Edited by

David J. Hoffman
Barnett A. Rattner
G. Allen Burton, Jr.
John Cairns, Jr.

Handbook of ECOTOXICOLOGY

Second Edition






LEWIS PUBLISHERS

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CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Version Date: 20131024

International Standard Book Number-13: 978-1-4200-3250-5 (eBook - PDF)

Visit the Taylor & Francis Web site at
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and the CRC Press Web site at
http://www.crcpress.com

Preface

The first edition of this book, a bestseller for Lewis Publishers/CRC Press, evolved from a series of articles on ecotoxicology authored by the editors and published in the journal *Environmental Science and Technology.* Ecotoxicology remains a rapidly growing field, with many components periodically being redefined or open to further interpretation. Therefore, this second edition of the *Handbook of Ecotoxicology* has expanded considerably in both concept and content over the first edition. The second edition contains 45 chapters with contributions from over 75 international experts. Eighteen new chapters have been introduced, and the original chapters have been substantially revised and updated. All of the content has been reviewed by a board of experts.

This edition is divided into five major sections: I. Quantifying and Measuring Ecotoxicological Effects, II. Contaminant Sources and Effects, III. Case Histories and Ecosystem Surveys, IV. Methods for Making Estimates, Predictability, and Risk Assessment in Ecotoxicology, and V. Special Issues in Ecotoxicology. In the first section, concepts and current methodologies for testing are provided for aquatic toxicology, wildlife toxicology, sediment toxicity, soil ecotoxicology, algal and plant toxicity, and landscape ecotoxicology. Biomonitoring programs and current use of bioindicators for aquatic and terrestrial monitoring are described. The second section contains chapters on major environmental contaminants and other anthropogenic processes capable of disrupting ecosystems including pesticides, petroleum and PAHs, heavy metals, selenium, polyhalogenated aromatic hydrocarbons, urban runoff, nuclear and thermal contamination, global effects of deforestation, pathogens and disease, and abiotic factors that interact with contaminants.

In order to illustrate the full impact of different environmental contaminants on diverse ecosystems, seven case histories and ecosystem surveys are described in the third section. The fourth section discusses methods and approaches used for estimating and predicting exposure and effects for purposes of risk assessment. These include global disposition of contaminants, bioaccumulation and bioconcentration, use of quantitative structure activity relationships (QSARs), population modeling, current guidelines and future directions for ecological risk assessment, and restoration ecology. The fifth section of this book identifies and describes a number of new and significant issues in ecotoxicology, most of which have come to the forefront of the field since the publication of the first edition. These include endocrine-disrupting chemicals in the environment, the possible role of contaminants in the worldwide decline of amphibian populations, potential genetic effects of contaminants on animal populations, the role of ecotoxicology in industrial ecology and natural capitalism, the consequences of indirect effects of agricultural pesticides on wildlife, the role of nutrition on trace element toxicity, and the role of environmental contaminants on endangered species.

This edition was designed to serve as a reference book for students entering the fields of ecotoxicology and other environmental sciences. Many portions of this handbook will serve as a convenient reference text for established investigators, resource managers, and those involved in risk assessment and management within regulatory agencies and the private sector.

David J. Hoffman
Barnett A. Rattner
G. Allen Burton, Jr.
John Cairns, Jr.

The Editors

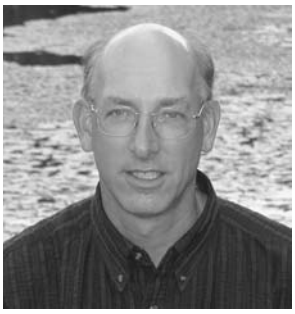
David J. Hoffman

David J. Hoffman is a research physiologist in the field of environmental contaminants at the Patuxent Wildlife Research Center, U.S. Geological Survey of the Department of the Interior. He is also an Adjunct Professor in the Department of Biology, University of Maryland at Frostburg. Dr. Hoffman earned a Bachelor of Science degree in Zoology from McGill University in 1966 and his Doctor of Philosophy Degree in Zoology (developmental physiology) from the University of Maryland in 1971. He was an NIH Postdoctoral Fellow in the Biochemistry Section of Oak Ridge National Laboratory from 1971 to 1973. Other positions included teaching at Boston College during 1974 and research as a Senior Staff Physiologist with the Health Effects Research Laboratory of the U.S. Environmental Protection Agency in Cincinnati from 1974 to 1976 before joining the Environmental Contaminants Evaluation Program of the Patuxent Wildlife Research Center.

Dr. Hoffman's research over the past 20 years has focused on morphological and biochemical effects of environmental contaminants including bioindicators of developmental toxicity in birds in the laboratory and in natural ecosystems. Key areas of study have included sublethal indicators of exposure to planar PCBs, lead, selenium, and mercury; embryotoxicity and teratogenicity of pesticides and petroleum to birds and impact on nestlings; interactive toxicant and nutritional factors affecting agricultural drainwater and heavy-metal toxicity; and measurements of oxidative stress for monitoring contaminant exposure in wildlife.

Dr. Hoffman has published over 120 scientific papers including book chapters and review papers and has served on eight editorial boards.

Barnett A. Rattner



Barnett A. Rattner is a research physiologist at the Patuxent Wildlife Research Center, U.S. Geological Survey of the Department of the Interior. He is also Adjunct Professor of the Department of Animal and Avian Science Sciences, University of Maryland. Dr. Rattner attended the University of Maryland, earning his Doctor of Philosophy degree in 1977. He was a National Research Council Postdoctoral Associate at the Naval Medical Research Institute in 1978 before joining the Environmental Contaminants Evaluation Program of the Patuxent Wildlife Research Center.

Dr. Rattner's research activities during the past 20 years have included evaluation of sublethal biochemical, endocrine, and physiological responses of wildlife to petroleum crude oil, various pesticides, industrial contaminants, and metals. He has investigated the interactive effects of natural stressors and toxic environmental pollutants, developed and applied cytochrome P450 assays as a biomarker of contaminant exposure, conducted risk assessments on potential substitutes for lead shot used in hunting, and compiled several large World Wide Web-accessible ecotoxicological databases for terrestrial vertebrates.

Dr. Rattner has published over 65 scientific articles and serves on four editorial boards and several federal committees including the Toxic Substances Control Act Interagency Testing Committee of the U.S. Environmental Protection Agency.

G. Allen Burton, Jr.

G. Allen Burton, Jr. is the Brage Golding Distinguished Professor of Research and Director of the Institute for Environmental Quality at Wright State University. He earned a Ph.D. degree in Environmental Science from the University of Texas at Dallas in 1984. From 1980 until 1985 he was a Life Scientist with the U.S. Environmental Protection Agency. He was a Postdoctoral Fellow at the National Oceanic and Atmospheric Administration's Cooperative Institute for Research in Environmental Sciences at the University of Colorado. Since then he has had positions as a NATO Senior Research Fellow in Portugal and Visiting Senior Scientist in Italy and New Zealand. Dr. Burton's research during the past 20 years has focused on developing effective methods for identifying significant effects and stressors in aquatic systems where sediment and stormwater contamination is a concern. His ecosystem risk assessments have evaluated multiple levels of biological organization, ranging from microbial to amphibian effects. He has been active in the development and standardization of toxicity methods for the U.S. EPA, American Society for Testing and Materials (ASTM), Environment Canada, and the Organization of Economic Cooperation and Development (OECD). Dr. Burton has served on numerous national and international scientific committees and review panels and has had over $4 million in grants and contracts and more than 100 publications dealing with aquatic systems.

John Cairns, Jr.

John Cairns, Jr. is University Distinguished Professor of Environmental Biology Emeritus in the Department of Biology at Virginia Polytechnic Institute and State University in Blacksburg, Virginia. His professional career includes 18 years as Curator of Limnology at the Academy of Natural Sciences of Philadelphia, 2 years at the University of Kansas, and over 34 years at his present institution. He has also taught periodically at various field stations.

Among his honors are Member, National Academy of Sciences; Member, American Philosophical Society; Fellow, American Academy of Arts and Sciences; Fellow, American Association for the Advancement of Science; the Founder's Award of the Society for Environmental Toxicology and Chemistry; the United Nations Environmental Programme Medal; Fellow, Association for Women in Science; U.S. Presidential Commendation for Environmental Activities; the Icko Iben Award for Interdisciplinary Activities from the American Water Resources Association; Phi Beta Kappa; the B. Y. Morrison Medal (awarded at the Pacific Rim Conference of the American Chemical Society); Distinguished Service Award, American Institute of Biological Sciences; Superior Achievement Award, U.S. Environmental Protection Agency; the Charles B. Dudley Award for excellence in publications from the American Society for Testing and Materials; the Life Achievement Award in Science from the Commonwealth of Virginia and the Science Museum of Virginia; the American Fisheries Society Award of Excellence; Doctor of Science, State University of New York at Binghamton; Fellow, Virginia Academy of Sciences; Fellow, Eco-Ethics International Union; Twentieth Century Distinguished Service Award, Ninth Lukacs Symposium; 2001 Ruth Patrick Award for

Environmental Problem Solving, American Society of Limnology and Oceanography; 2001 Sustained Achievement Award, Renewable Natural Resources Foundation, 2001.

Professor Cairns has served as both vice president and president of the American Microscopical Society, has served on 18 National Research Council committees (two as chair), is presently serving on 14 editorial boards, and has served on the Science Advisory Board of the International Joint Commission (United States and Canada) and on the U.S. EPA Science Advisory Board. The most recent of his 57 books are *Goals and Conditions for a Sustainable Planet*, *2002* and the Japanese edition of *Restoration of Aquatic Ecosystems: Science, Technology, and Public Policy*, 1999.

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Contents

CHAPTER 1

Introduction

David J. Hoffman, Barnett A. Rattner, G. Allen Burton, Jr., and John Cairns, Jr.,

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1.1 HISTORY

The term *ecotoxicology* was first coined by Truhaut in 1969 as a natural extension from toxicology, the science of the effects of poisons on individual organisms, to the ecological effects of pollutants.[1] In the broadest sense ecotoxicology has been described as toxicity testing on one or more components of any ecosystem, as stated by Cairns.[2] This definition of ecotoxicology can be further expanded as the science of predicting effects of potentially toxic agents on natural ecosystems and on nontarget species. Ecotoxicology has not generally included the fields of industrial and human health toxicology or domestic animal and agricultural crop toxicology, which are not part of natural ecosystems, but are rather imposed upon them. Yet there is a growing belief by some that humanity and its artifacts should be regarded as components of natural systems, not apart from them. More recently, Newman has defined ecotoxicology as the science of contaminants in the biosphere and their effects on constituents of the biosphere, which includes humans.[3] Ecotoxicology employs ecological parameters to assess toxicity. In a more restrictive but useful sense, it can be defined as the science of assessing the effects of toxic substances on ecosystems with the goal of protecting entire ecosystems, and not merely isolated components.

Historically, some of the earliest observations of anthropogenic ecotoxic effects, such as industrial melanism of moths, date back to the industrial revolution of the 1850s (see Table 1.1). In the field of aquatic toxicology Forbes was one of the first researchers to recognize the significance of the presence or absence of species and communities within an aquatic ecosystem and to report approaches for classifying rivers into zones of pollution based on species tolerance.[4] At the same

Table 1.1 Historical Overview: First Observations of Ecotoxic Effects of Different Classes of Environmental Contaminants

Date	Contaminant(s)	Effects
1850s	Industrial revolution; soot from coal burning	Industrial melanism of moths
1863	Industrial wastewater	Toxicity to aquatic organisms; first acute toxicity tests
1874	Spent lead shot	Ingestion resulted in death of waterfowl and pheasants
1887	Industrial wastewater	Zones of pollution in rivers established by species tolerance
1887	Arsenic emissions from metal smelters	Death of fallow deer and foxes
1907	Crude oil spill	Death of thousands of puffins
1924	Lead and zinc mine runoff	Toxicity of metal ions to fish
1927	Hydrogen sulfide fumes in oil field	Large die-off of both wild birds and mammals
1950s	DDT and organochlorines	Decline in American robins linked to DDT use for Dutch Elm disease; eggshell thinning in bald eagles, osprey, and brown pelicans linked to DDT; and fish-eating mammals at risk
1960s	Anticholinesterase pesticides	Die-offs of wild birds, mammals, and other vertebrate species
1970s	Mixtures of toxic wastes, including dioxins at hazardous waste sites	Human, aquatic, and wildlife health at risk
1980s	Agricultural drainwater containing selenium and other contaminants	Multiple malformations and impaired reproduction in aquatic birds in central California
1986	Radioactive substances from Chernobyl nuclear power station	Worst nuclear incident in peacetime, affecting a wide variety of organisms and ecosystems
1990s	Complex mixtures of potential endocrine disrupting chemicals, including PCBs and organochlorine pesticides	Abnormally developed reproductive organs, altered serum hormone concentrations, and decreased egg viability in alligators from contaminated lakes in Florida

Source: Adapted from: Hoffman, D. J., Rattner, B. A., Burton, G. A. Jr., and Lavoie, D. R., Ecotoxicology, in *Handbook of Toxicology,* Derelanko, M. J., and Hollinger, M. A., Eds., CRC Press, Boca Raton, FL, 2002.

time some of the earliest acute aquatic toxicity tests were first performed by Penny and Adams (1863)[5] and Weigelt, Saare, and Schwab (1885),[6] who were concerned with toxic chemicals in industrial wastewater. The first "standard method" was published by Hart et al. in 1945 and subsequently adopted by the American Society for Testing and Materials.[7] In this manner it has become generally recognized that the presence or absence of species (especially populations or communities) in a given aquatic ecosystem provides a more sensitive and reliable indicator of the suitability of environmental conditions than do chemical and physical measurements alone.

In the field of terrestrial toxicology reports of anthropogenic contaminants affecting free-ranging wildlife first began to accumulate during the industrial revolution of the 1850s. These included cases of arsenic pollution and industrial smoke stack emission toxicity. One early report described the death of fallow deer (*Dama dama*) due to arsenic emissions from a silver foundry in Germany in 1887, and another described hydrogen sulfide fumes near a Texas oil field that resulted in a large die-off of many species of wild birds and mammals,[8] thus affecting multiple species within an ecosystem. With the advent of modern pesticides, most notably the introduction of dichlorodiphenyltrichloroethane (DDT) in 1943, a marked decline in the population of American robins (*Turdus migratorius*) was linked by the early 1950s to DDT spraying to control Dutch Elm disease. It soon became evident that ecosystems with bald eagles (*Haliaetus leucocephalus*), osprey (*Pandion haliaetus*), brown pelicans (*Pelecanus occidentalis*), and populations of fish-eating mammals were at risk.[9,10]

More recent observations of adverse effects of environmental contaminants and other anthropogenic processes capable of disrupting ecosystems will be covered in subsequent chapters of this book. Exposure and adverse effects, sometimes indirect, of anticholinesterase and other pesticides used in agriculture, petroleum and polycyclic aromatic hydrocarbons (PAHs), manufactured and waste polyhalogenated aromatic hydrocarbons, heavy metals, selenium and other trace elements are included. Other processes and contaminants include nuclear and thermal processes, urban runoff, pathogens and disease, deforestation and global warming, mining and smelting operations, waste

and spent munitions, and released genotoxic and endocrine disruptive chemicals will be presented and discussed in detail.

This book is divided into five sections (I. Quantifying and Measuring Ecotoxicological Effects; II. Contaminant Sources and Effects; III. Case Histories and Ecosystem Surveys; IV. Methods for Making Estimates, Predictability, and Risk Assessment in Ecotoxicology; V. Special Issues in Ecotoxicology) in order to provide adequate coverage of the following general areas of ecotoxicology: (1) methods of quantifying and measuring ecotoxicological effects under controlled laboratory conditions and under natural or manipulated conditions in the field; (2) exposure to and effects of major classes of environmental contaminants and other ecological perturbations capable of altering ecosystems; (3) case histories involving disruption of natural ecosystems by environmental contaminants; (4) methods used for making estimates, predictions, models, and risk assessments; and (5) identification and description of a number of new and significant issues and methodologies, most of which have come to light since publication of the first edition of this book in 1995. The rationale and some of the key points and concepts presented in each of the five sections are presented below.

1.2 QUANTIFYING AND MEASURING ECOTOXICOLOGICAL EFFECTS

Current methodologies for testing and interpretation are provided for aquatic toxicology and design of model aquatic ecosystems, wildlife toxicology, sediment toxicity, soil ecotoxicology, algal and plant toxicity, and the concept of landscape. Identification of biomonitoring programs and current use of biomarkers and bioindicators in aquatic and terrestrial monitoring are also important chapters in this section.

Chapter 2, by Adams and Rowland, provides a comprehensive overview of aquatic toxicology with an emphasis on test methods to meet the requirements of various regulatory guidelines. The chapter describes recent efforts to develop protocols and identify species that permit full-life cycle studies to be performed over shorter durations (e.g., 7-day *Ceriodaphnia dubia* life cycle tests, two-dimensional rotifer tests) and to establish protocols that use sensitive species and life stages that generate accurate estimates of chronic no-effect levels. There has been an increasing need to assess the toxicity of various types of suspect samples in minutes to hours instead of days. The use of rapid assays during on-site effluent biomonitoring allows for the collection of extensive data sets. The expanded use of biomarkers in natural environments, where organisms are exposed to multiple stressors (natural and anthropogenic) over time, will allow better detection of stress and provide an early indication of the potential for population-level effects. Model aquatic ecosystems, known as microcosms and mesocosms, were designed to simulate ecosystems or portions of ecosystems in order to study and evaluate the fate and effects of contaminants. Microcosms are defined by Giesy and Odum[11] as artificially bounded subsets of naturally occurring environments that are replicable, contain several trophic levels, and exhibit system-level properties. Mesocosms are defined as larger, physically enclosed portions of natural ecosystems or man-made structures, such as ponds or stream channels, that may be self-sustaining for long durations. Chapter 3 by Kennedy et al. focuses on key factors in the experimental design of microcosm and mesocosm studies to increase their realism, reduce variability, and assess their ability to detect changes. The success in using such systems depends on the establishment of appropriate temporal and spatial scales of sampling. Emphasis is placed on the need to measure exposure as a function of life history using parameters of size, generation time, habitat, and food requirements. This chapter also addresses the utility of employing a suite of laboratory-to-field experiments and verification monitoring to more fully understand the consequences of single and multiple pollutants entering aquatic ecosystems.

With the advent of modern insecticides and the consequent wildlife losses, screening of pesticides for adverse effects has become an integral part of wildlife toxicology. Avian testing protocols

developed by the U.S. Fish and Wildlife Service and other entities include protocols required for regulatory and other purposes. These are described with respect to acute, subacute, subchronic, chronic, developmental, field, and behavioral aspects of avian wildlife toxicity (Hoffman, Chapter 4). Several unique developmental toxicity tests assess the potential hazard of topical contaminant exposure to bird eggs and the sensitivity of "neonatal" nestlings to contaminants, including chemicals used for the control of aquatic weeds, mosquitoes, and wild fires. Coverage of toxicity testing for wild mammals, amphibians, and reptiles is provided as well, although in somewhat less detail since development of such tests has been more limited in scope and requirement.

Sediments serve as both a sink and a source of organic and inorganic materials in aquatic ecosystems, where cycling processes for organic matter and the critical elements occur. Since many potentially toxic chemicals of anthropogenic origin tend to sorb to sediments and organic materials, they become highly concentrated. Sediment toxicity testing (Burton et al., Chapter 5) is an expanding but still relatively new field in ecological assessments. The U.S. Environmental Protection Agency has initiated new efforts in managing contaminated sediments and method standardization that will result in an even greater degree of sediment toxicity testing, regulation, and research in the near future. A number of useful assays have been evaluated in freshwater and marine studies in which the importance of testing multiple species becomes apparent in order to protect the ecosystem. The assay methods described are sensitive to a wide variety of contaminants, discriminate differing levels of contamination, use relevant species, address critical levels of biological organization, and have been used successfully in sediment studies.

The importance of soil ingestion in estimating exposure to environmental contaminants has been best documented in assessments of pesticides or wastes applied to land supporting farm animals. Soil ingestion tends to be most important for those environmental contaminants that are found at relatively high concentrations compared to concentrations in a soil-free diet. Chapter 6, by Beyer and Fries, is designed to relate the toxicological significance of soil ingestion by wild and domestic animals. Concepts covered include methods for determining soil intake, intentional geophagy in animals, soil ingestion by both domestic animals and wildlife, toxicity of environmental contaminants in soil or sediment to animals, relation of particle size of ingested soil to exposure to contaminants, bioavailability of organic and inorganic contaminants in soil, and applications to risk assessments.

Chapter 7, by Linder, Henderson, and Ingham, focuses on applications of ecological risk assessment (ERA) of contaminated soils on wildlife and habitat restoration, since at present there is little or no federal, state, or other guidance to derive soil cleanup values or ecological-based remedial goal options. Three components of this chapter include ERA tools used to characterize a lower bound, the role of bioavailability in critically evaluating these lower bound preliminary remedial goals, and remediation measures intended to address field conditions and modify soil in order to decrease a chemical's immediate bioavailability, while increasing the likelihood of recovery to habitats suitable for future use by fish and wildlife.

Evaluation of the phytotoxicity of a chemical is an essential component of the ecological risk assessment, since primary producers form an essential trophic level of any ecosystem. Since most chemicals introduced into the environment ultimately find their way into aquatic ecosystems, aquatic algal and plant toxicity evaluations are particularly critical. Klaine, Lewis, and Knuteson (Chapter 8) discuss the current state of phytotoxicity testing with particular attention to algal and vascular (both aquatic and terrestrial) plant bioassays. The algal bioassay section not only focuses on test methods developed over the relatively long history of algal toxicity testing, but also includes many adaptations to traditional laboratory methods to provide more realistic phytotoxicity estimates. The vascular plant section focuses on different species used for bioassays and the various endpoints used. Bioassay systems described include soil, hydroponics, foliar, petri dish, and tissue culture.

In recent years ecologists have established a need for studying natural processes not only at the individual, community, or ecosystem level, but over the entire landscape,[12–14] since quite often ecological studies may be too small both spatially and temporally to detect certain important natural

processes and the movement of pollutants across multiple ecosystems. Holl and Cairns (Chapter 9) discuss the concept of landscape ecology with a focus on (a) landscape structure, that is, spatial arrangement of ecosystems within landscapes; (b) landscape function, or the interaction among these ecosystems through flow of energy, materials, and organisms; and (c) alterations of this structure and function. Different types of landscape indicators in ecotoxicology are presented.

Biomonitoring data form the basis upon which most long-term stewardship decisions are made. These data often provide the critical linkage between field personnel and decision-makers. Data from biomonitoring programs have been very useful in identifying local, regional, and national ecotoxicological problems. Natural resource management decisions are being made that annually cost millions of dollars. These decisions should be supported by scientifically sound data. Chapter 10, by Breckenridge et al., discusses why monitoring programs are needed and how to design a program that is based on sound scientific principles and objectives. This chapter identifies many of the large-scale monitoring programs in the United States, how to access the information from the programs, and how this information can be used to improve long-term management of natural resources. Bioindicators are an important part of biomonitoring and reflect the bioavailability of contaminants, provide a rapid and inexpensive means for toxicity assessment, may serve as markers of specific classes of chemicals, and serve as an early warning of population and community stress. Melancon (Chapter 11) defines bioindicators as biomarkers (biochemical, physiological, or morphological responses) used to study the status of one or more species typical of a particular ecosystem. Systematically, the responses can range from minor biochemical or physiological homeostatic responses in individual organisms to major toxicity responses in an individual, a species, a community, or an ecosystem. Many currently used bioindicators of contaminant exposure/effect for environmental monitoring are discussed. Some of these bioindicators (e.g., inhibition of cholinesterase by pesticides, induction of hepatic microsomal cytochromes P450 by PAHs and polychlorinated biphenyls (PCBs), reproductive problems such as terata and eggshell thinning, aberrations of hemoglobin synthesis including the effects of lead on ALAD, and porphyria caused by chlorinated hydrocarbons) have been extensively field-validated. Other potentially valuable bioindicators undergoing further validation are discussed and include bile metabolite analysis, oxidative damage and immune competence, metallothioneins, stress proteins, gene arrays, and proteomics.

