis?
11. Describe the effects oxidants have on humans and animals.
12. What is adaptation to O₃?
13. Discuss the five mechanisms postulated for O₃ toxicity?
14. How does CO formation occur? What is an important physiological effect of CO?
15. Describe fluoride-induced effects in plants based on the four levels of biologic organization.
16. What are the principal mechanisms suggested as the mode of action of fluoride ion on plant metabolic enzymes?
17. What are the acute, chronic, and biochemical effects of fluoride on animals?
18. What are the chronic effects of fluoride accumulation in humans?

CHAPTER 8

Heavy Metals

Pollution caused by heavy metals is now 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 also are 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.

LEAD

Lead (Pb) is one of the ancient metals and has been used by humans for several thousands of years. Pb plays an important role in the economy of all industrialized countries in the world. In the U.S., 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 (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).

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.

Properties and Uses

Lead has a low melting point (326°C). It is a soft, malleable metal and it can be easily formed into a variety of shapes. It also can form alloys with many other metals. Other important industrial products containing Pb include pipes, paints, solders, glass, pottery glazes, rubber, plastics, and insecticides.

Exposure

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 into the air, soils, and vegetation growing nearby. However, the most common source of Pb contamination in ambient air until recently 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 U.S. decreased from 56,000 to 7100 metric tons per year between 1981 and 1990 (EPA 1991). While the atmospheric Pb pollution problem in other developed countries likewise has been significantly reduced, a similar trend has not occurred in many third-world countries.

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 the North America's lakes and ponds. A large number of waterfowl in the U.S. are poisoned or killed following ingestion of the shot.

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 might 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 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.

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 also is an important problem in areas surrounding metal mines. Earlier reports indicate that about 50% of the Pb liberated from motor vehicles in the U.S. was deposited within 30 m of the roadways (Ryan 1976) and the remainder was 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 are found in soil adjacent to 12 highways in the Minneapolis-St. Paul area (Ryan 1976). These levels were 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, CO contained as much as 3000 ppm Pb, while vegetable samples from gardens less than 50 ft from roads in Canandaigua, NY 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) studied 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 1975 to 1979. They concluded that the mandatory use of unleaded gasoline had significantly reduced the Pb concentrations in soils near highways.

Lead Toxicity

Effect on Plants

Plants exposed to high levels of Pb from ambient air and soils can accumulate the metal and manifest toxicity. The toxicity and presence of other trace metals vary greatly among plant species. Based on *in vitro* studies, toxicity sequences have been determined for several species. Barley plants were shown to be more sensitive to Pb than to Cr, Cd, Ni, or Zn (Oberlander and Roth 1978), and exposure to relatively high levels of Pb was shown to inhibit seed germination (Koeppel 1977; Yu 1991). The effect of Pb on germination, however, was found to be less severe compared to

several other metals such as Cd, As, and Hg (Koepppe; Fargasova 1994). It is important to note that, following plant uptake, Pb moves into the food chain and thus can affect animals and humans.

Effect on Animals

The effect of Pb on freshwater fish varies depending on the species of fish. Goldfish, for example, are relatively resistant to Pb, presumably due to their abundant gill secretion. As mentioned above, following the ingestion of expended lead shot in lakes or in the field, more than one million birds are estimated killed each year in the U.S. Lead absorbed by the bird paralyzes the gizzard leading to starvation, and death usually follows within several weeks after the exposure.

Effect on Humans

Daily intake of Pb in humans is estimated to range from 20 mg to 400 mg per person. The FAO/WHO Expert Committee established a Provisional Tolerable Weekly Intake (PTWI) of 3000 mg, approximately 500 mg/day. Only one-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/day and represents two-thirds of the total absorbed Pb. 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 Pb.

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 circulatory system, 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.

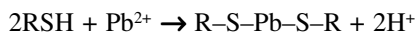
It is estimated that approximately 90% of Pb absorbed by humans is deposited in the bone (Aufderheide and Wittmers 1992). Bone, however, is no longer considered a sink for Pb in the body. Rather, it is recognized as a two-way process of active influx and efflux of Pb to and from the bone and blood stream (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.

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 (Koeppel and Miller 1970).

Lead, as mentioned above, is a systemic poison and can induce deleterious effects 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:



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 important in amino acid metabolism.

Second, divalent Pb is similar in many aspects to Ca and may exert a competitive action on 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 endplate potential. The miniature endplate 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 δ -aminolevulinic acid dehydratase (ALA-D) (Hernberg et al. 1970) and ferrochelatase (Tephly et al. 1978). These are two key enzymes involved in heme biosynthesis. 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 (Figure. 8.1). Lead inhibition of the two enzymes appears to be due to its interaction with Zn and Fe required in the process.

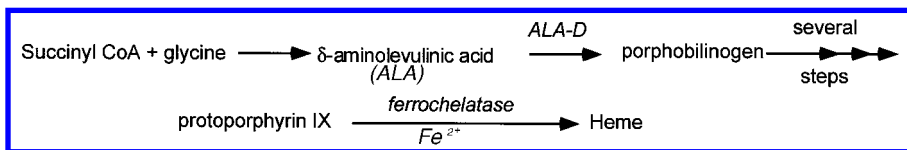


Figure 8.1 Steps in heme synthesis inhibited by lead.

CADMIUM

Cadmium (Cd) is a transition metal in Group IIb along with Zn and Hg. It is frequently associated with Zn. The U.S. is the world's largest producer of cadmium, with an annual output of about 5000 short tons. Mexico is an important producer of Cd-bearing dusts and fumes, but most of these are smelted in the U.S.

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, producing the colorless Cd²⁺ ion. Cadmium persists in the environment with a half-life of 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 manufacturing and semi-conductors. Because Cd strongly absorbs neutrons, it is also used in the control rods in nuclear reactors.

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 in the lungs is much greater than that from the gastrointestinal tract, smoking contributes significantly to the total body burden. Each cigarette on

the 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 it 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 onto agricultural soils. Other sources of Cd pollution are through rainfall and dry precipitation, 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.

Cadmium Toxicity

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 plants, 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 Pb, Cd inhibits seed germination under laboratory conditions (Koeppel 1977; Yu 1991; Fargasova 1994). Seedlings exposed to solutions of Cd salts exhibit decreased root elongation and development.

Effects on Animals and 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 the 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 liters (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 Japanese farmers suffering from the widely known "itai-itai" disease were reported to be from 600 to 1000 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, kidney, and, to a small amount, bone and muscle tissue.

The excretion of Cd appears minimal under normal exposure. Loss in the urine accounts for the 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 organ is 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.

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 PEP carboxylase, lipase, invertase (Yu 1998), 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 Zn and displacing it from metalloenzymes. Naturally, Cd also can 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.

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.

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 sealed switches and relays. Many metals dissolve in mercury to form amalgams (alloys).

In the U.S. the largest user of Hg is the chlor-alkali industry in which chlorine and caustic soda are produced by the electrolysis of a 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. High-pressure mercury-vapor lamps are now widely installed for street and highway lighting, and Hg compounds are added to paints as preservatives. Formerly, certain Hg compounds were widely used as pesticides in agriculture. Mercury has no known biological role and, as mentioned above, the metal and its compounds are toxic to all living organisms.

Sources of Mercury Pollution

Mercury contamination of the environment is caused by both natural and man-made sources. Natural sources include volcanic action and erosion of mercury-containing sediments. Some of the ways humans contaminate the environment with Hg is through mining, transporting and processing mercury ores; dumping industrial wastes into rivers and lakes; combustion of fossil fuels (e.g., 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.

Toxicity

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 1998).

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 also is taken up through the mucous layer and/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-h 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).

Wild birds concentrate the highest levels of Hg in the kidney and liver with less in the muscle tissues. Swedish ornithologists observed the first Hg-related ecological problems during 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 US. and Canada, elevated levels of Hg also were 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 U.S., pheasants and other wild birds can still accumulate relatively high levels of Hg.

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 shows 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 into 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 1975).

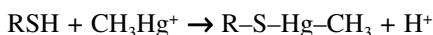
The critical organ concentration may differ for different stages of the human life cycle. The developing fetal (and newborn) brain may be the most sensitive organ (i.e., critical organ) in terms of human methylmercury toxicity. During the Japanese Minamata outbreak, 23 infants with severe psychomotor signs of brain damage were shown. They 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 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 d. 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.

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 with methylmercury in the following:



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 manifested. 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.

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STUDY QUESTIONS

1. Why are heavy metals toxic to organisms?
2. List four sources of lead exposure. Explain a source of Pb 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 what source? What other sources exist for Cd exposure?
8. List several effects of Cd 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 Hg?
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.

CHAPTER 9

Biotransformation, Detoxification, and Biodegradation

INTRODUCTION

As mentioned in Chapter 5, following the entry into a living organism and translocation, a foreign chemical may be stored, metabolized, or excreted (Figure 5.2). When the rate of entry is greater than the rate of metabolism and/or excretion, storage of the chemical often occurs. Storage or binding sites may not be the sites of toxic action, however. 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 produces harmful effects.

Some chemicals that are stored may remain in the body for a long time without exhibiting direct harmful effects. DDT may be considered as an example. 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 exemplified by fluorosis and lead and cadmium poisoning.

METABOLISM OF ENVIRONMENTAL CHEMICALS: BIOTRANSFORMATION

Subsequent to the entry of an environmental chemical into an organism such as a mammal, 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, kidney, skin, and liver.

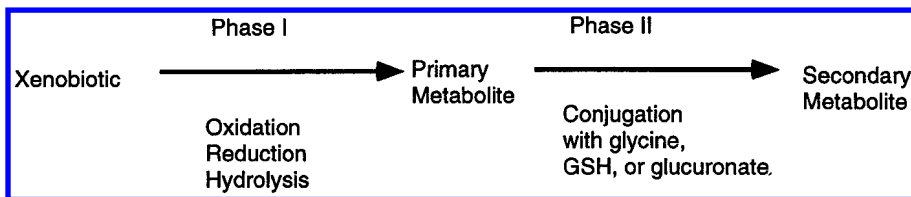


Figure 9.1 The two phases of xenobiotic metabolism.

By far the largest number 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 processes is probably to reduce the toxicity of chemicals, this does not prove to be always the case. Occasionally the metabolic process converts a xenobiotic to a reactive electrophile that is capable of causing injuries through interaction with liver cell constituents (Reynolds 1977).

Types of Biotransformation

The process of xenobiotic metabolism includes two phases commonly known as Phases I and II. The major reactions included in Phase I are oxidation, reduction, and hydrolysis, as shown in [Figure 9.1](#). Among the representative oxidation reactions are hydroxylation, dealkylation, deamination, and sulfoxide formation, whereas reduction reactions include azo reduction and addition of hydrogen. Such reactions as splitting of ester and amide bonds are common in hydrolysis. During Phase I, a chemical may acquire a reactive group such as OH, NH₂, COOH or SH.

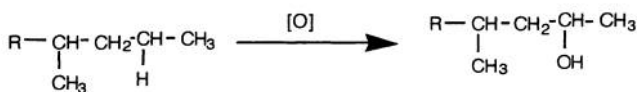
Phase II reactions, on the other hand, are synthetic or conjugation reactions. An environmental chemical may combine directly with an endogenous substance, or may be altered by Phase I and then undergo conjugation. The endogenous substances commonly involved in conjugation reactions include glycine, 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 9.2](#).

Mechanisms of Biotransformation

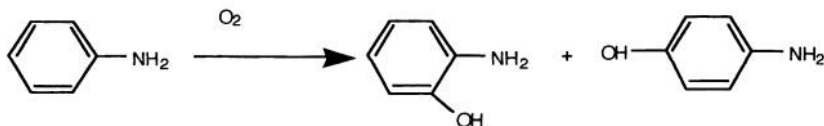
In the two phases of reactions shown in [Figure 9.1](#), the lipophilic foreign compound is first oxidized so that a functional group (usually a hydroxyl group) is introduced into the molecule. This functional group is then coupled by conjugating enzymes to a polar molecule so that the excretion of the foreign chemical is greatly facilitated.

Phase I reactions

Oxidation



Side Chain Oxidation



Aniline

Aromatic hydroxylation

o-Aminophenol

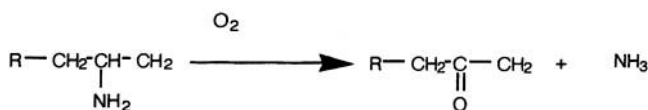
p-Aminophenol



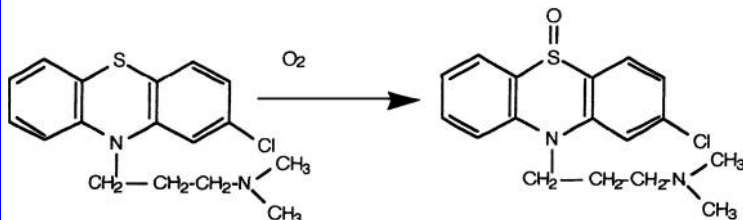
N-Dealkylation



O-Dealkylation



Deamination



Sulfoxide formation

Figure 9.2 Detoxification pathways.

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. Cytochrome P-450 system, localized in the smooth endoplasmic reticulum of cells of most mammalian tissues, is particularly abundant

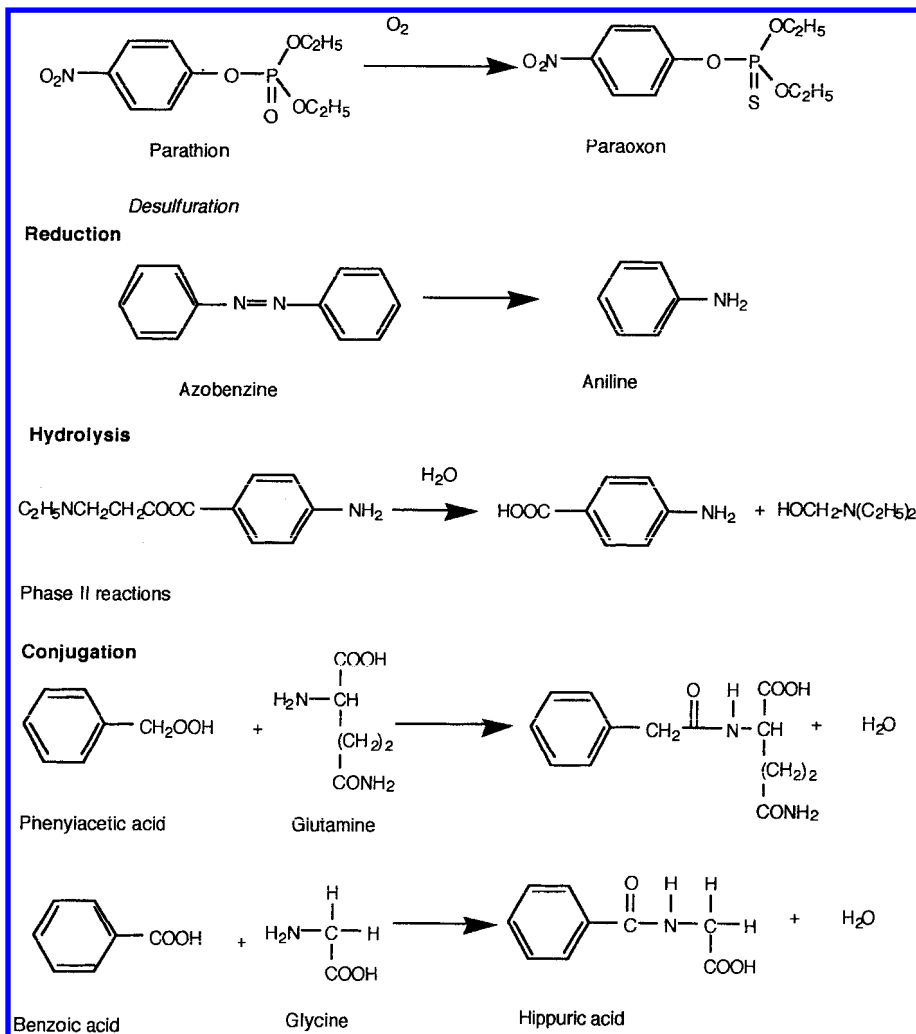


Figure 9.2 (continued)

in the liver. This system contains a number of 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 sites of cytochrome P-450 is an iron atom that, in the oxidized form, binds the substrate (SH) (Figure 9.3). Reduction of this enzyme-substrate complex then occurs, with an electron being transferred from NADPH via NADPH cytochrome P-450

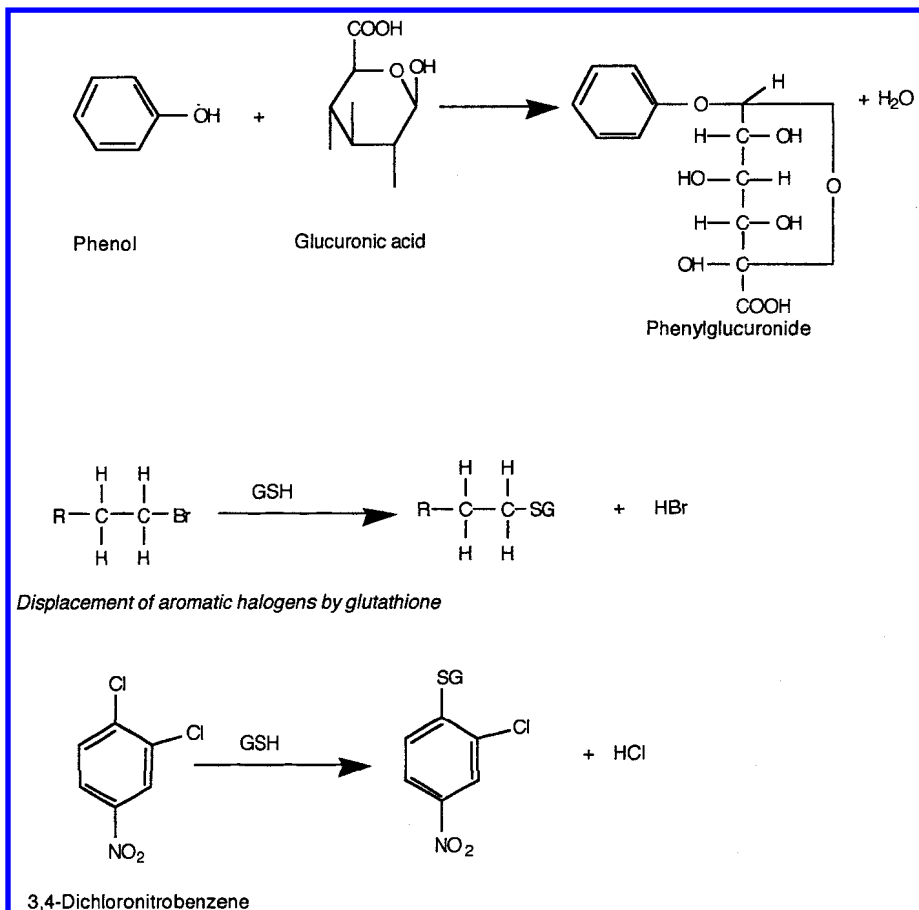


Figure 9.2 (continued)

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 therefore:



where SH is the substrate. As shown in the above equation, one atom from molecular oxygen is reduced to water and the other is incorporated into the substrate. The requirements for this enzyme system are oxygen, NADPH, and Mg^{2+} ions.

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, it is clear that

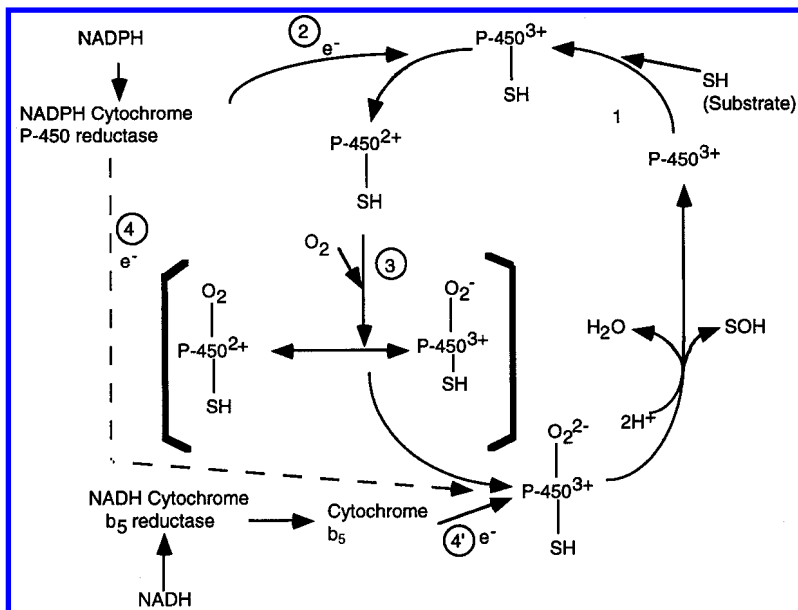


Figure 9.3 The cytochrome P-450 monooxygenase system. P-450³⁺: cytochrome P-450 with heme iron in oxidized state (Fe³⁺); P-450²⁺: cytochrome P-450 with iron in reduced state; S: substrate; e: electron. (Adapted from J.A. Trimbrell. 1982. *Principles of Biochemical Toxicology*. Taylor and Francis Ltd., London.)

adequate intracellular contents of cofactors such as NADPH, NADH, O₂, glucuronate, ATP, cysteine, and GSH are required for one or more reactions.