1.3 CONTAMINANT SOURCES AND EFFECTS

The purpose of this section is to identify and describe the effects of significant environmental contaminants and other anthropogenic processes capable of disrupting ecosystems. We have focused on major pesticides (including organophosphorus and carbamate anticholinesterases and persistent organochlorines), petroleum and PAHs, heavy metals (lead and mercury), selenium, polyhalogenated aromatic hydrocarbons, and urban runoff. Toxicity of other metals and trace elements is included in Chapter 40 on amphibian declines, Chapter 44 on trace element interactions, and in three of the case history chapters. Chapters in this section on other important anthropogenic processes include nuclear and thermal contamination, global effects of deforestation, pathogens and disease, and abiotic factors that interact with contaminants.

About 200 organophosphorus (OP) and 50 carbamate (CB) pesticides have been formulated into thousands of products that are available in the world's marketplace for control of fungi, insects, herbaceous plants, and terrestrial vertebrates following application to forests, rangelands, wetlands, cultivated crops, cities, and towns.[15,16] Though most applications are on field crops and other terrestrial habitats, the chemicals often drift or otherwise translocate into nontarget aquatic systems and affect a much larger number of species than originally intended. Hill (Chapter 12) provides an overview of the fate and toxicology of organophosphorus and carbamate pesticides. More attention is given to practical environmental considerations than interpretation of laboratory studies, which were detailed in the first edition of this book. Invertebrates, fish, amphibians, and reptiles are

exemplified as ecosystem components and for comparison with birds and mammals. The focus is on concepts of ecological toxicology of birds and mammals related to natural systems as affected by pesticidal application in agriculture and public health. The environmental fate of representative OP and CB pesticides, their availability to wildlife, and toxicology as related to ambient factors, physiological cycles and status, product formulations and sources of exposure are discussed.

It is unlikely that any other group of contaminants has exerted such a heavy toll on the environment as have the organochlorine (OC) pesticides. Blus (Chapter 13) discusses the nature and extent of ecotoxicological problems resulting from the use of organochlorine pesticides for over a half century as well as the future relevance of these problems. Toxicity of OCs is described as influenced by species, sex, age, stress of various kinds, formulations used, and numerous other factors. The eggshell-thinning phenomenon, depressed productivity, and mortality of birds in the field led to experimental studies with OCs, clearly demonstrating their role in environmental problems. An assessment of the environmental impact of OCs leads to the conclusion that the ecotoxicologist must integrate data obtained from controlled experiments with those obtained from the field. In this manner through the use of the "sample egg technique" and other such innovative procedures, controversies over whether DDE or dieldrin were more important in causing a decline of peregrine falcons and other raptors in Great Britain could have been resolved. Although most of the problem OCs have been banned in a number of countries, exposure, bioaccumulation, and ecotoxicological effects will linger far into the future because of the environmental persistence of many compounds and their continued use in a fairly large area.

Petroleum and individual PAHs from anthropogenic sources are found throughout the world in all components of ecosystems. Chapter 14 (Albers) discusses sources and effects of petroleum in the environment. Less than half of the petroleum in the environment originates from spills and discharges associated with petroleum transportation; most comes from industrial, municipal, and household discharges, motorized vehicles, and natural oil seeps. Recovery from the effects of oil spills requires up to 5 years for many wetland plants. Sublethal effects of oil and PAHs on sensitive larval and early juvenile stages of fish, embryotoxic effects through direct exposure of bird eggs, and acute effects in vertebrates are discussed. Evidence linking environmental concentrations of PAHs to induction of cancer in wild animals is strongest for fish. Although concentrations of individual PAHs in aquatic environments are usually much lower than concentrations that are acutely toxic to aquatic organisms, sublethal effects can be produced. Effects of spills on populations of mobile species have been difficult to determine beyond an accounting of immediate losses and, sometimes, short-term changes in local populations.

Lead (Pb) is a nonessential, highly toxic heavy metal, and all known effects of lead on biological systems are deleterious. According to Pattee and Pain (Chapter 15), present anthropogenic lead emissions have resulted in soil and water lead concentrations of up to several orders of magnitude higher than estimated natural concentrations. Consequently, lead concentrations in many living organisms, including vertebrates, may be approaching adverse-effect thresholds. The influence of the chemical and physical form of lead on its distribution within the environment and recent technology to accurately quantify low lead concentrations are described. The chapter also discusses the most significant sources of lead related to direct wildlife mortality and physiological and behavioral effects detected at tissue lead concentrations below those previously considered safe for humans.

The widespread geographic extent and adverse consequences of mercury pollution continue to prompt considerable scientific investigation. Globally increasing concentrations of methylmercury are found in aquatic biota, even at remote sites, as a consequence of multiple anthropogenic sources and their releases of mercury into the environment. For example, in the marine food web of the North Atlantic Ocean, analysis of feathers of fish-eating seabirds sampled from 1885 through 1994 have shown a steady long-term increase in concentration of methylmercury.[17,18] Wiener et al. (Chapter 16) characterize the environmental mercury problem, critically review the ecotoxicology of mercury, and describe the consequences of methylmercury contamination of food webs. Topics include processes and factors that influence exposure to methylmercury, the highly neurotoxic form.

This form readily accumulates in exposed organisms and can biomagnify in food webs to concentrations that can adversely affect organisms in upper trophic levels. Emphasis is given to aquatic food webs, where methylmercury contamination is greatest.

Reproductive impairment due to bioaccumulation of selenium in fish and aquatic birds has been an ongoing focus of fish and wildlife research, not only in the western United States but also in other parts of the world. Selenium is a naturally occurring semimetallic trace element that is essential for animal nutrition in small quantities, but becomes toxic at dietary concentrations that are not much higher than those required for good health. Thus, dietary selenium concentrations that are either below or above the optimal range are of concern. Chapter 17, by Ohlendorf, summarizes the ecotoxicology of excessive selenium exposure for animals, especially as reported during the last 15 years. Focus is primarily on freshwater fish and aquatic birds, because fish and birds are the groups of animals for which most toxic effects have been reported in the wild. However, information related to bioaccumulation by plants and animals as well as to effects in invertebrates, amphibians, reptiles, and mammals is also presented.

PCBs, dioxins (PCDDs), and dibenzofurans (PCDFs) are all similar in their chemistry and manifestation of toxicity, including a high capacity for biomagnification within ecosystems. Mammals, birds, and fish all have representative species that are highly sensitive, as well as highly resistant, to dioxin-like adverse effects, especially chronic reproductive and developmental/endocrine effects. Aquatic food chain species (seals, dolphins, polar bears, fish-eating birds, and cold-water fish species) with high exposure potential through biomagnification are particularly vulnerable. Rice, O'Keefe, and Kubiak (Chapter 18) review the fate of these environmentally persistent compounds and their toxicity, which is complex and often chronic rather than acute. As for PCBs the complexity begins with the large number of compounds, with varying toxicities, that are regularly detected in the environment (100 to 150). With dioxin- and dibenzofuran-related compounds there are fewer commonly measured residues (< 20). However, environmental problems are confounded since they are not directly manufactured but occur as unwanted impurities in manufacturing and incineration.

Urban runoff investigations, which have examined mass balances of pollutants, have concluded that this process is a significant pollutant source. Some studies have even shown important aquatic life impacts for streams in watersheds that are less than 10% urbanized. In general, monitoring of urban stormwater runoff has indicated that the biological beneficial uses of urban receiving waters are most likely affected by habitat destruction and long-term exposures to contaminants (especially to macroinvertebrates via contaminated sediment), while documented effects associated with acute exposures of toxicants in the water column are less likely. Pitt (Chapter 19) recommends longer-term biological monitoring on a site-specific basis, using a variety of techniques, and sediment-quality analyses to best identify and understand these impacts, since water column testing alone has been shown to be very misleading. Most aquatic life impacts associated with urbanization are probably related to long-term problems caused by polluted sediments and food web disruption.

In addition to natural background radiation, irradiation occurs from the normal operation of nuclear power plants and plutonium production reactors, nuclear plant accidents, nuclear weapons testing, and contact with or leakage from radioactive waste storage sites. Assessing the impacts of nuclear power facilities on the environment from routine and accidental releases of radionuclides to aquatic and terrestrial ecosystems is important for the protection of these ecosystems and their species component. The impacts of power-plant cooling systems — impingement, entrainment, elevated water temperatures, heat shock, and cold shock — on aquatic populations and communities have been intensively studied as well. Discussion in Chapter 20 (Meyers-Schone and Talmage) focuses on basic radiological concepts and sources as well as the effects of radiation on terrestrial and aquatic populations and communities of plants and animals. Radiation effects in this chapter focus on field studies, with supporting information from relevant laboratory investigations. Selected examples attempt to relate estimated doses or tissue levels to potential effects; however, dose estimates in the field are often imprecise, and observations are further confounded by the presence of other contaminants or stressors. Thermal toxicity is related to power-plant cooling systems.

Nearly 17 million ha of tropical forests are being cleared each year for new agricultural lands, equivalent to clearing an area the size of the state of Georgia or Wisconsin annually.[19] Global effects of deforestation include irreplaceable loss of species, emissions into the atmosphere of chemically active and heat-trapping trace gases (carbon dioxide, methane, nitrous oxide, and carbon monoxide), and consequent global warming. Current emissions of greenhouse gases from deforestation account for about 25% of the global warming calculated to result from all anthropogenic emissions of greenhouse gases. Continued emissions of greenhouse gases from both deforestation and industrial sources will raise global mean temperature by an estimated 1 to 3.5°C by the end of this century. Houghton (Chapter 21) reviews the contribution of deforestation and subsequent land use with respect to the increasing concentrations of greenhouse gases in the atmosphere and projected global warming. Suggested remedial and preventative actions include (1) a large (≥60%) reduction in the use of fossil fuels through increased efficiency of energy use and a much expanded use of renewable energy sources, (2) the elimination of deforestation, and (3) reforestation of large areas of land, either to store carbon or to provide renewable fuels to replace fossil fuels.

Pathogenic organisms are life forms that cause disease in other life forms; they are components of all ecosystems. Although ecotoxicology is often considered to be the study of chemical pollutants in ecosystems, pathogenic organisms and their diseases are relevant in this context in at least several different ways, as described by Leighton (Chapter 22). Pathogens can be regarded as pollutants when they are released by humans into ecosystems for the first time or when they are concentrated in certain areas by human activity. Four situations in which human activities can alter the occurrence of diseases in the environment include: (1) translocation of pathogens, including manmade ones, host species, and vectors, to new environments; (2) concentration of pathogens or host species in particular areas; (3) changes in the environment that can alter host-pathogen relationships; and (4) creation of new pathogens by intentional genetic modification of organisms.

Environmental factors have long been shown to influence the toxicity of pollutants in living organisms. Drawing upon controlled experiments and field observations, Rattner and Heath (Chapter 23) provide an overview of abiotic environmental factors and perspective on their ecotoxicological significance. Factors discussed include temperature, salinity, water hardness, pH, oxygen tension, nonionizing radiation, photoperiod, and season. Free-ranging animals simultaneously encounter a combination of environmental variables that may influence, and even act synergistically, to alter contaminant toxicity. It is not possible to rank these factors, particularly since they are oftentimes interrelated (e.g., temperature and seasonal rhythms). However, it is clear that environmental factors (particularly temperature) may alter contaminant exposure and toxicity (accumulation, sublethal effects, and lethality) by more than an order of magnitude in some species. Accordingly, it is concluded that effects of abiotic environmental variables should be considered and factored into risk assessments of anthropogenic pollutants.

1.4 CASE HISTORIES AND ECOSYSTEM SURVEYS

To illustrate the full impact of different environmental contaminants on diverse ecosystems, seven case histories and ecosystem surveys are presented. These include effects of the nuclear meltdown of Chernobyl, agricultural pesticides on migratory birds in Argentina and Venezuela, impact of mining and smelting on several river basins in the western United States, white phosphorus from spent munitions on waterfowl, and effects of PCBs on the Hudson River.

The partial meltdown of the 1000 Mw reactor at Chernobyl in the Ukraine released large amounts of radiocesium and other radionuclides into the environment, causing widespread contamination of the northern hemisphere, particularly Europe and the former Soviet Union. Eisler (Chapter 24) provides a concise review of the ecological and toxicological aspects of the Chernobyl accident, with an emphasis on natural resources. The most sensitive local ecosystems and organisms are

discussed, including soil fauna, pine forest communities, and certain populations of rodents. Elsewhere, reindeer in Scandinavia were among the most seriously afflicted by fallout since they are dependent on lichens, which absorb airborne particles containing radiocesium. Some reindeer calves contaminated with ^{137}Cs from Chernobyl showed ^{137}Cs-dependent decreases in survival and increases in frequency of chromosomal aberrations. The full effect of the Chernobyl nuclear reactor accident on natural resources will probably not be known for at least several decades because of gaps in data on long-term genetic and reproductive effects and on radiocesium cycling and toxicokinetics.

Hooper and co-authors (Chapter 25) describe how recent events in Argentina and Venezuela have shown that pesticide effects transcend national borders with respect to migratory birds exposed to potentially damaging pesticides throughout their range. Despite its withdrawal from the United States, monocrotophos, one of the most acutely toxic pesticides to birds, remained the second highest use OP throughout the world through the mid-1990s, resulting in the death of an estimated 20,000 Swainson's hawks (*Buteo swainsoni*) in Argentina. What has been learned since, as demonstrated by the risks that face many trans-border avian migrants, has clarified the need for greater international cooperation and harmonization of pesticide use. Where a large portion of a species population occupies a small geographical area, either in the course of its migration or on wintering grounds, any localized contaminant or noncontaminant impact can have potentially serious consequences for that population.

Studies conducted in the vicinity of mining operations and smelters have provided some of the most revealing examples of environmental damage caused by metals and associated contaminants. Metal-contaminated soils eroded from exposed and disturbed landscapes and tailings generated during processing may be released to the environment and are associated with increased metal concentrations in surface water and groundwater. Similarly, dispersed sediments often become deposited as alluvial materials in riparian areas and can result in soil metal concentrations greatly exceeding predepositional conditions. Henny (Chapter 26) reviews the history and cause of waterfowl mortality in the Coeur d'Alene (CDA) River Basin of Idaho related to mining sediment containing high concentrations of lead and other metals. Diagnostic procedures and techniques to assess lead poisoning are discussed. Beyer and co-workers[20] concluded that exposure of waterfowl to lead in the CDA River Basin was principally related to the amount of ingested sediment, since the relative amount of lead in vegetation and prey was small. Following this logic and the fact that most raptors neither ingest sediment nor digest bones of prey species that are a major storage site for lead, it becomes clear why the ospreys, hawks, and owls in the CDA River Basin were less contaminated than waterfowl with lead from mining sources. Along the Arkansas River, lead concentrations in livers of nestling tree swallows conclusively demonstrated that lead from sediments is bioavailable to this species prior to fledging (Custer and co-authors, Chapter 28). Lead was detected in most tree swallow livers at two sites along an 11-mile stretch, the most sediment-contaminated section of the Arkansas River. The proportion of livers with detectable lead was less both downstream and upstream of the 11-mile stretch, but with a site-related upstream/downstream gradient in lead concentrations. Additionally, the mean half-peak coefficient of variation of DNA content (HPCV) indicative of possible chromosomal damage was positively correlated with both liver and carcass cadmium concentrations. Linder, Woodword, and Pascoe (Chapter 30) summarize ecological risk-assessment studies focused on metal-contaminated soil, sediment, and surface water for a series of Superfund sites located in the Clark Fork River (CFR) watershed of western Montana. Aquatic, terrestrial, and wetland resources at risk including benthic invertebrates, fish, earthworms, plants, and animals are evaluated.

Eagle River Flats (ERF), located on the Cook Inlet of Anchorage, Alaska, is used by waterfowl and shorebirds throughout the spring and summer and is particularly important as a spring and fall staging or stopover area for more than 75 species of migratory ducks, geese, swans, raptors, gulls, shorebirds, and passerines. Massive waterfowl mortalities due to ingestion of particles of white phosphorus (P4) originating from the firing of munitions into the area occurred and involved over

2000 ducks and swans each year. Sparling (Chapter 27) provides a discussion on toxicity of white phosphorus, a hazard assessment model, efforts to remediate the problem, and how ERF serves as a model for the effects and remediation of P4 on at least 71 other sites where the contaminant has been found.

The Hudson River dominates the history and landscape of eastern New York and surrounding states and has come to symbolize the difficulties associated with finding solutions to the problems of widespread contamination by persistent organic compounds. McCarty (Chapter 29) reviews the history of PCB contamination in the context of the Hudson River ecosystem, describing the patterns of PCB contamination in the biota of the Hudson, the risk contaminants pose to fish and wildlife, and attempts to mitigate those risks.

1.5 METHODS FOR MAKING ESTIMATES, PREDICTABILITY, AND RISK ASSESSMENT IN ECOTOXICOLOGY

Ecological risk assessment is a process that evaluates the likelihood that adverse ecological effects will occur as a result of exposure to one or more stressors; it is receiving increasing emphasis in ecotoxicology. Suter[21] defines risk assessment as the process of assigning magnitudes and probabilities to the adverse effects of human activities or natural catastrophes. Examples and uses of ecological risk assessment occur in chapters throughout all sections of this book. However, the fourth section of this book is focused on describing methods and approaches used for estimating and predicting the outcome of potentially ecotoxic events for purposes of risk assessment. These include global disposition of contaminants, bioaccumulation and bioconcentration of contaminants, use of quantitative structure activity relationships (QSARs) for predicting ecological effects of organic chemicals, and population modeling in contaminant studies. Another important part of this section is the current guidelines and future directions of the U.S. Environmental Protection Agency for ecological risk assessment, followed by an exemplary chapter of an ecological risk assessment. The final chapter of this section describes the relationship between restoration ecology and ecotoxicology and quantifies how damaged ecosystems can be restored.

The sources of many environmental contaminants are relatively easy to identify. While short-lived contaminants are most readily identified close to the source, the more persistent substances, such as heavy metals and PCBs, may achieve a truly global distribution due to atmospheric transport and deposition to soils and surface waters. The interim period between emission or discharge of an environmental contaminant and ultimate contact with a specific ecosystem or representative species often contains many varied and interesting processes. Harrison, Harrad, and Lead (Chapter 31) describe some of the more important processes involved in pollutant transport and removal from the environment and discuss how such processes influence the distribution of pollutants. Included are processes leading to the transfer of chemical substances between environmental compartments such as water to air and air to soil. Bioaccumulation and bioconcentration are terms describing the transfer of contaminants from the external environment to an organism. In aquatic organisms bioaccumulation can occur from exposure to sediment (including pore water) or via the food chain (termed trophic transfer). Bioconcentration is the accumulation of waterborne contaminants by aquatic animals through nondietary exposure routes. Biomagnification is defined as the increase in contaminant concentration in an organism in excess of bioconcentration. Biomagnification appears most significant for benthic-based food webs and for very hydrophobic contaminants resistant to biotransformation and biodegradation. As reviewed by Barron (Chapter 32) concern for the bioaccumulation of contaminants arose in the 1960s because of incidents such as toxicity from methyl mercury residues in shellfish and avian reproductive failure due to chlorinated pesticide residues in aquatic species. Bioaccumulation models were first developed in the 1970s to account for processes such as the partitioning of hydrophobic chemicals from water to aquatic organisms. To regulate new and existing chemicals laws such as the Federal Insecticide,

Fungicide and Rodenticide Act contain stringent requirements for bioaccumulation testing. This chapter presents an overview of the principles and determinants governing bioaccumulation from sediments and water and biomagnification in aquatic-based food webs. Organic and metal contaminants are discussed, with an emphasis on hydrophobic organics. The objective of this chapter is to elucidate concepts relating to bioaccumulation, rather than simply present an exhaustive review of the literature.

Structure activity relationships (SARs) are comparisons or relationships between a chemical structure, chemical substructure, or some physical or chemical property associated with that structure or substructure and a biological (e.g., acute toxicity) or chemical (e.g., hydrolysis) activity. When the result is expressed quantitatively, the relationship is a quantitative structure activity relationship (QSAR). Most SARs have been developed for predicting ecological effects of organic chemicals. For SARs that predict ecological effects Walker and Schulz (Chapter 33) provide examples, developmental approaches, universal principles, applications, and recommendations for new QSARs to predict ecotoxicological effects. Since most QSARS have been developed for freshwater aquatic organisms, it is recommended that additional QSARs be developed for predicting effects of chemicals on terrestrial and sediment-dwelling organisms. There is a critical need for these QSARs, especially given the high exposure potential of terrestrial wildlife to pesticides that are intentionally dispersed and to persistent industrial chemicals that are toxic and undergo long-range transport.

The inconvenience and hardship resulting from ecological failures in ecosystem services (e.g., waste processing, provision of potable water, and food production) motivated early attempts at proactive management of the environment, including prediction and mitigation of damage. Cairns and Niederlehner (Chapter 34) discuss the science of predictive ecotoxicology, emphasizing that prediction of environmental outcome is different from appraisal of existing damage and that prediction is solely dependent upon modeling. There is often no way to verify the accuracy of prediction through field survey, yet accuracy checks are essential in assuring that predictive techniques are adequate to management needs. Validation studies compare predictions to appraisals of damage in natural systems. Through these comparisons the magnitude and significance of predictive errors can be evaluated. Ways in which predictive models can be improved are discussed.

A population model is a set of rules or assumptions, expressed as mathematical equations, that describe how animals survive and reproduce. Ecology has a rich history of using models to gain insights into population dynamics. Population models provide a means for evaluating the effects of toxicants in the context of the life cycle of an organism. By developing a model and estimating demographic parameters effects of toxicants on demographic parameters of population growth rates and model stability can be assessed. Also, modeling allows one to identify what portions of the life cycle are most sensitive to toxicants and can guide future data collection and field experiments.

Chapter 35 (Sauer and Pendleton) reviews how population modeling has been used to provide insights into theoretical aspects of ecology and addresses practical questions for resource managers about how population dynamics are affected by changes in the environment. Specific concepts include (1) use of modeling procedures that group populations into discernible age classes, with survival and fecundity rates measured at various intervals for these groups; (2) methods for analyzing the stable population attributes of these models; (3) methods for assessing the effects of changes in the parameters of the models; and (4) applications of the models in evaluating the effects of changes in the demographic parameters.

ERA has been used to evaluate a wide variety of environmental issues of interest such as water-regime management, chemical and biological stressors used to control harmful insects, and toxic chemicals used in industrial processes or present at hazardous waste sites. Chapter 36 (Norton and co-authors) describes recent and ongoing guidelines established by the U.S. Environmental Protection Agency for ERA. Taken into consideration are effects on multiple populations, communities, and ecosystems and the need to consider nonchemical as well as chemical stressors. Problem formulation, characterization of exposure and ecological effects, risk characterization, and case

studies and interaction between risk assessment and risk management are discussed. Applications are described in terms of assessing ecological risks from chemicals using probabilistic methods, conducting ecological risk assessment of biological stressors, expanding concepts of exposure, developing management objectives for ERA (including watershed management), and integrating ecological risk assessment with economic, human health, and cultural assessments. This chapter is followed by an exemplary chapter (Byron and co-authors, Chapter 37) describing an actual ERA involving selenium exposure in waterfowl and shorebirds feeding in ephemeral pools in the vicinity of Kesterson Reservoir in central California.

Cairns (Chapter 38) concludes that the adoption of a no-net-ecological-loss policy will require ecological restoration of systems damaged by accidental spills, cumulative impact of anthropogenic stresses over a long period of time, and even ecoterrorism. Ecotoxicology as a scientific discipline will remain essential to ensure that the management practices associated with potentially toxic materials are well understood. Illustrative examples of where the relationship between restoration ecology and ecotoxicology might be most effective include rivers chronically and cumulatively impacted by hazardous materials or by unexpected spills of hazardous materials. For terrestrial systems the Superfund sites in the United States, where accumulations of hazardous materials pose a threat to the surrounding environment and human health, provide an example. Although this chapter emphasizes the relationship between restoration ecology and ecotoxicology, other disciplines should be engaged in order to generate a long-term solution to a complex multivariate problem.

1.6 SPECIAL ISSUES IN ECOTOXICOLOGY

The purpose of the fifth section of this book is to identify and describe a number of new and significant issues and approaches in ecotoxicology, most of which have come into focus since the publication of the first edition of this book. These include endocrine-disrupting chemicals and endocrine active agents in the environment, the possible role of contaminants in the worldwide decline of amphibian populations, potential genetic effects of contaminants on animal populations, the role of ecotoxicology in industrial ecology and natural capitalism, the consequences of indirect effects of agricultural pesticides on wildlife, the role of nutrition on trace element toxicity in fish and wildlife, and the role of environmental contaminants on endangered species.

Over the last 5 years there has been a surge of reports in wildlife of suspect endocrine-disruptor-related effects based primarily on adverse reproductive and developmental effects.[22–24] Collectively, there is some evidence of altered reproductive and developmental processes in wildlife exposed to endocrine disruptors, and in the United States, Congress has passed legislation requiring the Environmental Protection Agency to develop, validate, and implement an Endocrine Disruptor Screening Program (EDSP) for identifying potential endocrine-disrupting chemicals. A wide variety of chemicals have been reported as potential endocrine disruptors and are described by Gross and co-workers in Chapter 39 of this book. This chapter reviews and selectively summarizes methods for screening and monitoring of potential endocrine disruptors, the current evidence for endocrine-disrupting effects, and chemical classes in vertebrate wildlife and their potential modes of action. Classes of chemicals include polycyclic aromatic hydrocarbons; polychlorinated and polybrominated biphenyls, dibenzo-*p*-dioxins, dibenzo-*p*-furans; organochlorine pesticides and fungicides; some nonorganochlorine pesticides; complex environmental mixtures; and a few metals.

Over the past decade widespread population declines of amphibians have been documented in North America, Europe, Australia, and Central and South America.[25–27] Population declines in eastern Europe, Asia, and Africa have been suggested as well but are not as well documented. Based on comparative toxicities of organic compounds and metals between amphibians and fish, the overall conclusions were that there was great variation among amphibian species in their sensitivity to metal and organic contaminants, that amphibians generally were more sensitive than fish, and that water-quality criteria established for fish may not be protective of amphibians.

Contaminants may be involved with amphibian population declines, including their possible interaction with other factors discussed by Sparling in Chapter 40.

Understanding changes to the genetic apparatus of an organism exposed to contaminants in the environment is essential to demonstrating an impact on parameters of ecological significance such as population effects. Genetic ecotoxicology attempts to identify changes in the genetic material of natural biota that may be induced by exposure to genotoxicants in their environment and the consequences at various levels of biological organization (molecular, cellular, individual, population, etc.) that may result from this exposure. Shugart, Theodorakis, and Bickham (Chapter 41) describe two major classes of effects studied in genetic ecotoxicology: (1) direct exposure to genotoxicants that have the potential to lead to somatic or heritable (genotoxicological) disease states and that could lower the reproductive output of an affected population, and (2) indirect effects from contaminant stress on populations that lead to alterations in the genetic makeup, a process termed evolutionary toxicology.[28] These latter types of effects alter the inclusive fitness of populations such as by the reduction of genetic variability.

Industrial ecology is the study of the flows of materials and energy in the industrial environment and the effects of those flows on natural systems. Natural capitalism refers to the increasingly critical relationship between natural capital (i.e., natural resources), living systems and the ecosystem services they provide, and manmade capital. Natural capitalism and two of its subdisciplines, industrial and municipal ecology, are essential components in developing a sustainable relationship with natural systems and protecting both natural capital and the delivery of ecosystem services. Cairns (Chapter 42) discusses the role of ecotoxicologists in this sustainability.

Agricultural pesticides have been identified as contributing to the decline of farmland wildlife, although the impact is often exacerbated by other farm practices associated with intensive agriculture. Many species of farmland birds are in decline in the United Kingdom, and there is considerable evidence for the indirect effects of pesticides as the cause. Sotherton and Holland (Chapter 43) discuss how changes in chick survival drive the population size of the once common United Kingdom farmland grey partridge, and conclude that the timing and magnitude of changes in population size and chick survival are consistent with having been caused by the increased use of pesticides, which reduce the insect foods available for partridge chicks. Indirect effects are also likely to impact upon a wide variety of farmland wildlife that are dependent on the same food chain as the grey partridge, and evidence of this is starting to appear for some passerines.

Nutrition of test organisms is one of the most important variables in the conduct of any biological experiment. Deficiencies of vitamins, minerals, and other nutrients in the diet of captive and free-ranging fish and wildlife can result in skeletal deformities, cataracts, histological lesions, abnormal behavior, and many other abnormalities. Excessive amounts of vitamins and minerals have also resulted in abnormalities. The quality of commercial or experimentally prepared diets of captive animals as well as diets consumed by wild animals can influence the acute and chronic toxicity of test compounds. Chapter 44 (Hamilton and Hoffman) examines interactions between nutrition and potentially toxic trace elements and interactions among trace elements. Limited information from dietary studies with trace elements, especially selenium, reveals that diet can have a profound effect on toxicity observed in contaminated ecosystems, yet water-quality standards are rarely derived taking this factor into account. Incorporation of dietary criteria into national criteria for trace elements will occur only after a sufficient database of information is generated from dietary toxicity studies. Recent findings with environmentally relevant forms of mercury (methylmercury) and selenium (selenomethionine) in birds have shown that mercury and selenium may be mutually protective to the toxicity of each other in adult birds but synergistic in combination to the reproductive process in embryos. Further studies are needed to examine the relationship between selenium, nutrition, and other trace elements that may be toxic by compromising cellular antioxidative defense mechanisms. There is also a need for comparative interaction studies in species of wild mammals.

The speed, severity, and taxonomic diversity of declining species is a major concern to ecologists because extinctions are taking place at a rate of approximately 100 species per day.[29] Previously,

Wilson[30] projected the loss of species at more than 20% of the planet's total biodiversity in 20 years. The last chapter (45) of this section by Pattee, Fellows, and Bounds examines the role of contaminants/pollution in the decline of species. Habitat destruction is the primary factor that threatens species and is listed as a significant factor affecting 73% of endangered species. The second major factor causing species decline is the introduction of nonnative species. This affects 68% of endangered species. Pollution and overharvesting were identified as impacting 38% and 15%, respectively, of endangered species. No other contaminant has impacted animal survival to the extent of DDT, which remains one of the few examples of pollution actually extirpating animal species over a significant portion of DDT's range. Once a species is reduced to a remnant of its former population size and distribution, its vulnerability to catastrophic pollution events increases, frequently exceeding or replacing the factors responsible for the initial decline. Therefore, large-scale environmental events, such as global warming, acid rain, and sea-level rise, attract considerable attention as speciation events, adversely affecting some species populations while causing other species to flourish.

The editors of this book conclude that with increasing loss of habitat the quality and fate of the remaining habitat becomes increasingly critical to the survival of species and ecosystems. Species that are endangered or at risk and that occupy a very limited geographical area could be easily decimated by a single event such as an oil or chemical spill or misapplication of pesticides. Furthermore, on a temporal basis where a large portion of a species population occupies a small geographical area, either in the course of its migration or on wintering grounds, any localized impact, whether pesticide-related (e.g., as reported by Hooper and co-authors, Chapter 25) or not, has the potential for serious consequences to populations. For these reasons the balance between shrinking habitat and anthropogenic stressors becomes increasingly crucial to sustain both ecosystems and species diversity.

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SECTION I

Quantifying and Measuring Ecotoxicological Effects

CHAPTER 2

Aquatic Toxicology Test Methods

William J. Adams and Carolyn D. Rowland

CONTENTS

2.1 INTRODUCTION

Aquatic toxicology is the study of the effects of toxic agents on aquatic organisms. This broad definition includes the study of toxic effects at the cellular, individual, population, and community levels. The vast majority of studies performed to date have been at the individual level. The intention

of this chapter is to provide an overview of aquatic toxicology with an emphasis on reviewing test methods and data collection to meet the requirements of various regulatory guidance.

2.2 HISTORICAL REVIEW OF THE DEVELOPMENT OF AQUATIC TOXICOLOGY

Aquatic toxicology grew primarily out of two disciplines: water pollution biology and limnology. The development of these disciplines spanned about 130 years in Europe and the United States. Early studies included basic research to define and identify the biology and morphology of lakes, streams, and rivers. These studies included investigations on how plants, animals, and microorganisms interact to biologically treat sewage and thus reduce organic pollution. For example, the role of bacteria in the nitrification process was demonstrated in 1877 by Schoesing and Muntz. Stephen Forbes is generally credited as one of the earliest researchers of integrated biological communities (Forbes, 1887).[1] Kolwitz and Marsson[2,3] and Forbes and Richardson[4] published approaches for classifying rivers into zones of pollution based on species tolerance and their presence or absence. It has become an accepted belief that the presence or absence of species (especially populations or communities) living in a given aquatic ecosystem provides a more sensitive and reliable indicator of the suitability of environmental conditions than do chemical and physical measurements. Thus, a great deal of effort has been expended over many years in the search for organisms that are sensitive to environmental factors and changes in these parameters. This effort has been paralleled by similar attempts to culture and test sensitive organisms in laboratory settings. The underlying belief has been that organisms tested under controlled laboratory conditions provide a means to evaluate observed effects in natural ecosystems and to predict possible future effects from human-made and natural perturbations. The science of aquatic toxicology evolved out of these studies and has concentrated on studying the effects of toxic agents (chemicals, temperature, dissolved oxygen, pH, salinity, etc.) on aquatic life.

The historical development of aquatic toxicology up to 1970 has been summarized by Warren.[5] Most early toxicity tests consisted of short-term exposure of chemicals or effluents to a limited number of species. Tests ranged from a few minutes to several hours and occasionally 2 to 4 days. There were no standardized procedures. Some of the earliest acute toxicity tests were performed by Penny and Adams (1863)[6] and Weigelt, Saare, and Schwab (1885),[7] who were concerned with toxic chemicals in industrial wastewaters. In 1924 Kathleen Carpenter published the first of her notable papers on the toxicity to fish of heavy metal ions from lead and zinc mines.[8] This was expanded by the work of Jones (1939)[9] and has been followed by thousands of publications over the years on the toxicity of various metals to a wide variety of organisms.

Much of the work conducted in the 1930s and 1940s was done to provide insight into the interpretation of chemical tests as a first step into the incorporation of biological effects testing into the wastewater treatment process or to expand the basic information available on species tolerances, metabolism, and energetics. In 1947 F.E.J. Fry published a classical paper entitled *Effects of the Environment on Animal Activity.*[10] This study investigated the metabolic rate of fish as an integrated response of the whole organism and conceptualized how temperature and oxygen interact to control metabolic rate and hence the scope for activity and growth. Ellis (1937)[11] conducted some of the earliest studies with *Daphnia magna* as a species for evaluating stream pollution. Anderson (1944, 1946)[12,13] expanded this work and laid the groundwork for standardizing procedures for toxicity testing with *Daphnia magna*. Biologists became increasingly aware during this time that chemical analyses could not measure toxicity but only predict it. Hart, Doudoroff, and Greenbank (1945)[14] and Doudoroff (1951)[15] advocated using toxicity tests with fish to evaluate effluent toxicity and supported the development of standardized methods. Using aquatic organisms as reagents to assay effluents led to their description as aquatic bioassays. Doudoroff's 1951 publication[15] led to the first standard procedures, which were eventually included in *Standard Methods for the Examination of Water and Wastewater.* [16] Efforts to standardize aquatic tests were renewed, and the Environmental

Protection Agency (EPA) sponsored a workshop that resulted in a document entitled *Standard Methods for Acute Toxicity Test for Fish and Invertebrates*.[17] This important publication has been the primer for subsequent aquatic standards development and has been used worldwide.

The concept of water quality criteria (WQC) was formulated shortly after World War II. McKee (1952)[18] published a report entitled *Water Quality Criteria* that provided guidance on chemical concentrations not to be exceeded for the protection of aquatic life for the State of California. A second well-known edition by McKee and Wolf (1963)[19] expanded the list of chemicals and the toxicity database. WQC are defined as the scientific data used to judge what limits of variation or alteration of water will not have an adverse effect on the use of water by man or aquatic organisms.[1] An aquatic water quality criterion is usually referred to as a chemical concentration in water derived from a set of toxicity data (criteria) that should not be exceeded (often for a specified period of time) to protect aquatic life. Water quality standards are enforceable limits (concentration in water) not to be exceeded that are adopted by states and approved by the U. S. federal government. Water quality standards consist of WQC in conjunction with plans for their implementation.

In 1976 the EPA published formal guidelines for establishing WQC for aquatic life that were subsequently revised in 1985.[20] Prior to this time WQC were derived by assessing available acute and chronic aquatic toxicity data and selecting levels deemed to protect aquatic life based on the best available data and on good scientific judgment. These national WQC were published at various intervals in books termed the Green Book (1972),[21] the Blue Book (1976),[22] the Red Book (1977),[23] and the Gold Book (1986).[24] In some cases WQC were derived without chronic or partial life-cycle test data. Acute toxicity test results (LC_{50} — lethal concentration to 50% of the test organisms) were used to predict chronic no-effect levels by means of an application factor (AF). The acute value was typically divided by 10 to provide a margin of safety, and the resulting chronic estimate was used as the water quality criterion. It was not until the mid-1960s that chronic test methods were developed and the first full life-cycle chronic toxicity test (with fathead minnows) was performed.[25]

The AF concept emerged in the 1950s as an approach for estimating chronic toxicity from acute data.[26] Stephan and Mount (1967)[27] formalized this AF approach, which was revised by Stephan (1987)[28] and termed the acute-to-chronic ratio (ACR). This approach provides a method for calculating a chronic-effects threshold for a given species when the LC_{50} for that species is known and the average acute-to-chronic ratio for two or more similar species is also available. Dividing the LC_{50} by the ACR provides an estimate of the chronic threshold for the additional species. The approach has generally been calculated as the $LC_{50} \div GMCV$, where GMCV = the geometric mean of the no-observed effect concentration (NOEC) and the lowest observed effect level (LOEC), termed the chronic value (CV). Before the ACR method was published, the AF concept was not used consistently. Arbitrary or "best judgment" values were often used as AFs to estimate chronic thresholds (CVs). Values in the range of 10 to 100 were most often used, but there was no consistent approach. The chronic value has also been alternatively referred to as the geometric mean maximum acceptable toxicant threshold (GM-MATC).

The passage of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA, 1972), the Toxic Substances Control Act (TSCA, 1976), and the Comprehensive Environmental Compensation Liabilities Act (CERCLA, 1980) as well as the incorporation of toxicity testing (termed biomonitoring) as part of the National Pollution Discharge Elimination System (NPDES, 1989)[29] have increased the need for aquatic toxicological information. Standard methods now exist for numerous freshwater and marine species, including fishes, invertebrates, and algae, that occupy water and sediment environments.

2.3 TEST METHODS

The fundamental principle upon which all toxicity tests are based is the recognition that the response of living organisms to the presence (exposure) of toxic agents is dependent upon the dose

(exposure level) of the toxic agent. Using this principle, aquatic toxicity tests are designed to describe a concentration-response relationship, referred to as the concentration-response curve when the measured effect is plotted graphically with the concentration. Acute toxicity tests are usually designed to evaluate the concentration-response relationship for survival, whereas chronic studies evaluate sublethal effects such as growth, reproduction, behavior, tissue residues, or biochemical effects and are usually designed to provide an estimate of the concentration that produces no adverse effects.

2.3.1 Acute Toxicity Tests

Acute toxicity tests are short-term tests designed to measure the effects of toxic agents on aquatic species during a short period of their life span. Acute toxicity tests evaluate effects on survival over a 24- to 96-hour period. The American Society for Testing and Materials (ASTM), Environment Canada, and the U.S. EPA have published standard guides on how to perform acute toxicity tests for water column and sediment-dwelling species for both freshwater and marine invertebrates and fishes. A list of the standard methods and practices for water-column tests for several species is presented in Table 2.1. The species most often used in North America include the fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), sheepshead minnows (*Cyprinodon variegatus*), *Daphnia magna, Ceriodaphnia dubia*, amphipods (*Hyalella azteca*), midges (*Chironomus* sp.), duckweed (*Lemna* sp.), green algae (*Selenastrum capricornutum*), marine algae (*Skeletonema costatum*), mayflies (*Hexagenia* sp.), mysid shrimp (*Mysidopsis bahia*), penaid shrimp (*Penaeus* sp.), grass shrimp (*Palaemonetes pugio*), marine amphipods (*Rhepoynius aboronius and Ampleisca abdita*), marine worms (*Nereis virens*), oysters (*Crassotrea virginica),* marine mussel *(Mytilus edulis),* and marine clams (*Macoma* sp.). Use of particular species for different tests, environmental compartments, and regulations is discussed in the following sections.

Acute toxicity tests are usually performed by using five concentrations, a control, and a vehicle (i.e., solvent) control if a vehicle is needed, generally with 10 to 20 organisms per concentration. Most regulatory guidelines require duplicate exposure levels, although this is not required for pesticide registration. Overlying water quality parameters are generally required to fall within the following range: temperature, ±1°C; pH, 6.5 to 8.5; dissolved oxygen, greater than 60% of saturation; hardness (moderately hard), 140 to 160 mg/L as $CaCO_3$. For marine testing, salinity is controlled to appropriate specified levels. All of the above variables, as well as the test concentration, are typically measured at the beginning and end of the study and occasionally more often. This basic experimental design applies for most regulations and species.

2.3.2 Chronic Toxicity Tests

Chronic toxicity tests are designed to measure the effects of toxicants to aquatic species over a significant portion of the organism's life cycle, typically one tenth or more of the organism's lifetime. Chronic studies evaluate the sublethal effects of toxicants on reproduction, growth, and behavior due to physiological and biochemical disruptions. Effects on survival are most frequently evaluated, but they are not always the main objective of the study. Examples of chronic aquatic toxicity studies have included: brook trout (*Salvelinus fontinalis*), fathead and sheepshead minnow, daphnids, (*Daphnia magna), (Ceriodaphnia dubia)*, oligochaete (*Lumbriculus variegatus), midge (Chironomus tentans),* freshwater amphipod *(Hyalella azteca),* zebrafish (*Brachydanio rerio*), and mysid shrimp (*Americamysis bahia*). Algal tests are typically 3 to 4 days in length and are often reported as acute tests. However, algal species reproduce fast enough that several generations are exposed during a typical study, and therefore these studies should be classified as chronic studies. Currently, many regulatory agencies regard an algal EC_{50} as an acute test result and the NOEC or the EC_{10} as a chronic test result.

Table 2.1 Summary of Published U.S. Environmental Protection Agency (U.S. EPA), the American Society for Testing and Materials (ASTM), and Environment Canada (EC) Methods for Conducting Aquatic Toxicity Tests

Test Description	Reference
Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians	EPA-660/3-75-009
Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms	EPA/600/4-90/027F
Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms	EPA/600/4-91/002
Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast and Marine and Estuarine Organisms	EPA/600/R-95/136
Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms	EPA/600/4-91/003
Methods Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR Part 136)	EPA/821/B-00/004
Methods for Aquatic Toxicity Identification Evaluations: Phase I. Toxicity Characterization Procedures	EPA-600/6-91/003
Methods for Aquatic Toxicity Identification Evaluations: Phase II. Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity.	EPA-600/R-92/080
Methods for Aquatic Toxicity Identification Evaluations: Phase III. Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity.	EPA-600/R-92/081
Toxicity Identification Evaluation: Characterization of Chronically Toxic Effluents, Phase I	EPA-600/6-91/005F
Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Mollusks	ASTM E 724-98
Conducting Acute Toxicity Tests on Materials with Fishes, Macroinvertebrates, and Amphibians	ASTM E 729-96
Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians	ASTM E 729-88
Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Mollusks	ASTM E 1022-94
Assessing the Hazard of a Material to Aquatic Organisms and Their Uses	ASTM E 1023-84
Life-Cycle Toxicity Tests with Saltwater Mysids	ASTM E 1191-97
Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians	ASTM E 1192-97
Conducting *Daphnia magna* Life Cycle Toxicity Tests	ASTM E 1193-97
Using Brine Shrimp Nauplii as Food for Test Animals in Aquatic Toxicology	ASTM E 1203-98
Conducting Static 96-h Toxicity Tests with Microalgae	ASTM E 1218-97a
Conducting Early Life-Stage Toxicity Tests with Fishes	ASTM E 1241-97
Using Octanol-Water Partition Coefficient to Estimate Median Lethal Concentrations for Fish Due to Narcosis	ASTM E 1242-88
Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*	ASTM E 1295-89
Standardized Aquatic Microcosm: Fresh Water	ASTM E 1366-96
Conducting Static Toxicity Tests with *Lemna gibba* G3	ASTM E 1415-91
Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX)	ASTM E 1439-98
Acute Toxicity Tests with the Rotifer Brachionus	ASTM E 1440-91
Conducting Static and Flow-Through Acute Toxicity Tests with Mysids from the West Coast of the United States	ASTM E 1463-92
Conducting Sexual Reproduction Tests with Seaweeds	ASTM E 1498-92
Conducting Acute, Chronic and Life-Cycle Aquatic Toxicity Tests with Polychaetous Annelids	ASTM E 1562-94
Conducting Static Acute Toxicity Tests with Echinoid Embryos	ASTM E 1563-98
Conducting Renewal Phytotoxicity Tests with Freshwater Emergent Macrophytes	ASTM E 1841-96
Conducting Static, Axenic, 14-day Phytotoxicity Tests in Test Tubes with the Submersed Aquatic Macrophyte *Myriophyllum sibiricum* Komarov	ASTM E 1913-97
Conducting Toxicity Tests with Bioluminescent Dinoflagellates	ASTM E 1924-97
Algal Growth Potential Testing with *Selenastrum capricornutum*	ASTM D 3978-80
Acute Lethality Test Using Rainbow Trout	EPS 1/RM/9
Acute Lethality Test Using Threespine Stickleback	EPS 1 RM/10
Acute Lethality Test Using *Daphnia* ssp.	EPS 1/RM/11
Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*	EPS 1/RM/21
Test of Larval Growth and Survival Using Fathead Minnows	EPS 1/RM/22
Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)	EPS 1/RM/24

Table 2.1 Summary of Published U.S. Environmental Protection Agency (U.S. EPA), the American Society for Testing and Materials (ASTM), and Environment Canada (EC) Methods for Conducting Aquatic Toxicity Tests *(Continued)*

Test Description	Reference
Growth Inhibition Test Using the Freshwater Alga (*Selenastrum capricornutum*)	EPS 1/RM/25
Fertilization Assay with Echinoids (Sea Urchin and Sand Dollars)	EPS 1/RM/27
Toxicity Testing Using Early Life Stages of Salmonid Fish (Rainbow Trout) – Second Edition	EPS 1/RM/28
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte – *Lemna minor*	EPS 1/RM/37
Reference Method of Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/1

Note: EPS = Environmental Protection Series (Environment Canada).

Partial life-cycle studies are often referred to as chronic studies; however, frequently only the most sensitive life stages are utilized for exposure in these studies and they should therefore not be considered true chronic studies. Hence, they are often referred to as partial chronic or subchronic studies. Common examples of partial life-cycle studies are the fish early-life-stage studies with fathead and sheepshead minnows, zebrafish, and rainbow trout. These studies generally expose the most vulnerable developmental stage, the embryo and larval stage (30 to 60 days post-hatch), to a toxicant and evaluate the effects on survival, growth, and sometimes behavior. Recently, procedures have been developed for an abbreviated fathead minnow life-cycle test to assess the potential of substances to affect reproduction.[30] This test was developed in response to a need to screen for endocrine-disrupting chemicals. Likewise, a partial life-cycle test with *Xenopus laevis* that evaluates tail resorption as a screen for thyroid active substances was recently developed.[31]

2.3.3 Static Toxicity Tests

Effluent, sediment, and dredged-materials tests are often performed in static or static renewal systems. Static toxicity tests are assays in which the water or toxicant in test beakers is not renewed during the exposure period. Static toxicity tests are most frequently associated with acute testing. The most common static acute tests are those performed with daphnids, mysids, amphipods, and various fishes. Renewal tests (sometimes called static renewal tests) refer to tests where the toxicant and dilution water is replaced periodically (usually daily or every other day). Renewal tests are often used for daphnid life-cycle studies with *Ceriodaphnia dubia* and *Daphnia magna* that are conducted for 7 and 21 days, respectively. Renewal tests have also been standardized for abbreviated early-life-stage studies or partial life-cycle studies with several species (e.g., 7- to 10- day fathead minnow early-life-stage studies).