Consequence of Biotransformation

Although hepatic enzymes that catalyze Phase I and II reactions convert the lipid-soluble xenobiotic to a more water-soluble metabolite, 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 hydrogen sulfide formed by anaerobic bacteria in the intestinal tract. It can be seen, therefore, that chemicals or conditions that influence the activity of the Phase I and 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. 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, 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.

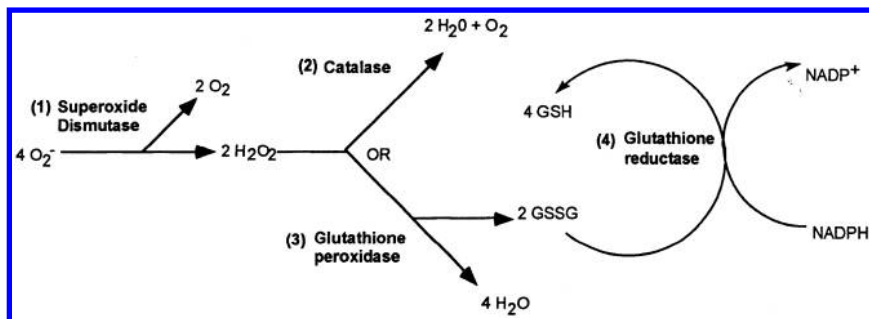


Figure 9.4 The four important enzymatic components of the cellular antioxidant defense system. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide (O_2^-) to peroxide. Catalase reduces peroxide to H_2O . GSH peroxidase also detoxifies peroxide by reducing it to H_2O . GSH reductase re-reduces 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 N.K. Mottet, Ed. *Environmental Pathology*. Oxford University Press, New York, 1985.)

Although liver cells are dependent on the detoxification enzymes for protection against reactive electrophilic species produced during biotransformation, endogenous antioxidants such as vitamins C and E and glutathione also provide protection. As mentioned in Chapter 5, these substances are 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 also can donate its sulfhydryl hydrogen to a reactive free radical (GS). The glutathione radical formed can then react with another glutathione radical to form stable oxidized GSSG. The GSSG can be reduced back to GSH through an NADPH-dependent reaction catalyzed by glutathione reductase. The NADPH is generated in reactions involved in the pentose phosphate pathway.

In addition to vitamin E and C and GSH, there are enzymatic systems that are important in the defense against free radical-mediated cellular damage. These include superoxide dismutase (SOD), catalase, and GSH peroxidase. Figure 9.4 shows the interrelationship between these enzymatic components.

MICROBIAL DEGRADATION

Microbial degradation of xenobiotics is crucial in the prediction of the longevity and thereby the long-term effects of the toxicant and also may be crucial in the actual remediation of a contaminated site. 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

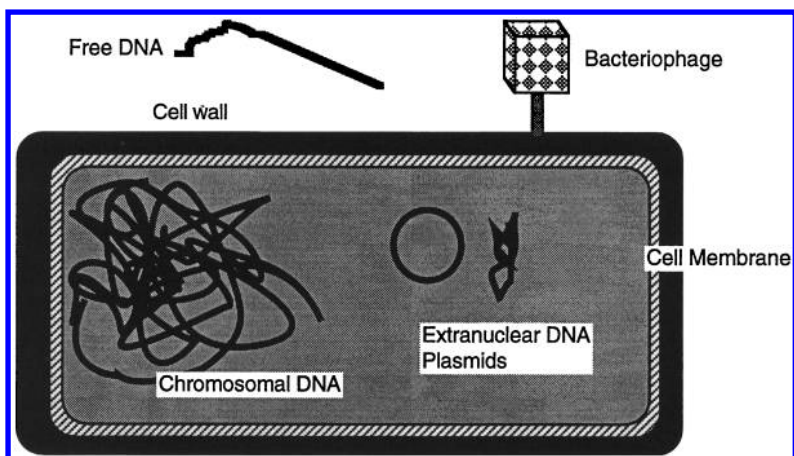


Figure 9.5 Schematic of a typical prokaryote. Genetic information and thereby coding for the detoxification and degradation of a xenobiotic may be available from a variety of sources.

prognosis, but also to 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, 70s ribosomes, a chromosome that is not membrane bound, various inclusions and vacuoles, and extrachromosomal DNA or plasmids. Eucaryotic microorganisms are equally varied with a variety of forms, many are photosynthetic or harbor photosynthetic symbionts. Many eucaryotic cells contain prokaryotic endosymbionts, some of which contain their own set of plasmids. Given the variety of eucaryotic 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 nutrients 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 frequently the degradation of xenobiotics. Plasmids may be obtained through a variety of processes including conjugation, infection, and the absorption of free DNA from the environment (Figure 9.5).

Eucaryotic 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 resembles prokaryotic genomes.

Many microbial also contain prokaryotic and eucaryotic 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 when 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 the 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, such as aquatic, marine, ground water, soil, and even in the Arctic. many are found in extreme environments, from tundra to the superheated smokers at sites of seafloor 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 criteria is that the metabolism of the material results in a material able to enter the tricarboxylic acid or 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 pathways for the metabolism of aromatic compounds with the resulting product acetyl-CoA and succinic acid, materials that easily enter into the TCA cycle (Figure 9.6). 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 that exist between bacteria and fungi.

Often the coding process for degradation of a xenobiotic is contained on both the extrachromosomal DNA, the plasmid, and the chromosome. 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 complimentary sequences to the degradation genes, it should be possible to follow the frequency and thereby the population genetics of degradative plasmids in procaryotic communities.

In procaryotic 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 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

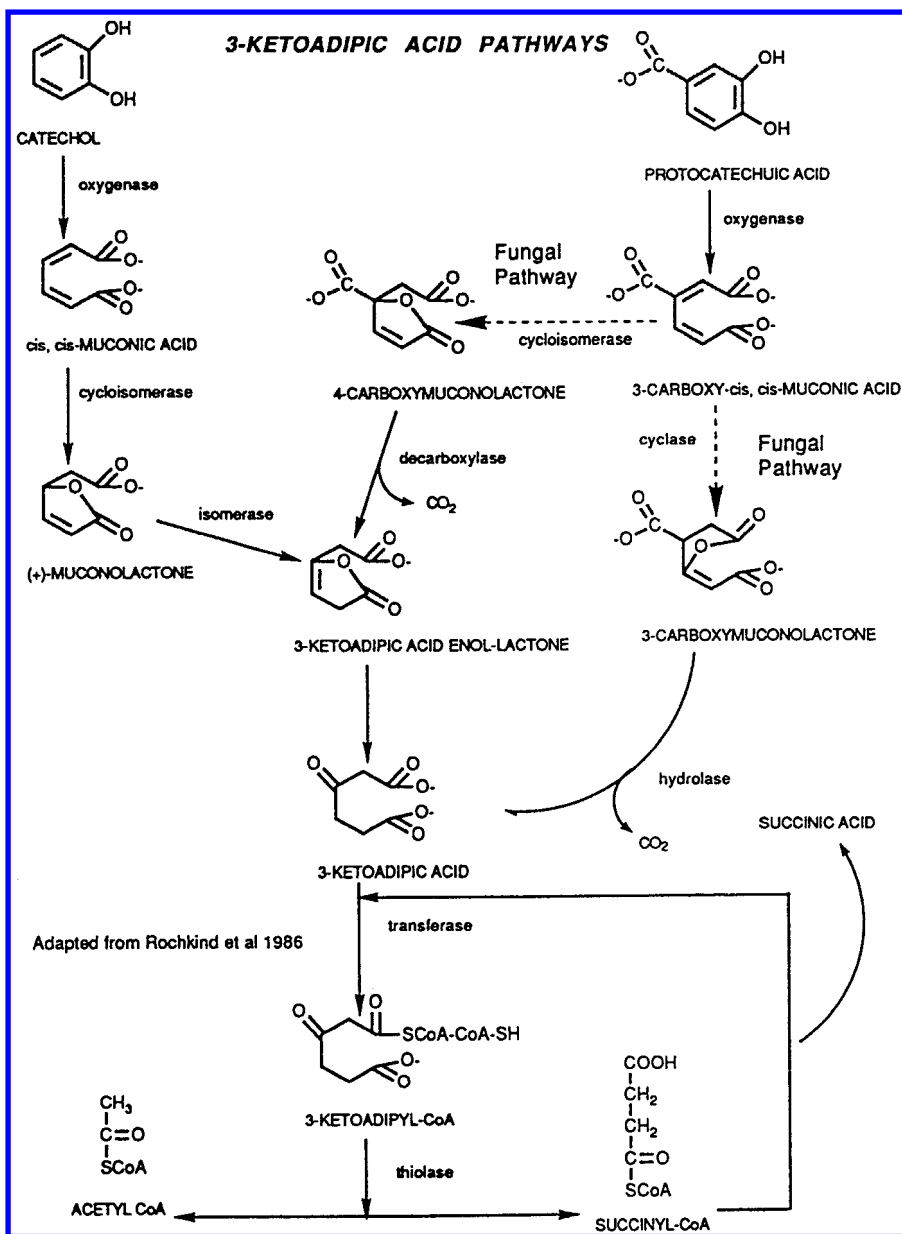


Figure 9.6 The 3-ketoadipic acid pathway.

radiolabelled metabolic products. Mineralization of the substrate also is 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 process 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 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 waste water 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 9.1](#) provides a compilation from a recent review giving both the compound and the strains that have so far been found that are responsible for the degradation. Only a few examples will be discussed below.

Substituted benzenes are commonly occurring xenobiotics. In [Figure 9.7](#) 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 to water ([Figure 9.8](#)). The resulting epoxide can be further hydrolyzed to form an intermediate ultimately ending with a transhydroxy compound. The epoxide also can isomerize to form a variety of phenols. Both of these mechanisms occur in the degradation of naphthalene by the fungus *Cunninghamella elagans*.

A particularly widespread environmental contaminant is the pesticide pentachlorophenol (PCP). PCP has been used as a bactericide, insecticide, fungicide, herbicide, and molluscicide 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 9.9](#). 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.

Table 9.1 Examples of Organic Compounds and Degradative Bacterial Strains

Aniline	<i>Fratureia</i> sp. ANA-18 <i>Nocardia</i> sp. <i>Pseudomonas</i> sp. <i>Pseudomonas multivorans</i> AN1 <i>Rhodococcus</i> sp. AN-117 <i>Rhodococcus</i> sp. SB3
Anthracene	<i>Beijerinckia</i> sp. B836 <i>Cunninghamella elegans</i> <i>Pseudomonas</i> sp. <i>Pseudomonas putida</i> 199
Benzene	<i>Achromobacter</i> sp. <i>Pseudomonas</i> sp. <i>Pseudomonas aeruginosa</i> <i>Pseudomonas putida</i>
Benzoic acid	<i>Alcaligenes eutrophus</i> <i>Aspergillus niger</i> <i>Azotobacter</i> sp. <i>Bacillus</i> sp. <i>Pseudomonas</i> sp. <i>Pseudomonas acidovorans</i> <i>Pseudomonas testosteroni</i> <i>Pseudomonas</i> sp. strain H1 <i>Pseudomonas</i> PN-1 <i>Pseudomonas</i> sp. WR912 <i>Rhodopseudomonas palustris</i> <i>Streptomyces</i> sp. By consortia of bacteria
2-Chlorobenzoic acid	<i>Aspergillus niger</i>
3-Chlorobenzoic acid	<i>Acinetobacter calcoaceticus</i> Bs5 (grown on succinic acid and pyruvic acid) <i>Alcaligenes eutrophus</i> B9 <i>Arthrobacter</i> sp. (grown on benzoic acid) <i>Aspergillus niger</i> <i>Azotobacter</i> sp. (grown on benzoic acid) <i>Bacillus</i> sp. (grown on benzoic acid) <i>Pseudomonas aeruginosa</i> B23 <i>Pseudomonas putida</i> (w/plasmid p AC25) <i>Pseudomonas</i> sp. B13 <i>Pseudomonas</i> sp. H1 <i>Pseudomonas</i> sp. WR912 By consortia of bacteria
4-Chlorobenzoic acid	<i>Arthrobacter</i> sp. <i>Arthrobacter globiformis</i> <i>Azotobacter</i> sp. (grown on benzoic acid) <i>Pseudomonas</i> sp. CBS 3 <i>Pseudomonas</i> sp. WR912 <i>Chlamydomonas</i> sp. A2
4-Chloro- 3,5-Dinitrobenzoic acid	
2,5-Dichlorobenzoic acid	By consortia of bacteria
3,4-Dichlorobenzoic acid	By consortia of bacteria
3,5-Dichlorobenzoic acid	<i>Pseudomonas</i> sp. WR912
	By consortia of bacteria
2,3,6-Trichlorobenzoic acid	<i>Brevibacterium</i> sp. (grown on benzoic acid)

Table 9.1 (Continued) Examples of Organic Compounds and Degradative Bacterial Strains

Biphenyl	<i>Beijerinckia</i> sp. <i>Beijerinckia</i> sp. B836 <i>Beijerinckia</i> sp. 199 <i>Cunninghamella elegans</i> <i>Pseudomonas putida</i> By consortia of bacteria
Catechol	<i>Pyrocatechase I</i>
4-Chlorocatechol	<i>Achromobacter</i> sp.
3,5-Dichlorocatechol	<i>Achromobacter</i> sp.
Chlorobenzene	<i>Pseudomonas putida</i> (grown on toluene) unidentified bacterium, strain WR1306
Chlorocatechol	<i>Pyrocatechases</i>
3,5-Dichlorocatechol	<i>Achromobacter</i> sp. (grown on benzoic acid)
Chlorophenol	<i>Arthrobacter</i> sp.
2-Chlorophenol	<i>Alcaligenes eutrophus</i> <i>Nocardia</i> sp. (grown on phenol) <i>Pseudomonas</i> sp. B13
3-Chlorophenol	<i>Nocardia</i> sp. (grown on phenol) <i>Pseudomonas</i> sp. B13 <i>Rhodotorula glutinis</i>
4-Chlorophenol	<i>Alcaligenes eutrophus</i> <i>Arthrobacter</i> sp. <i>Nocardia</i> sp. (grown on phenol) <i>Pseudomonas</i> sp. B13 <i>Pseudomonas putida</i>
2,4,6-Trichlorophenol	<i>Arthrobacter</i> sp.
2,3,4,6-Tetrachlorophenol	<i>Aspergillus</i> sp. <i>Paecilomyces</i> sp. <i>Penicillium</i> sp. <i>Scopulariopsis</i> sp.
Chlorotoluene	<i>Pseudomonas putida</i> (grown on toluene)
Gentisic acid	<i>Trichosporon cutaneum</i>
Guaiacols (<i>o</i> -methoxyphenol)	<i>Arthrobacter</i> sp.
3,4,5-Trichloroguaiacol	<i>Arthrobacter</i> sp. 1395
Homoprotocatechuic acid	<i>Trichosporon cutaneum</i>
Naphthalene	<i>Cunninghamella elegans</i> <i>Oscillatoria</i> sp. Pseudomonads
Pentachlorophenol (PCP)	<i>Arthrobacter</i> sp. <i>Coniophora pueana</i> <i>Mycobacterium</i> sp. <i>Pseudomonas</i> sp. Saprophytic soil corynebacterium KC3 isolate Mutant ER-47 Mutant ER-7 <i>Trichoderma viride</i>
Phenanthrene	<i>Aeromonas</i> sp. Fluorescent and nonfluorescent pseudomonad groups Vibrios

Table 9.1 (Continued) Examples of Organic Compounds and Degradative Bacterial Strains

Protocatechuic acid	<i>Neurospora crassa</i>
Sodium pentachlorophenate (Na-PCP)	<i>Trichosporon cutaneum</i>
	<i>Trichoderma</i> sp.
	<i>Trichoderma virgatum</i>
Tetrachlorohydroquinone	KC3
Toluene	<i>Achromobacter</i> sp.
	<i>Pseudomonas</i> sp.
	<i>Pseudomonas aeruginosa</i>
	<i>Pseudomonas putida</i>
4-amino-3,5-dichlorobenzoic acid	By consortia of bacteria
2,4,5-Trichlorophenoxyacetic acid	<i>Pseudomonas cepacia</i> AC1100

List Compiled from: Rochkind, M.L., J.W. Blackburn, and G.S. Saylor. 1986. *Microbial Degradation of Chlorinated Aromatic Compounds*. Environmental Protection Agency 160012-861090, pp. 45-98.

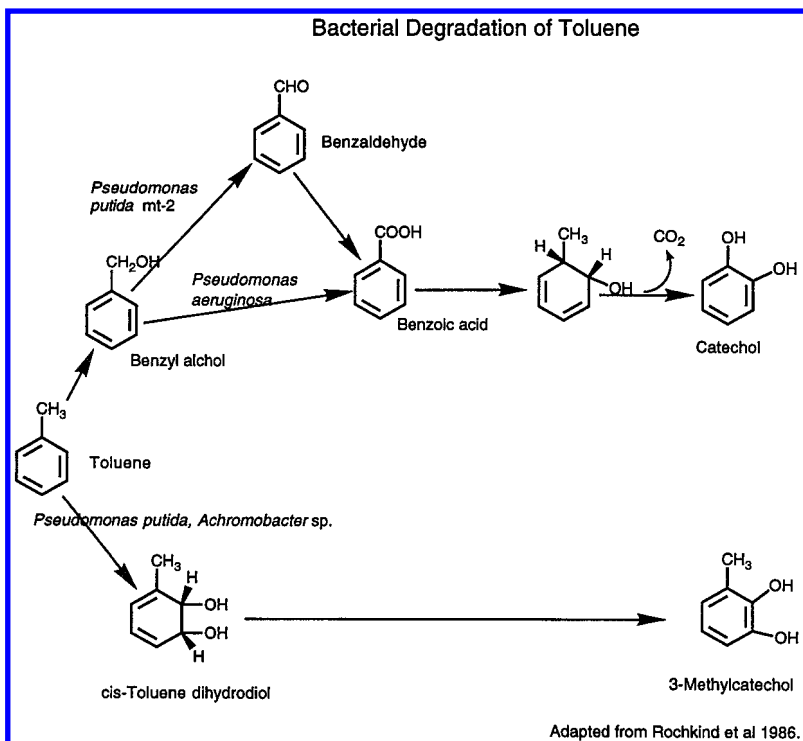


Figure 9.7 Alternate pathways for the degradation of a substituted benzene, toluene, (Adapted from Rochkind et al. 1986.)

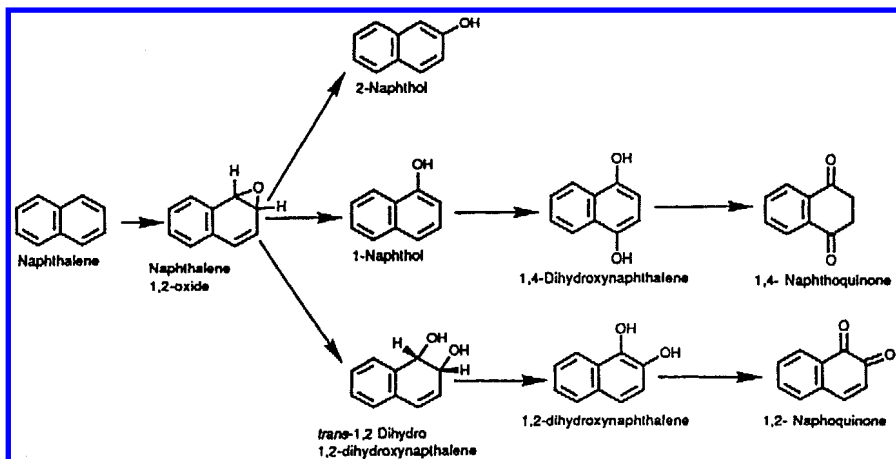


Figure 9.8 Biodegradation of naphthalene by *Cunninghamella elegans*. (Adapted from Rochkind et al. 1986.)

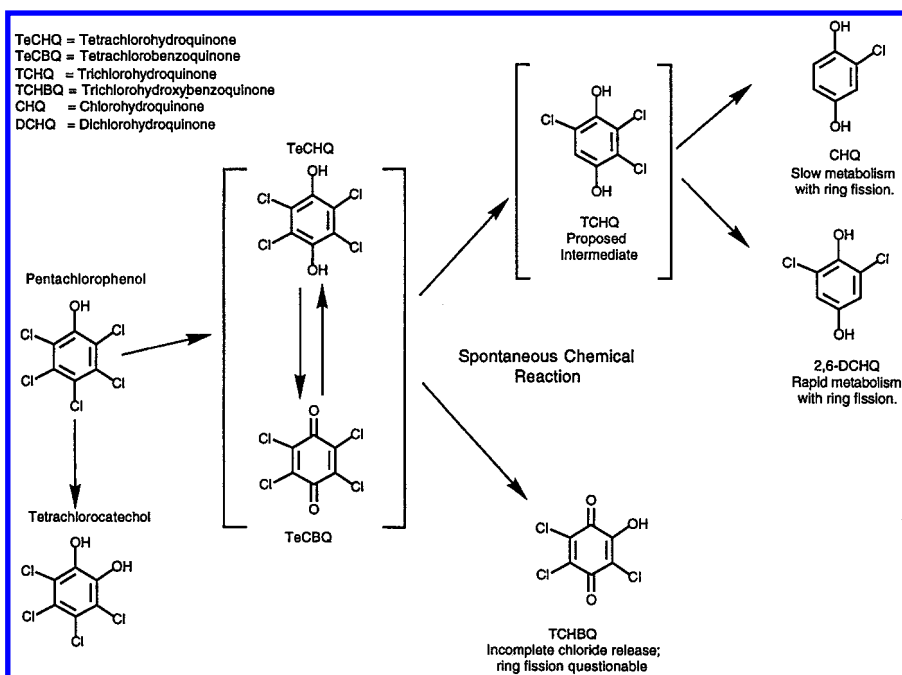


Figure 9.9 Possible mechanisms for the degradation of pentachlorophenol by *Pseudomonas* sp. (Adapted from Rochkind et al. 1986.)