Static and renewal tests are usually not an appropriate choice if the test material is unstable, sorbs to the test vessel, is highly volatile, or exerts a large oxygen demand. When any of these situations is apparent, a flow-through system is preferable. Static-test systems are usually limited to 1.0 g of biomass per liter of test solution so as not to deplete the oxygen in the test solution. More detail on fundamental procedures for conducting aquatic toxicity bioassays can be found in Sprague, 1969, 1973 and Rand, 1995.[32–34]

2.3.4 Flow-Through Toxicity Tests

Flow-through tests are designed to replace toxicant and the dilution water either continuously (continuous-flow tests) or at regular intermittent intervals (intermittent-flow tests). Longer-term studies are usually performed in this manner. Flow-through tests are generally thought of as being superior to static tests as they are much more efficient at maintaining a higher-level of water quality,

ensuring the health of the test organisms. Static tests designed to provide the same organism mass to total water test volume as used in a flow-through study can maintain approximately the same water quality. Flow-through tests usually eliminate concerns related to ammonia buildup and dissolved oxygen usage as well as ensure that the toxicant concentration remains constant. This approach allows for more test organisms to be used in a similar size test system (number of organisms/standing volume/unit time) than do static tests.

There are many types of intermittent-flow diluter systems that have been designed to deliver dilution water and test for chemical presence in intermittent-flow toxicity tests. The most common system is that published by Mount and Brungs.[35] Continuous-flow systems provide a steady supply of dilution water and toxicant to the test vessels. This is achieved with a diluter system that utilizes flow meters to accurately control the delivery of water and metering pumps or syringes to deliver the toxicant.[36]

2.3.5 Sediment Tests

The science of sediment-toxicity testing has rapidly expanded during the past decade. Sediments in natural systems and in test systems often act as a sink for environmental contaminants, frequently reducing their bioavailability. Bioavailability refers to that fraction of a contaminant present that is available for uptake by aquatic organisms and capable of exerting a toxic effect. The extent to which the bioavailability is reduced by sediments is dependent upon the physical-chemical properties of the test chemical and the properties of the sediment. Past studies have demonstrated that chemical concentrations that produce biological effects in one sediment type often do not produce effects in other sediments even when the concentration is a factor of 10 or higher. The difference is due to the bioavailability of the sediment-sorbed chemical.

The ability to estimate bioavailability is a key factor in ultimately assessing the hazard of chemicals associated with sediments. Much progress has been made in this area recently. It is now widely recognized that the organic carbon content of the sediment is the component most responsible for controlling the bioavailability of nonionic (nonpolar) organic chemicals.[37, 38] This concept has been incorporated into an approach termed the "Equilibrium Partitioning Approach" and is being used by the EPA for establishing sediment quality criteria.[39] For some metals (cadmium, copper, nickel, and lead, silver, and zinc) the acid volatile sulfide (AVS) content of the sediments has recently been shown to control metal bioavailability in sediments with sufficient sulfide. AVS is a measure of the easily extractable fraction of the total sulfide content associated with sediment mineral surfaces. Metal-sulfide complexes are highly insoluble, which limits the bioavailability of certain metals. When the AVS content of the sediment is exceeded by the metal concentration (on a molar ratio of 1:1), free metal ion toxicity may be expressed.[40] Recent research shows that toxicity is frequently not expressed when SEM exceeds AVS due to the fact that metal ions are sorbed to sediment organic carbon or other reactive surfaces such as iron and manganese oxides.[41] Approaches for additional classes of compounds such as polar ionic chemicals have been proposed.[42] Recently, an approach was developed for assessing the combined effects of multiple PAHs sorbed to sediments based on equilibrium partitioning, narcosis toxicity theory, and the concept that chemicals within a given class of compounds with the same mode of action act in a predictive and additive manner.[43, 44]

The recognition that sediments are both a sink and a source for chemicals in natural environments has led to increased interest in sediments and to the development of standard testing methods for sediment-dwelling organisms. Until recently, most sediment tests were acute studies. Greater emphasis is now placed on chronic sediment-toxicity tests with sensitive organisms and sensitive life stages. For example, partial life-cycle test procedures are available for several species of amphipods and the sea urchin. Full life-cycle tests can be performed with the marine worm *Nereis virens,* freshwater midges (*C. tentans* and *Paratanytarsus disimilis*), and freshwater amphipods (*H. azteca*) (Table 2.2). Partial and full life-cycle tests can be performed with epibenthic species such

Table 2.2 Summary of Published U.S. Environmental Protection Agency (U.S. EPA), the American Society for Testing and Materials (ASTM) and Environment Canada (EC) Methods for Conducting Sediment Toxicity Tests

Test Description	Reference
Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates.	EPA/600/R-99/064
Standard Guide for Conduction of 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods	ASTM E 1367-92
Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing	ASTM E 1391-94
Standard Guide for Designing Biological Test with Sediments	ASTM E 1525-94a
Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater invertebrates	ASTM E 1706-95b
Standard Guide for Conduction of Sediment Toxicity Tests with Marine and Estuarine Polychaetous Annelids	ASTM E 1611
Standard Guide for Determination of Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates	ASTM E 1688-00
Acute Test for Sediment Toxicity Using Marine and Estuarine Amphipods	EPS 1/RM/26
Test for Survival and Growth in Sediment Using Freshwater Midge Larvae *Chironomus tentans* or *riparius*	EPS 1/RM/32
Test for Survival and Growth in Sediment Using Freshwater Amphipod *Hyalella azteca* I	EPS 1/RM/33
Test for Survival and Growth for Sediment Using a Marine Polychaete Worm	EPS 1/RM/*
Reference Method for Determining Acute Lethality of Sediments to Estuarine or Marine Amphipods	EPS 1/RM/35
Reference Method of Determining Sediment Toxicity Using Luminescent Bacteria	EPS 1/RM/*
Sediment-Water Chironomid Toxicity Test Using Spiked Sediment	218
Sediment-Water Chironomid Toxicity Test Using Spiked Water	219

Note: EPS = Environmental Protection Series (Environment Canada).

* Document in preparation.

as *D. magna* and *C. dubia*. These species can be tested with sediments present in the test vessels. Porewater (interstitial water) exposures offer a potentially sensitive approach to the toxicity of the freely dissolved fractions of contaminants. The interstitial water is extracted from the sediment, usually by centrifugation, and subsequently used in toxicity tests with a wide variety of test organisms and life stages. The use of porewater allows for the testing of fish early-life stages as well as invertebrates. An extensive review of porewater testing methods and utility of the data was recently summarized at a SETAC Pellston Workshop.[45] Available sediment-assessment methods have been reviewed by Adams et al.[46] Guidance for conducting sediment bioassays for evaluating the potential to dispose of dredge sediment via open ocean disposal has been summarized in the EPA-Corps of Engineers (COE) Green Book.[47]

Typical sediment bioassays are used to evaluate the potential toxicity or bioaccumulation of chemicals in whole sediments. Sediments may be collected from the field or spiked with compounds in the laboratory. Spiked and unspiked sediment tests are performed in either static or flow-through systems, depending on the organism and the test design. Flow-through procedures are most often preferred. Between 2 and 16 replicates are used, and the number of organisms varies from 10 to 30 per test vessel. Sediment depth in the test vessels often ranges from 2 to 6 cm and occasionally as deep as 10 cm. Test vessels often range from 100 to 4000 mL in volume. Sediment tests for field projects are not based on a set number of test concentrations but rely on a comparison of control and reference samples with sediments from sites of interest. Care must be exercised in selecting sites for testing, collecting, handling, and storing the sediments. [48,49] Likewise, special procedures have been devised for spiking sediments with test substances. A reference sediment from an area known to be contaminant-free and that provides for good survival and growth of the test organisms is often included as an additional control in the test design. Guidance for selecting reference samples and sites can be found in the EPA-COE Green Book.[47]

2.3.6 Bioconcentration Studies

Bioconcentration is defined as the net accumulation of a material from water into and onto an aquatic organism resulting from simultaneous uptake and depuration. Bioconcentration studies are performed to evaluate the potential for a chemical to accumulate in aquatic organisms, which may subsequently be consumed by higher trophic-level organisms including man (ASTM E 1022–94, Table 2.1). The extent to which a chemical is concentrated in tissue above the level in water is referred to as the bioconcentration factor (BCF). It is widely recognized that the octanol/water partition coefficient — referred to as $K_{ow,}$ Log K_{ow} or Log *P* — can be used to estimate the potential for nonionizable organic chemicals to bioconcentrate in aquatic organisms. Octanol is used as a surrogate for tissue lipid in the estimation procedure. Equations used to predict BCFs have been summarized by Boethling and Mackay.[50] While the use of K_{ow} is useful for estimating the bioconcentration potential of nonpolar organics, it is not useful for metals or ionizable or polar substances. Additionally, it should be recognized that the use of BCFs have limited utility for metals and other inorganic substances that may be regulated to some extent and that typically have BCFs that are inversely related to exposure concentration. For these substances the BCF value is not an intrinsic property of the substance.[51,52]

Methods for conducting bioconcentration studies have been described and summarized for fishes and saltwater bivalves by ASTM (Table 2.1) and TSCA (Table 2.3). To date, the scientific community has focused its efforts on developing methods for fishes and bivalves because these species are higher trophic-level organisms and are most often consumed by man. In general, the approach for determining the BCF for a given chemical and species is to expose several organisms to an environmentally relevant chemical of interest that is no more than one tenth of the LC_{50} (lethal concentration) for the species being tested. At this exposure level mortality due to the test chemical can usually be avoided. The test population is sampled repeatedly, and tissue residues (usually in the fillet, viscera, and whole fish) are measured. This is most often done with C^{14} chemicals to facilitate tissue residue measurements. The study continues until apparent steady state is reached (a plot of tissue chemical concentrations becomes asymptotic with time) or for 28 days. At this point the remaining fish are placed in clean water, and the elimination (depuration) of the chemical from the test species is measured by analyzing tissues at several time intervals.

Apparent steady state can be defined as that point in the experiment where tissue residue levels are no longer increasing. Three successive measurements over 2 to 4 days showing similar tissue concentrations are usually indicative of steady state. When steady state has been achieved, the uptake and depuration rates are approximately equal. It has been shown that 28 days is adequate for most chemicals to reach steady state. However, this is not true for chemicals with a large K_{ow} (e.g., DDT, PCBs). An estimate of the time required to reach apparent steady state can be made for a given species based on previous experiments with a similar chemical or using K_{ow} for nonionizable chemicals that follow a two-compartment, two-parameter model for uptake and depuration. The following equation is used: $S = \{\ln[1/(1.00 - 0.95)]\}/k_2 = 3.0/k_2$, where: S = number of days, ln = logarithm to the base e, k_2 = the first-order depuration constant (day^{-1}) and where k_2 for fishes is estimated as antilog ($1.47 - 0.414 \log K_{ow}$).[53] The use of K_{ow} for estimating the BCF or time to equilibrium is not useful for polar substances or inorganic substances such as metals.

Two additional terms of interest are bioaccumulation and biomagnification. The first refers to chemical uptake and accumulation in tissues by an organism from any external phase (water, food, or sediment). Biomagnification is the process whereby a chemical is passed from a lower to successively higher trophic levels, resulting in successively higher residue at each trophic level. Biomagnification is said to occur when the trophic transfer factor exceeds 1.0 for two successive trophic levels (e.g., algae to invertebrates to fish). Biomagnification is generally thought to occur only with chemicals with a large K_{ow} (>4.0) and does not occur for inorganic substances.[54] Specific tests and standard guidelines have been developed for measuring bioaccumulation of sediment associated contaminants in the freshwater oligochaete *L. variegatus* (EPA and ASTM).[55, 56]

Table 2.3 Summary of the Toxicity Test Requirements by Regulatory Guideline

Regulatory Guideline	Type of Testing Required
Clean Water Act (CWA)	Aquatic Tests for the Protection of Surface Waters
U.S. EPA NPDES Regulations	Effluent Biomonitoring Studies
	Toxicity Identification and Reduction Evaluations
Water Quality Standards	Aquatic Tests for the Development of Water Quality Criteria (WQC)
Toxic Substances Control Act (TSCA)	Industrial and Specialty Chemicals: Aquatic Assessments
Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)	
Premanufacture Notification, PMN	Algae, daphnid, and one fish species
Section Four Test Rule	Data set requirements may include multiple acutes with fish algae and invertebrates, freshwater and marine; followed by 1–3 chronic or partial life-cycle studies. A sediment study with midge and a bioconcentration study may be required if low $K_{ow} > 3.0$.
Adams et al. (1985)	Midge partial life cycle test with sediments
TSCA and FIFRA Aquatic Test Guideline Numbers	
Series 850 OPPTS Ecological Effects Test Guidelines	
(Aquatic Test Guideline Number):	
850.1010	Aquatic invertebrate acute toxicity test, freshwater daphnids
850.1012	Gammarid acute toxicity test
850.1025	Oyster acute toxicity test (shell deposition)
850.1035	Mysid acute toxicity test
850.1045	Penaeid acute toxicity test
850.1055	Bivalve acute toxicity test (embryo larval)
850.1075	Fish acute toxicity test freshwater and marine
850.1085	Fish acute toxicity test mitigated by humic acid
850.1300	Daphid chronic toxicity test
850.1350	Mysid chronic toxicity test
850.1400	Fish early-life state toxicity test
850.1500	Fish life cycle toxicity test
850.1710	Oyster BCF
850.1730	Fish BCF
850.1735	Whole sediment acute toxicity invertebrates, freshwater
850.1740	Whole sediment acute toxicity invertebrates, marine
850.1790	Chironomid sediment toxicity test
850.1800	Tadpole/sediment subchronic toxicity test
850.1850	Aquatic food chain transfer
850.1900	Generic freshwater microcosm test, laboratory
850.1925	Site-specific microcosm test, laboratory
850.1950	Field testing for aquatic organisms
850.4400	Aquatic plant toxicity test using *Lemna* spp.
850.4450	Aquatic plants field study, Tier III
850.5100	Soil microbial community toxicity test
850.5400	Algal toxicity, Tiers I and II
850.6200	Earthworm subchronic toxicity test
Adams et al. (1985)	Midge partial life cycle test with sediments
Food and Drug Administration (FDA)	New Drug Environmental Assessments
Environmental Effects Test Number:	
4.01	Algal test
4.08	*Daphnia magna* acute toxicity
4.09	*Daphnia magna* chronic toxicity
4.10	*Hyalella azteca* acute toxicity
4.11	Freshwater fish acute toxicity
4.12	Earthworm subacute toxicity

Table 2.3 Summary of the Toxicity Test Requirements by Regulatory Guideline *(Continued)*

Regulatory Guideline	Type of Testing Required
Organization of Economic Cooperation and Development (OEDC) and European Economic Community (EEC)	European Community Aquatic Testing Requirements
Aquatic Effects Testing:	
201	Algal growth inhibition test
202	*Daphnia magna* Acute Immobilization Test and Reproduction Test
203	Fish, Acute Toxicity Test: 14-Day Study
204	Fish, Prolonged Toxicity Test: 14-Day Study
210	Fish, Early Life-Stage Toxicity Test
211	*Daphnia magna* Reproduction Test
212	Fish, Short-Term Toxicity Test on Embryo and Sac-Fry Stages
215	Fish, Juvenile Growth Test
221	*Lemna* sp. Growth Inhibition Test
305	Bioconcentration: Flow-Through Fish Test
PARCOM European Community: Paris Commission	Offshore Chemical Notification/Evaluation
—	Algal growth inhibition test (*Skeletonema costatum* or *Phaeodactylum tricornutum*)
—	Invertebrate acute toxicity test (*Acartia tonsa*, *Mysidopsid* sp., *Tisbe* sp.)
—	Fish Acute Toxicity Test (*Scophthalmus* sp.)
	Sediment Reworker Test (*Corophium volutator*, *Nereis virens*, and *Abra alba*)

Note: — Indicates no guideline number available.

2.4 TOXICOLOGICAL ENDPOINTS

Toxicological endpoints are values derived from toxicity tests that are the results of specific measurements made during or at the conclusion of the test. Two broad categories of endpoints widely used are assessment and measures of effect. Assessment endpoints refer to the population, community, or ecosystem parameters that are to be protected (e.g., population growth rate, sustainable yield). Measures of effect refer to the variables measured, often at the individual level, that are used to evaluate the assessment endpoints. The measures of effect describe the variables of interest for a given test. The most common measures of effect include descriptions of the effects of toxic agents on survival, growth, and reproduction of a single species. Other measures of effect include descriptions of community effects (respiration, photosynthesis, diversity) or cellular effects such as physiological/histopathological effects (backbone collagen levels, ATP/ADP levels, RNA/DNA ratios, biomarkers, etc.). In each case the endpoint is a variable that can be quantitatively measured and used to evaluate the effects of a toxic agent on a given individual, population, or community. The underlying assumption in making toxicological endpoint measurements is that the endpoints can be used to evaluate or predict the effects of toxic agents in natural environments. EPA risk-assessment guidelines provide information on how endpoints can be used in the environmental risk-assessment process.[56]

2.4.1 Acute Toxicity Tests

Endpoints most often measured in acute toxicity tests include a determination of the LC or EC_{50} (median effective concentration), an estimate of the acute no-observed effect concentration (NOEC), and behavioral observations. The primary endpoint is the LC or EC_{50}. The LC_{50} is a lethal concentration that is estimated to kill 50% of a test population. An EC_{50} measures immobilization

or an endpoint other than death. The LC and EC_{50} values are measures of central tendency and can be determined by a number of statistical approaches.[57] The Litchfield-Wilcoxen approach is most often used[58] and consists of plotting the survival and test chemical concentration data on log-probability paper, drawing a straight line through the data, checking the goodness of fit of the line with a chi-squared test, and reading the LC or EC_{50} directly off the graph. Various computer packages are also available to perform this calculation. Other common methods include the moving-average and binomial methods. The latter is most often used with data sets where the dose-response curve is steep and no mortality was observed between the concentrations where zero and 100% mortality was observed.

The NOEC (no-observed effect concentration — acute and chronic tests) is the highest concentration in which there is no significant difference from the control treatment. The LOEC (lowest observed effect concentration — acute and chronic tests) is the lowest concentration in which there is a significant difference from the control treatment. The NOEC and LOEC are determined by examining the data and comparing treatments against the control in order to detect significant differences via hypothesis testing. The effects can be mortality, immobilization, reduced cell count (algae), or behavioral observations. These endpoints are typically determined using t-tests and analysis of variance (ANOVA) and are most often associated with chronic tests. NOECs/LOECs are concentration-dependent and do not have associated confidence intervals. Sebaugh et al. demonstrated that the LC_{10} could be used as a substitute for the observed no-effect concentration for acute tests.[59] This provides a statistically valid approach for calculating the endpoint and makes it possible to estimate when the lowest concentration results in greater than 10% effects. It should be noted, however, that the confidence in the estimated LC decreases as one moves away from 50%. Regression analysis, as opposed to hypothesis testing, is gaining favor as a technique for evaluating both acute and chronic data. The advantage is that it allows for the calculation of a percentage of the population of test organisms affected, as opposed to ANOVA, which simply determines whether or not a given response varies significantly from the control organisms. EC and LC values are readily incorporated into risk-assessment models and are particularly useful in probabilistic risk assessments.[60,61]

2.4.2 Partial Life-Cycle and Chronic Toxicity Tests

In partial life-cycle studies the endpoints most often measured include egg hatchability (%), growth (both length and weight), and survival (%). Hatchability is observed visually; growth is determined by weighing and measuring the organisms physically at the termination of the study. Computer systems are available that allow the organisms to be weighed and measured electronically and the data to be automatically placed in a computer spreadsheet for analysis. In chronic studies, reproduction is also evaluated. Endpoints include all the parameters of interest, i.e., egg hatchability, length, weight, behavior, total number of young produced, number of young produced per adult, number of spawns or broods released per treatment group or spawning pair, physiological effects, and survival. In partial and full life-cycle studies, the endpoints of interest are expressed as NOEC/LOEC or LC_x values. The geometric mean of these two values has traditionally been referred to as the maximum acceptable toxicant concentration (MATC). More recently, the term MATC has been referred to as the chronic value (CV), which is defined as the concentration (threshold) at which chronic effects are first observed. Other endpoints (LC or EC_{50}) may be estimated in chronic and subchronic studies, but they are of lesser interest. It is the CV that is compared to the LC or EC_{50} to determine the acute-to-chronic ratio for a given species and toxicant.

The approach for assessing the aforementioned endpoints is based upon selecting the appropriate statistical model for comparing each concentration level to the control. Dichotomous data (hatchability or survival expressed as number dead and alive) require the Fisher's exact or chi-square test.[62] For continuous data (growth variables, e.g., length and weight; reproductive variables, e.g.,

number of spawns; hatchability or survival data expressed as percentages) the Dunnett's means-comparison procedure would be used based on an analysis of variance (ANOVA).[62] The type of ANOVA, such as one-way or nested, and the error term used, such as between chambers or between aquaria, should correspond to the experimental design and an evaluation of the appropriate experimental unit. Typically, a one-tailed test is used because primary interest is in the detrimental negative effects of the compound being tested and not on both a negative and a positive effect (two tails). Although parametric ANOVA procedures are robust, a nonparametric Dunnett's test should be performed if there are large departures from normality within treatment groups or large departures from homogeneous variance across treatment groups.

For studies that provide continuous data that are analyzed by calculating a percent change from the control, the most appropriate approach is to plot the percent change against the logarithm of the test concentration. The resulting regression line can be used to calculate a percent reduction of choice along with its corresponding confidence interval. It is common to calculate a 25% reduction and express the value either as an ICp (inhibition concentration for a percent effect) or as an EC. Probit analysis of these data is not appropriate. Expressing the data as an ICp, as opposed to an EC, is probably a better approach because it does not have as its basis the concept of a median effect concentration, which is dependent on dichotomous data as opposed to continuous data.

2.5 REGULATORY ASPECTS OF AQUATIC TOXICOLOGY IN THE UNITED STATES

2.5.1 Clean Water Act (CWA)

The CWA was passed in 1972 and has been amended several times since then. A primary goal of this regulation was to ensure that toxic chemicals were not allowed in U.S. surface waters in toxic amounts. The passage of this act had a major impact on environmental engineering and aquatic toxicology, which led to formalized guidelines for deriving water quality criteria.[20] These criteria were used to develop federal water quality standards that all states adopted and enforced. To date, 24 WQC have been developed in the United States.[63] The aquatic tests required to derive WQC are listed in Table 2.4. Additionally, 129 priority pollutants have been identified, and discharge enforceable limits that cannot be exceeded have been set.

Under the authority of the Clean Water Act, the EPA, Office of Water, Enforcement Branch, established a system of permits for industrial and municipal dischargers (effluents) into surface waters. This permit system is termed the National Pollutant Discharge Elimination System (NPDES). Chemical producers are classified according to the type of chemicals they produce (organic chemicals, plastics, textiles, pesticides, etc.). Each chemical industry category has a list of chemicals and corresponding concentrations that are not to be exceeded in the industry's wastewater effluent. These chemical lists apply to all producers for a given category and are part of each producer's NPDES permit. Each producer also has other water-quality-parameter requirements built into their permit that are specific to their operations. These usually include limitations on the amount (pounds) of chemical that is permitted to be discharged per month and may include items such as total organic carbon, biochemical oxygen demand, suspended solids, ammonia, and process-specific chemicals.

The NPDES permit system incorporates biomonitoring of effluents, usually on a monthly, quarterly, or yearly basis.[29] A toxicity limit is built into the discharger's permit for both industrial and municipal dischargers that must be achieved. If the toxicity limit is exceeded, the permittee is required to identify the chemical responsible for the excess toxicity and take steps to eliminate the chemical, reduce the toxicity, or both. Effluent biomonitoring most often consists of acute toxicity tests with *daphnia* (*Ceriodaphnia dubia*) and fathead minnow (*Pimephales promelas*). Seven-day life-cycle and partial life-cycle studies are required in some cases. An extensive set of procedures (toxicity identification evaluation, TIE) for identifying the substance or substances responsible for

Table 2.4 Aquatic Toxicity Tests Required by U.S. EPA for the Development of Water Quality Criteria

Type of Testing	Recommended Aquatic Tests
Acute Toxicity Tests	Eight different families must be tested for both freshwater and marine species (16 acute tests): *Freshwater* 1. A species in the family Salmonidae 2. A species in another family of the class Osteichthyes 3. A species in another family of the phylum Chordata 4. A plankton species in class Crustacea 5. A benthic species in class Crustacea 6. A species in class Insecta 7. A species in a phylum other than Chordata or Arthropoda 8. A species in another order of Insecta or in another phylum *Marine* 1. Two families in the phylum Chordata 2. A family in a phylum other than Arthropoda or 3. Chordata 4. Either Family Mysidae or Penaeidae 5. Three other families not in the phylum Chordata (may include Mysidae or Penaeidae, whichever was not used above) 6. Any other family
Chronic Toxicity Tests	Three chronic or partial life-cycle studies are required: One invertebrate and one fish One freshwater and one marine species
Plant Testing	At least one algal or vascular plant test must be performed with a freshwater and marine species.
Bioconcentration Testing	At least one bioconcentration study with an appropriate freshwater and saltwater species is required.

toxicity in effluents and sediments has been developed over the past decade.[64–75] Present tie research efforts are focused primarily on freshwater and marine sediments.

2.5.2 Toxic Substances Control Act (TSCA)

The Toxic Substances Control Act (TSCA) was established by Congress on October 8, 1976 as public law 94–469 to regulate toxic industrial chemicals and mixtures. The goal of Congress was to establish specific requirements and authorities to identify and control chemical hazards to both human health and the environment. The office of Pollution Prevention and Toxics (OPPT) is the lead office responsible for implementing the Toxic Substances Control Act, which was established to reduce the risk of new and existing chemicals in the marketplace.

There are approximately 80,000+ compounds listed on the TSCA Chemical Inventory that are approved for use in the United States.[76] The detection of polychlorinated biphenyls (PCBs), an industrial heat transformer and dielectric fluid found in aquatic and terrestrial ecosystems in many parts of the United States, emphasized the need for controlling industrial chemicals not regulated by pesticide or food and drug regulations. From the viewpoint of aquatic testing, this regulation has focused on two areas: test requirements for new chemicals and existing chemicals. Under TSCA Section 5, notice must be given to the Office of Pollution Prevention and Toxics (OPPT) prior to manufacture or importation of any new or existing chemical. No toxicity information is required for the Premanufacture Notification (PMN). OPPT has 90 days to conduct a hazard/risk assessment and may require generation of toxicity information. Toxicity testing is required only if a potential hazard or risk is demonstrated.[76]

Existing chemicals listed on the TSCA inventory register prior to 1976 are not required to undergo a PMN review. However, the EPA, through the Interagency Testing Committee (ITC), reviews individual chemicals and classes of chemicals to determine the need for environmental and human health data to assess the safety of the chemicals. If the ITC determines that a potential exists for significant chemical exposure to humans or the environment, they can require the manufacturers

to provide additional data for the chemicals to help assess the risk associated with the manufacture and use of the product. TSCA empowers the EPA to restrict chemical production and usage when the risk is considered severe enough. Data collection is accomplished through Section 4 of TSCA by means of developing a legally binding consent order on a Test Rule. The Test Rule spells out the reasons for the testing and identifies which tests are required. Aquatic tests that are most often required for PMNs or by Test Rules are listed in Table 2.3.

The Chemical Right-to-Know Initiative was begun in 1998 in response to the finding that very little toxicity information is publicly available for most of the high production volume (HPV) commercial chemicals made and used (more than 1 million lbs/yr) in the United States. Without this basic hazard information, it is difficult to make sound judgments about what potential risks these chemicals could present to people and the environment. An ambitious testing program has been established, especially for those chemicals that are persistent, bioaccumulative, and toxic (PBT), or which are of particular concern to children's health.[77]

2.5.3 Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)

Under FIFRA the EPA is responsible for protecting the environment from unreasonable adverse effects of pesticides.[78] This legislation is unique among environmental protection statutes in that it licenses chemicals known to be toxic for intentional release into the environment for the benefit of mankind. Most regulatory statutes are designed to limit or prevent the release of chemicals into the environment. The FIFRA licensing process regulates three distinct areas: (1) labeling, (2) classification, and (3) registration. To fulfill its responsibility the EPA requires disclosure of scientific data regarding the effects of pesticides on humans, wildlife, and aquatic species.

By statutory authority the EPA assumes that pesticides present a risk to humans or to the environment. The pesticide registrant is responsible for rebutting the EPA's presumption of risk. To accomplish this the EPA recommends a four-tiered testing series.[79] The tests become progressively more complex, lengthy, and costly, going from Tier I to Tier IV.[80] Studies in Tiers I and II evaluate a substance for acute toxicity and significant chronic effects, respectively. Higher-tier tests evaluate long-term chronic and subchronic effects. In Tier IV, field and mesocosm studies can be required. The need to perform successively higher-tiered tests is triggered by the degree of risk the pesticide presents to the environment. Risk is determined by the quotient method, i.e., by comparing the expected environmental concentration with the measured levels of biological effect. The difference between the two levels is referred to as the margin of safety. When the margin of safety is small in Tiers I and II, additional higher-tier studies are required to rebut the presumption of risk to the environment.

2.5.4 Federal Food, Drug, and Cosmetics Act (FFDCA)

The FFDCA of 1980 is administrated by the Food and Drug Administration (FDA). This act empowers the FDA to regulate food additives, pharmaceuticals, and cosmetics that are shipped between states. The intent of this act is to protect the human food supply and to ensure that all drugs are properly tested and safe for use. The FDA enforces pesticide tolerance and action levels set by the EPA. This can result in a ban or food consumption advisory for fish and seafood from certain areas. The FDA also regulates drugs that are used for animals, including fish, as well as human drugs. The use of drugs to treat fish diseases has drawn national and international attention since the FDA has begun to restrict the use of certain drugs that have not been properly tested for potential environmental effects. These drugs are used in significant quantities in commercial fishery operations.

The U.S. FDA is responsible for reviewing the potential environmental impact from the intended use of human and veterinary pharmaceuticals, food or color additives, Class III medical devices, and biological products. To evaluate the potential effects of a proposed compound the FDA requires

the submission of an Environmental Assessment (EA). The National Environmental Protection Act, passed by Congress in 1969, provides the statutory authority for the FDA to conduct EA requirements.

EAs are required for all New Drug Applications as well as for some supplementary submissions and communications. Previously, an EA required little more than a statement that a compound had no potential environmental impact; however, changes within the FDA have increased and intensified the EA review and approval process. Under current policy the FDA requests quantitative documentation of a compound's potential environmental impact. The EA must contain statistically sound conclusions based on scientific data obtained through studies conducted under Good Laboratory Practices (GLPs). These changes have significantly impacted the content, manner of data acquisition, and preparation of an EA. Details relative to the FDA statutory authority and information required for an EA submission are contained in the Code of Federal Regulations.[81] The specific aquatic toxicity tests recommended by the FDA for inclusion with the EA submission are shown in Table 2.3.

2.5.5 Comprehensive Environmental Response, Compensation, Liability Act

Superfund is the name synonymous with the Comprehensive Environmental Response, Compensation, Liability Act (CERCLA, 1980). This act requires the EPA to clean up uncontrolled hazardous waste sites to protect both human health and the environment. CERCLA provides the statutory authority for the EPA to require environmental risk assessment as part of the Superfund site assessment process. Part of risk assessment includes evaluating the potential for risk to aquatic species, if appropriate, for a given site. Additional authority comes from the National Oil and Hazardous Materials Contingency Plan, which specifies that environmental evaluations shall be performed to assess threats to the environment, especially sensitive habitats and critical habitats of species protected under the Endangered Species Act.

The Superfund program provides (1) the EPA with the authority to force polluters to take responsibility for cleaning up their own wastes; (2) the EPA with the authority to take action to protect human health and the environment, including cleaning up waste sites, if responsible parties do not take timely and adequate action; and (3) a Hazardous Substance Response Trust Fund to cover the cost of EPA enforcement and cleanup activities. The Superfund process consists of: site discovery, preliminary assessment (PA)/site assessment (SA), hazard ranking/nomination to National Priorities List (NPL), remedial investigation (RI)/feasibility study (FS), selection of remedy, remedial design, remedial action, operation and maintenance, and NPL deletion.[82]

Environmental risk assessment is conducted as part of the PA/SA investigation and as part of the RI/FS studies. Sites that have the potential for contaminants to migrate to surface waters and sediments require aquatic assessment. Risk assessment procedures have been evolving, and guidance in the selection of tests and species is available.[83–85] Many of the tests for TSCA and FIFRA assessments are acceptable (Table 2.3). Most often, aquatic tests are performed on soils/sediments, which are shipped to an aquatic testing facility for studies with amphipods, midges, and earthworms. Most studies are static acute or static renewal partial life-cycle studies.

2.5.6 Marine Protection, Research and Sanctuaries Act (MPRSA)

The MPRSA of 1972 requires that dredged material be evaluated for its suitability for ocean disposal according to criteria published by the EPA (40 CFR 220–228) before disposal is approved. The maintenance of navigation channels requires dredging, and the disposal of that dredged material is a concern. For ocean disposal the dredged material must be evaluated to determine its potential for impact to the water column at the disposal site. In 1977 the EPA and COE developed the manual, "Ecological Evaluation of Proposed Discharge of Dredged Material into Ocean Waters," which contains technical guidance on chemical, physical, and biological procedures to evaluate the acceptability of dredged material for ocean disposal.[86] A similar manual was developed

in 1998 for freshwater systems entitled "Evaluation of Dredged Material Proposed for Discharge in Waters of the U.S."[87]

The manual outlines a tiered testing procedure for evaluating compliance with the limiting permissible concentration (LPC) as defined by ocean dumping regulations. The liquid-phase or water-column LPC must not exceed applicable marine water quality criteria or a toxicity threshold (0.01 times the acutely toxic concentration). The suspended and solid-phase LPCs must not cause unreasonable toxicity or bioaccumulation. The document describes four levels (tiers) of evaluation. Tiers I and II utilize existing information, which is often available for recurring disposals of dredged materials from channel maintenance, to determine the appropriateness for ocean disposal. Tier III contains most laboratory bioassays, and Tier IV includes some tests of bioaccumulation and a range of possible field investigations. The evaluation also recommends using a reference site, specifically a site that is free of contamination, as a source of sediments for comparison testing with the dredged materials. Examples of aquatic marine species that are acceptable to evaluate the suitability of dredge materials for ocean disposal via water-column, solid-phase, and bioaccumulation tests are presented in Table 2.5 (taken from the Green Book).[47]

2.5.7 European Community (EC) Aquatic Test Requirements

The European Community (EC) also requires toxicity testing as part of their chemical environmental assessment process. The EC is managed by four institutions — the Commission, the Council of Ministers, the Parliament, and the Court of Justice. The Commission proposes regulations to the Council of Ministers, who make final rulings. Actions taken by the Council have the force of law and are referred to as regulations, directives, decisions, recommendations, and opinions. Most actions taken relative to chemical environmental assessment have taken the form of directives. Directives are binding on member countries; however, member countries may choose the method of implementation.

Critical directives that mandate aquatic toxicity tests are the Pesticide Registration Directive[88] and the Sixth and Draft Seventh Amendments of Directive 67/548/CEE, Classification, Packaging, and Labeling of Dangerous Substances. Additionally, the Paris Commission was established to prepare guidelines to ensure that offshore (North Sea) oil exploration would not endanger the marine environment. The directives of the Paris Commission as well as the previously mentioned directives require aquatic toxicity tests as part of environmental assessments.

2.5.8 Organization for Economic Cooperation and Development (OECD)

The published list of aquatic test methods and species required to be used when fulfilling the data requirements of EC directives is shown in Table 2.3. Test guidelines are listed as EEC (European Economic Community) or OECD (Organization of Economic Cooperation and Development). The OECD operates as a methods-generating and standardization body, whereas the EEC formally adopts test guidelines that become the legally binding method to be used. Relevant internationally agreed-upon OECD test methods used by government, industry, and independent laboratories have been published and are available as a compendium of guidelines[89, 90] (Table 2.6).

2.6 SUMMARY AND FUTURE DIRECTION OF AQUATIC TOXICOLOGY

The field of aquatic toxicology has grown out of the disciplines of water pollution biology and limnology. Aquatic toxicology studies have been performed for the past 120 years. Studies evolved from simple tests conducted over intervals as short as a few hours to standard acute lethality tests lasting 48 or 96 hours, depending on the species. Acute toxicity tests were followed by the

Table 2.5 Examples of Appropriate Test Species for Use with Dredge Material when Performing Water Column, Solid-Phase Benthic, and Bioaccumulation Effects Testing

Type of Testing and Recommended Species

Water Column Toxicity Tests

Crustaceans

Mysid shrimp, *Americamysis bahia* sp.*
Neomysis sp.*
Holmesimysis sp.*
Grass shrimp, *Palaemonetes* sp.
Oysters, *Crassostrea virginica**
Commercial shrimp, *Penaeus* sp.
Oceanic shrimp, *Pandalus* sp.
Blue crab, *Callinectes sapidus*
Cancer crab, *Cancer* sp.

Zooplankton

Copepods, *Acartia* sp.*
Larvae of:
Mussels, *Mytilus edulis**
Oysters, *Crassostrea virginica**
Ostrea sp.*
Sea urchin, *Stronglyocentrotus purpuratus*
Lyetechinus pictus

Fish

Silversides, *Menidia* sp.*
Shiner perch, *Cymatogaster aggregata**
Sheepshead minnow, *Cyprinodon variegatus*
Pinfish, *Lagodon rhomboides*
Spot, *Leiostomus xamthurus*
Sanddab, *Citharichys stigmaeus*
Grunion, *Leuresthes tenuis*
Dolphinfish, *Coryphaena hippurus*

Bivalves

Mussel, *Mytilus* sp.
Oyster, *Crassostrea* sp.

Benthic Solid-Phase Toxicity Tests

Infaunal Amphipods

Ampelisca sp.*
Rhepoxynius sp.*
Eohaustorius sp.*
Grandiderella japonica
Corophium insidiosum

Crustaceans

Mysid shrimp, *Americamysis bahia* sp.
Neomysis sp.
Holmesimysis sp.
Commercial shrimp, *Penaeus* sp.
Grass shrimp, *Palaemonetes* sp.
Sand shrimp, *Crangon* sp.
Blue crab, *Callinectes sapidus*
Cancer crab, *Cancer* sp.
Ridge-back prawn, *Sicyonia ingentis*

Burrowing Polychaetes

Neanthes sp.*
Nereis sp.*
Nephthys sp.*
Glycera sp.*
Arenicola sp.*
Abarenicola sp.*

Fish

Arrow gobi, *Clevelandia ios*

Mollusks

Yoldia clam, *Yoldia limatula* sp.
Littleneck clam, *Protothaca staminea*
Japanese clam, *Tapes japonica*

Bioaccumulation Tests

Polychaetes

Neanthes sp.*
Nereis sp.*

Mollusks

Macoma clam, *Macoma* sp.
Yoldia clam, *Yoldia limatula* sp.

Table 2.5 Examples of Appropriate Test Species for Use with Dredge Material when Performing Water Column, Solid-Phase Benthic, and Bioaccumulation Effects Testing *(Continued)*

Type of Testing and Recommended Species	
Polychaetes	***Mollusks***
Nephthys sp.*	Nucula clam, *Nucula* sp.
Arenicola sp.*	Littleneck clam, *Protothaca staminea*
Abarenicola sp.*	Japanese clam, *Tapes japonica*
	Quahog clam, *Mercenaria mercenaria*
Fish	***Crustaceans***
Arrow gobi, *Clevelandia ios*	Ridge-back prawn, *Sicyonia ingentis*
Topsmelt, *Atherinops affinis*	Shrimp, *Peneaus* sp.

Note: Information is taken from the EPA-COE Green Book.[47]

* Recommended species.

development of various short sublethal tests (e.g., behavior or biochemical studies) and tests with prolonged exposures such as partial life-cycle studies and full life-cycle studies. Early studies were performed in the absence of regulatory requirements by individuals with a high degree of scientific curiosity. Today, aquatic toxicology studies are done for research purposes or environmental risk assessments and are required by many regulatory agencies for product registration, labeling, shipping, or waste disposal.

The cost and length of time required to perform full life-cycle tests have encouraged scientists to search for sensitive test species and sensitive life stages. Full life-cycle fish studies have, for the most part, been replaced by embryo-larval studies (partial life-cycle studies).[91] A major effort has been expended to identify species that allow full life-cycle studies to be performed in much shorter periods (e.g., 7-day *Ceriodaphnia dubia* life cycle tests,[92] two-dimensional rotifer tests[93]) or tests that use sensitive species and sensitive life stages. For example, a 7-day fathead minnow embryo-larval growth and survival study is used to evaluate effluents.[94] The goal of these tests is to quickly provide accurate estimates of chronic no-effect levels. It is important to remember that these tests estimate chronic results, not duplicate them. The estimated value is often within a factor of 2 to 4 of the chronic value and, depending on the use of these data, may provide adequate accuracy.

During the last decade significant effort has been expended in developing rapid toxicity assays. There has been an increasing need to assess the toxicity of various sample types in minutes to hours instead of days. For example, effluent toxicity identification evaluation (TIE) procedures require multiple toxicity tests on successive days. The use of assays (such as the Microtox [95]assay) can speed up the TIE process considerably. The use of rapid assays during on-site effluent biomonitoring allows for collection of a more extensive data set during the limited testing time available.

Table 2.6 Adopted and Draft OECD Test Guidelines Harmonized with OPPTS since 1990

Test Guideline No.	Guideline Title	Date of Adoption as an Original or as an Updated Version and Draft Date
203	Fish, Acute Toxicity Test.	July 17, 1992
210	Fish, Early-Life Stage Toxicity Test	July 17, 1992
211	*Daphnia magna* Reproduction Test	September 21, 1998
212	Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages	September 21, 1998
215	Fish, Juvenile Growth Test	January 21, 2000 Draft Guideline, July 1999
202	*Daphnia* sp., Acute Immobilization Test	Draft
305	Bioconcentration; Flow-Through Fish Test	June 14, 1996

In recent years the increasing desire to link exposure to effect has drawn considerable attention to the "biomarker approach." Because chemical contaminants are known to evoke distinct measurable biological responses in exposed organisms, biomarker-based techniques are currently being investigated to assess toxicant-induced changes at the biological and ecological levels.[96] Collectively, the term biomarker refers to the use of physiological, biochemical, and histological changes as "indicators" of exposure and effects of xenobiotics at the suborganism or organism level. [97] However, indicators or biomarkers can be defined at any level of biological organization, including changes manifested as enzyme content or activity, DNA adducts, chromosomal aberrations, histopathological alterations, immune-system effects, reproductive effects, physiological effects, and fertility at the molecular and individual level, as well as size distributions, diversity indices, and functional parameters at the population and ecosystem level. In the field of ecotoxicology, the use of biomarkers has emerged as a new and very powerful tool for detecting both exposure and effects resulting from environmental contaminants.[97–104] Unlike most chemical monitoring, biomarker endpoints have the potential to reflect and assess the bioavailability of complex mixtures present in the environment as well as render biological significance. Biomarkers provide rapid toxicity assessment and early indication of population and community stress and offer the potential to be used as markers of specific chemicals.

Chemical effects are thought to be the result of the interaction between toxicant and biochemical receptor. Therefore, biochemical responses are expected to occur before effects are observed at higher levels of biological organization. Biomarker response frequently provides a high degree of sensitivity to environmental impacts, thereby providing an "early warning" to potential problems or irreversible effects. In natural environments, where organisms are exposed to multiple stresses (natural and anthropogenic) over time, biomarkers reflect this integrated exposure of cumulative, synergistic, or antagonistic effects of complex mixtures. A myriad of recent studies have demonstrated the utility of biomarker techniques in the assessment of contaminants ranging from single compounds to complex mixtures in both the laboratory and the field.[105–109]

To date, biomarker assays have not been standardized or incorporated into regulatory guidelines as part of chemical environmental risk assessments. It is expected that in the future a variety of specific biomarkers will be sufficiently validated as predictors of whole organism and population effects; however, it is unlikely that they will therefore tell us if an ecosystem is in danger of losing its integrity or if compensation to a particular insult is possible. A more reasonable application would be use as either part of a tiered assessment or as measurement by some standard of predefined ecological health. The trend toward more sensitive, biologically relevant test methods predictive of early ecosystem stress will continue, and biomarkers are expected to play a role as surrogate measures or predictors of ecosystem well-being.

ACKNOWLEDGMENTS

We wish to thank Jerry Smrchek for critically reviewing this manuscript.

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CHAPTER 3

Model Aquatic Ecosystems in Ecotoxicological Research: Considerations of Design, Implementation, and Analysis

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CONTENTS

3.1 INTRODUCTION

A number of research studies have made use of model aquatic ecosystems of varying design and complexity for evaluating the fate and effects of contaminants in aquatic ecosystems. These systems are designed to simulate ecosystems or portions of ecosystems. As research tools, model ecosystems contribute to our understanding of the ways in which contaminants affect natural ecosystems.[1] These systems are a tool for allowing ecologists to address hypotheses on a manageable scale and with control or reference systems. They also provide ecotoxicologists with models of ecosystem functioning, in the absence of perturbation, so that direct and indirect effects might be better separated from natural events such as succession or inherent variation.[2]

Traditionally, model ecosystems have been categorized as either microcosms or mesocosms. The distinction between microcosms and mesocosms has been somewhat subjective, with researchers establishing their own criteria, but has mainly been a function of size.[3] The degrees of organizational complexity and realism will often vary when these systems are established, depending largely on study goals and endpoints selected by the researchers.

Giesy and Odum[4] define microcosms as artificially bounded subsets of naturally occurring environments that are replicable, contain several trophic levels, and exhibit system-level properties. Mesocosms are defined as either physical enclosures of a portion of a natural ecosystem or manmade structures such as ponds or stream channels.[5] Voshell[5] further specifies that the size and complexity of mesocosms are sufficient for them to be self-sustaining, making them suitable for long-term studies. In this regard they differ from microcosms, where smaller size and fewer trophic levels do not allow for long study durations, particularly in laboratory systems. Cairns,[6] however, does not distinguish between microcosms and mesocosms because "both encompass higher levels of biological organization and have high degrees of environmental realism." The lack of a defined distinction between microcosm and mesocosm systems has caused some confusion among researchers around the world. The organizing committee of the European Workshop on Freshwater Field Tests (EWOFFT) operationally described microcosms on the basis of size, defining outdoor lentic microcosms as those surrogate ecosystems whose volume contain less than 15 m^3 of water and mesocosms as ponds of 15 m^3 or larger. Experimental stream channels were also characterized on the basis of size, defining microcosms as smaller and mesocosms as larger than 15 m in length. Such designations are useful categories for standardizing terminology. These distinctions are often used when comparing studies conducted throughout the world, and this paper will define model systems based on the EWOFFT definitions, when needed.

The use of "model" systems in aquatic research has grown considerably since the use of replicated ponds in community structure analysis by Hall, Cooper, and Werner[7] in the late 1960s and the pesticide studies of Hurlbert et al.[8] Studies prior to or concurrent with these, such as Eisenberg's[9] studies of density regulation in pond snails, used experimentally manipulated natural systems. Aspects such as community composition and spatial heterogeneity can be controlled to a greater extent in model (constructed) systems relative to natural ones. Model ecosystems are logistically more manageable and replicable for statistical analyses. In addition, model systems are effective tools in aquatic research because they act as surrogates for important cause-and-effect pathways in natural systems[6,10] yet retain a high degree of environmental realism relative to laboratory single-species bioassays.[6] These tests should be viewed as part of a tiered testing sequence and not as replacements of single-species bioassays.[11] Single-species tests, however, are inadequate when chemical fate is altered significantly under field conditions, when organismal behavior can affect exposure to a toxicant, or when secondary effects occur due to alterations in competitive or predator-prey relationships.[12]

Model ecosystems in ecotoxicological research are used to study the fate and potential adverse effects of chemicals. The ability to detect and accurately measure these effects can be influenced by both system and experimental design that influence variability. This paper addresses key factors that can influence the ability of model systems to accomplish these tasks.

3.2 HISTORICAL PERSPECTIVE

The concept of the microcosm was introduced early in ecological thinking through the writings of Forbes.[13] In his work on lake natural history the basic principles of ecological synergism, variability, and dynamic equilibrium as well as the complex interactions of predator and prey were discussed. Though speaking of the lake itself and not of the surrogate systems routinely employed in aquatic research today, Forbes[13] touched upon the rationale for the use of artificial systems in both toxicological and ecological research: "It forms a little world within itself — a microcosm within which all the elemental forces are at work and the play of life goes on in full, but on so small a scale as to bring it easily within the mental grasp."