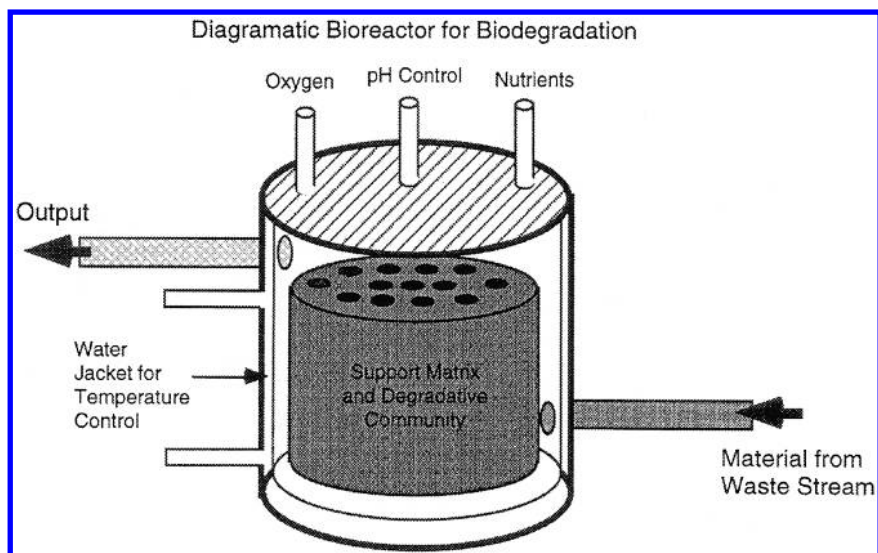


Figure 9.10 Schematic of a bioreactor for the detoxification of a waste stream or for inclusion in a pump and water treatment process.

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, in others the data are not so clear.

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 9.10](#), 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 increase, a consortia of several organisms or of an entire degradative community may be necessary. Consortia also can be established in a bioreactor-type setting.

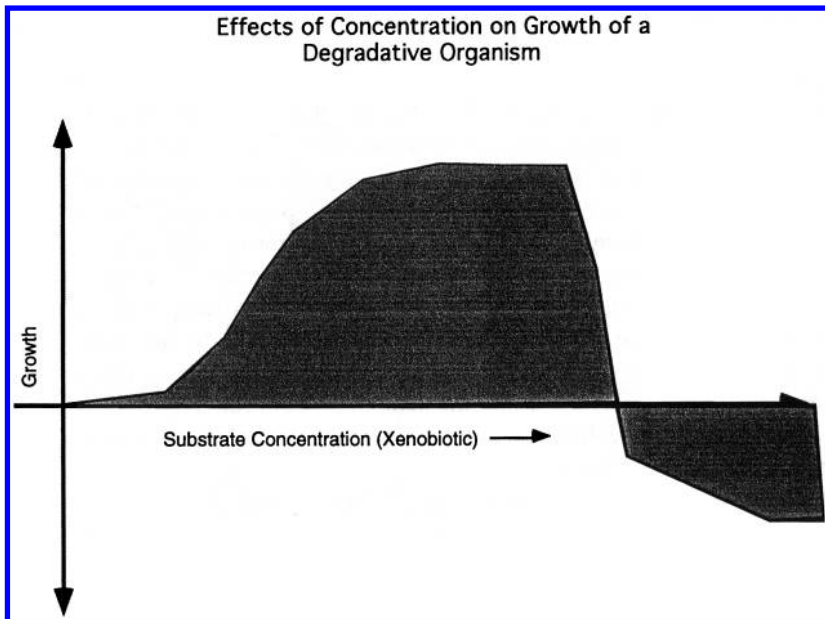


Figure 9.11 Degradative growth curve. At low concentrations, degradation may not occur due to the lack of nutritive content of the xenobiotic as substrate. Eventually a maximal rate of degradation and also growth may occur with a plateau. Eventually the concentration of the toxic material overwhelms the ability of the organism to detoxify the material and death ensues.

Concentration of the toxicant is essential in determining the success of the bioremediation attempt. As shown in [Figure 9.11](#), 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 the 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, they must be able to compete effectively with other microflora and escape predation.

To enhance degradation frequent plowing and fertilization of a terrestrial site may be done to ensure proper aeration of the soil. Ground water is often nutrient- and oxygen-limited and both of these materials can be introduced. Often hydrogen peroxide is pumped into ground water as an effective means of delivering oxygen as the hydrogen peroxide decomposes.

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 or not 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 9.12). Again, it may take long periods for evolution of a degradative organism or community to arise.

As the degradative organism or consortia is 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 hosts chromosome. If the desire is to place the organisms into a field situation, basic survival traits also must be maintained.

THE GENETICS OF DEGRADATIVE ELEMENTS

Once formed, a degradative element can suffer a number of fates (Figure 9.13). 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

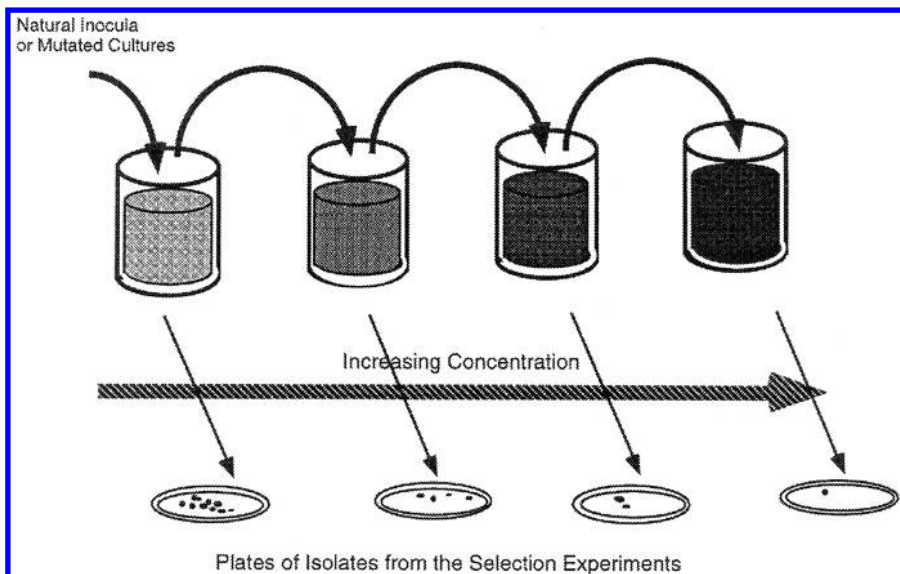


Figure 9.12 Selection protocol for the isolation of degradative microorganisms.

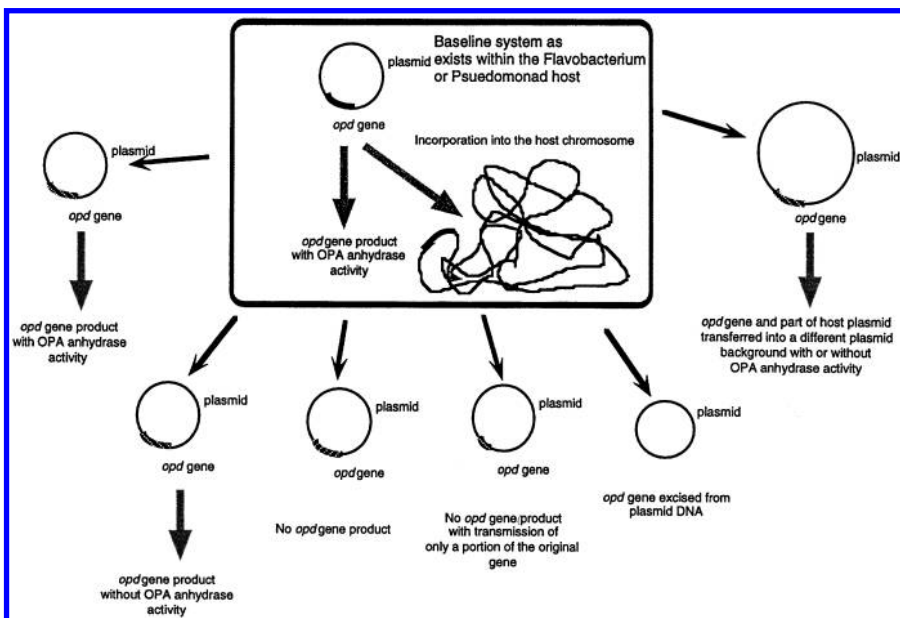


Figure 9.13 Outcomes in the evolution of a degradative element in a prokaryote.

reproduce as an independent plasmid. This scenario is similar to that for the life cycle of lambda phage.

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 miss match 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, thereby, the derivative protein will be produced.

A deletion event also may 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 be recovered unless recombination with a plasmid containing the degradative element occurs.

Of course, many procaryotes 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.

AN EXAMPLE OF A DETOXIFICATION ENZYME — THE OPA 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 anhydrolases. Enzymes that may aid in the understanding of organophosphate intoxication and also may provide a means for the detoxification and bioremediation of these materials.

An interesting example of a series of enzymes able to hydrolyze a variety of organophosphates are the organophosphorous acid anhydrolases (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 recently 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. 1989).

Two categories of organofluorophosphate OPA anhydrolases have been recognized in the literature (Hoskin et al. 1984). Typically, Mazur type is characterized

as being stimulated by Mn^{2+} , hydrolyzing soman faster than DFP, nontolerant of ammonium sulfate precipitation, and is usually found to be dimeric with a molecular weight of approximately 62,000 D (Strokebaum and Witzel 1975) and is competitively or reversibly inhibited by Mipafox (Hoskin 1985). Mipafox is a structural analog to DFP (Figure 9.14). 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.) 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.), and does not demonstrate stereospecificity towards the hydrolysis of soman. Squid-type OPA anhydrase is present in nerve (optic ganglia, giant nerve axon), hepatopancreas, and salivary glands of cephalopods (Hoskin et al.). Cephalopods also contain OPA anhydrase resembling the Mazur-type in other tissues. Table 9.2 lists the characteristics of several of the different OPA anhydrolases studied to date.

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 recently been shown that the *opd* OPA anhydrase (also called phosphotriesterase) has the capability to hydrolyze DFP and perhaps other organofluorophosphates (Dumas et al. 1989; Donarsky et al. 1988). Until recently, 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.

The *opd* OPA anhydrase is coded by a plasmid-borne gene of 1079 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 (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.

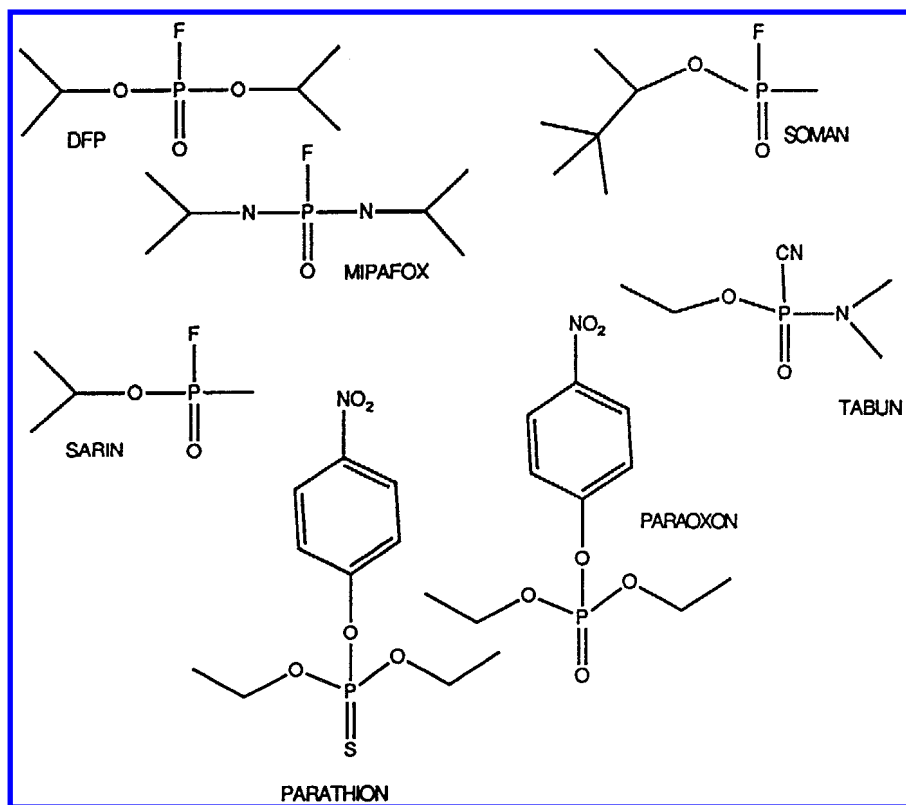


Figure 9.14 Structures of several common substrates and an inhibitor used to study the mechanisms of OPA anhydase activity. DFP (diisopropylfluorophosphate), mipafox (N,N'-diisopropylphosphorodiamidofluoridate), tabun (N,N-dimethylethylphosphoramidocyanidate) soman(0-1,2,2-trimethylpropylethylphosphonofluoridate), paraoxon (diethyl 4-nitrophenyl phosphate), and parathion.

In an elegant series of experiments, the mechanism of the *opd* OPA anhydase 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 inline displacement by an activated water molecule at the phosphorus center of the substrate (Figure 9.15). It is significant that this same enzyme also was able to hydrolyze DFP and other related organofluorophosphates.

Attaway et al. (1987) have screened a number of bacterial isolates for OPA anhydase activity including strains of *Psuedomonas diminuta*, *P. aeruginosa*, *P. putida*, *Vibrio alginolyticus*, *V. parahaemolyticus*, *Escherichia coli*, and *Flavobacterium* sp. m. Chettur et al. (1988) and Hoskin et al. (1989) have recently published findings on the OPA anhydase activities of the OT (obligate thermophile) organism, also known as JD.100, from the DeFrank collection. The thermophilic bacteria were isolated by J. DeFrank from soil samples from the Edgewood Area of the Aberdeen Proving Ground, MD. OT has been identified as a strain of *Bacillus stearothermophilus*. The OPA anhydase activity was purified using a Pharmacia G-100 column

Table 9.2 Comparison of Several Aquatic OPA Anhydrase Activities with Typical Squid and Mazur-type OPA Anhydrolases

Characteristic activity	Substrate hydrolysis			
	mw ¹	Soman/DFP ratio	Mn ²⁺ stimulation	Mipafox inhibition
<i>T. thermophila</i>				
<i>Tt</i> DFPase-1	80,000	1.12	2.5–4.0	+
<i>Tt</i> DFPase-2	75,000	1.26	2.0	+
<i>Tt</i> DFPase-3	72,000	0.71	1.7–2.5	+
<i>Tt</i> DFPase-4	96,000	1.95	17–30	nt
<i>R. cuneata</i>				
<i>Rc</i> opa-1	19–35,000	nt	1	—
<i>Rc</i> opa-3	82–138,000	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 ⁴	0.5	3–5	nt
<i>opd</i> gene product (parathion hydrolase) ⁴	60–65,000 (35,418 subunits)	nt	+	
Squid-type opa anhydrase (<i>Loligo pealei</i>)	23–30,000	0.25	1	—
Mazur-type OPA anhydrase (hog kidney)	62–66,000 (30,000 subunits)	6.5	2	+

Note: The enzymes vary in molecular weight, reaction to ions, and in the Soman/DFP ratios.

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 Mn²⁺. Dimebu hydrolysis also was stimulated but less stimulation by Mn²⁺ 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, UT. Two OPA anhydrase activities were present; however, 90% of the activity was represented by one of the enzymes, OPA-2. According to SDS-PAGE electrophoresis and gel permeation chromatography, the molecular weight has been estimated at approximately 62,000 D. OPA-2 is stimulated by Mn²⁺ and hydrolyzes soman and 4-nitrophenyl ethyl [phenyl] phosphinate (NPEPP). 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, UT and also contained OPA anhydrase

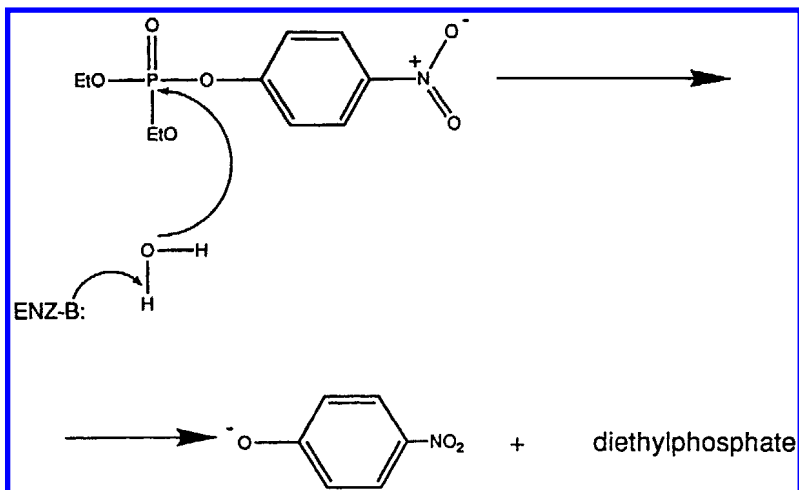


Figure 9.15 Mechanism of hydrolysis of parathion by the *opd* OPA anhydase as determined by Lewis et al. The reaction is a single displacement using a base at the active site to activate a water molecule. The activated water attacks the phosphorus, producing diethyl phosphate and 4-nitrophenol. The same active site is able to hydrolyze DFP (Dumas et al. 1989) and related organofluorophosphates (Dumas et al. 1990). (Modified with permission from Lewis et al. 1988.)

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 anhydases 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 9.3.

Eucaryotic 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 using 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 9.2 Molecular

weights of the *Tetrahymena* OPA anhydases range from 67,000 D 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 2 to 4 fold, 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 anhydases.

Mipaflox inhibition is reversible and competitively inhibits *Tt* DFPase-1, *Tt* DFPase-2 and *Tt* DFPase-3 (Landis et al. 1989). Hydrolysis of the mipaflox by partially purified *Tetrahymena* extract was only 13% the rate of DFP.

Of all the conventionally recognized OPA anhydases, 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 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 of the four stereoisomers of soman with some stereospecificity in rates.

Mipaflox 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.

Although the primary investigation into the OPA anhydases 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).

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 anhydases, 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/soman ratio. Conversely, *Aplysia*, *Spisula*, and *Homarus* (lobster) hydrolyze 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 9.3](#). *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/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 more than one OPA anhydrase system may be present.

Table 9.3 Comparison of DFP and Soman Hydrolysis Ratio and Stimulation by Mn^{2+} For Aquatic Organisms

Enzyme source	Soman/DFP ratio		Stimulation by Mn^{2+}	
	Mn^{2+}	no Mn^{2+}	DFP	Soman
<i>Proteus vulgaris</i>	19	22	1.5	1.3
<i>Saccharomyces cerevisiae</i>	8	4	0.5	1.0
<i>Homarus</i> (lobster) nerve	11	9.1	2.9	3.4
<i>Spisula</i> (surf clam) nerve	6.1	3.0	1.0	2.0
<i>Electrophorus electricus</i> (torpedo fish) liver	16	14	1.7	1.8
<i>T. thermophila</i> (crude extract)	20	10	1.0	2.0

Note: Interestingly, *S. cerevisiae*, *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+} .

Modified from Hoskin et al. 1984.

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, lowest in the foot muscle (Anderson et al.). 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. 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 Mazur-type OPA anhydrase.

Characteristics of the Fish Activities

Hogan and Knowles (1968) examined the OPA anhydases of liver homogenates from the bluegill sunfish, *Lepomis macrochirus*, and the channel catfish, *Ictalurus 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 h 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 1mM 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 also were able to hydrolyze paraoxon and methyl 3-hydroxy- α -cronate, dimethyl phosphate (mevinphos), although at a very slow rate. K_m s 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.

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+} is 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 D 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 9.2), 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 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 also has been demonstrated.

To date, the active site of the 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: 4-nitrophenyl ethyl [phenyl] phosphinate (NPEPP) is readily hydrolyzed by the *Tetrahymena* OPA anhydrases, but its close analog 4-nitrophenyl [isopropyl] phenylphosphinate (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.

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 *Psuedomonas* 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 9.16](#)), 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; Kitteridge and Roberts; Rouser et al.; Simon and Rouser; Quin and Shelburn; Neidleman and Geigert). 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; Landis et al. 1987; Landis et al. 1989). That hypothesis must be expanded as some OPA anhydrases also may be important in dehalogenation of naturally occurring halogenated organic compounds.