This assertion — that artificial systems simulate processes that occur in nature enough to be viable surrogates for natural systems — is central to the underlying basis for using microcosms (and mesocosms) in ecotoxicological research.

The initial applications of artificial aquatic systems, such as laboratory microcosms, artificial ponds, and various *in situ* enclosures, were historically utilized in ecological studies of productivity,[7,14–16] community metabolism,[17–19] and population dynamics.[20] The earliest of these experiments, using laboratory microcosms, were those of Woodruff[21] and Eddy,[22] who examined protozoan species succession in hay infusions, and the slightly later studies of Lotka,[23,24] Volterra,[25,26] and Gause,[20] which formed the basis of the now standard quadratic population models in competition and predator-prey interaction. Gause[20] conducted his classic experiments on protozoan competition in glass dish microcosms from which his mathematical theory of competitive exclusion was derived. Gause[20] sought to address important ecological issues in these systems while being cognizant of their (potential) limitations. In discussing earlier studies conducted in laboratory microcosms Gause writes:

> However, in experiments of this type there exists a great number of different factors not exactly controlled, and a considerable difficulty for the study of the struggle for existence is presented by the continuous and regular changes in the environment. It is often mentioned that one species usually prepares the way for the coming of another species. Recollecting what we have said in Chapter II it is easy to see that in such a complicated environment it is quite impossible to decide how far the supplanting of one species by another depends on the varying conditions of the microcosm which oppress the first species, and in what degree this is due to direct competition between them.

The above-cited research helped lay the groundwork for understanding how biotic processes function in artificially bounded and maintained systems. A fundamental knowledge of the ecology of the systems is necessary if there is to be any understanding of how they may be altered by an introduced perturbation. There has been considerable concern and debate over whether model systems, such as microcosms, simulate natural systems closely enough to be used as ecosystem surrogates. Microcosms tend not to closely simulate natural systems at all levels of ecological organization. Traditionally, this has not been viewed as a problem, as the system selected will vary with the research goals and the endpoints of choice. The presence of higher levels of organization may not be necessary to demonstrate effects with some endpoints.

The use of surrogate systems in toxicological research, particularly those encompassing any appreciable scale and complexity, has been relatively recent (ca. 1960). Concern over the effects of insecticides used to control mosquito populations in California prompted a series of field studies on the consequences of chemical control methods on nontarget species such as mosquito fish and waterfowl. Keith and Mulla[27] and Mulla et al.[28] used replicated artificial outdoor ponds to examine the effects of organophosphate-based larvicides on mallard ducks. Hurlbert et al.[8] conducted subsequent studies in the same systems, examining the impact on a greater number of species within several broad taxa (phytoplankton, zooplankton, aquatic insects, fish, and waterfowl). Essentially, system-level impacts were being assessed, with subsequent evaluation of indirect effects

(e.g., changes in prey species densities in the absence of a predator species or the effects of emigration from a system) attributable to the pesticide application.

The broad application of microcosms and mesocosms in toxicological studies largely followed the realization that single-species toxicity tests alone were inadequate for predicting effects at the population and ecosystem levels.[29,30] Multispecies tests have the potential to demonstrate effects not evident in laboratory tests that use a single, presumably (most) sensitive, species.[31] As the goals of environmental protection focus on ecosystem-level organization, testing within more complex systems involves less extrapolation, apparently enhancing the prediction of impacts on natural systems. Model ecosystems in ecotoxicological research are seen primarily as a way of studying potential contaminants in systems that simulate parts of the natural environment but that are amenable to experimental manipulations.[1]

An assessment of the ecological risk of pesticides is required under the United States Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). A tiered data-collecting process that results from a progression of increasingly complex toxicity tests is considered together with an estimation of environmental exposure to make an assessment of whether a chemical may pose an unacceptable risk to the aquatic environment. Following tests conducted for each tier, data are evaluated, and risk to the aquatic environment is determined. Based on the outcomes of testing at each tier, the decision is made whether to stop testing or to continue to the next tier. Initial tiers are in the form of laboratory bioassays. The final tier (Tier IV) involves field testing. A description of the tests required at each tier and criteria for their implementation by the Environmental Protection Agency (EPA) is given in the Report of the Aquatic Effects Dialogue Group (AEDG).[32] Registrants may be required by the EPA to conduct higher-tier tests, or they may opt for this level of testing to refute the presumption of unacceptable environmental risk indicated by a lower-tier test.

Prior to the EPA's adoption of the mesocosm technique as part of the ecological risk assessment of pesticides, Tier IV tests were conducted in natural systems that were exposed to the agricultural chemical during the course of typical farming practices. Although these types of studies provided realism in terms of environmental fate of the compound and exposure to the aquatic ecosystem, they were difficult to evaluate, in part because of insufficient or no replication, a high degree of variability associated with the factors being measured, and influences of uncontrollable events such as weather. In the mid-1980s the EPA adopted the use of mesocosms (experimental ponds) as surrogate natural systems in which ecosystem-level effects of pesticides could be measured (Tier IV tests) and included in the ecological risk-assessment process.[33]

Although no longer part of the regulatory requirements in the United States, mesocosm test requirements have stimulated an increased worldwide interest in the use of surrogate ecosystems for the evaluation of the fate and effects of contaminants in aquatic ecosystems, as evidenced by the number of symposia[5,34–38] and workshops[1,39–41] over the last decade.

3.3 BIOMAGNIFICATION

Barron[42] presents an overview of the principles and determinants of biomagnification in aquatic food webs. Environmental contaminants affect organisms that are part of an aquatic food chain. Biomagnification is the increase in contaminant body burden (tissue contaminant) caused by the transfer of contaminant residues from lower to higher trophic levels.[43] Rasmussen et al.[44] showed that PCBs in lake trout increased with the length of benthic-based food web and with the lipid content of tissue. Simon et al.[45] have analyzed the trophic transfers of metals (cadmium and methylmercury) between the Asiatic clam *Corbicula fluminea* and crayfish *Astacus astacus*. Their experimental data suggest a small risk of Cd transfer between the crayfish and predators, humans included. However, methylmercury distribution in muscle and accumulation trends in this tissue represent an obvious risk of transfer.

3.4 MODEL ECOSYSTEMS

A wide variety of model ecosystems have been developed and used for fundamental and applied aquatic ecological research. Review articles describing these systems are available for microcosms,[46] freshwater mesocosms,[47,48] marine mesocosms,[49–51] and artificial streams.[35] Kennedy et al.[52] review and summarize in table format representative examples of experimental designs used by researchers to study the fate and effects of xenobiotic chemicals in freshwater and marine aquatic environments.

3.4.1 Microcosms

Microcosms have been employed extensively in studies of contaminant effects on community-level structure and function. These systems can be viewed as an intermediate to laboratory tests and larger-scale mesocosms. Microcosms — whether indoor or outdoor — may not accurately parallel natural systems at all levels of organization, but important processes such as primary productivity and community metabolism can be studied in them, even in cases where systems cannot support all of the trophic levels found in larger systems.

Outdoor microcosms have taken a variety of forms including small enclosures in larger ponds[53–56] and free-standing tanks of sizes ranging from small aquaria (12 L)[57] suspended in a natural pond to vessels constructed of fiberglass,[58–63] stainless steel,[64] or concrete[65–67] or excavated from the earth.[68,69] Other researchers have used plastic wading pools[70] and temporary pond microcosms.[71]

3.4.2 Mesocosms

Likewise, an assortment of mesocosm ecosystems have been devised. Most mesocosm systems can be categorized into one of several systems based on their construction.

3.4.3 Enclosures

Limnocorrals — large enclosure systems in open-water areas of lakes — have been used extensively by Canadian researchers. These are systems designed to partition and encompass natural planktonic populations in order to study their responses to perturbations. Kaushik et al.[72] described limnocorral construction. The impact of pesticides on plankton populations has been a frequent focus of enclosure studies.[72–79]

Littoral enclosures, which border the edge of a pond or lake, have been developed and used by the U.S. EPA Research Laboratory at Duluth, MN. These systems (5 m × 10 m surface area) have been used to study the fate and effects of pesticides on water quality parameters, zooplankton, phytoplankton, macroinvertebrates, and fish.[80] Brazner et al.[81] described littoral enclosure construction and endpoints studied and discussed variability (coefficients of variation) of different indicators.

3.4.4 Pond Systems

Replicated pond mesocosms have been used extensively to evaluate pesticide fate and toxicological effect relationships.[82] Most ponds used for this purpose are dug in the earth and range in size from 0.04 to 0.1 hectares in surface area.

3.4.5 Artificial Streams

Unlike lentic mesocosms, there have been no attempts to standardize the conduct of lotic experimental systems, even though experimental stream ecosystems have been employed to test chemical effects (Tables 3.1 and 3.2). Invariably, the use of these constructed stream ecosystems

Table 3.1 Use of Stream Mesocosms: Physical Parameters

References	Circulation	Length/Size	Volume/Flow
Austin et al.[93]	FT	0.245 m	18.0 L/min
Belanger et al.[94]	FT	20.0 L	1.6 L/min
Clements et al.[95]	FT	0.76 m	1.6 L/min
Clements[96]	FT	0.76 m	1.0 L/min
Farris et al.[242]			
Belanger et al.[243]	FT	12.0 m	166.0 L/min
Guckert et al.[244]			
Lee et al.[245]			
Dorn et al.[97–99]	FT	4.9 m	77.0 L/min
Gillespie et al.[100–102]			
Harrelson et al.[103]			
Kline et al.[104]			
Haley et al.[105]	FT	110.0 m	1241.0 L/min
Hall et al.[106]			
Hermanutz et al.[107]	FT	520.0 m	0.57 m^3/min winter, 0.76 m^3/min
Kreutzweiser and Capell[108]	FT	6.0 m	14.0 L/min
Crossland et al.[109]	PRC	5.0 m	10.0 L/h
Maltby[110]			
Mitchell et al.[111]			
Pascoe et al.[112]			
Richardson and Kiffney[113]	FT	2.5 m	0.1–0.2 L/s

Note: RC = recirculating; FT = flow-through; PRC = partially recirculating. Single-spaced references imply use of the same systems.

has involved studying the responses of macrobenthic communities to multiple chemicals, chosen to be "typical" of what might be expected in natural streams. Response variables have differed among previous lotic studies and depend on the research questions asked and approaches taken (Table 3.2). Presently, there are relatively few such systems in the world. Costs associated with building and operating lotic mesocosm systems often limit the number of experimental units. Thus, most stream mesocosm studies have evaluated single chemicals at multiple concentrations with or without treatment replication. The designs range from small recirculating streams[83] to large, in-ground flow-through streams of 520 m in length.[84] Most constructed streams are 3 or 4 m long and around 50 cm wide. Volume flows range considerably and are usually selected to approximate the regional conditions.

The endpoints selected for study are almost always functional and structural endpoints of algae or benthic invertebrate populations (Table 3.2). The size and scale of the artificial streams preclude the use of predator fish, except for the very large systems. For the short term, studies pools may be constructed downstream to place herbivorous minnows or larval predators such as bluegill or bass.

Regression designs are common and suggested for use in risk assessment when experimental units are scarce.[85,86] Despite problems associated with pseudoreplication,[87] lack of replication may be justified because intraunit variability due to treatments can be substantially more important than interunit variability.[88] Limited experimental stream studies have used factorial designs or addressed issues of multiple stressors.[89,90] Factorial designs use ANOVA (requires replication), are efficient, and allow investigation of multiple-factor interactions (multiple stressors).[91,92] Tables 3.1 and 3.2 present representative examples of experimental designs and endpoints used in outdoor stream mesocosms.

3.5 DESIGN CONSIDERATIONS

There are many problems to be considered when designing and implementing studies using model systems. These range from the pragmatic (funding, time constraints, etc.) to the heuristic

Table 3.2 Use of Stream Mesocosm Chemicals Tested and Response Variables

References	Chemical(s)	Structural	Functional
Austin et al.[93] (periphyton)	Herbicide	A, biomass	
Belanger et al.[94] (clams)	Cu		Mortality, growth, & bioaccumulation
Belanger et al.[246] (clams)	Surfactant		Mortality, growth, reproduction, cellulolytic enzyme activity, larval colonization
Belanger et al.[247] (invertebrates)	Surfactant	A, biomass, H′, trophic functional feeding group	Drift
Belanger et al.[243] (periphyton, protozoa, invertebrates)	Surfactant	A, biomass, H′	Productivity, biodegradation of test chemical
Clements et al.[96] (invertebrates)	Cd, Cu, Zn	A	Invertebrate survival, drift, & predation rate
Clements et al.[95] (invertebrates)	Cu	A	
Crossland et al.[109] (invertebrates)	Effluent	A	Feeding rates and drift
Dorn et al.[97, 98, 99] (fish, inverts, macrophytes, periphyton)	LAE	A, biomass	Drift, mortality, growth, reproduction, chlorophyll & pheophytin
Farris et al.[242] (clams and snails)	Zn		Cellulolytic enzyme activity bioaccumulation
Gillespie et al.[106, 108] (invertebrates.)	LAE	A	Drift
Gillespie et al.[100] (invertebrates)	LAE	A	Drift, feeding rates
Guckert et al.[244] (periphyton, protozoa, invertebrates)	Surfactant	A, functional group composition, H′	Primary production, drift, recruitment
Haley et al.[105] (fish, invertebrates, periphyton)	Effluent	A, biomass, H	Mortality, growth, histopathology, chlorophyll, production
Hall et al.[106] (fish, invertebrates, periphyton)	Effluent	a, biomass	Mortality, growth, histopathology, reproduction, chlorophyll, production
Harrelson et al.[103] (fish)	LAE		Mortality, growth, reproduction, behavior
Hermanutz et al.[107] (fish)	Se		Bioaccumulation, mortality, growth, development, reproduction
Kline et al.[104] (fish, zooplankton)	Surfactant	A	Mortality, growth, reproduction, swimming performance
Kreutzweiser and Capell[108] (Invertebrates)	Pesticides		Mortality, drift
Lee et al.[245] (periphyton)	Surfactants	A, community biovolume	Surfactant biodegradation, heterotrophic respiration
Maltby[110] (invertebrate)	Effluent		Scope for growth
Mitchell et al.[111] (invertebrates, periphyton)	Lindane	A	Drift, feeding rates, photosynthesis rate
Pascoe et al.[112] (invertebrates, periphyton)	Cu, lindane, 3,4-dichloroaniline (DCA)	A, biomass	Drift, growth, precopula disruption, photosynthesis, chlorophyll
Richardson and Kiffney[113] (invertebrates, periphyton)	Cu, Zn, Mn, Pb	A	Emigration (drift), chlorophyll, bacterial respiration

Note: H′ = invertebrate diversity; A = abundance; LAE = linear alkyl ethoxylate surfactant.

(What are the study goals? What levels of realism are desired?). The physicochemical and biotic features of a model system will determine to what extent, if any, the system will represent a natural one. These factors also influence contaminant fate and effects in model ecosystems. System design is therefore important in defining what inferences may be drawn from the results of surrogate systems and extrapolated to natural aquatic ecosystems. Using results from the scientific literature on model ecosystems, the following sections seek to provide a synthesis of some key experimental design considerations.

3.5.1 Scaling Effects in Artificial System Research

The question of whether artificial aquatic systems are reliable surrogates for natural ones is strongly linked to system scale. Scale includes not only size and physical dimensions of a microcosm or mesocosm but also its spatial heterogeneity and attendant biotic components. Crucial physical and chemical processes behave differently as both a function of, and contributor to, scale. Thus, scaling effects can have implications for community structure and the resultant functional attributes of the system.

The choice of spatial and temporal scales in an experiment may determine whether changes in selected endpoints can be detected during a study and is, therefore, vital to the research methodology. Frost et al.[114] stated that "typically scale has not been incorporated explicitly into sampling protocols and experimental designs." The choice of appropriate time scales, for example, in model aquatic system research must be considered in the selection of both study duration and sampling frequency intervals between sampling events. Both temporal elements should consider life cycles and periodicities of important system species. Sampling intervals should also consider the temporal behavior of key physicochemical processes (often related to pesticide fates and half-lives) and, ultimately, the longevity of the surrogate system as well.

Microcosms, particularly laboratory ones, require little or no equilibration time prior to their use as test systems. Results can be observed quickly, but the systems are not self-sustaining and tend to become unstable over time. Because laboratory microcosms can sustain only a limited number of trophic levels, usually composed of small organisms with short lifespans (days to weeks) and rapid turnover times, frequent sampling regimes and short study durations are required. Unfortunately, frequent sampling in small systems may be damaging to the system and its biotic contingent.[32]

The size and overall dimensions of systems in ecotoxicological research have idiosyncratic implications in the outcome of the project. Dudzik et al.[115] cite the prevalence of biological and chemical activity on the sides and bottoms of microcosms as one of the most important problems in microcosm research. Edge effects have been noted and discussed in enclosure studies as well,[116,117] but the ecological implications of such scaling ramifications in ecotoxicological and ecological studies have yet to be resolved. These concerns present a unique challenge in the toxicological arena, as scaling effects may ultimately hinder the validation process, which is becoming increasingly critical in decision-making, policy-making, enforcement, and litigation issues.

The cause of some edge effects in ecotoxicological work pertain to materials from which littoral and pelagic enclosures are constructed, since they may serve as sorption sites for some toxins (via adsorption).[118–120] This problem was also linked to physical scale and system dimensions, as the ratio of the wall surface area to water volume is greater in smaller test systems. Smaller enclosures and microcosms may remove disproportionate amounts of pesticide from the water column via absorption to container walls.[32]

A study investigating the role of spatial scale on methoxychlor fate and effects in three sizes of limnocorrals found pesticide dissipation was more rapid than expected in the smallest enclosures.[74] These findings were associated with less severe impacts and quicker recovery of zooplankton populations in the smallest enclosures. Such studies are, in part, contingent upon an understanding of the role of spatial factors in biotic organization.

In an attempt to address such concerns, Stephenson et al.[116] studied the spatial distributions of plankton in limnocorrals of three sizes (equal depths) in the absence of perturbations to assess the viability of such systems in community-level toxicant research. The most predominant edge effects were reported in the largest enclosures, where macrozooplankton occurred in significantly higher numbers than microzooplankton. Perhaps differentially sized edge zones contributed to distributional differences (edge zones constituted 100% of the volume of the smallest enclosures), or circular currents that occurred only in the largest enclosures could have affected zooplankton distributions. The actual cause of the zooplankton distribution differences was not determined, but basic research of this type provides important "background" data to better recognize treatment effects once disturbance has been introduced.

The limited size and accompanying physical homogeneity of many microcosms creates additional problems: (1) they are particularly susceptible to stochastic, often catastrophic, events from which system recovery may be highly variable relative to mesocosms,[32] and (2) the often limited species compositions of microcosms induce overly strong biotic couplings, resulting in drastic population oscillations and competitive exclusion events.[114] This latter problem was established early in ecological study with the research of Lotka[24] and Gause[20] when only a limited number of population cycles could be established in small microcosms.

As discussed previously, large outdoor systems, such as pond mesocosms, require colonization and equilibration times of months to years because they may incorporate many trophic levels, and an extensive number of interactions occur as a function of greater physical scale. Frequent (i.e., daily) sampling for many selected parameters may not be logistically feasible or even necessary to detect effects at the population or community levels. Study durations are by necessity and design much longer, since impacts at higher levels of organization, particularly indirect effects, may not be immediately evident. Such systems are presumably self-sustaining enough to permit the study periods necessary for detecting effects at these higher levels.

A variety of scales are to be considered when designing studies using surrogate systems because the scales discussed herein will affect the outcome of research whether the experimenter acknowledges them or not. Most researchers are aware of the implications of system size in fate and effects research, though indirect results in these studies may not always be perceived or attributed to their actual causes. Temporal aspects are also recognized, though the interaction of timing and spatial factors is still not well understood. The treatment of these scaling considerations in a more integrated fashion will ultimately enhance the predictive value and ecological relevance of the results.

3.5.2 Variability

Variability is inherent in any biological system, but the limiting of variability is often critical to the scientific process wherein the ultimate goal is prediction. Variability occurs within and among systems such as microcosms or mesocosms. Replication of treatments and the use of controls are necessary to distinguish natural variation from the effects of treatment.[83] Sampling replication can assess intrasystem heterogeneity resulting from spatiotemporal variation in community structure and physicochemical parameters.

Studies of stream benthos, however, indicate that the number of samples required to obtain adequate representation of the community would be quite high and no doubt impractical.[121–123] There is also the risk that accepted sampling regimes in lentic and marine research may similarly underestimate inherent variability in these more "homogeneous" systems. Assessing variability through such methods as coefficients of variation[123] and determining the number of sampling replicates that would be adequate to ensure representative sampling become critical in ecological research. Green[124] emphasizes the importance of conducting pilot studies in ecological research and having adequate replication, both in treatment and sampling. Unfortunately, even though the number of replicates needed to detect changes of a given magnitude can be determined *a priori*,

such estimates do not always match the availability of research resource personnel, space, or time.[125] Therefore, sampling must be focused on those variables that convey scientific meaning and provide investigators with resolving power for detecting differences. At the present time these variables are primarily structural.[126]

An alternative to increased sampling replication has been to employ less diverse systems as a means of reducing intrasystem variation. This approach may result in simplification to the extent that model systems will not resemble the natural systems they are attempting to "mimic," thereby affecting predictability[4] and applicability. However, mimicking natural systems may not provide the best experimental models, according to Maciorowski,[127] who further emphasizes that the challenge in ecotoxicological research is to find those phenomena that can be simplified to several salient interactions. Extrapolating the results of model system research to natural systems remains one of the major areas of contention regarding their use.

In any manipulative experiment the assumption is made that observed effects (i.e., significant differences) are due to the treatment. Often, however, observed differences among treatment levels, or even among replicates within a treatment level, may be influenced by factors other than those being tested.[87] When this occurs, it is impossible to separate the covariates, and the hypotheses being tested at the onset may be invalidated. Variability among systems is a frequent contributor to this phenomenon.

Sources of variability may be structural, physicochemical, or biotic. Biotic variability can occur at a variety of levels within the ecosystem and markedly affect system-level processes such as productivity and respiration. Variability can be due to differences among systems prior to study initiation, or it may result from changes that occur during the study. Hurlbert[87] discusses both initial or inherent variability among systems and the temporal changes that occur within systems.

The confounding influence of system variability in ecotoxicological studies involving microcosms and mesocosms has long been recognized,[115] but no uniform approach to a solution has been reached. Some researchers[128,129] have attempted to assess inherent variability and determine the amount of sampling replication required to detect treatment effects. Other solutions involve establishing more stable communities in the hopes that equilibrium within systems will occur, enhancing both similarity among systems and increasing system realism. Giesy and Odum[4] suggest that higher trophic levels assert a controlling influence on lower trophic levels in microcosms being used for effects studies. Giddings and Eddlemon[130] have attempted to assess microcosm variability for the purpose of determining the validity of using such model systems in toxicological research.

Methods of limiting intersystem variability sometimes employ design features. One routinely applied method in mesocosm — and sometimes in microcosm — research circulates water among the systems prior to study commencement.[60,131–134] Heimbach[135] developed outdoor microcosms in which three interconnected tanks were joined via wide locks (passageways). Water exchange was allowed during an acclimation period, followed by isolation prior to pesticide application. Systematic "seeding" of the systems with biota and sediments from mature ponds may minimize variability resulting from nonuniform distributions of macroinvertebrates and macrophytes.[136]

3.5.3 Colonization and Acclimation

Ecological maturity of mesocosms affects the degree of variability of both physicochemical and biological parameters used to investigate the impact of contaminants.[137] The establishment of biological organism communities is a critical part of microcosm and mesocosm experiments. Adequate time is required to establish a number of interacting functional groups.[4] The colonization methods used in microcosm and mesocosm research will vary predominantly as functions of system size, the type of study, whether it is fate- or effect-oriented, and the endpoints of interest.[138] Studies using limnocorrals and littoral enclosures usually have no acclimation period because it is assumed these systems enclose established communities.[73,119,139] In stream mesocosms stabilization periods of 10 days,[140] 4 weeks,[109] or 1 year[141] have been reported.