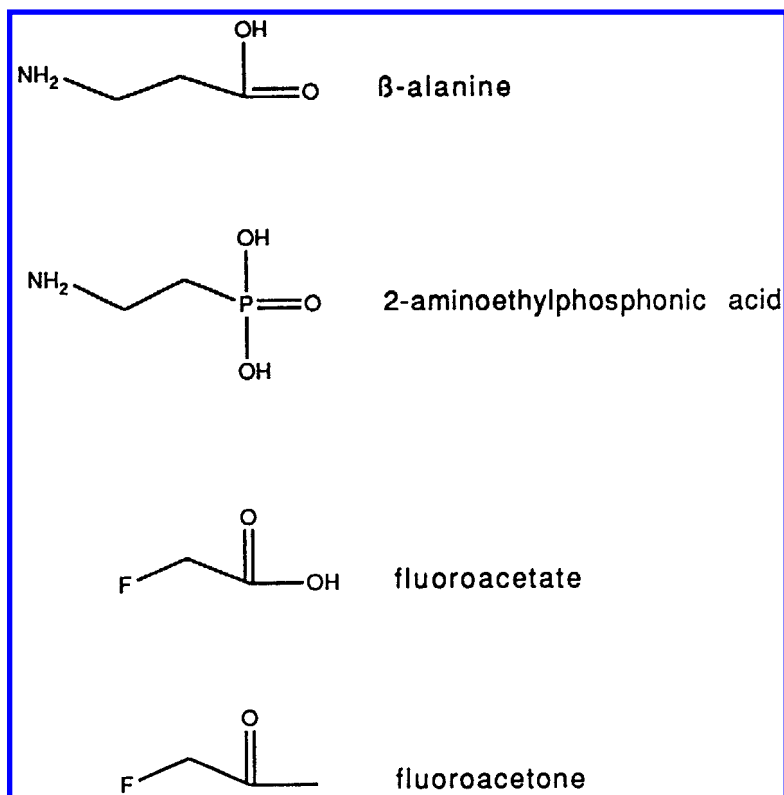


Figure 9.16 Natural substances similar to the substrates of the OPA anhydases. AEP, naturally synthesized, is an organophosphate analog to the amino acid β -alanine. Several naturally synthesized fluorometabolites are known, fluoroacetate and fluorocitrate are two examples. OPA anhydases may be involved in the metabolism of these or similar compounds.

A wide variety of halogenated organics also are naturally occurring. Neidleman and Geigert 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 are the 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 iodosome-tabolites. Fluorometabolites are not as common but do occur, especially in higher plants. Fluoroacetate and fluorocitrate are synthesized by a number of plants (Figure 9.16). Fluorinated fatty acids are found in the seeds of *Dichapetalum toxicarium*. The fungi *Streptomyces calvus* produces the fluorinated antibiotic nucleocidin, a 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). 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 also reviewed 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 also may be mediated by the production of halogenated organics. Detection of 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.

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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 use?
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 anhydrolase?
16. Summarize the hypothesized natural role of the OPA anhydrolases.

CHAPTER 10

Measurement and Interpretation of the Ecological Effects of Toxicants

INTRODUCTION

This chapter deals with perhaps the most difficult topic in environmental toxicology, how to measure and then evaluate the impact of toxicants at ecological levels of organization. The chapter starts with an evaluation of methods and ends with a discussion of the responses of ecosystems to chemical stressors.

MEASUREMENT OF ECOLOGICAL EFFECTS AT VARIOUS 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 as to the predictions and protections derived from the elaborate series of tests and our understanding presented in previous chapters 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 PCBs in terrestrial mammals and birds are examples of this application of biomonitoring. Effects monitoring looks at various levels of biological organization 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.

In the biomonitoring process, there is the problem of balancing specificity with the reliability of seeing an impact ([Figure 10.1](#)). 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

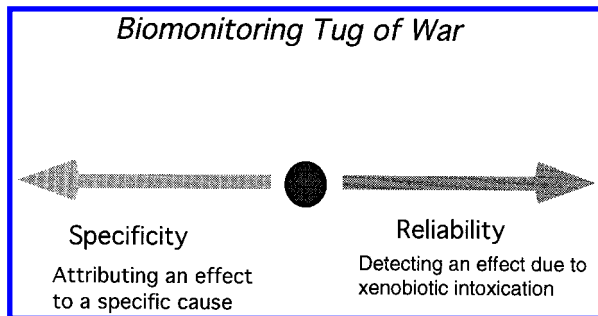


Figure 10.1 The tug of war in biomonitoring. An organismal or community structure monitoring system may pick up a variety of effects but lack the ability to determine the precise cause. On the other hand, a specific test, such as looking at the inhibition of a particular enzyme system, may be very specific but completely miss other modes of action.

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 are depicted in [Figure 10.2](#).

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.

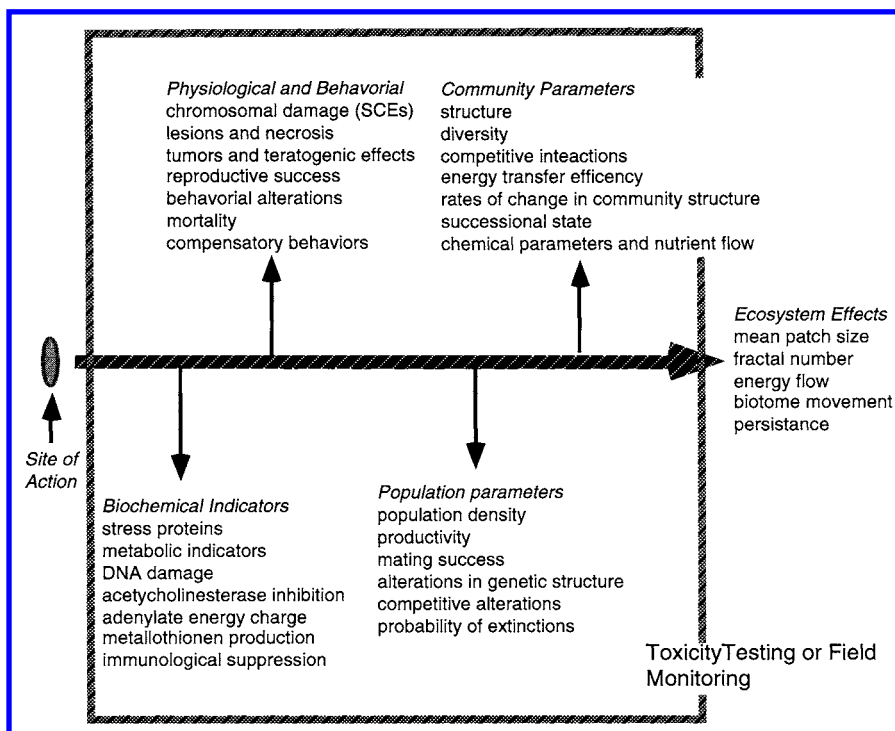


Figure 10.2 Methods and measurements used in biomonitoring for ecological effects. A number of methods are used both in a laboratory situation and in the field to attempt to classify the effects of xenobiotics upon ecological systems. Toxicity tests can be used to examine effects at several levels of biological organization and can be performed with species introduced as monitors for a particular environment.

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

$$E = \frac{U_i}{B_i} \quad (10.1)$$

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 effect or 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.

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 lipophilic 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 as new analytical tools. 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 lay 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.

MOLECULAR AND PHYSIOLOGICAL INDICATORS OF CHEMICAL STRESS BIOMARKERS

A great deal of research has been done recently 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 organization. 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 indications 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, that 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.

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 drawbacks to using blood samples are 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 also can 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 past 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 to 60 and 20 to 30 kDa. A related protein, ubiquitin, has an extremely small molecular weight, 7kDa. 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 also are known. The glucose regulated proteins are 100 to 75 kDa molecular weight and form another group of proteins that respond to a variety of stressors.

The stress-related proteins discussed above 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 toxins bind directly to the DNA causing an adduct to form. Classical mutagens can actually change the sequence of the nucleotides, cause 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. The passage of 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.

Physiological and Histological Indicators

Physiological and behavioral indicators of impact within a population are the classical means by which the health of populations are 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 is apparent 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 also can 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 indicate 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 are those that are tumorigenic. Tumors in fish, especially flatfish, have been extensively studied as indicators of oncogenic materials in marine sediments. Oncogenesis also has 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 in the blood plasma due to the damage and subsequent lesion of organs. Several enzymes such as the LDHs are specific as to the tissue. 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 mitotic 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 ecological relevant manner for that particular species. Biotransformation and detoxification process 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 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 is essential to distinguish natural from anthropogenic stressors. Shugart 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, EROD (ethoxyresorufin-*O*-deethylase activity), liver and spleen somatic indexes, and DNA adducts and strand breaks as examples. Importantly, patterns of the biomarkers were similar in the laboratory bluegills to 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 that is important.

Toxicity Tests and Population Level Indicators

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 and 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 as *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 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 also can 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 question of the ecological relevance of such subtle physiological markers can be questioned. However, sensitive measure of toxicity measures 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 evaluations (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 the 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 also have 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. Soils elutriates can be tested using species such as the fathead minnow. Earthworms are a popular test organism for soils and have proven 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 test. 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.

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. Lower and Kendall (1990) have recently published a book of these methods for terrestrial systems.

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. Radio telemetry 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.

The introduction of sentinel organisms also has 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 would 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, Bennett, and Landis (Matz 1992; Matz, Bennett, and Landis 1994), bob-white 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 also are available for residue measurements; can be assayed for the 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.

POPULATION PARAMETERS

A variety of endpoints have been used to characterize the stress upon populations. Population numbers or density have 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 stripped 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 *Drosophilla*. 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, with 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 also may 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.

ASSEMBLAGE AND COMMUNITY PARAMETERS

The structure of biological communities has always been a commonly used indicator of stress in a biological 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 is diverse, each with advantages and disadvantages, as described in the following.

Species abundance curves — Plotting the relative abundance of species ranking from most to least abundant (Newman 1995). These measurements may be most useful in a comparative mode, as the rankings and distribution change over time.

Species richness, diversity, and equability — Perhaps the most commonly measured aspects of communities has been the number of species, evenness of the composition, and diversity. These measures are not measures of toxicant stress, but 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, stability 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 review of these

methods can be found in Matthews et al. (1997). 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 dataset.

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) as 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 lead 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 10.1](#) and [10.2](#).

The utility of a measure such as the IBI is that it is transferable with modifications to other fish assemblages and to 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, that also is a disadvantage.

Table 10.1 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

^a Original IBI metrics for midwest U.S.

^b Generalized IBI metrics (see Miller et al. 1988).

Modified from Karr, J.R. 1991. *Ecol. Appl.* 1:66-84.

All indices collapse in a somewhat arbitrary fashion 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 upon professional judgment. Indices can be fooled, and quite different systems can result in indices of comparable scores. Interpretation of such score 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.

INTERPRETATION OF EFFECTS AT THE POPULATION, COMMUNITY, AND ECOSYSTEM LEVELS OF ORGANIZATION

Related to diversity is the notion of static and dynamic stability in ecosystems. Traditional dogma stated that diverse ecosystems were more stable and, therefore,

Table 10.2 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
^b	No fish	Repeated sampling finds no fish

^a Sites with values between classes assigned to appropriate integrity class following careful consideration of individual criteria/metrics by informed biologists.

^b No score can be calculated where no fish were found.

Modified from Karr, J.R. 1991. *Ecol. Appl.* 1: 66–84.

healthier than less rich ecosystems. May’s work in the early 1970s did much to test these, at the time, almost unquestionable assumptions about properties of ecosystems. Biological diversity may be important, but diversity itself may be an indication of the longevity and size of the habitat rather than the inherent properties of the ecosystem. Rarely are basic principals, such as island biogeography and evolutionary time, 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.

The impacts 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 rational for studying indirect effects emerges.

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

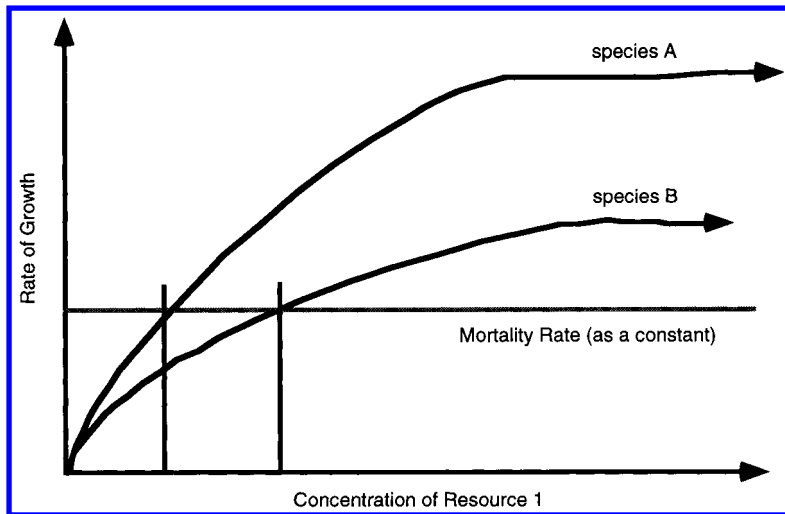


Figure 10.3 Rate of growth and resource supply. As the supply of resource increases so does the reproductive rate of an organism until a maximum is reached. At one point the rate of growth exceeds the rate of mortality and the population increases. As long as the resource concentration exceeds this amount the population grows; below this amount, extinction will occur.

found in Tilman's excellent monograph. This brief review demonstrates the utility of resource competition to the prediction, or at least explanation, of community level impacts.

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 10.3 illustrates growth curves for species A and 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; 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.

In order to describe the uptake of the toxicant by the organism, a resource consumption vector is constructed. Figure 10.4 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.

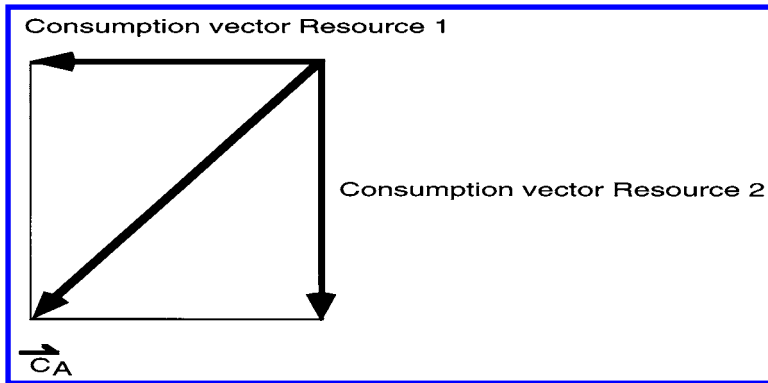


Figure 10.4 Consumption vector. Consumption vector for species A. $\hat{C}_A$ is the sum of the vectors for the rate of consumption of resource 1 and resource 2. The consumption vector determines the path of the concentrations of resources as it moves through the resource space. In the one species case, the eventual equilibrium of resources occurs where the sum of the utilization vectors and the $\hat{C}_A$ is zero.

The zero net growth point expanded to the two-dimensional resource space produces a zero net growth isocline (ZNGI) as illustrated in Figure 10.5. 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 $\hat{U}_1$, can be derived 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:

$$\hat{C}_A + \hat{U}_{1,2} \quad (10.2)$$

Tilman has shown that this point exists and is stable. Metaphorically speaking, the C1 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 10.6). Region 1 is the area in which the

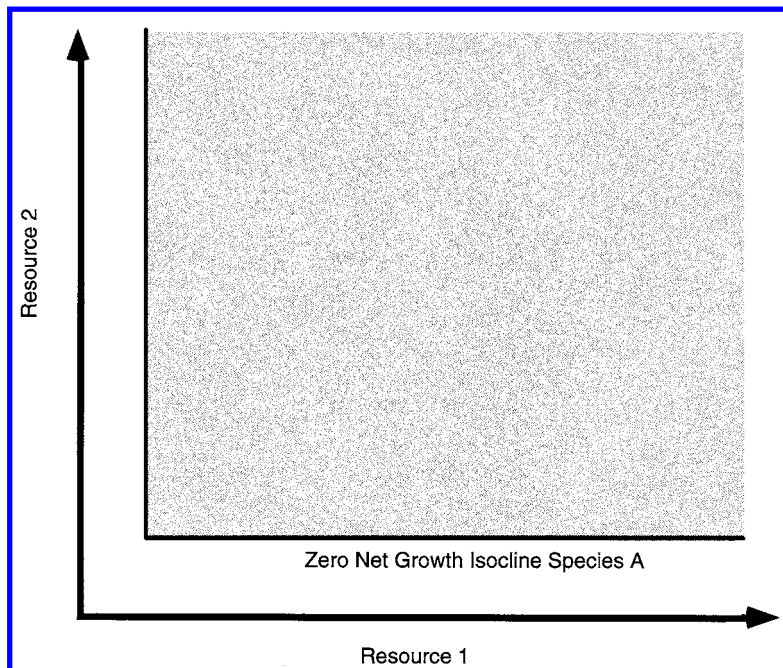


Figure 10.5 Zero net growth isocline (ZNGI). The ZNGI is the line in the resource space that represents the lowest concentration of resources that can support a species. In an equilibrium situation, the equilibrium will eventually be drawn to a point along the ZNGI. In the shaded area of the resource space, the population will grow. In the whiter area extinction will eventually occur.

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 is 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 is altered. In the following discussions the implications of these changes on examples using essential resources are depicted.

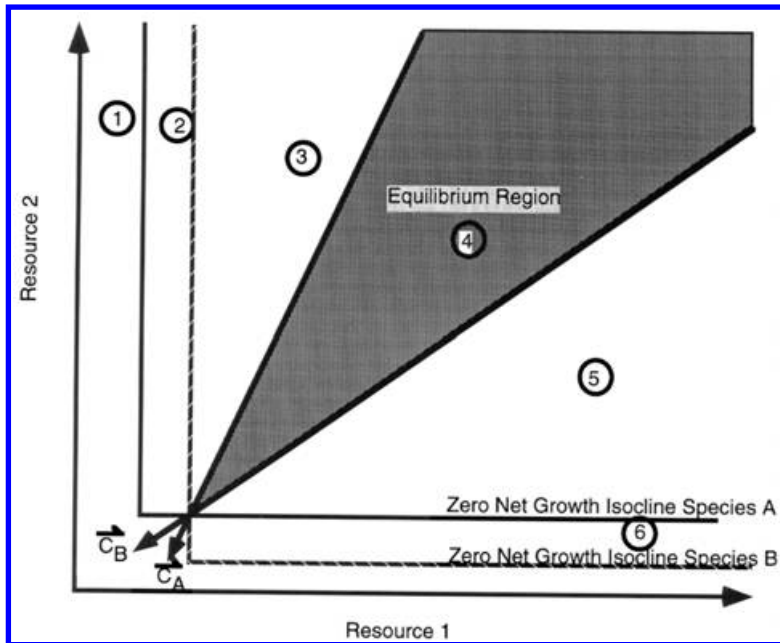


Figure 10.6 Two species graph. The $\hat{C}_A$ and ZNGI for each species is incorporated into the graph. Six regions of the resource space are created. In region 1, neither species can exist, in region 2, only species A can survive; in region 3, species A and species B can survive, but B is driven to 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.

Case 1

In the first example, the initial conditions are the same as used to illustrate the two-species resource competition model with essential resources (Figure 10.7). The toxicant alters the ability of species B to use resource 1. The slope of $\hat{C}_B$ increases and the ZNGI and the $\hat{C}_B$ shift the equilibrium point and reduce the area of the equilibrium region. The resource supply point A, that 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 still 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 $\hat{C}_A$ as the ability of species A to use resource 1 is altered. In Figure 10.8(A) the toxicant has forced the ZNGIA to a near overlap with the ZNGIB in the utilization of resource 1. In only a small region can species A drive species B to extinction. As the ZNGI_A

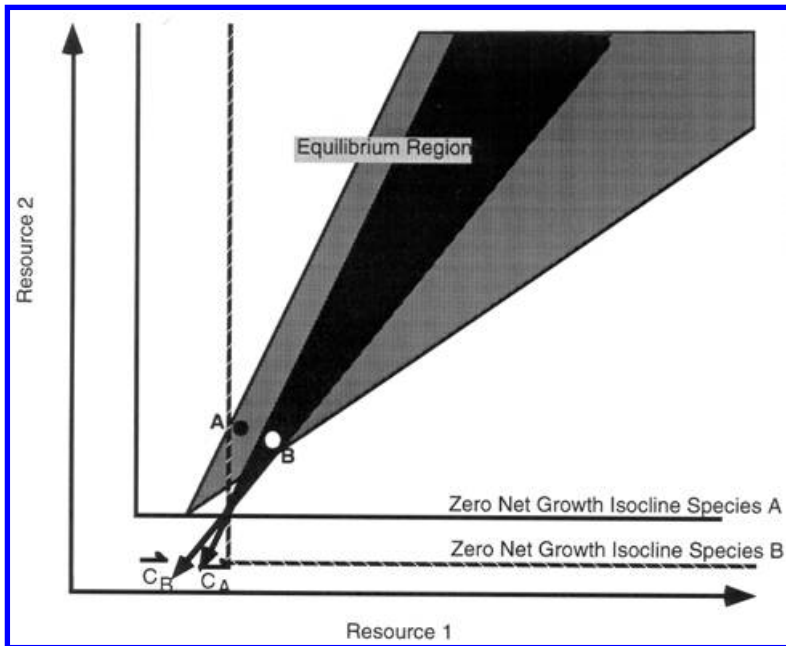


Figure 10.7 Case 1: toxicant impacts on species B. The introduction of a toxicant alters the ability of species B to use resource 1. The slope of the consumption vector is altered and the ZNGI shifts compared to the initial condition. The equilibrium point moves and the equilibrium region shifts and shrinks. With a smaller equilibrium region, the probability of coexistence of the two species also is decreased.

and $ZNGI_B$ overlap in regards to resource 1, the equilibrium region would be at a maximum. The addition of more toxicant 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 10.9). 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 these results cannot be missed. 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

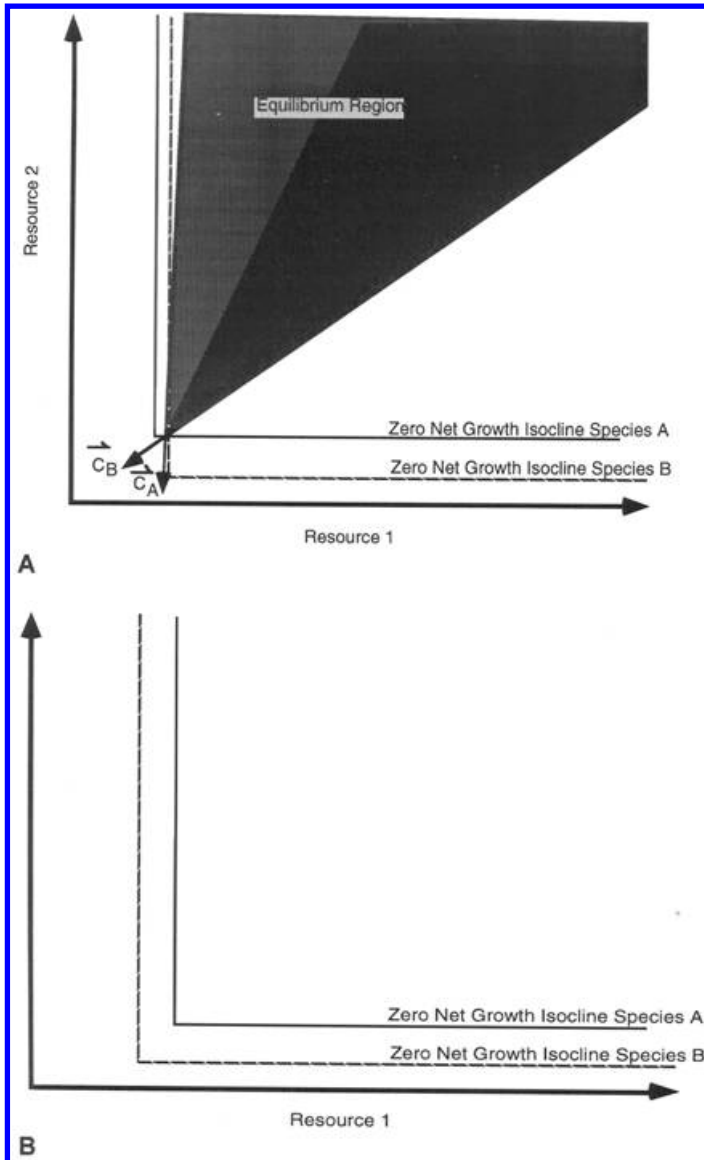


Figure 10.8 Case 2: toxicant impacts on species A. The delivery of the toxicant impacts upon the ability of species A to use resource 1. In this case, the equilibrium point has not moved but the equilibrium region has greatly increased thus increasing the opportunities for a coexistence of the two species (A). However, an increase in the equilibrium and an increase in species diversity does not mean that the system is less stressed. (B) the addition of a toxicant has forced the ZNGIA inside the ZNGIB, resulting in the eventual extinction of species A.

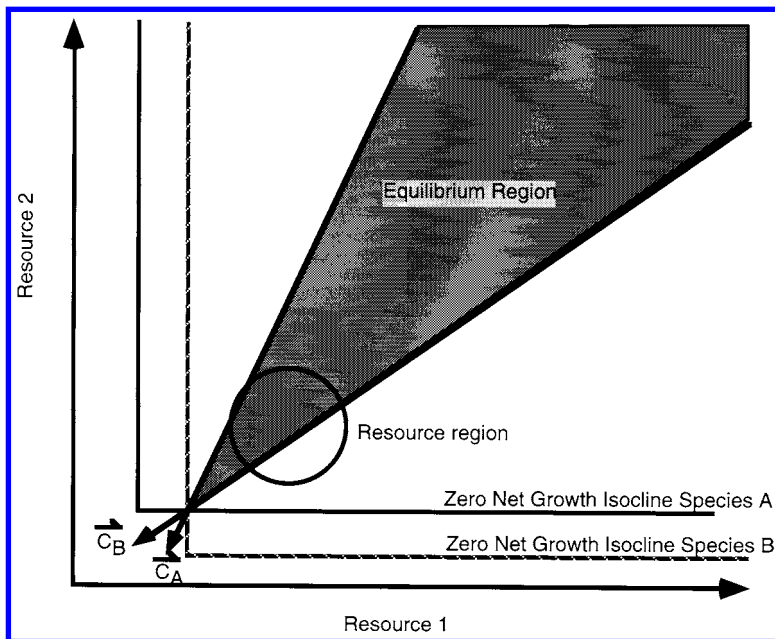


Figure 10.9 Resource heterogeneity. The heterogeneity of the resource can be represented by two dimensional 95% intervals projected upon the graph. The placement of the circle can help to predict the dynamics of the system and describe the occasional extinction of one species and the coexistence of the two.

in species composition are expected and the limitations of one-time sampling are well known. However, the confidence interval also can be expressed over space as well. 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 10.10](#), 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 confidence region is now outside the equilibrium region and species B becomes extinct.

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 10.11](#) 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

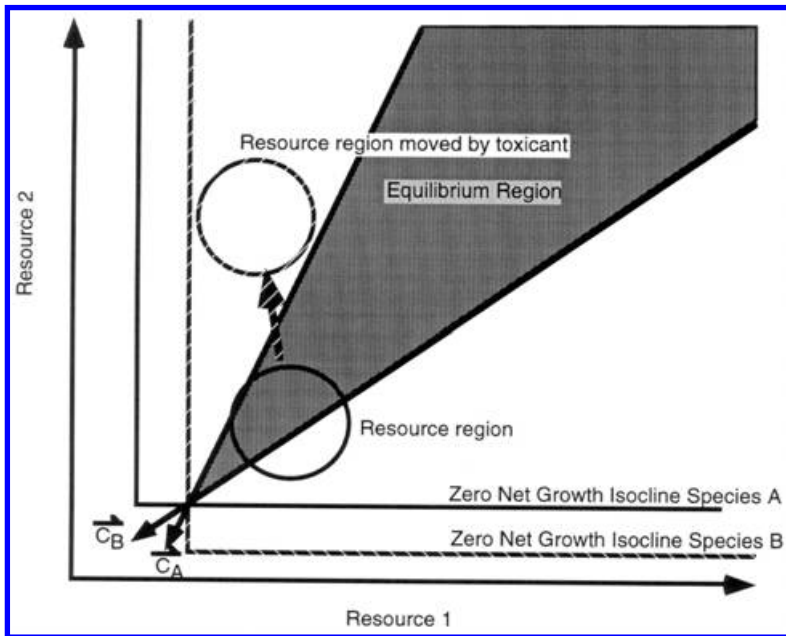


Figure 10.10 Shifting of the confidence interval of resources. The addition of a toxicant that impacts organisms that act as resources for other organisms can have dramatic effects without any direct impact upon the consumers. A shift in the resource region due to a shift in competitive interactions 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.

within the population. Guttman and colleagues have attempted to document these changes by following changes in allelic frequencies in polluted and so-called reference sites. The approach may have promise, but the difficulty of sorting natural variation from toxicant-induced selection can be daunting.

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 10.12). 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.

Reproduction — Teratogenicity and the reduction of reproductive capacity are well-known effects of toxicants, especially in vertebrate systems.

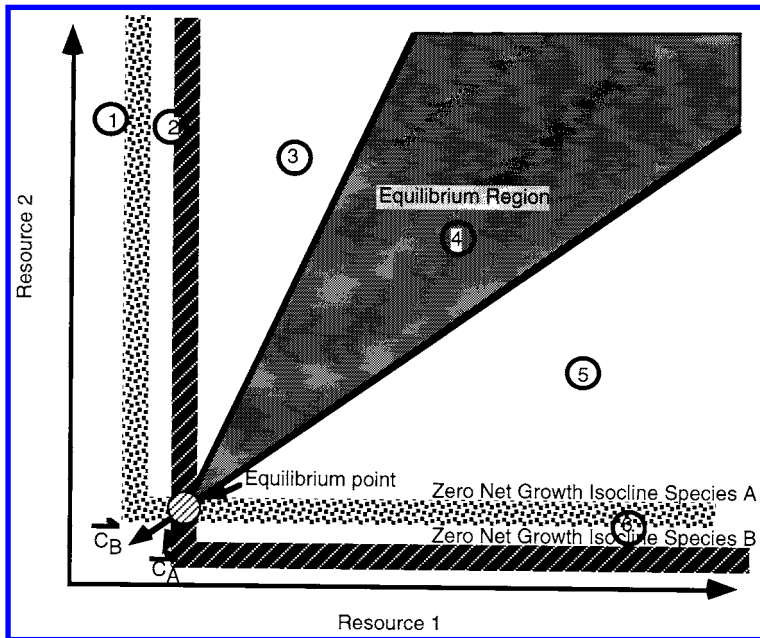


Figure 10.11 Genetic diversity. The genetic diversity of a population will alter the sharp lines of the ZNGIs into bars representing 95% confidence intervals. The consumption vectors can be similarly altered, although for this diagram they are still conventionally represented. The equilibrium point and equilibrium region then become probabilistic.

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

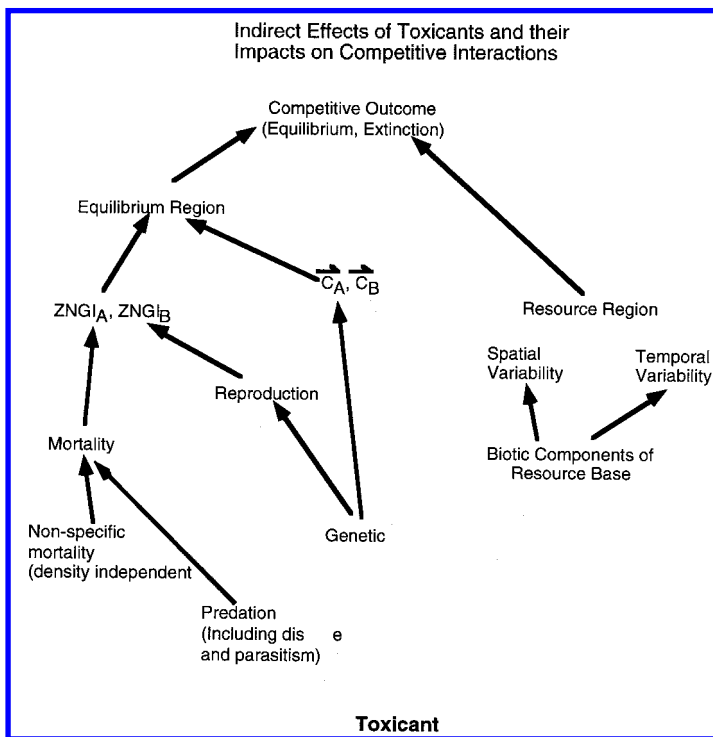


Figure 10.12 Impacts of toxicants upon the components of resource competition. The relationships among the factors incorporated into resource competition models can be affected in several ways by a toxicant. Only the density independent factors governing mortality escape.

same stressor. History of the system, therefore, plays a large part in determining the response of a community to a stressor.

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 between 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.

Population Biology, Nonlinear Systems, and Chaos

A great deal of interest has been sparked by the realization that simple models for the description of population dynamics of organisms with nonoverlapping generations. May (1973), and May and Oster (1976) demonstrated that the use of difference equations such as that for population growth:

$$N_{t+1} = N[1+r[1 - N/K]] \quad (10.3)$$

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 set 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, i.e., they appear chaotic.

The investigation of chaotic dynamics also has spread to weather forecasting and the physical sciences. An excellent popularization by Gleick (1987) reviews the discovery of the phenomena, from the butterflies of Lorenz in the modeling of weather to the complexity theory. What follows is only a brief introduction.

Figure 10.13 compares the outcomes. In one instance, r is set at 2.0, the carrying capacity 10,000 and the $N = 2500$. 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 that the r value is 3.0. The system does initially climb towards 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 the Equation 10.2. 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 10.2 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 10.14 provides an example. Using Equation 10.2, the initial N in Figure 10.14A is 9999, with a carrying capacity of 10,000. Overlaid on this figure in Figure 10.14B is the dynamic of a population whose initial $N = 10,001$. Notice that after 10 time intervals that the two systems have dramatically diverged from each other. An error in 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

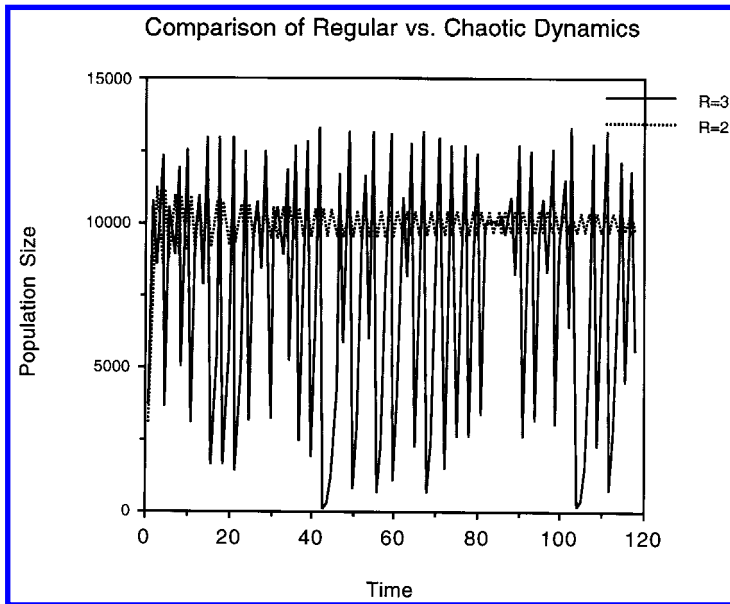


Figure 10.13 Comparison of the population dynamics of two systems that begin at the same initial conditions but with different rates of increase.

differentiated from a truly stochastic system. [Figure 10.15](#) 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 is deterministic and the other chaotic. However, there is a simple way to differentiate these two alternatives: the phase space plot.

[Figure 10.16A](#) is the phase space plot for the $N = 10,001$ graph. In this plot N vs. the N at an arbitrary yet constant time interval are plotted against each other. For these illustrations, N is plotted vs. 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 10.16B](#), the phase space plot of the randomly generated plot, no such 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 recently Tilman and Weldin (1991). Chaotic dynamics certainly are not universal, but have been found in several ecological and epidemiological contexts as described in [Table 10.3](#).

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 most recent studies that has particular relevance to environmental toxicology is the demonstration that grass populations

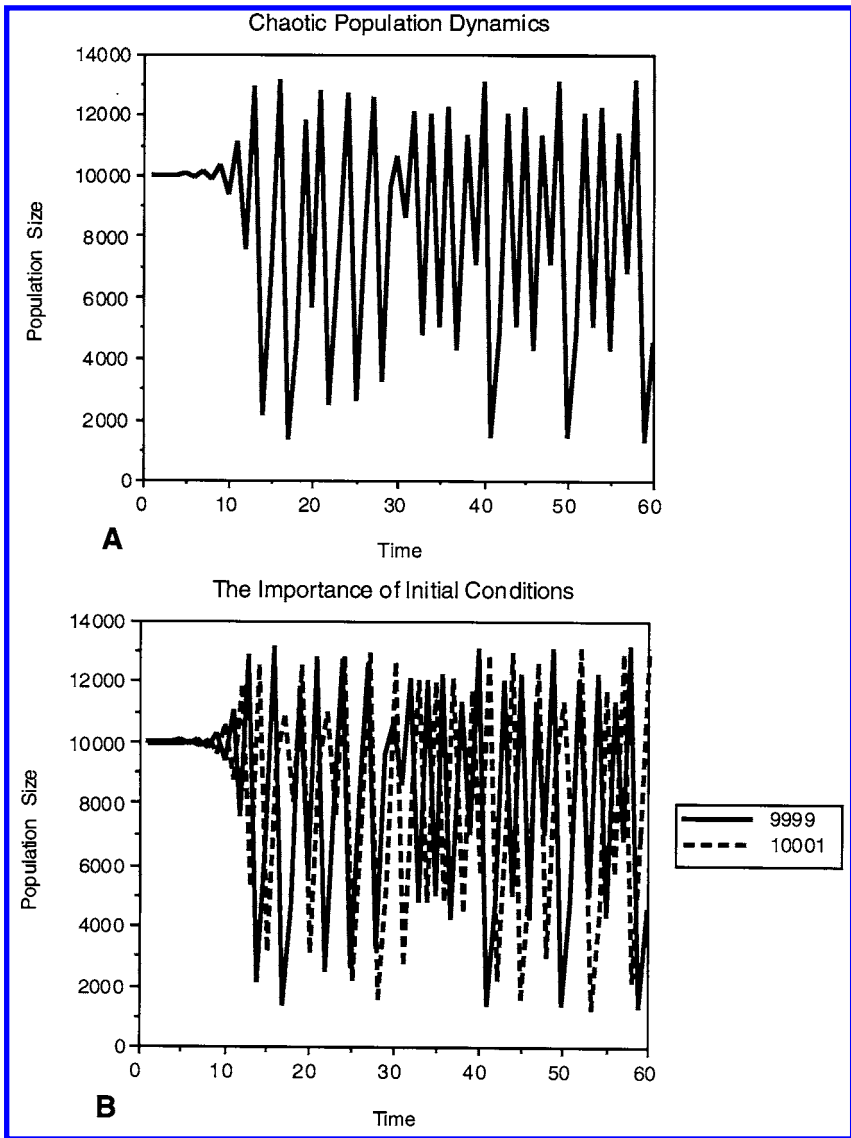


Figure 10.14 The importance of initial conditions. Although the equations governing the populations are identical, a slight 1/10,000 difference in the initial conditions results in very different dynamics.

studied by Tilman and Wedin 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 the 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 tenants of ecological theory depend on an assumption of equilibrium, they may be

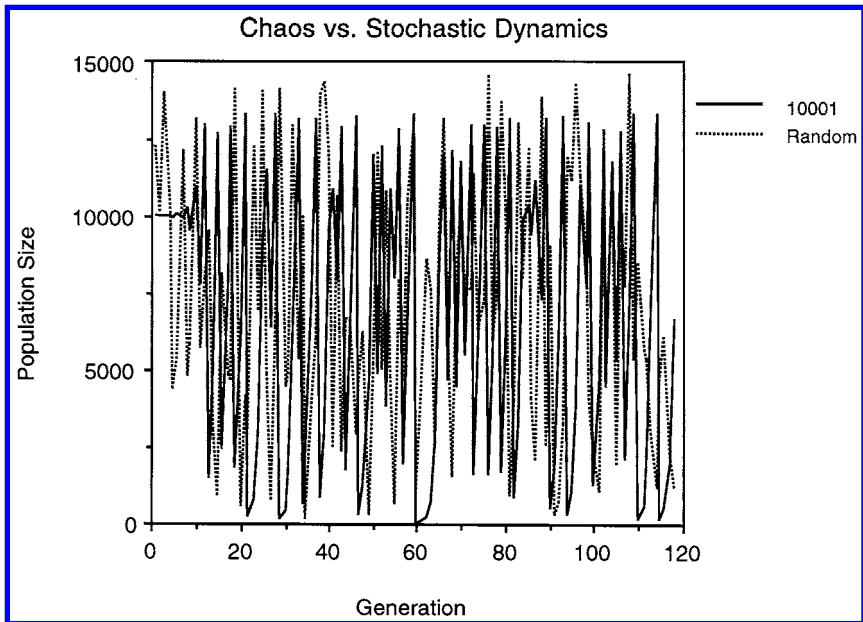


Figure 10.15 Comparison of chaotic vs. random population dynamics.

misleading. Schaffer and Kot make a stronger statement, “Our own opinion is that what passes for fundamental concepts in ecology is as mist before the fury of the storm; in this case, a full, nonlinear storm.” 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.

The implications of nonlinear dynamics in environmental toxicology have recently been discussed by Landis et al. (1993, 1994). 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 unique dynamics that are likely unique for each situation. As stated by Schaffer and Kot, 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 replicability of field studies also can 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.