The duration of the maturation period for pond mesocosms varies from 1 to 2 months[39] to 2 years.[7] Following initial system preparation a period of acclimation is usually required to allow the various biotic components to adjust to the new environment and establish interspecific and abiotic interactions. Duration of acclimation time depends on system size and complexity. Systems with more trophic levels will form more complex interactions that may require much more time to equilibrate than small systems with fewer species. The time needed to equilibrate will increase with initial system complexity, although the use of natural sediments usually shortens the duration of the stabilization period because natural maturation processes are enhanced.[52] During this acclimation period in outdoor systems the initial preparation of the systems is typically controlled, and natural colonization by insects and amphibians will contribute to biotic heterogeneity and system realism. Continuous colonization, however, presents further problems in that each system tends to follow its own trajectory through time. These trends are most apparent in small-scale systems and in systems that have been in operation longer.[35] Circulation of water between the different systems has frequently been proposed as a way to limit intersystem variability during this period.[142,143,68]

3.5.4 Macrophytes

Aquatic vascular plants play a key role in system dynamics within natural lakes, and their presence in model ecosystems makes them more representative of littoral zones in natural systems. However, once introduced into model ecosystems, macrophyte growth is difficult to control and may vary greatly among replicates. This is of particular concern in field studies because macrophytes can influence the fate of chemicals, the occurrence and spatial distribution of invertebrates, and, if present, the growth of fish. Thus, variations of plant density and diversity in model ecosystems can be a major contributor to system variability and subsequent inability to detect changes in ecosystem structure and function.

Macrophyte densities can affect chemical fate processes by increasing the surface area available for sorption of hydrophobic compounds. The pyrethroid insecticide deltamethrin accumulated rapidly in aquatic plants and filamentous algae during a freshwater pond chemical fate study.[144] Caquet et al.[137] reported the residues of deltamethrin and lindane in the macrophyte samples for 5 weeks after treatment but never in the sediment. A microcosm study with permethrin demonstrated similar results, with extensive partitioning to macrophytes.[145] Weinberger and others[146] evaluated fenitrothion uptake by macrophytes in freshwater microcosms and found that pesticide accumulation was two- to fivefold greater in the light compared with microcosms in the dark. They concluded that both uptake and degradation of fenitrothion appeared to be photocatalyzed.

Macrophytes can also affect physicochemical composition in surrounding waters, influencing the distribution and community structures of many aquatic organisms.[147] In addition, macrophytes provide three-dimensional structure within constructed ecosystems, which affects organism distribution and interactions. Brock et al.[148] in a study with the insecticide Dursban 4E observed considerable invertebrate taxa differences between Elodea-dominated and macrophyte-free systems. Other workers have shown that macroinvertebrate community diversity is influenced by patchy macrophyte abundance[149] and specific macrophyte types.[150,151] Cladoceran communities are also associated with periphytic algae on aquatic macrophytes.[152]

Impacts of chemicals on macrophytes densities may cause indirect effects on organisms by influencing trophic linkages such as predator-prey interactions between invertebrates and vertebrates. Bluegill utilization of epiphytic prey may be much greater than predation upon benthic organisms.[153] Excessive macrophyte growth may force fish that normally forage in open water to feed on epiphytic macroinvertebrates, where the energy returns may not be as great.[154] Fish foraging success on epiphytic macroinvertebrates depends on macrophyte density[155] and plant growth form (i.e., cylindrical stems vs. leafy stems).[107,156–158] Dewey[159] studied the impacts of atrazine on aquatic insect community structure and emergence. Decreases in the number of insects in this study were

correlated with reductions in aquatic macrophytes and associated algae and were not a direct effect of the pesticide.

The wide range of chemical, structural, and biotic interactions dependent upon macrophyte type and density as outlined above emphasizes the central role of this part of the community in lentic systems. It is apparent that the design of surrogate ecosystems needs to consider plant density and diversity as a contributor to system variability and the inability to detect ecosystem changes.

3.5.5 Fish

Whether to include fish, what species or complex of species to select, the loading rates, and their potential for reproduction are critical factors to consider in experiment design. Fish populations are known to have direct and indirect effects on ecosystem functioning. Fish predation is known to alter plankton community composition,[160–162] and the presence of fish in limnocorral or microcosm experiments may alter nutrient dynamics and cycling.[163,164] For example, during an outdoor microcosm experiment, Vinyard and others[162] found that filter feeding cichlids altered the "quality" of nitrogen (shifting dominant form) and decreased limnetic phosphorus levels via sedimentation of fecal pellets. Additionally, unequal fish mortality among replicate microcosms may influence nutrient levels independently of any other treatment manipulations.[165]

In separate limnocorral studies Brabrand et al.[166] and Langeland et al.[167] both concluded that fish predation alters planktonic communities in eutrophic lakes and that the very presence of certain fish species may contribute to the eutrophication process. These studies offered a number of interesting hypotheses regarding fish effects in limnetic systems; unfortunately, the experimental designs of these studies lacked treatment replication, limiting their inferential capability.

Many studies completed in the United States from 1986 through 1992 under U.S. EPA guidelines[33] for pesticide studies require that mesocosms include a reproducing population of bluegill sunfish (*Lepomis macrochirus* Rafinesque). Presumably, these fish and their offspring are integrators of system-level processes, and differences in numbers, biomass, and size distribution between pesticide exposure levels provide requisite endpoints for risk-management decisions. Chemical registration studies by Hill et al.,[47,168] Giddings et al.,[169] Johnson et al.,[65] Morris et al.,[170] and Mayasich et al.[171] have determined that the abundance of young bluegill in mesocosm experiments obscured or complicated the evaluation of pesticide impacts on many invertebrate populations. This is consistent with Giesy and Odum's[4] suggestion that higher trophic levels assert a controlling influence on lower trophic levels in microcosms being used for effects studies. Ecological research with freshwater plankton and pelagic fish communities indicates that both "top-down" and "bottom-up" influences affect planktonic community structure and biomass.[172–174] These relationships have not been investigated to the same degree in littoral zone communities, and the role of benthic macroinvertebrates in these trophic relationships requires further study. Along these lines Deutsch et al.[175] stocked largemouth bass in pond mesocosms in order to control unchecked bluegill population growth, thereby potentially limiting intersystem variability and provide a more natural surrogate system. However, the desirability of adding bass to mesocosms must be balanced against possible increases in experimental error variances that may result from differential predation on bluegill if variable bass mortality occurs in the ponds.[176] The only way to control variability in predation of bluegill would be to maintain equal levels of predator mortality in all ponds.

The requirement of using a single test-fish species (bluegill sunfish) in mesocosm experiments may not be sufficiently protective of natural fish communities, for a number of reasons. First, the inherent sensitivity of other fishes compared with bluegill is not known with any degree of certainty. Second, due to a variety of life history adaptations, other fish might experience differential exposure to chemicals. For example, surface-dwelling fish, such as top-minnows, would potentially be exposed to high initial pesticide concentrations found in the surface layer following treatment. Alternatively, contaminants that sorb to sediments (including many pesticides) might be expected to impact bottom-feeding fish selectively. Drenner et al.[177] studied the effects of a pyrethroid

insecticide on gizzard shad, *Dorosoma cepedianum*, in outdoor microcosms. These fish are filter feeders and commonly have large amounts of bottom sediments and detritus in their digestive systems. This study[177] is unique in the use of "nonstandard" fish species. Similar field studies utilizing other fish species should be pursued in order to evaluate the influence of feeding behavior and habitat selection on chemical exposure. Following appropriate research it is conceivable that a multispecies assemblage (i.e., surface feeder, water-column planktivore, and bottom feeder) might eventually be used to better represent potential impacts to natural fish communities.

The reader is cautioned, however, that additional research in this area is needed. Scaling is an important consideration, and criteria for fish stocking levels are highly dependent on system size. The fish population should not exceed the "carrying capacity" of the test system.[168] Biomass densities should generally not exceed 2 g/m^3.[178]

It may be useful to stock the mesocosm with a low adult density and remove adults and larvae after spawning. However, the life stage, number, and biomass of fish added will depend on the purpose of the test. For example, should the emphasis be on an insecticide, larval fish may be added to monitor their growth in relation to the invertebrate food base.

3.6 DOSING CONTAMINANT EXPOSURE

3.6.1 Chemical Fate Considerations

The primary assessment of the potential that a chemical has to affect an aquatic ecosystem is the prediction of its environmental fate. This includes how it is transported, its persistence, its distribution or partitioning among various environmental compartments, and an estimation of its bioavailability and potential to bioaccumulate.[179] Various chemical characteristics affecting fate are currently measured in the laboratory such as solubility, octonal/water and soil/water partitioning, and bioaccumulation in different organisms. More comprehensive estimates of the fate of the chemical are manifested in mathematical and physical models of aquatic ecosystems. Boyle[179] provided a list of examples of different representative types of mathematical models from the literature used to determine the fate of a potential contaminant. Rand et al.[63] described the design, specific techniques, and fate of pyridaben in microcosms and discussed the usefulness of microcosms to study the fate of a chemical under environmental conditions that are more representative of the field.

3.6.2 Application Method and Dosing

Test chemicals, such as pesticides and other toxicants, are commonly applied to treatment mesocosms, with application method, frequency, and concentration of test chemical used being the major considerations.[137] The method used for application of the test chemical can have considerable effect on its fate and the exposure of organisms.[137]

Because of their scale microcosms usually lend themselves to somewhat less complicated methods of chemical application compared to similar mesocosm experiments. Microcosm experiments have used systems to distribute the test material that range from simply pouring the solution into the test chamber and stirring,[180] to a continuous-flow system.[181] Stay et al.[180] poured in the selected concentration of the chemical and used a magnetic stirring bar to thoroughly mix the contents of the microcosm before any measurements or samples were taken. Staples et al.[181] used a flow-through system in which dilution water and the chemical mixture flowed into a mixing tube and dispensed at three subsurface levels. A stirring paddle was employed to consistently mix the chemical solution in the microcosm. In their edge-of-field runoff study Huckins et al.[182] placed topsoil in a flask, spiked it with pesticide, and mixed it thoroughly. Then water was added, and mixing was achieved using a magnetic stirring bar.

Preparing a stock solution in a diluter or mixing chamber of some type and pumping it into the system is also common in these studies.[181,183,184] For example, experiments by Cairns et al.[183] and Pratt et al.[184] demonstrated this application procedure in which the dilutent flowed to a headbox, into a mixing chamber where the toxicants were added, and delivered to the microcosm test chambers with a peristaltic pump. Koerting-Walker and Buck[185] added their chemical to sediment samples to use in their 135 mm × 15 mm sediment tube microcosms.

Outdoor microcosm experiments also demonstrate a variety of dosing procedures. Pratt et al.[184] added and mixed chlorine in 130-L sediment, and water-filled polyethylene bags that were floating in a lake. Lehtinen et al.[186] used a continuous-flow system with 400-L fiberglass tanks to which effluent was continuously pumped. In his simulated wetland microcosms Johnson[187] prepared and poured a soil/water slurry onto the water surface to simulate field runoff. Similar methods were used by researchers at the University of North Texas Water Research Field Station,[65,170] where a pesticide/water solution and pesticide/soil/water slurry were prepared and poured onto the water surface of concrete microcosms to simulate spray drift and runoff events, respectively.

Complexity of dosing methods for mesocosm studies varies with the purpose of the study. The contaminant may be added to the water surface or subsurface or on the sediments by pouring the active ingredient or a mixture of soil and toxicant surface,[188–191] spraying with hand-held sprayers and spanners that release the solution onto the water surface,[192–198] or pumping via a flow-through system.[199–201] Subsurface dosing can also be achieved by placing the spray nozzle or hand-held sprayer below the water level.[131,202]

Some application methodologies are quite innovative. Wakeham et al.[203] spiked the water column of their fiberglass tank mesocosms with volatile organic compounds (VOCs), using Teflon tubing that released the VOC at about mid-tank depth, while the tank was mixed for several hours to ensure uniform VOC dispersal in the water column. Stephenson and Kane[204] applied their stock solution by allowing it to run out a separating funnel through a diffuser that was raised and lowered within the water column. De Noyelles et al.[195] used a boat to achieve access to multiple portions of a pond and dispensed a herbicide through a fine screen just below the water surface so that undissolved portions would be finely dispersed. Lay et al.[205] soaked strips of polyethylene in p-chloroaniline and placed these in the mesocosm to achieve a slow-release technique type of application. Giddings et al.[169] used a circulating system of reservoirs and tanks to simulate a typical runoff event. A stock-solution reservoir was metered to ensure the desired concentration passed into the mixing tank. The mesocosm water was then circulated into the mixing tank and pumped back into the mesocosm at three different places to ensure that each test system received a similar hydrologic treatment.

Reviewing the literature, one comes to realize that there are nearly as many application methods as there are researchers designing microcosm and mesocosm studies. It should be noted that the method chosen for the application of the test material can have considerable influence on its fate and subsequent exposure to organisms. For example, the size of droplets reaching the water surface from a spray nozzle held near the water surface of an experimental system may differ from that of droplets deposited on a natural body of water following agricultural application to adjacent land.[1] In turn, droplet size may be critical since volatilization from the water-surface microlayer can be a very rapid process and may be a major route of dissipation.[206] Thus, the decision to either spray a chemical on the water surface or inject it underneath can have a major influence on its half-life. Clearly, the method of test material application must be chosen so that realistic exposures are obtained.

3.7 EXPERIMENTAL DESIGN AND STATISTICAL CONSIDERATIONS

3.7.1 Experimental Design Considerations

Key issues in designing microcosm and mesocosm tests that need attention are replication of treatments, sample size and power, optimization criteria in design selection, choice of number and

spacing of dose levels, inference on "safe dose," and defining the dose-response curve.[207] Biological variables measured in field studies have a large amount of variability associated both within and between test systems that can decrease our ability to detect ecosystem effects.[208] One approach to improving designs is by reducing variation. While this may be done by increasing the number of microcosms, this is not always possible due to cost. Information that can be gathered through power analysis can be used to maximize manpower resources and project expenditures to produce the best possible sample design as well as to determine which biological parameters should be included in a study protocol.[208]

3.7.2 Endpoint Selection

Toxicological endpoints are values derived from toxicity tests that are the results of specific measurements made during or at the conclusion of the test.[209] Two broad categories of endpoints are widely used: assessment and measurement endpoints. The determination, selection, and measurement of assessment and measurement endpoints are among the most critical factors in conducting an ecological risk assessment.[210] Assessment endpoints refer to the population, community, and ecosystem parameters that are to be protected, such as population growth rate, or something specific and quantifiable, such as eutrophication.[209–211] Measurement endpoints refer to the variables measured, often at the individual level, that are used to evaluate the assessment endpoints.[209] The measurement endpoints describe the variables of interest for a given test. The most common measurement endpoints include descriptions of the effects of toxic agents on survival, growth, and reproduction of a single species. Other measurement endpoints include descriptions of community effects (respiration, photosynthesis, or diversity) or cellular effects. In each case the endpoint is a variable that can be quantitatively measured and used to evaluate the effects of the toxic agent on a given individual, population, or community. Sometimes it is not possible to examine the assessment endpoint directly. In this case measurement endpoints are used to describe the organism or entity of concern.[210] The underlying assumption in making toxicological endpoint measurements is that the endpoints can be used to evaluate or predict the effects of toxic agents in natural environments. Suter[211] discussed the endpoints for the different levels of organization: suborganismal endpoints, organismal endpoints, population endpoints, and ecosystem endpoints. EPA risk assessment guidelines provide information on how endpoints can be used in the environmental risk-assessment process.[212]

3.7.3 Level of Taxonomic Analysis

Frost et al.[114] discussed as a scale of concern the taxonomic or functional levels to which organisms are identified or the degree of resolution. In this context organisms may be analyzed in trophic levels or functional groups or at some taxonomic level depending on the research focus and the questions proposed. Theoretically, species-level identifications have the greatest potential for identifying impacts of chemicals on aquatic organisms.[213] However, from a practical and technological standpoint, our ability to identify many organisms to the lowest taxonomic levels is limited. Many endpoints measured in mesocosm and microcosm studies require the identification of invertebrates. Increasing the taxonomic resolution used in a study increases the expertise and time needed to complete a study. As a result, taxonomic resolution used in field monitoring studies has traditionally been determined by budgetary considerations, the familiarity of the researchers with critical taxa, and the availability of reliable identification guides.

Decisions regarding the appropriate level of identification should be made with some knowledge of an organism's habitat and life history, combined with information regarding the fate of the chemical. Coarser identifications may obscure results and failure to identify organismal responses to stressors. Taxonomic sufficiency has been defined as the highest level of identification where toxic response is similar to that occurring at the lower levels of identification.[214,215] In a mesocosm

study Kennedy et al.[216] demonstrated that identification to the family level for a dominant macrobenthic group failed to detect statistical differences when compared to the reference populations that were evident if subfamily identifications were determined. However, identification of these invertebrates to the genus and species level failed to detect statistical differences. This observation demands that we consider the influence counts of organisms made at lower taxonomic levels can have on statistical tests. As diverse populations are identified to lower taxonomic levels progressively lower counts and greater variability result at each level. Lower counts and increased variability results in tests of lower statistical power, which compromises our ability to detect statistical differences between populations.[208,217] If preliminary studies indicate that greater taxonomic resolution is needed, then specialized sampling methods and strategies need to be developed to increase sampling effectiveness, thereby increasing counts and reducing variability.

3.7.4 Species Richness, Evenness, Abundance, and Indicator Organisms

The presence of species and their relative abundance are used as a measure of the degree of contamination of an aquatic habitat.[218,219] These parameters are often used to calculate diversity indices. Although diversity indices have been shown to be insensitive to slight to moderate perturbations,[220,221] they are still reported in biological monitoring.[222,223] Species richness (the number of different species) and evenness (the distribution of individuals among species present) have been shown to better reflect impacts to aquatic communities than diversity indices.[224] The abundance of species has been a standard measure for "good quality" habitat since early studies of habitat perturbation.[225]

3.7.5 Univariate Methods

Univariate techniques, particularly analysis of variance (ANOVA) using parametric or log (x + 1) transformed data, are the most commonly used analysis method, with either Dunnett's or the Student-Newman-Keuls (SNK) being the most common post hoc test.[208] Linear regression and correlation have also been used, but with less frequency.[226] When the assumptions of parametric tests, normality, and homogeneity cannot be met, nonparametric tests, such as Spearman, Wilcoxon rank statistic, and the Kruskal-Wallis tests, have been employed.[208]

However, 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 powers of the statistical tests are quite low due to the few replicates, the high inherent variance of many of the biotic variables, and the zero counts for some organisms that were eradicated during the experiment.[227] Ammann et al.[217] and Kennedy et al.[208] proposed a statistical program, TAXALLN.Q, that overcomes the problems of high variability and zero counts as well as provides a measurement of statistical power that is an important design criterion in experimental studies. 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 the statistical power and the concentrations chosen by the experimenter that they are artifacts of the experimental design rather than reflections of the intrinsic hazard of the toxicant.[228]

3.7.6 Multivariate Methods

Highly variable data are common in aquatic mesocosm studies. This can be a problem when univariate statistical procedures are used to analyze these data. The statistical power to detect effect is so low that the usefulness of conducting the analysis is questionable because even if effects exist, they may not be detected.[208,217,228] However, even if the univariate procedures are performed with satisfactory power, the interactions between species, populations, or communities are usually not

considered and are therefore inadequate to elucidate ecological effects.[124] Additionally, standard univariate statistical methods can only properly analyze the information on a limited number of taxa (usually the abundant ones).[229,230] Furthermore, with the vast number of potentially confounding variables that can affect population dynamics, such approaches can lead to problems in determining cause and effect.[231]

A variety of multivariate techniques offer potential solutions to these analytical and interpretational problems.[232] Analyzing ecotoxicological field studies with multivariate techniques has some clear advantages. Community-level approaches have more ecological relevance than studies at lower levels of biological organization, and so far no compelling evidence suggests that they are any less sensitive at detecting the biological effects of pollution, especially when multivariate analyses are applied. Multivariate statistics analyzes all available data, and it is more likely to discriminate between treatments than simple univariate summaries of the same data.[210] Consequently, these approaches may be more helpful in determining the ecological significance of toxicant impacts and may help the evaluator of the study reach conclusions based on ecologically important effects, a fundamental responsibility in field studies.[233] Cost effectiveness is important, and costs can be reduced dramatically by considering taxonomic sufficiency and sampling design appropriate for the subsequent statistical analysis.[234] Multivariate techniques are also ideal for handling large amounts of data and endpoints more effectively. Kedwards et al.[67] showed how multivariate techniques can aid in the interpretation of biological monitoring studies, which present difficulties related to the sometimes semiquantitative nature of the data and the unavailability of true control sites, replication, and experimental manipulation.

Ludwig and Reynolds[235] provide an introduction to the assumptions, derivations, and use of several multivariate techniques commonly used for the analysis of ecological communities. Van Wijgaarden et al.[236] compare DCA, PCA, and RDA and their usage in mesocosm research in more detail. Van den Brink et al.[237] proposed a multivariate method based on redundancy analysis (RDA). Clarke[238] showed the use of nonmetric multivariate analysis in community-level ecotoxicology, which does not require the restrictive assumptions of parametric techniques. Multivariate techniques have become more accessible and user-friendly with the availability of software such as the principle response curves method[233] and the routines in the PRIMER software package.[238] Major steps have also been taken to produce outputs readily interpretable by both ecologists and environmental managers and regulators. Multivariate techniques now provide ecotoxicologists with powerful tools to visualize and present impacts at the community and ecosystem level.

3.8 SUMMARY

This chapter has focused on key factors that need to be considered in the experimental design of outdoor model ecosystem studies to increase their realism, reduce variability, and ultimately assess the ability of these systems to detect changes. The success in using such systems depends on the establishment of appropriate scales of sampling, both temporal and spatial. As systems need to be sampled with response times for species taken into account, so sampling programs should reflect the variance in activities, life span, and reproductive potential of the species of interest. The failure to observe patterns (predicted or otherwise) or establish equilibrium conditions in experimental plots is often a result of the scales selected.

In performing a model ecosystem study it is important to determine the ecological relevance of effects identified in linked laboratory studies. The studies therefore include several species, functional groups, or habitat types. Interpretation of the field study focuses on effects at the community level, potential indirect effects, and the recovery potential of aquatic populations and communities. A second important reason for conducting a model ecosystem study is to measure consequences of the chemical under environmentally realistic exposure conditions (realistic fate and distribution). Such conditions could include partitioning to sediments and plants, photolysis, and other processes

that may influence the fate of the pesticide. Moreover, these studies incorporate natural abiotic conditions (temperature, light, pH, etc.) that may influence the response of certain organisms.[40]

Based on the discussions and examples given in the previous pages it should be evident that there is no single "best" experimental design or test system.[46] There are a number of options that can be chosen depending on available budgets and facilities. The experimental design needs to address the objectives of the study and must consider the characteristics of the contaminant being studied and the ecosystem being impacted. Outdoor meso- or microcosm studies can be performed with artificial tanks or ponds or in parts of existing ecosystems that are enclosed in a way that causes minimal disturbance. The size of the mesocosm depends on the nature of the study and size and habitat of the organisms of interest. Typically, for "pond" studies, volumes of 1 to 20 m^3 are usually regarded as appropriate for outdoor meso- or microcosm studies. In situations in which planktonic species are the main concern microcosms of 100 to 1000 L may also be appropriate. The size to be selected for a meso- or microcosm study will depend on the objectives of the study and the type of ecosystem that is to be simulated. In general, studies with smaller tanks (about 1 to 5 m^3) are more suitable for shorter studies (up to about 1 month), and larger volumes are more suitable for longer studies (e.g., up to 6 months or longer). Benthic and planktonic invertebrates are often added to mesocosms with sediments and water. Invertebrates typically include rotifers, cladocera, copepods, annelids, benthic crustaceans, gastropods, and insect larvae.

In general, approaches to ecosystem-level testing using surrogate systems have been overly simplistic. Continued development of innovative approaches to data collection and analysis are needed. Historically, mesocosm tests have been viewed as a "series" of single-species tests (the ANOVA statistical approach is currently favored). Ecosystem-level studies often require the prediction of responses of many biological variables given information on the state of environmental or other biological variables.[239]

Methods that evaluate endpoints in a more integrated and holistic fashion should be applied to these studies. Multivariate statistics are one tool for viewing the "big picture." Multivariate techniques, however, are not a panacea for data analysis[240] but should be part of an integrated approach that encompasses both reductionist component analysis and other holistic approaches such as modeling.[127] Ultimately, the value of research using surrogate systems lies in their potential to provide prediction of and probabilities for ecosystem responses to contaminants.