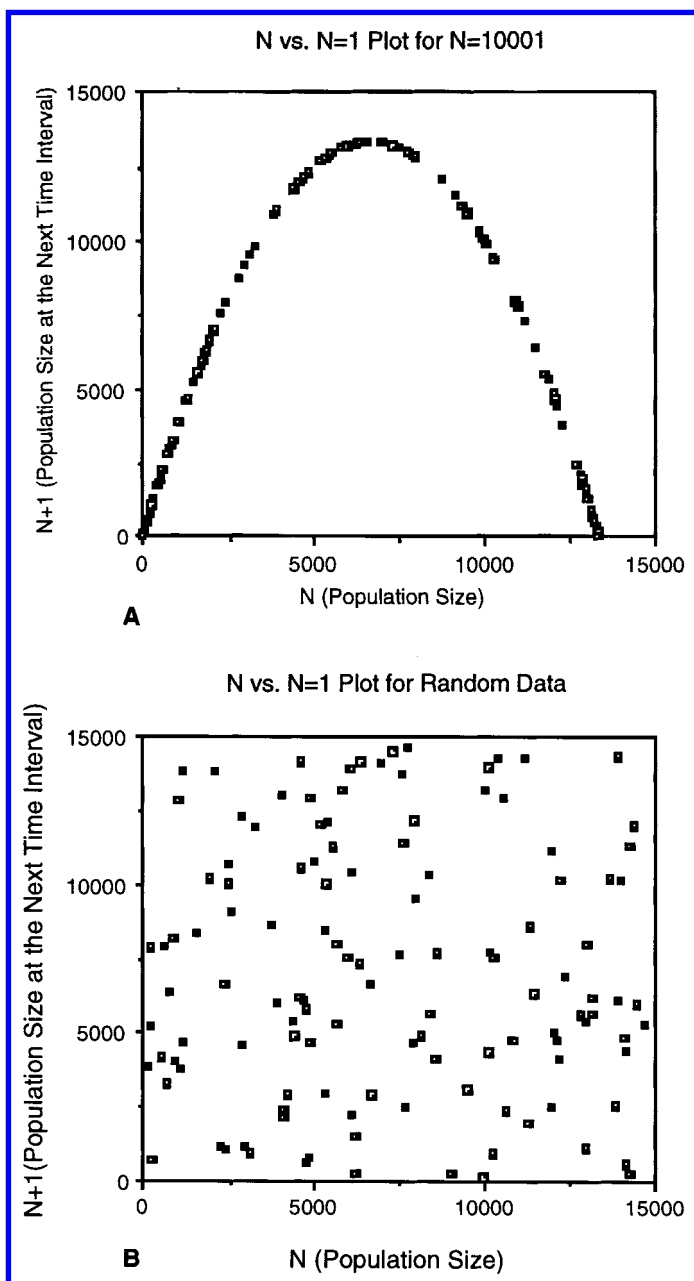


Figure 10.16 Plots of the population size vs. the population size at a specific time interval reveals the structure of a chaotic system. (A) Derived from the deterministic yet stochastic looking dynamics, a pattern readily forms that is characteristic of the underlying equation. (B) A shotgun blast or random pattern is revealed.

Table 10.3 Examples of Chaotic Dynamics in Ecological Systems

Organism (as compiled by Schaffer 1985)	Chaotic dynamics observed
Mammals	
Canadian Lynx	Yes
Muskrat	No
Insects	
Thrips	Yes
<i>Leucoptera coffeina</i>	Yes
<i>L. meyricki</i>	Yes
Blowflies	Yes
Human Diseases	
Chickenpox-New York City	No
Chickenpox-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	No
Sugihara, G., B. Grenfell, and R. M. May (1990)	
Measles City by City (UK)	Yes
Measles (Country wide)	No — Noisy 2-year cycle
Tilman, D. and D. Weldin (1991)	
Perennial grass <i>Agrostis</i>	Yes

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.

Metapopulation Dynamics

Environmental heterogeneity and the patchy distribution of organisms in space also can be considered in evaluating the risks to populations. Metapopulation dynamics is a useful tool in evaluating the consequences of a stress over both time and space.

A metapopulation is a “population of populations” (Levins, 1969) connected through immigration and emigration. The general assumptions are that there is a minimum viable population 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 (1991) derived the “rescue effect”, a population that is below the minimum viable population 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 if only 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 that 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 recently 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.

Spromberg, Johns, and Landis have used modified versions of the Wu et al. (1993) model to examine the impact of a contaminated patch on a three-patch metapopulation of varying arrangements (Figure 10.17). The simulation models also allow the recognition of a variety of dynamics and rates of population growth. A simple stochastic function for exposure to the toxicant was incorporated. Linear and circular three-patch models were used and the distance between each patch could be varied. No toxicant was allowed to cross between patches. In some instances the toxicant was persistent, in other simulation the toxicant was degraded over time. Three important sets of findings were made.

The first finding is that populations in patches removed from the contamination were affected by the presence of the toxicant. 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 10.18). In the simulations when the dosed patch was at a EC_{100} , organisms could still be found due to immigration from other patches. Due to the stochastic nature of the exposure between the toxicant and the organism, the simulations are repeatable only in type of outcome, not in the specific dynamics.

The second finding is that in the simulations that incorporated toxicant degradation, several discrete outcomes are available from the same set of initial conditions. 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

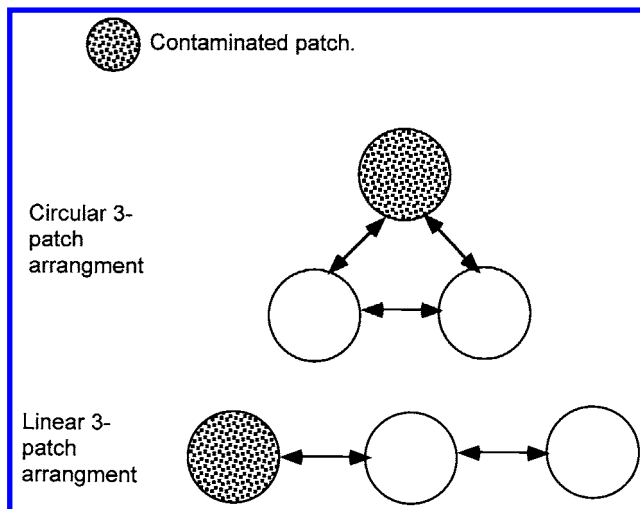


Figure 10.17 Arrangement of patches in the metapopulation model. In the discussion, the distances between the patches are assumed to be equidistant. The exposure of the toxicant to the organisms is modeled using a Poisson distribution.

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 by 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 (MVP). 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 populations 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.

Third, it is apparent that not just the chemical concentration, but also the population sizes in the connected patches determine the probabilities of outcomes. Small differences in initial population sizes can drastically alter these probabilities, and these are very simple ecological models.

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. Reference patches cannot be linked by migration to the contaminated patch; if connected, the reference patch can be affected by the toxicant
3. Multiple discrete outcomes can occur from the same set of initial conditions

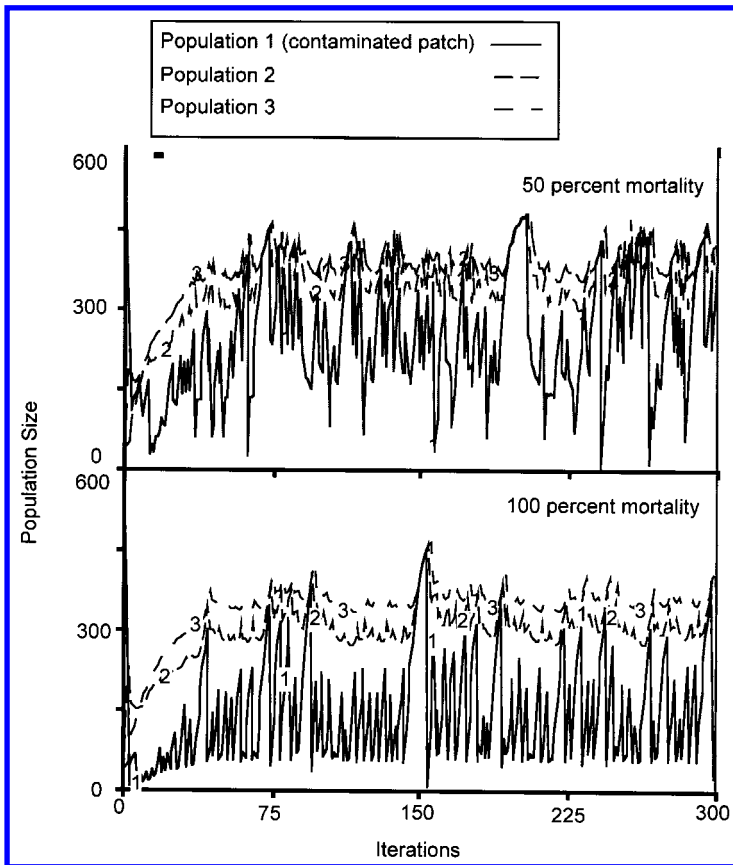


Figure 10.18 Action at a distance. In the simulation where the contaminated patch has a toxicant concentration equal to an EC_{50} , the dosed patch has wide fluctuations. However, there is an occasional overlap among all three populations. The populations in nondosed patches are below the carrying capacity of 500. Even at a EC_{100} in the dosed patch, organisms are still extant and occasionally reach numbers comparable to the nondosed patches.

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 the status of the population that determines relative risk

These conclusions are all based on relatively simple models that in the most part do not consider more than one population. Of course, ecological risk assessment attempts to predict effects on ecological structures, both their structure and function.

COMMUNITY AND ECOSYSTEM EFFECTS

The difficulty of measuring community and ecosystem effects has been extensively discussed in the literature (Suter 1993). Ecological systems can be perceived

as mechanisms for energy flow, materials cycling, and as assemblages of species. Ecosystem properties also may be examined.

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 a n -dimensional hypervolume is 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 1976). 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, problems with Type II error and the difficulty of graphically representing the dataset have been problematic. Conquest and Taub (1989) developed a method to overcome some of the problems of classical 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 SAM and is applicable to other datasets. 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.

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 end-points 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.

Classification

Ordination, classification, and clustering techniques are among the most useful methods for examining changes in structural components and also may include

abiotic factors. Classifier systems attempt to fix rules that discriminate among points in datasets. Classification is a two-step process. First, a training dataset is used to derive algorithms for determining which point in a dataset 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, and Matthews, unpublished results) has found this to be the case in microcosm datasets.

Ludwig and Reynolds 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 component analysis (PCA) and its derivatives. PCA attempts to find orthogonal linear combinations of variables that account for the variance within a dataset. 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 (DPC) use a polynomial expression to remove the nonlinear relationships from the PCA axes. DPC is useful for datasets 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 recently fashionable technique derived from a principal component 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.

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 also are relevant to this discussion. Other techniques, such as COBWEB (Fisher 1987) and RIFFLE (Matthew and Hearne 1991), 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. 1995; G. 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 datasets (Matthews, Landis, and Matthews 1996).

Ideally, multivariate statistical tests used for evaluating complex datasets 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 dataset 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.

APPLICATION OF MULTIVARIATE TECHNIQUES

The application of these methods have been examined in a series of 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

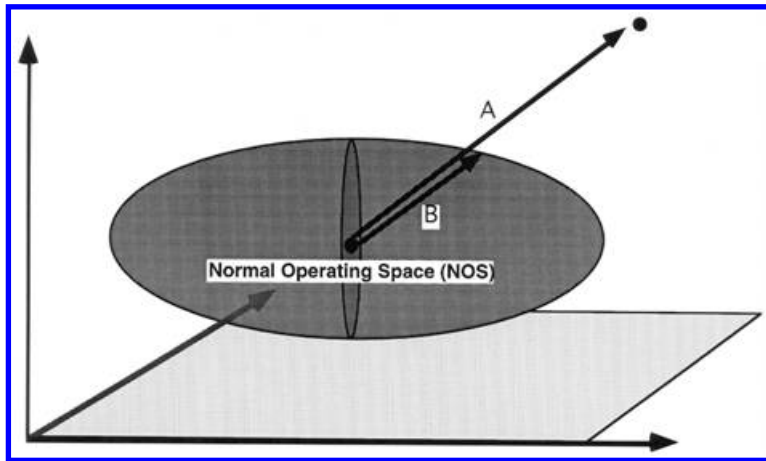


Figure 10.19 Normalized ecosystem strain.

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 developed by Matthews et al.

Normalized Ecosystem Strain

Normalized Ecosystem Strain (NES) (Figure 10.19) was developed by Kersting (1984, 1988) as a means of describing the impacts of several materials to the three compartment microecosystems containing an autotrophic, herbivore, and decomposer subsystems. These variables in the unperturbed control systems are used 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 Mahalanobis 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 an 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 is 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 sensitivity 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.

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. 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 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 this 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 in determining cleanup standards. In a follow-up

publication Johnson 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 datasets 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 Johnson and Kersting was the computational effort required. Computational requirements are not the limiting factor that they may have once been, even for large datasets.

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 using 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 previous 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 become less, the metric measures do not exhibit the same magnitudes of separation.

Nonmetric Clustering and Association Analysis

Multivariate methods have proved promising as a method of incorporating all of the dimensions of an ecosystem. Both of these methods presented above have the advantage of examining the multispecies test systems as a whole and can track such process 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 ml, where low numbers are in the 10^6 range. Random data indiscriminately incorporated with large metrics may overwhelm important variables with a different metric. Recently 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, are 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.

Before we can determine whether a toxin has affected a group of organisms or the dynamics of an ecological community, we must first determine what types of changes would occur that are independent of the toxin. In field situations, this is usually attempted by using a reference site, monitoring the changes that occur at that site, and comparing this with the changes that occur in organisms at the dosed site.

However, one of the most difficult analytical challenges in ecology is to identify patterns of change in large ecological datasets. 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(s).

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.

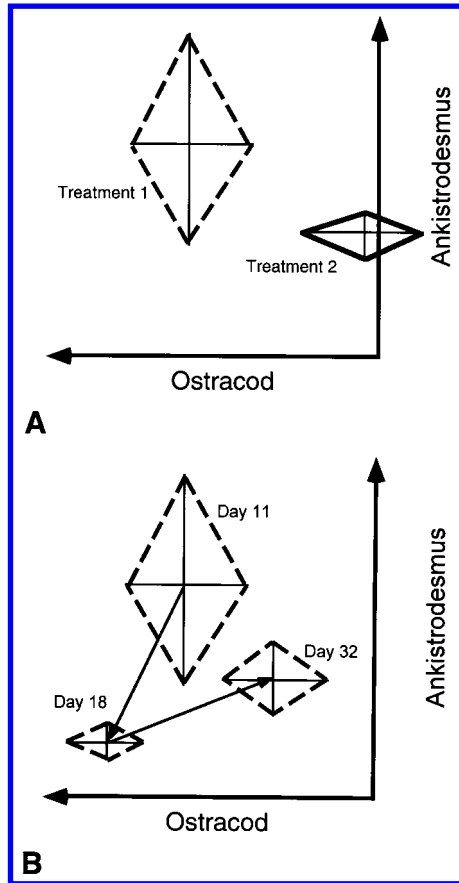


Figure 10.20 Construction of a space-time worm (STW). (A) The average values for the variables Ankistrodesmus and Ostracod are plotted along with a box plot to represent one standard deviation. Each treatment group can be represented and compared. If time is added (B), then a plot for each sampling date can be represented but the diagram becomes much harder to interpret.

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. We have developed a method we call space-time worms (STWs).

STWs were developed to more easily visualize the dynamic relationships between the variables in microcosm experiments (Landis, Matthews, and Matthews 1997). Matthews and 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 10.20 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 researchers intuition. The mean of the replicates is then plotted and the standard deviation along each axis represented (Figure 10.21A). Whiskers to the box may be added to represent

minimum and maximum values or other characteristics of the dataset. The position and variability within a treatment group can then be compared to 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 10.21B). 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 10.21A). 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 10.22B portrays the dynamics along the small daphnia, 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 d. 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 ecosystem (pH and small daphnia) 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.

There is another method of 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 then can be joined in a manner similar to that portrayed in Figure 10.21. A newer method has been to use a redundancy analysis to construct the axes. Kersting and van den Brink have used this method to present the results from a series of ditch experiments (Figure 10.22). Using such a projection the convergence or divergence of the treated systems can be observed. The drawback to these methods is that the best PCA or RDA axes are likely to change 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.

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 multivariate techniques were used, generally Euclidean and cosine distances (Figure 10.23), principal components, and nonmetric clustering and association analysis.

Matthews et al. (1991a, 1991b) have compared several types of multivariate techniques to evaluate two types of ecological data, a limnological dataset that included spatial and temporal changes in water chemistry and phytoplankton populations, and

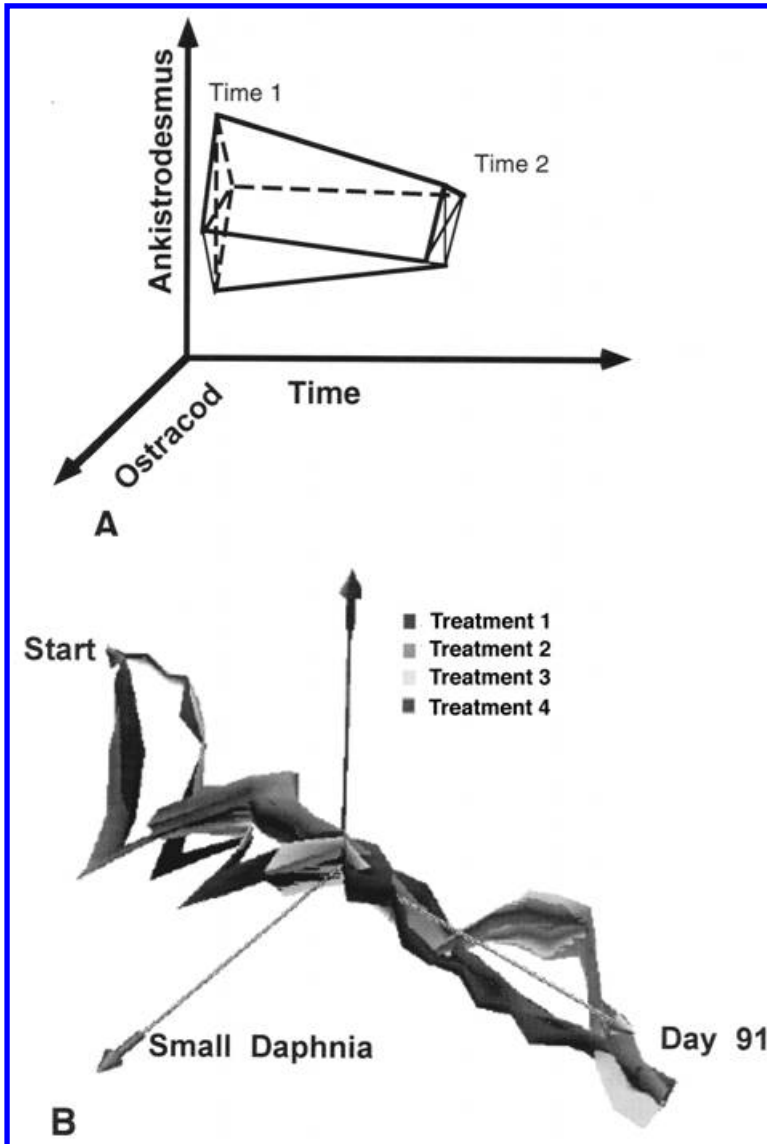


Figure 10.21 STW construction. (A) Time is added as a third axes and the measurements for the two sampling days connected. When this is done for an entire experiment (B), the relative dynamics of the systems becomes readily apparent.

a stream dataset 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

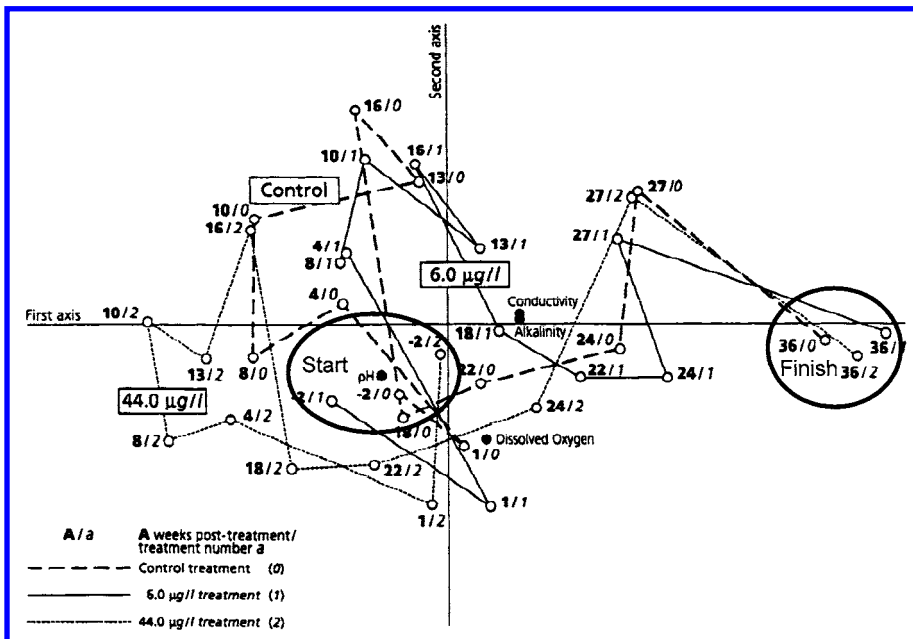


Figure 10.22 A RDA projection of an artificial stream experiment. The trajectories of the three treatments can be followed throughout the course of the experiment. (Modified from Kersting and van den Brink 1997, SETAC, Pensacola, FL. With permission.)

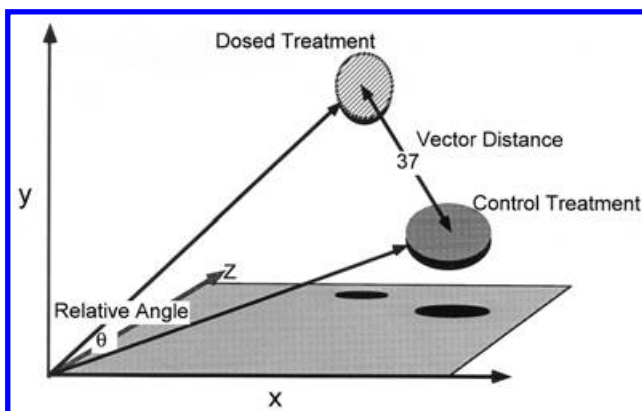


Figure 10.23 Measures of distance between clusters. Two of the commonly used measures of separation of clusters in an n-dimensional space are the cosine of the angle and the vector distance. Each method has advantages and disadvantages. In order to visualize the data as accurately as possible, several measures should be employed. (From Kersting, K. and P.J. van den Brink. 1997. *Environ. Toxicol. Chem.* 16: 251-259. With permission.)

(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 dataset 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 recently been used to examine a series of multispecies toxicity tests. Described below are the data analyses from two recently published tests using methodology derived from the Standardized Aquatic Microcosm (SAM) (ASTM E1366-91). 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. 1993). *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 environment. Toxicity tests of the riot control material demonstrated that although *A. denitrificans* CR-1 eliminated the toxicity of a CR solution towards 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 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 dataset used to analyze the CR microcosm experiment was presented in a blind fashion for analysis. Neither the purpose 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 analysis. 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. 1993). 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 3l.

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.

In [Figure 10.24](#), the average cosine distances within the control group and between the control group and each of the three treatment groups are plotted on a log scale. The initial, strong effect, from day 11 to day 25, is easily seen as a large distance from the treatment 1 (control) and treatment 2, together, to both treatment groups 3 and 4, initially, but then treatment 3 moves closer to the control. The period of no significant difference, from day 35 to day 46, is also clear. During the second period of significant difference, from day 49 to 59, a perfect dose-response for all three treatments is seen, with higher doses becoming more distant from the control. This dose-response relationship is consistently maintained over a period of 11 days, for 4 sampling dates, days 49, 53, 56, and 59. A dose-response relationship like this was not observed earlier, although the magnitude of the distance was considerably greater.

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 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 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 a 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 microcosm 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:

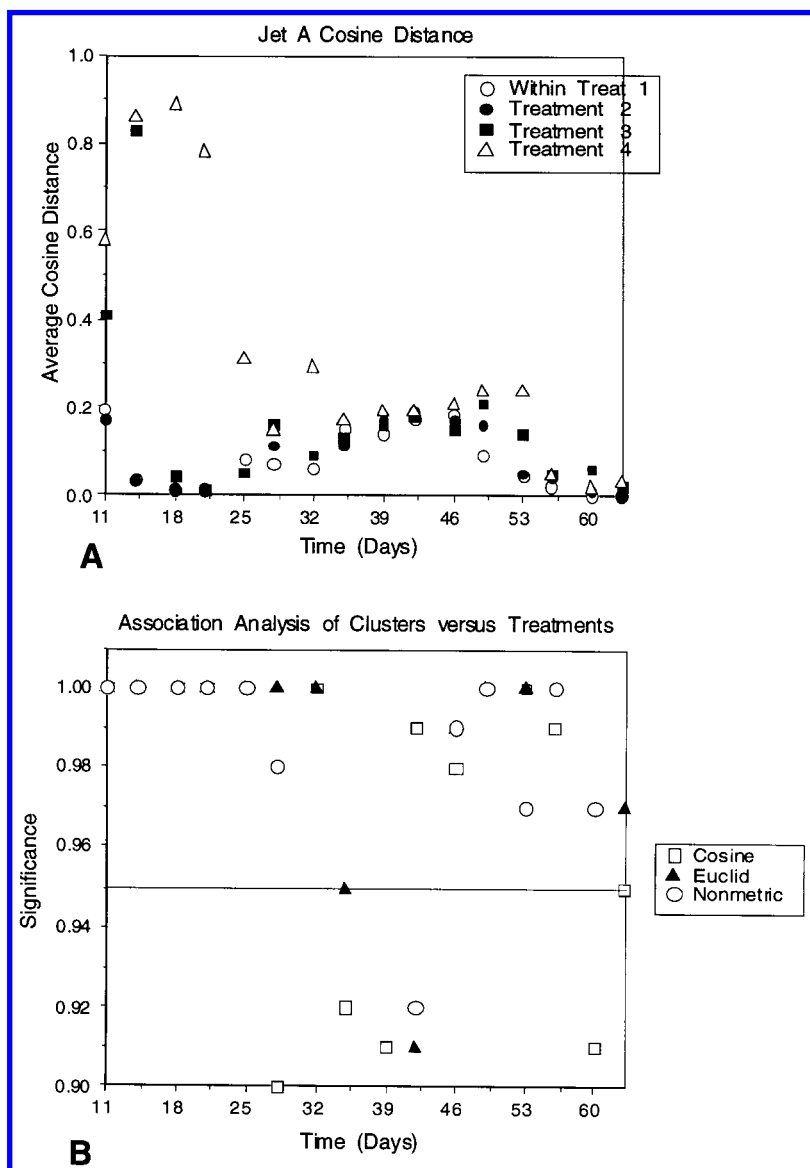


Figure 10.24 Multivariate analysis of the impact of Jet-A in the SAM test system. (A) The cosine distance from the control group to each of the treatments for each sampling day. Note that large differences are apparent early in the SAM. During the middle part of the 63-d experiment the distances between the replicates of treatment 1, the control group, is 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 are presented. Note that there are two periods, early and late ones, where the clustering into treatment groups is significant at the 95% confidence level or above.

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 that is 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 latter stages. The variable Ostracods 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.

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 future of ecosystems.

First, the apparent recovery or movement of a dosed system towards 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 indexes 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 10.25](#). In [Figure 10.25A](#), the dosed and the reference systems

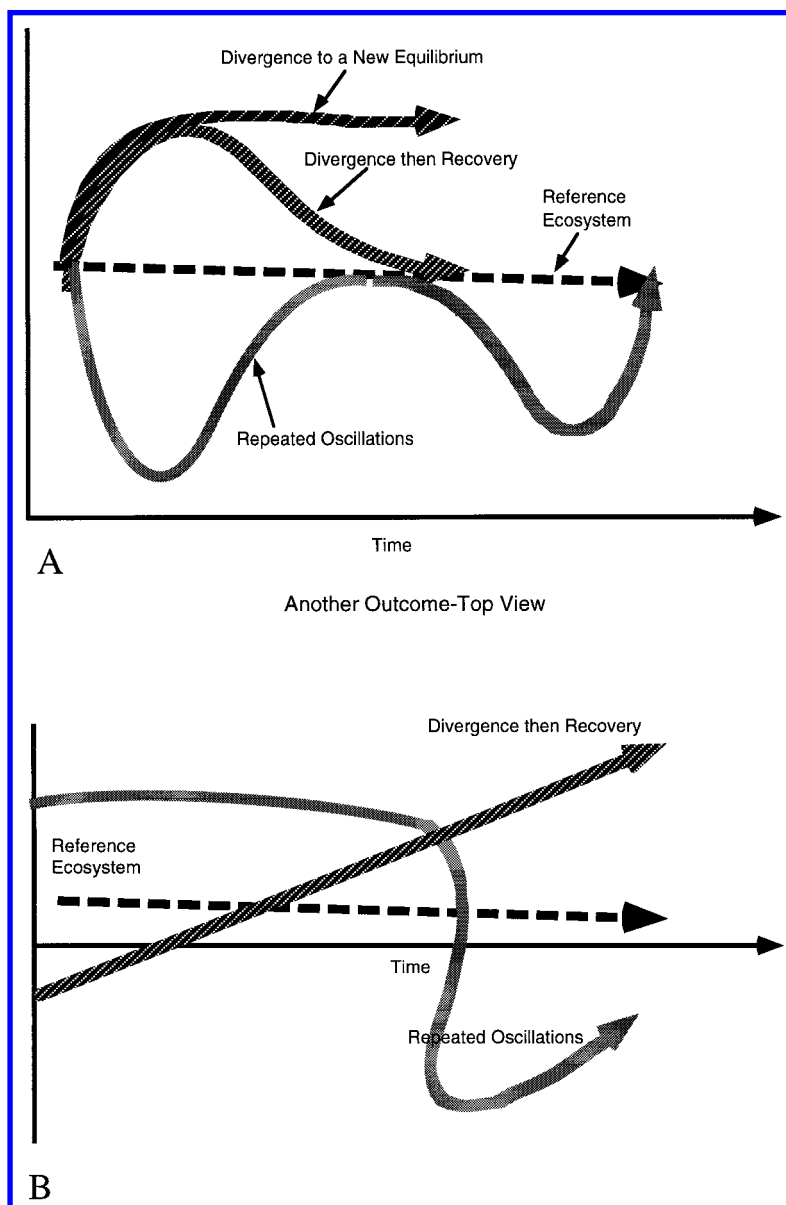


Figure 10.25 Two views of the dynamics of ecosystems. (A) It appears that in some instances the system returns to a control state or is in a stable oscillation. (B) Looking at the same system from the top indicates that the systems are moving in quite different directions.

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 10.25B](#) 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 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 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 with 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 principals also apply to spatial distributions of populations as recently 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 ranging from static “crystal lattice” patterns, spiral waves, chaotic variation, or 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. Katz 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 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 recently suggested independently by Dickson et al. (1992) and Matthews and Matthews. 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. eliminates 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 dataset. 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 errors 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 makes discussion of such parameters as ecosystem stability difficult to accurately determine. In [Figure 10.26](#) a system that hits a perturbation is depicted. Although the distances that each have 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 prevent a return to the original state, recovery cannot be considered an inherent property of the system.

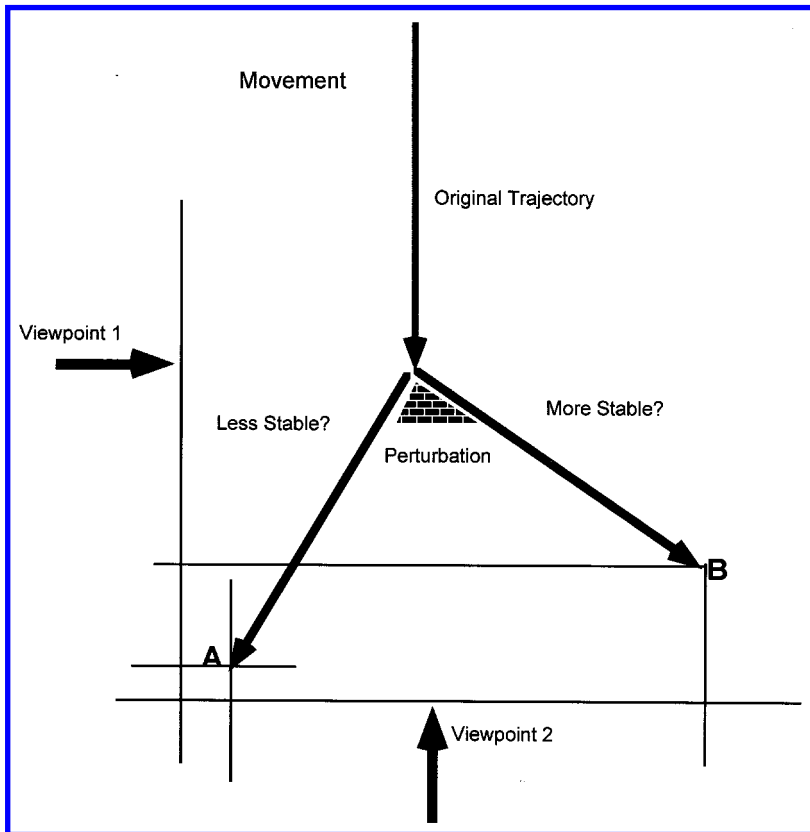


Figure 10.26 Apparent change in an ecosystem depends upon the point of view of the observer. An observer at viewpoint 1 sees the system moving steadily along until the perturbation occurs. At the perturbation, two outcomes may occur, A and B. Both arrows are the same length, indicating that the change 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 than with 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.

The dynamics in the research discussed above make a metaphor such as ecosystem health inappropriate and misleading. In a recent 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 lead 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 are quite different than those of organisms.

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, Landis, and Matthews 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 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 [Figures 10.25](#) and [10.26](#). 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 significance of community conditioning can be summarized by 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 to its genetic make up.
- You cannot even try. If the underlying dynamics are nonlinear the system is unlikely to reoccur. Even if regular cycles are possible the chance of the systems being in phase may be low. Even if major efforts, such a 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. Reasons discussed in 1 and 2 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 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 timespan. 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. Several potential outcomes may be possible, but not every outcome. Perhaps as a better understanding of the assembly of ecosystems is developed then we can even predict the probabilities of the outcomes. Prediction of ecological impacts will resemble more the weather forecast than the Newtonian dynamics.

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 with cosine of vectors distance (Good 1982; Smith et al. 1990). The third test used nonmetric clustering and association analysis (Matthews et al. 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, $\bar{\mathbf{x}} = \langle \mathbf{x}_1 \dots \mathbf{x}_n \rangle$, with one value for each of the measured biotic parameters, allows multivariate distance functions to be computed. $\bar{\mathbf{x}}$ and $\bar{\mathbf{y}}$ is computed as

$$\sqrt{\sum_i (x_i - y_i)^2}$$

The cosine of the vector distance between the points $\bar{\mathbf{x}}$ and $\bar{\mathbf{y}}$ is computed as

$$1 - \frac{\sum_i x_i y_i}{\sqrt{\sum_i x_i^2 \sum_i y_i^2}}$$

Subtracting the cosine from one 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 $\bar{\mathbf{x}}$ 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, each point in treatment group i and cluster j being counted as a point in frequency cell ij . 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 taken from Press et al. (1990).

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STUDY QUESTIONS

1. What are the two categories of biomonitoring programs?
2. List the six current organizational levels of biomonitoring and explain.
3. Discuss some examples of means by which past and current exposure to toxic xenobiotics are detected.
4. Of what value are biomarkers as predictors of the effects of toxicants?
5. 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.
6. Describe physiological and behavioral indicators of toxicant impact.
7. What are toxicity identification and reduction evaluations?
8. What are the advantages and disadvantages to the toxicity tests given as examples in the text?
9. Define and discuss sentinel organisms as ecosystem monitors. What are advantages of using this method?
10. Discuss alternations in genetic structure as a means of measuring xenobiotic effects on a population.
11. How can species diversity indicate stress on an ecosystem? What drawbacks does the structure of biological communities have as an indicator of stress?
12. What questions should biological diversity raise if it is used as an indicator of xenobiotic impacts upon biological communities?

13. What is resource competition? What is a resource consumption vector? What is a ZNGI?
14. Describe a two-species resource space graph.
15. Describe how resource heterogeneity can be incorporated into a two-species resource space graph.
16. How can toxicant input vs. natural variation be evaluated when community structure has altered?
17. Discuss nonlinear systems and their role in modeling xenobiotic impacts to ecological systems.
18. Describe the characteristics necessary in multivariate statistical tests used for evaluating complex datasets.
19. What is normalized ecosystem strain? What did Kersting find occurring with the NES as time increased after a perturbation?
20. Explain Johnson's state space of ecosystems.
21. What is the major difficulty with the Johnson and Kersting methods?
22. What is nonmetric clustering and what statistical importance does it have for ecosystem analysis?
23. What is one of the most difficult analytical challenges in ecology?
24. Discuss an assessment baseline and measurement endpoints as analysis of ecological risk assessment.
25. What would be a critical development in the formulation of risk assessment methodologies?
26. Discuss the benefits evolving from the use of multivariate techniques.
27. What illusions could give rise to a concept of recovery in ecosystems?
28. What are the main components of community conditioning?

Ecological Risk Assessment

INTRODUCTION

A great deal of environmental toxicology is performed with the eventual goal of performing a risk assessment. A great deal 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 is necessary. Appendix B contains a reprint of the recent U.S. EPA document “A Framework for Ecological Risk Assessment”. This document 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 1993, 1996). 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 as 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.

Table 11.1 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

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.

After Suter, G.W., II. 1990. In *Aquatic Toxicology and Risk Assessment*: 15th Volume. ASTM STP 1096. W.G. Landis and W.H. van der Schalie, Eds., American Society for Testing and Materials, Philadelphia, PA, pp. 5-15.

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.

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 LD₅₀ or 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 also may provide clues to dosage.

A stressor poses no risk to an environment unless there is 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 11-1](#) compares the two assessments as outlined in Suter (1990).

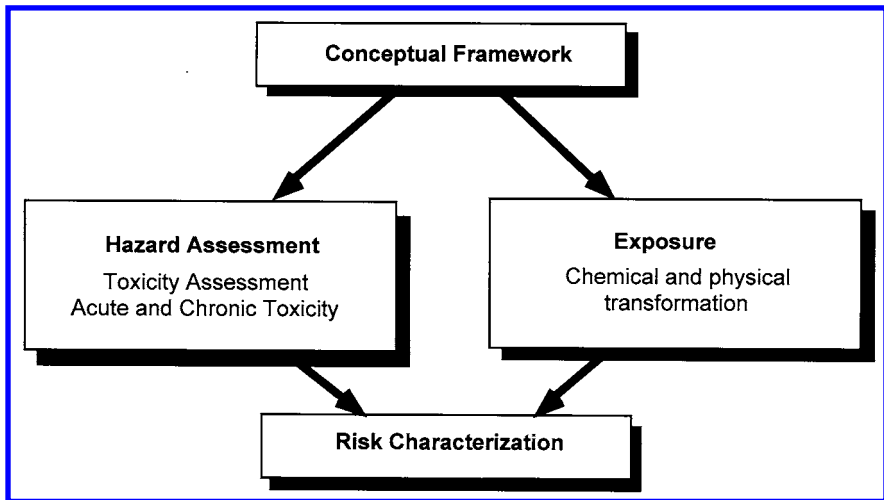


Figure 11.1 Classical risk assessment paradigm. Originally developed for human health risk assessment, this framework does not include the close interaction between effects and exposure in ecosystems.

ECOLOGICAL RISK ASSESSMENT

Two basic frameworks for ecological risk assessment have been proposed over the past 10 years. The first was based on the National Academy of Sciences' report detailing risk assessments for federal agencies. Though simple, this framework forms the basis of human health and ecological 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 11.1](#). Basically, four boxes contain the critical steps in the risk assessment. First, conceptual framework 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.

Recently, 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 ecosystem is affected

by the chemical, the ecosystem is altering the material. In light of this and other considerations a revised framework was presented in 1992.

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 11.2](#).

As before, the ecological risk assessment itself is characterized by a problem formulation process, analysis containing characterizations of exposure and effects, and a risk characterization process. 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.

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 11.3](#)): 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.

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 such as protection of endangered species, protection of a fishery, or the even vaguer preserve the structure and function of an ecosystem. The interaction between the risk assessor and the risk manager can aid 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

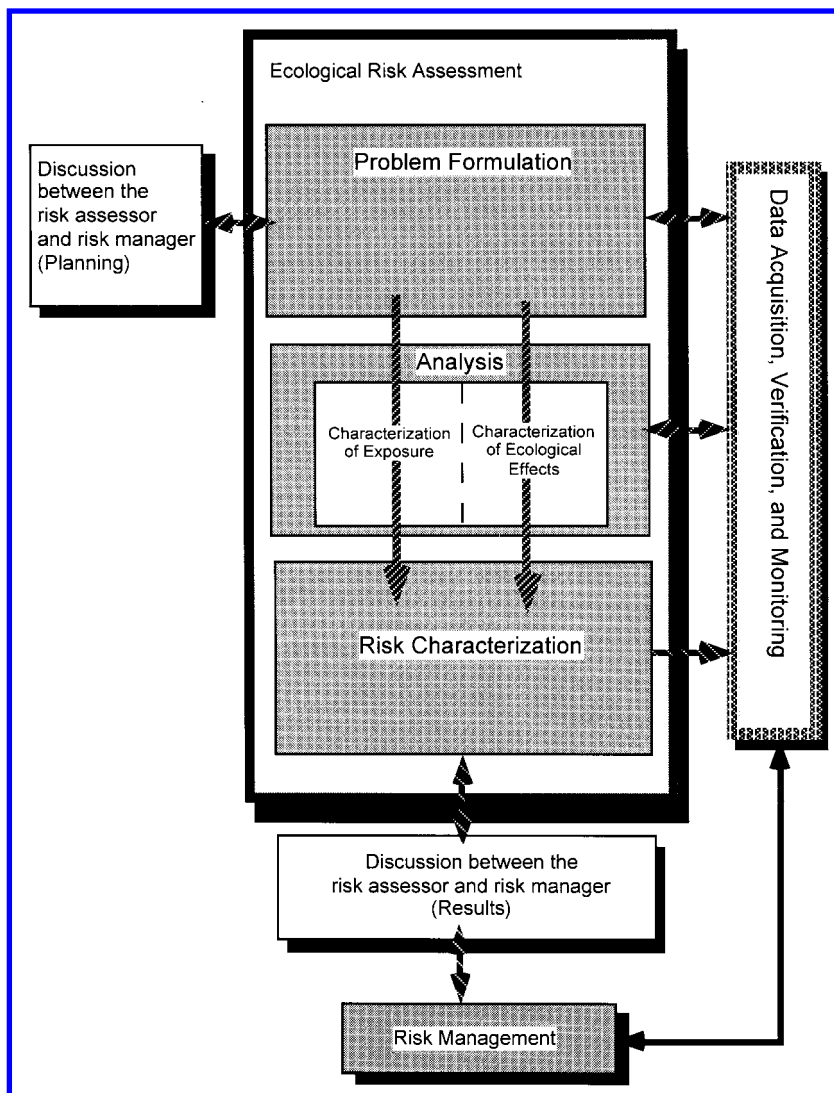


Figure 11.2 Schematic of the framework for ecological risk assessment (U.S. EPA 1992). Especially important is the interaction between exposure and hazard and the inclusion of a data acquisition, verification, and monitoring component. Multivariate analyses will have a major impact upon the selection or assessment and measurement endpoints as well as playing a major role in the data acquisition, verification, and monitoring phase.

together. Radionucleotides often produce ionizing radiation and also can produce toxic effects. Plutonium is not only radioactive but also is highly toxic.

Stressors vary not only in their composition but in other characteristics derived in part from their use patterns. These characteristics are usually listed as intensity (concentration or dose), duration, frequency, timing, and scale. Duration, frequency,

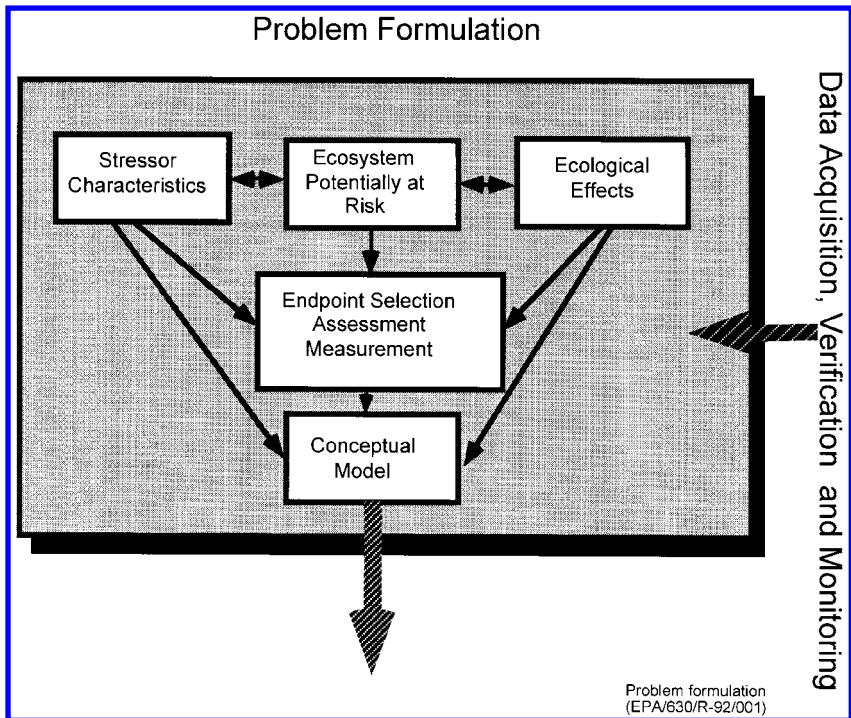


Figure 11.3 Problem formulation. This part of the risk assessment is critical because of the selection of assessment and measurement endpoints. The ability to choose these endpoints generally relies upon professional judgment and the evaluation of the current state of the art. However, *a priori* selection of assessment and measurement endpoints may lock the risk assessor from consideration of unexpected impacts.

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 local because 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, i.e., 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 systems 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 also is an important variable influencing species number and system complexity. All of the characteristics 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 10.2 in Chapter 10 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, as well as 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 past 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 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 are 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 also must 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 the 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; 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

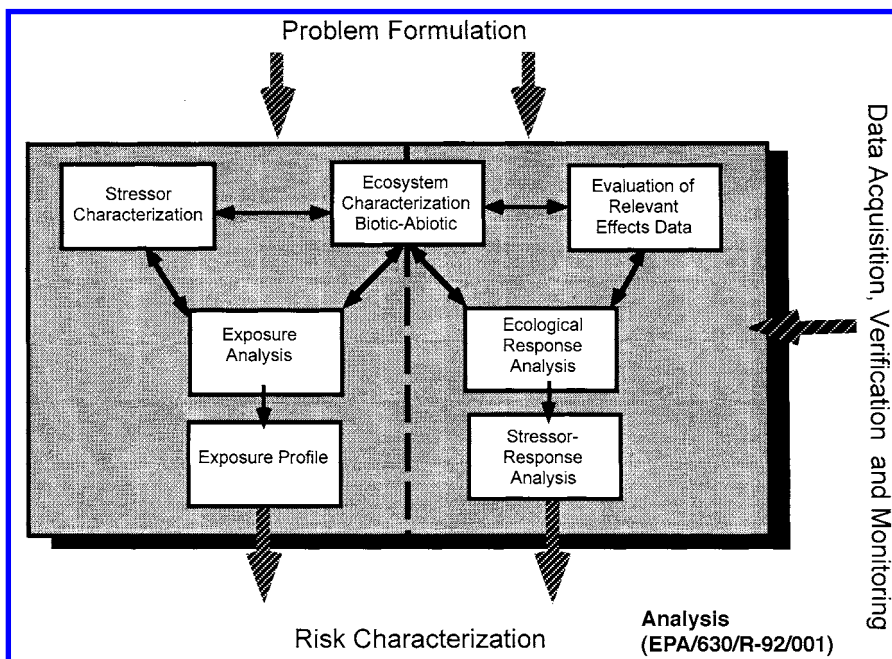


Figure 11.4 Analysis. Although separated into different sides of the analysis box, exposure and ecological responses are intimately connected. Often the biological response to a toxicant alters the exposure for a different compartment of the ecosystem.

their power in predicting and corroborating assessment endpoints is essential to the development of better methodologies.

Analysis

As the problem formulation aspect of the risk assessment is completed, an analysis of the various factors detailed above comes into play (Figure 11.4). 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 predominately terrestrial systems have aquatic components that play a major role in nutrient and toxicant input. Ecosystems also are 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 needs 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 also is not clear what part ecosystem stability plays 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 have been incorporated into the analysis component. However, as have 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.

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, is 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 make up of the populations, plus the presence in the past or present of additional stressors, all 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 chemicals and the detection ranges are often in the $\mu\text{g/l}$ 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 also can 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.

Last, 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 of our imperfect understanding of exposure and should be tested whenever possible against comparable datasets. 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.

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 with one of the crucial factors the relevance of the information to the endpoints selected during problem formulation. 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 is 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.

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. Phylogenetic extrapolation — Relationship of toxicity data from one species to another or perhaps more often, class to class. Often only a 96-h green algal toxicity test is available to use as a representative of all photosynthetic eucaryotes.
2. Response extrapolation — Relationship between two toxicity endpoints such as the NOAEL and the EC₅₀.
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 a herbicide will eventually eliminate the invertebrate herbivores and the fish that rely upon them as a food source.
6. Organizational levels — Examines 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 elimination of a 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, a consideration must often be given to the strength of the cause-effect relationship. Such relationships are relatively straightforward in single species.

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 level, 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 climatological 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.

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.

RISK CHARACTERIZATION

Risk characterization is the final stage of the risk assessment process (Figure 11.5). This aspect of a risk assessment is comprised of a risk estimation and a risk

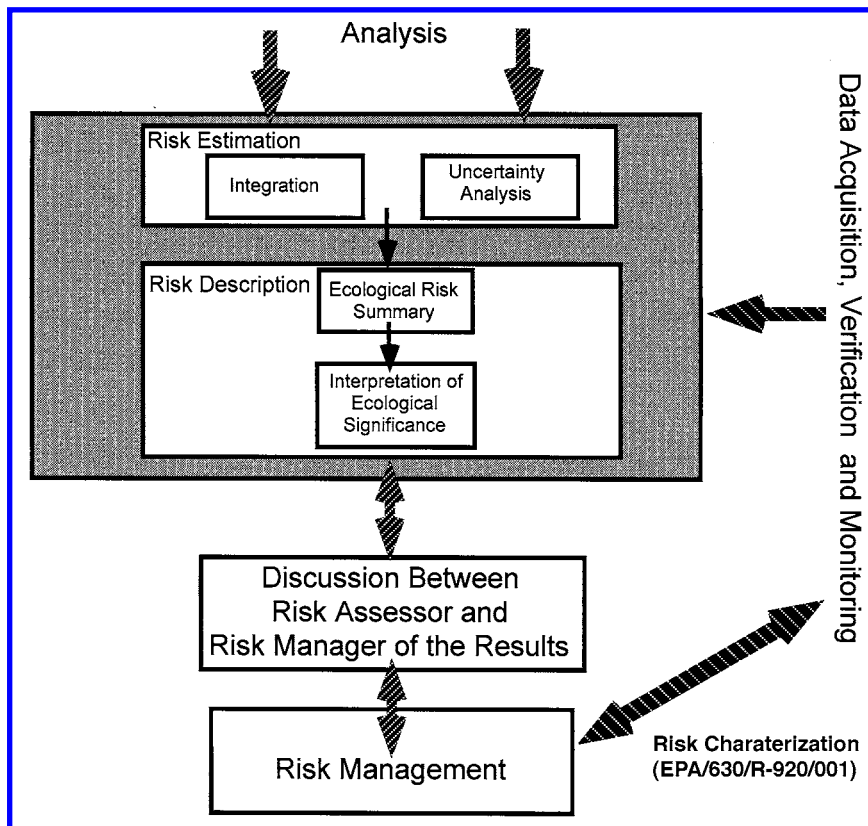


Figure 11.5 Risk characterization. This compartment is comprised of the risk estimation and risk description boxes. The integration of the exposure and effects data from the analysis compartment is reconciled in the risk estimation process.

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 that 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. Similarly 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.

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 LD₅₀ 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

$$\text{Quotient} = \frac{\text{Expected environmental concentration}}{\text{Concentration producing an unacceptable environmental effect}}$$

Of course, the equation produces a ratio which 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 also should 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 followup validation of the risk assessment is it likely that these types of errors will be eliminated. 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 contributes to the uncertainty associated with the risk assessment. Toxicological data routinely vary according to the strain or test organism used. 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 these data in a report may reflect more the nature of the researcher than the presence or absence of the effect.

Risk Description

The next step in this framework is the 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:

- Sufficiency of the data
- Corroborative information
- Evidence of causality

Sufficiency of the data relates to the quality of the data and its 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 due to chance may occur. It also may 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.

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 the terms of the assessment endpoints.

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.

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. Ptolmeic (Earth-centered) astronomy accurately predicted many aspects of the stars and planets and served to make accurate predicts 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 Ptolmeic 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 know. Recently, several researchers have proposed different methods of visualizing ecosystems and the risks associated with xenobiotic inputs.

DEVELOPMENTS 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 section discusses an approach for estimating ecological risks to regions that have a variety of stressor and habitat types.

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):

$$RQ = \frac{EEC}{TRV} \quad (11.1)$$

This has been the classical model for calculating risk. The basic equation requires two factors: the expected environmental concentration (EEC) and the toxicity reference value (TRV). 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, including NOELS and 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 be used only as a screening tool. If the RQ exceeds 1, the value gives no indication of where on the site or at what time the event 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, nor 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.

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 on 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

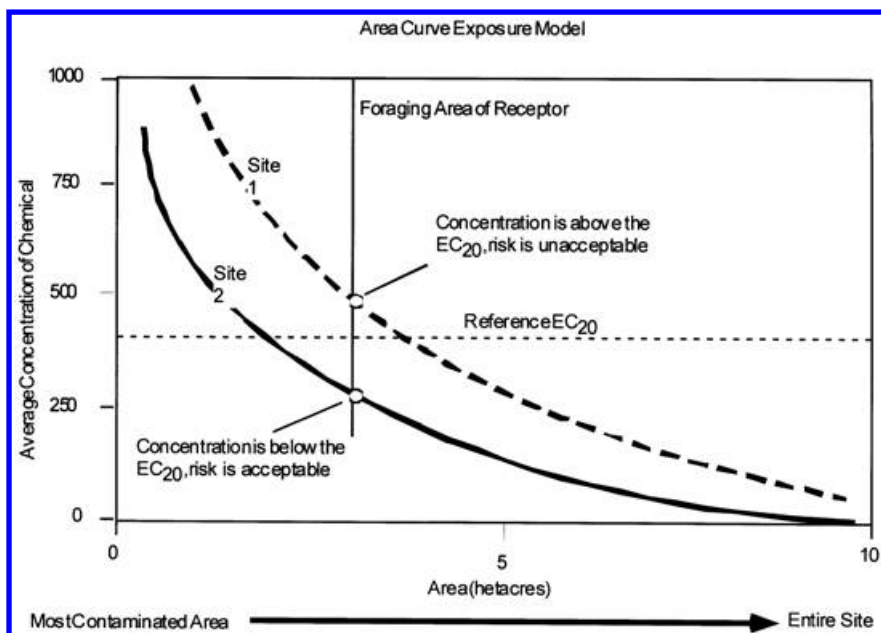


Figure 11.6 Curve exposure model. Site 1 exceeds the EC₂₀. Site 2, with a slightly different average concentration curve, is now below the EC₂₀ when it crosses the size of the foraging area.

present the entire derivation and an adapted step-by-step progression is presented below (Figure 11.6). 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 horizontal lines to the graph 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 cut-off limit for effects.

An additional use of this approach is that it can 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 vs. sites that are not as contaminated but are of a larger surface area. Such a plan can be used in the mitigation section of the final report.

Spatially Distinct Risk Quotients

RQs should be calculated using Equation 11.1 for each site that an environmental sample was collected and 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 also can be estimated from this information by:

$$\frac{\text{Number of RQs } > 1 \text{ or } 100}{\text{Total number of RQs}} \times 100 \quad (11.2)$$

A common assumption is that RQs can be added together to get a total risk. Of course, 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 for all species over all locations will be an approximation of overall risk.

A RANKING APPROACH TO MULTIPLE STRESSOR, WIDE-AREA ECOLOGICAL RISK ASSESSMENT

One of the emerging problems in environmental toxicology and ecological risk assessment is the problem of multiple receptors and multiple stressors over a broad region or landscape. This question is only partially addressed in the recent U.S. EPA proposed guidance (1996). 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, 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 Wiegiers (1997) have recently 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, AK. This fiord 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.

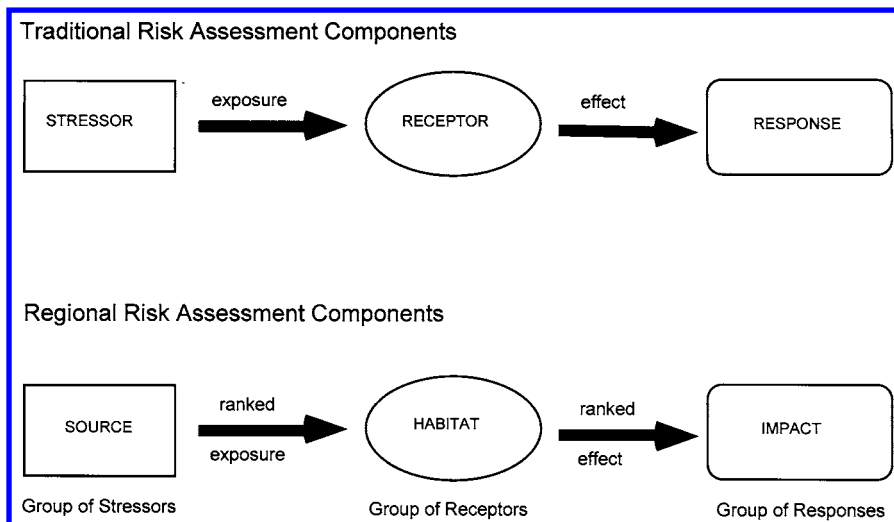


Figure 11.7 Comparison of risk components applied at the traditional and the regional levels. At the regional level, the source releases the stressor to the habitat. The habitat is explicitly and spatially defined within the region. If one of the organisms that constitute an assessment endpoint or other ecological properties of concern exist within that habitat, then an impact can occur. (After Landis and Wiegers 1997.)

EcoRA methods traditionally evaluate the interaction of three environmental components (Figure 11.7): **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.

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 11.7). 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 the locations, and combine this information to predict the relative risk in these areas. The number 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 11.8).

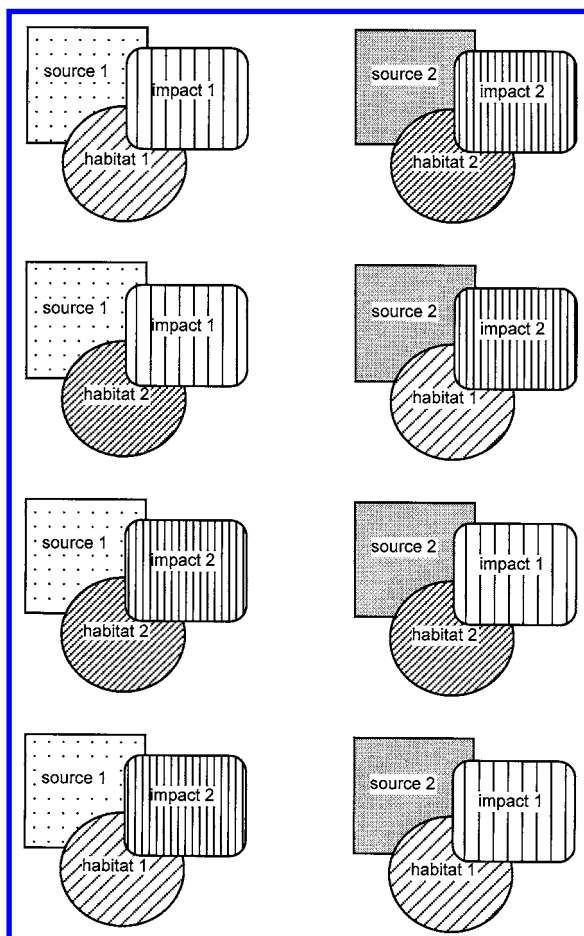


Figure 11.8 Possible combinations characterizing risk from two sources, two habitat types, and two potential impacts to assessment endpoints. Eight potential combinations are possible and each needs to be evaluated. (After Landis and Wiegers 1997.)

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 11.9). If a source generates stressors that affect habitats important to the assessment endpoints the ecological risk is high (Figure 11.9a). A minimal interaction between the components results in a low risk (Figure 11.9b). If one component does not interact with one of the other two components, there is no risk (Figure 11.9c). 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).

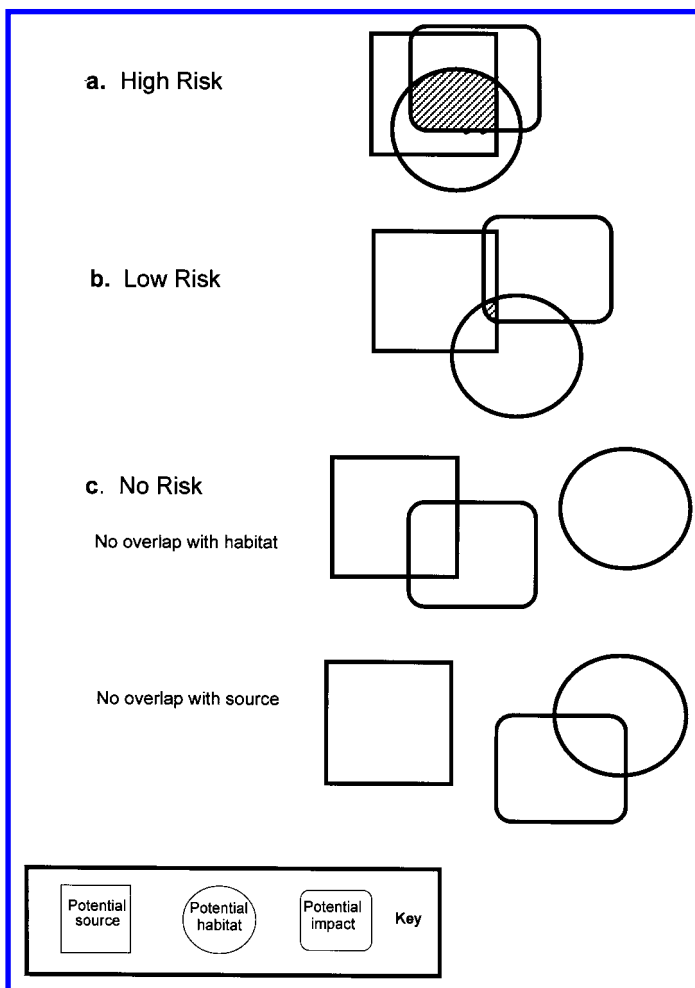


Figure 11.9 The ecological risk resulting from the interaction between sources, habitats, and assessment endpoints in the environment. The assumption is that risk is increasingly proportional to the overlap of source, habitat, and impact.

Impacts can be due to a variety of combinations of stressors and habitats. Integrating these combinations together demonstrates that impact No. 1 is actually the result of many combinations of sources and habitats (Figure 11.9). It is also apparent that the interactions that lead to impact No. 1 are different from those that lead to impact No. 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 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.

Simple Example

Consider a coastal inlet with a single-source type as a concern, i.e., 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 were 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 and some of each habitat

The shape and size of each subarea incorporates 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.

	Sources ranked	Habitats 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 ranks 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 crabs:	Subtidal	0	4	0
	Shoreline	0	0	0
	Rivers	0	0	0
	Wastewater			

The results for each subarea are totalled to determine the relative risks within the region. The risk of contamination is greatest for crabs in Subarea B. Crabs are at a lower risk than clams 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.

Advantages and Dangers of the Ranking Approach

A major advantage of the above categorization and ranking approach is the intrinsic simplicity of the approach. Few assumptions are needed, the most basic being that the more stressors that can affect more habitats will result in an increase in the probability of an assessment endpoint being affected. No reference or control site is necessary, nor are 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 decisionmaking is constructed by the ranking procedure.

In a properly constructed ranking analysis each assumption has to be documented. A sensitivity analysis can be performed that investigates the impacts of ranking decisions upon the final outcome. Uncertainties also can be quantified and data gathered to make the ranking based more 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 decendent of the nonmetric clustering approach described in Chapter 10.

No technique is without drawbacks. First and foremost is the danger that the ranks being 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 an regression anymore 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.

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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 11.1](#).
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 which 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?