A number of ecotoxicological studies with similar experimental designs have been completed in North America and Europe for purposes of pesticide registration. The existence of such a large number of similarly designed and conducted large-scale model ecosystem studies provides a unique opportunity for further research. If results of these studies were compiled into a common database, analysis of these data could help identify common results, allowing generalization for given classes of stressors. Evaluation of these results in the light of current ecological theory should allow for the formulation of alternative hypotheses, future study designs to test these hypotheses, and subsequent validation (or refutation) of these ideas. A clear-cut, systematic synthesis of the existing information will help enable model ecosystems to reach their full potential as a tool for predicting impacts (as opposed to simply effects assessment). Until this happens, regulators, the manufacturing industries, and researchers will continue to argue over the meaning of community and ecosystem responses measured in these studies. As Cairns[241] succinctly stated: "If environmental toxicology is to come of age, it must begin to ask more searching questions, develop broader hypotheses involving natural systems, and develop models that are validated in landscapes, not laboratories."

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CHAPTER 4

Wildlife Toxicity Testing

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CONTENTS

4.1 INTRODUCTION AND HISTORICAL BACKGROUND

Wildlife toxicology is the study of potentially harmful effects of toxic agents on wild animals. Fish and aquatic invertebrates are usually not included as part of wildlife toxicology since they fall within the field of aquatic toxicology, but collectively both fields often provide insight into one another and both are integral parts of ecotoxicology. Wildlife toxicology endeavors to predict the effects of toxic agents on nontarget wildlife species and, ultimately, populations in natural environments. Wildlife toxicology has often focused on highly visible species, including certain birds and a few mammals, that are of aesthetic or economic interest to humans.[1] However, during the past decade wildlife toxicology has expanded to include the effects of environmental contaminants on reptiles, amphibians, and terrestrial invertebrates.

Reports of anthropogenic environmental contaminants affecting free-ranging wildlife began to accumulate during the industrial revolution of the 1850s. Early reports included cases of arsenic and lead-shot ingestion and industrial smokestack-emission toxicity. One report described the death of fallow deer (*Dama dama*) due to arsenic emissions from a silver foundry in 1887 in Germany,[2] with subsequent reports of widespread killing of game animals, including deer and foxes, by arsenic emissions from metal smelters.[3] Another report described hydrogen sulfide fumes in the vicinity of a Texas oil field that resulted in a large die-off of many species of wild birds and mammals.[2] Mortality in waterfowl and ring-necked pheasants (*Phasianus colchicus*) from ingestion of spent lead shot was recognized as early as 1874 when lead-poisoned birds were reported in Texas and North Carolina.[4] Waterfowl mortality in the vicinity of mining and smelting operations was first reported in the early 1900s and subsequently linked to metallic or lead poisoning.[5] At about the same time the potentially devastating effects, including the death of seabirds such as puffins, of crude oil spills were noticed.[6]

Prior to World War II most agricultural and household pesticides were relatively simple derivations of naturally occurring minerals and plant products. The advent of synthetic organic insecticides evolved from World War II. Many of these post-war insecticides exhibited vast biological activity and were remarkably persistent in the natural environment. Initially, this persistence seemed desirable, especially from an economical perspective, i.e., long-term pest control. However, within a few years insects began to show resistance to many of these "modern" pesticides, and even trivial amounts of some impaired reproduction in certain wildlife species. For example, dichlorodiphenyltrichloroethane (DDT) was introduced in 1943, and by the end of the decade ecological problems other than incidents of acute mortality began to surface. Farsighted scientists, such as Lucille Stickel and others at the Patuxent Wildlife Research Center, Laurel, Maryland, cautioned users of the potential hazards of application of DDT to wildlife.[7,8] Little attention was paid to ecological hazards of pesticides until Rachel Carson published *Silent Spring*[9] in 1962. This seminal treatise effectively sensationalized many ecologically significant happenings such as a decline in the population of American robins (*Turdus migratorius*) by the early 1950s, which was linked to DDT spraying to fight Dutch Elm disease, and evidence that bald eagles (*Haliaeetus leucocephalus*), osprey (*Pandion haliaetus*), and populations of fish-eating mammals were at risk. Research then revealed that DDT and other chlorinated hydrocarbon insecticides, including dichlorodiphenyldichloroethane (DDD), endrin, aldrin, and dieldrin, when incorporated into the diets of pheasants and quail, impaired reproductive success without necessarily having other adverse effects on adults.[10] Eggshell thinning, related to DDT and ultimately dichlorodiphenyldichloroethylene (DDE), was determined to be an important factor in reproductive failure in European and North American birds of prey[11–13] as well as in brown pelicans (*Pelecanus occidentalis*).[14] This and similar research played a major role in the cancellation of many highly persistent pesticides in the United States.

Even pesticides that are comparatively labile in a natural system may be problematic. For example, many wildlife losses have been documented due to organophosphorus and carbamate insecticides.[15–18] These poisonings of many species of birds and mammals are due to acute lethal toxicity from cholinesterase inhibition. Agricultural practices other than pesticide application that

have received focus due to adverse effects on wildlife include subsurface drainage of irrigation water, wherein bioaccumulation of selenium and other trace elements in the aquatic food chain has proven highly embryotoxic and teratogenic to numerous species of waterbirds.[19–22]

Certainly, factors other than agricultural practices may pose toxic hazards to wildlife. For example, concern has arisen over globally increasing concentrations of methylmercury in aquatic biota, even evident at remote and semiremote sites, as a consequence of multiple anthropogenic sources and their emitting mercury into the environment.[23] A case in point — in the marine food web of the North Atlantic Ocean — is the steady long-term increase in concentration of methylmercury in feathers of fish-eating seabirds sampled from 1885 through 1994.[24] This increase has averaged 1.9% per year in Cory's shearwater (*Calonectris diomedea borealis*) and 4.8% per year in Bulwer's petrel (*Bulweria bulwerii*). These increases are attributed to global trends in mercury contamination rather than local or regional sources. Mercury concentrations have also increased over the past century in other species of seabirds. [25]

Another concern is endocrine disruption in wildlife species.[26–28] Many of the endocrine disruptor reports in wildlife are based upon observed adverse reproductive and developmental effects rather than direct evidence of endocrine-modified function or defined endocrine pathways. A wide variety of chemicals have been reported as potential endocrine disruptors and are described by Gross et al. in Chapter 39 of this book. These include polycyclic aromatic hydrocarbons; polychlorinated and polybrominated biphenyls, dibenzo-*p*-dioxins and dibenzo-*p*-furans; organochlorine pesticides and fungicides; some nonorganochlorine pesticides; complex environmental mixtures; and a few metals. Collectively, there is strong evidence of altered reproductive and developmental processes in wildlife exposed to endocrine disruptors. The U.S. Congress has passed legislation (listed in federal register notice, 63 FR 71542) requiring the U.S. Environmental Protection Agency (EPA) to develop, validate, and implement an Endocrine Disruptor Screening Program (EDSP) for identifying potential endocrine-disrupting chemicals.

Wildlife toxicology involves the integration of three principal strategies for understanding effects of toxic agents on wildlife.[1,29] The first strategy is *chemical screening*. A variety of toxicological tests are performed in the laboratory or in outdoor pens. Representative species are tested to help predict potential effects of a given chemical in natural populations of the same or closely related species. The second strategy is the *controlled field* or *mesocosm study.* Wildlife species of interest are exposed to operational chemical applications in a confined environment, simulating a natural system. The third strategy is *field ecology assessment.* Natural populations are studied in environments subjected to high levels of contamination.

With the advent of synthetic insecticides as well as the release of industrial pollutants and consequent wildlife losses, screening of pesticides and other chemicals for adverse effects has become an integral part of wildlife toxicology. A wide variety of wildlife testing protocols have been developed for regulatory use by the U.S. EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA). The U.S. EPA has established a unified library of test guidelines issued by the Office of Prevention, Pesticides and Toxic Substances (OPPTS), for the Series 850-Ecological Effects Test Guidelines. This document outlines testing requirements and protocols for review by the U.S. EPA under TSCA and FIFRA. The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among test procedures that must be performed to meet the U.S. EPA data requirements. These guidelines are a compilation of the testing guidance and requirements of the Office of Pollution Prevention and Toxics (OPPT; Title 40, Chapter I, Subchapter R of the Code of Federal Regulations), the Office of Pesticide Programs (OPP), and the Organization for Economic Cooperation and Development (OECD). Table 4.1 summarizes the guidelines for terrestrial wildlife in this series and the above sources from which they were derived.

This chapter provides a summary of the toxicity tests commonly used in wildlife toxicology. The focus is on avian studies because birds have served as primary models for terrestrial wildlife toxicology since the 1950s. In contrast, though mammalian wildlife species were considered

Table 4.1 Summary of Ecological Effects Test Guidelines for Terrestrial Wildlife, Soil Microbes, and Environmental Chemistry

OPPTS[a] Number	Name of Test	OTS[b]	OPP[c]	OECD[d]	EPA Pub. No.
	Terrestrial Wildlife Tests				
850.2100	Avian acute oral toxicity test	797.2175	71–1	none	712-C-96–139
850.2200	Avian dietary toxicity test	797.2050	71–2	205	712-C-96–140
850.2300	Avian reproduction test	797.2130, .2150	71–4	206	712-C-96–141
850.2400	Wild mammal acute toxicity	none	71–3	none	712-C-96–142
850.2450	Terrestrial (soil-core) microcosm test	797.3775	none	none	712-C-96–143
850.2500	Field testing for terrestrial wildlife	none	71–5	none	712-C-96–144
	Beneficial Insects and Invertebrates Tests				
850.3020	Honey bee acute contact toxicity	none	141–1	none	712-C-96–147
850.3030	Honey bee toxicity of residues on foliage	none	141–2	none	712-C-96–148
850.3040	Field testing for pollinators	none	141–5	none	712-C-96–150
Toxicity to Microorganisms Tests					
850.5100	Soil microbial community toxicity test	797.3700	none	none	712-C-96–161
	Chemical Specific Tests				
850.6200	Earthworm subchronic toxicity test	795.150	none	207	712-C-96–167
850.6800	Modified activated sludge, respiration inhibition test for sparingly soluble chemicals	795.170	none	209	712-C-96–168
	Field Test Data Reporting				
850.7100	Data reporting for environmental chemistry methods	none	none	none	712-C-96–348

[a] Office of Prevention, Pesticides and Toxic Substances
[b] Office of Toxic Substances (for TSCA)
[c] Office of Pesticide Programs (for FIFRA)
[d] Organization for Economic Cooperation and Development

important, it was generally accepted that the array of baseline tests routinely conducted with laboratory mammals would usually suffice for at least provisional intertaxa comparisons. The main avian tests described herein are acute, subacute, subchronic, chronic, developmental, field, and behavioral (Figure 4.1). Coverage of toxicity testing for wild mammals, amphibians, and reptiles is also provided but in somewhat less detail since the present body of information on these is more limited in scope and requirement than avian wildlife toxicity testing.

4.2 SINGLE-DOSE ACUTE ORAL AND SHORT-TERM SUBACUTE DIETARY AVIAN TOXICITY TESTS

Basic protocols with lethality as the principal endpoint have been used for first-line toxicity testing with birds by the U.S. Fish and Wildlife Service, the U.S. Geological Survey, and the U.S. EPA. These include experiments designed to estimate the acute oral median lethal dosage (LD_{50}), the 5-day median lethal dietary concentration (LC_{50}), and relevant statistical parameters.[30]

4.2.1 Single-Dose Acute Oral

Reports on single-oral-dose avian LD_{50}s contain data for adults of nearly 75 species of birds and more than 1000 chemicals tested.[31–33] Full-scale acute oral toxicity tests are required by

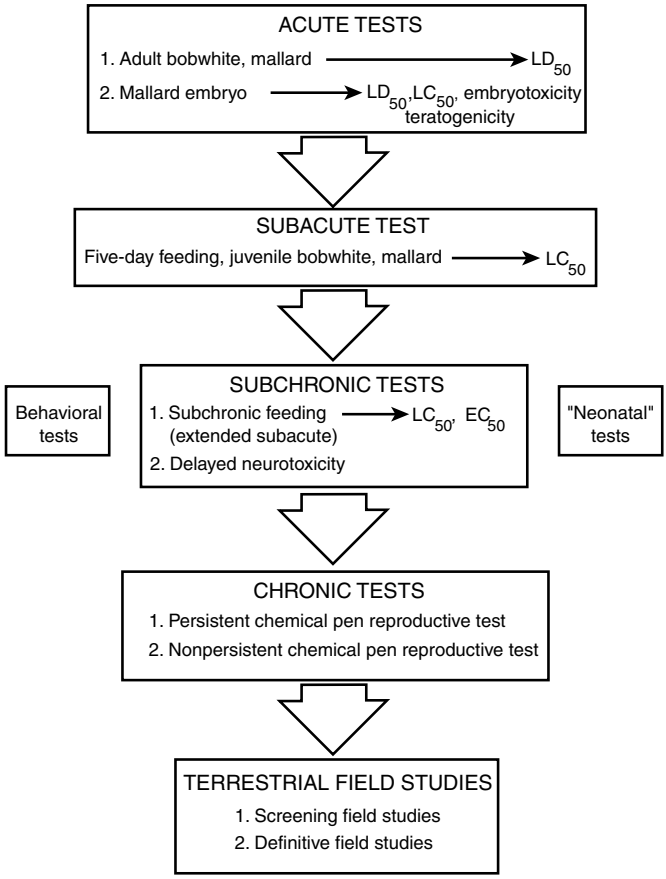

Figure 4.1 Protocols used in avian toxicity testing.

regulatory guidelines for pesticide registration in the United States (Table 4.1). Testing is with one of two species of birds, usually the mallard (*Anas platyrhynchos*) or the northern bobwhite (*Colinus virginianus*), and provides the LD_{50} and associated statistics including the dose-response curve.[34] Acute oral tests provide a preliminary indication of the lethal hazard of a substance.

These tests are rapid, uncomplicated, inexpensive, statistically reliable within the lethality curve (i.e., ± 1 SD of the LD_{50}), and, most importantly, provide insight necessary for further hazard evaluation.[35] Overnight-fasted birds receive a single dose of test substance at midmorning, usually administered by gavage or by capsule, at each of five or six geometrically arranged dosage levels that were predetermined from a preliminary study to span the expected 10 to 90% mortality levels.[30,35] Feed is provided immediately postdosing, and observations for signs of intoxication are continued throughout the day. Special attention is given to the length of time to first evidence of toxicity, death, and recovery. Observations are continued twice daily, or more often as indicated, for 14 days posttreatment, or longer if toxic signs persist. Gross necropsy should be performed on all birds that die and on a subsample of survivors.

Optimal use of the acute test requires statistical estimation of the lethality curve and its midpoint and descriptive information on toxic response. The LD_{50}, expressed as mg (active ingredient)/kg of body mass, its 95% confidence interval, and the slope and error of the dose-response curve are derived by probit, logit, or other appropriate analysis. When only the general order of acute toxicity is desired for wide-scale initial comparisons of many species or finished product formulations, then a choice of several approximate tests of lethality may be used instead (e.g., up-and-down, moving averages).[31,32,35]

Approximate tests conserve test animals. For example, as few as three groups of three to five birds are tested against a standardized fixed-dosage arrangement; toxicity statistics are derived from published tables.[31] The up-and-down procedure may use even fewer animals and is based on dosing single subjects at a time and adjusting each subsequent dose up or down, depending on the response of the preceding subject. Approximate tests provide an estimate of the LD_{50} and its 95% confidence interval but do not provide a statistical estimate of the dose-response curve.

The single acute oral test is best suited for substances whose principal action is lethality (e.g., insecticides, certain heavy metals, natural toxins), but (with less statistical confidence) the LD_{50} can be used as a toxicity marker for any substance with substantial bioactivity. Thus, the LD_{50} provides a statistical basis for comparisons among contaminants and species as well as with measured or estimated amounts of pesticide or other contaminant residue in the terrestrial or aquatic environment that could be consumed by wildlife in a single oral exposure.

However, care must be taken in how acute data are evaluated and whether the test is being used generally for regulatory purposes or specifically for risk assessment or research planning. For example, an avian database was compiled for 147 cholinesterase-inhibitor pesticides, with 837 acceptable LD_{50} determinations, and 733 noncholinesterase-inhibitor pesticides with 1601 acceptable LD_{50} values.[33] These authors found that when fitting a distribution to LD_{50}s expressed as mg/kg body weight, the LD_{50} values were overestimated for small-body birds. Therefore, the use of scaling factors for body weight has been recommended to improve cross-species comparisons of acute toxicological sensitivity.[36] After the protection level had been arbitrarily fixed at the 5th percentile of the species distribution (termed HD_5; Hazardous Dose 5%), it was shown that of all of the above pesticides, 34 had an HD_5 less than 1 mg/kg, of which 24 were cholinesterase inhibitors. Of the remaining ten, two were insecticides (including the very new pyrrole insecticide chlorfenapyr), two were fungicides, and the others were rodenticides, including three of the coumarin anticoagulant products.

In another example generally similar acute toxicities between mallards and laboratory rats were reported for insecticides and herbicides.[37] However, subsequent evaluations have revealed important and major differences in acute sensitivity to pesticides between birds and mammals (laboratory rats) and have been reviewed by Walker[38] and by Hill.[35] To illustrate, the organophosphorus pesticides dimethoate, fenitrothion, and temephos have LD_{50}s of, respectively, 215, 740, and 8600 mg/kg of body mass in rats, but are extremely toxic to both pheasants and blackbirds with LD_{50}s of 7 to 42 mg/kg (Table 4.2.). It was concluded that the laboratory rat is not a good model for the prediction of acute toxicity to birds, even for chemicals that elicit their toxicity in the same manner.

Table 4.2 Acute Avian Toxicity Testing of Organophosphorus Pesticides of Widely Variable Mammalian Toxicity[a]

Pesticide	Rat[b]	Pheasant[c]	Blackbird[d]
	Single-Dose Oral LD_{50}		
Phorate	2	7	1
Azinphos-methyl	13	75	8
Ethion	65	1297	45
Dimethoate	215	20	7
Fenitrothion	740	26	25
Temephos	8600	35	42

[a] Derived from reference 35.
[b] Sherman strain male laboratory rats, 3 months old, n = 50–60 per test.
[c] Farm-reared male and female ring-necked pheasants, 3 to 4 months old, n = 8–29 per test.
[d] Wild-captured pen-conditioned male and female red-winged blackbirds, adult, n = 8–28 per test.

Such remarkable differences between birds and rats clearly justify the need for acute avian toxicity testing and confirm that reliance on widely existing rat data is not adequate for prediction of avian hazard. Wide variation in sensitivity to acute exposure to pesticides exists even among avian species. For example, the red-winged blackbird (*Agelaius phoeniceus*) was one of the most sensitive of seven species tested with ten different anticholinesterase pesticides.[35] In another study adult red-winged blackbirds were 137 times more sensitive to terbufos and 65 times more sensitive to diazinon than adult European starlings (*Sturnus vulgaris*). [39] Furthermore, a difference of nearly 70-fold in sensitivity was detected between American kestrels (*Falco sparverius*) and eastern screech owls (*Otus asio*) for the anticholinesterase insecticide EPN; screech owls were relatively tolerant (LD_{50} = 274 mg/kg), but kestrels were not (LD_{50} = 4 mg/kg).[40]

LD_{50} tests have also been used to demonstrate practical differences in acute hazard of technical grade and liquid and granular formulations of pesticides.[35,41–44] While the toxicity of liquid formulations was found to be equal or somewhat more toxic than the technical grade, granulars were generally less toxic than the technical grade. However, the hazard involved in risk assessment of granulars was more dependent upon which avian species (size and feeding behavior) was likely to inhabit a treated area rather than on actual application rate.[45] If ingestion is haphazard, then the application rate becomes more critical, but if ingestion is selective, then even the most stringent attempts to reduce granule availability may fail to reduce the hazard. Recent studies with bobwhite in planted enclosures have suggested that ground-feeding birds are more susceptible to granular insecticides than flowable applications.[46] The color, size, texture, granule base, taste, and application rate are all factors for consideration in reduction of granular hazard to wildlife.[47–54]

4.2.2 Subacute Dietary

The subacute test (LC_{50}), a 5-day feeding trial, is required for two species, including upland game birds and waterfowl, to support registration of pesticides (Table 4.1). This guideline was modified from 40 CFR 797.2050 and OPP 71–2 to be harmonized with OECD guideline 205, allowing the use of coturnix (Japanese quail, *Coturnix japonica*) as an acceptable test species. This test serves as a composite indicator of vulnerability to a contaminated diet, allowing for metabolic changes that occur over time. The test was developed to quantify the toxicity of dietary residues that were considered an important source of exposure of wildlife to environmental contaminants.[43,44,54] This test was optimized with young precocial birds, including ducks and quail, but almost any species can be tested provided it can be maintained in captivity in good health and cannot survive for 5 days without eating.[35] Mortality and signs of intoxication are monitored at least twice daily, and food consumption is measured at 24-hour intervals. After the fifth day, all feed is replaced with untreated feed, and the study is continued for at least 3 days or until complete remission of overt toxic signs.

The LC_{50} is expressed as mg (active ingredient)/kg of feed (or ppm) with its 95% confidence interval and slope with error of the dose-response curve as done for acute tests. Results of tests on more than 200 pesticides with young northern bobwhite, coturnix, ring-necked pheasants, and mallards have been published.[43,44] When LC_{50} tests are compared with LD_{50} tests, subacute LC_{50} results often describe relationships among species and chemicals that are quite different from those for LD_{50}s because LC_{50} tests measure ability of birds to cope with toxic feed for a set duration.[35] LC_{50}s must be used carefully when comparing the toxicity of pesticides among species because there is no assurance that the species of interest have been equally challenged by the test protocol.[35] For example, if one species has a greater tendency to refrain from eating the test diet, or if a portion of the population can survive severely reduced nutriment for the duration of the test, then responses may vary considerably.

Age is an important consideration when evaluating LC_{50}s. In precocial species there is generally an increase in resistance to chemicals with increasing age during early growth.[55,56] This increase occurs across a given class of chemical or pesticide and appears to be the result of changes in

ability to cope with a toxic diet for the exposure duration, where older (larger) chicks that eat less proportional to body mass are better able to survive the 5-day trial by reducing food consumption and hence toxic exposure.[56] During the first 21 days average increases in LC_{50}s in coturnix for nine pesticides (three organophosphorus and two each of carbamate, chlorinated hydrocarbon, and methylmercury) were 36% for days 1–7, 43% for days 7–14, and 28% for days 14–21.[56] This corresponded to reductions of normal food consumption in controls of about 35, 23, and 21% per week from hatch to 3 weeks of age. In contrast, acute oral LD_{50}s show a dichotomy of change with age. For example, mallard LD_{50}s for anticholinesterases that require activation for maximum potency tend to decrease between hatch and 7 days and then increase through adulthood, whereas the opposite pattern occurs for direct-acting organophosphorus and carbamate anticholinesterases.[57]

4.3 AVIAN SUBCHRONIC DIETARY TOXICITY TEST

This test was developed as an extension of the subacute LC_{50} test but with greater emphasis on sublethal indicators of toxicity. The test was designed as a precursor to provide a biological indication of the necessity for conducting a full-scale reproductive trial and to provide a possible hazard index based on the ratio of sublethal to lethal toxicity values.[30] The first test of this kind was conducted to compare the effects of organic and inorganic mercury on various physiological parameters, including indicator enzymes and blood chemistries, in coturnix through 9 weeks of age, which is full maturity in this species; calculation of periodic EC_{50}s (median effective concentration) for each responding variable was used to develop hazard indices relating the EC_{50} to the oral LD_{50} and 5-day LC_{50}.

Other special studies of a subchronic nature (exposure period generally 1 to 3 months) have been conducted on pesticides and other environmental contaminants using farm-reared mallards and quail, as well as wild-captured species of birds. The emphasis of these studies has been on biochemical indicators of toxicity and toxicokinetics. For example, when studying the effects of an organic form of selenium, selenomethionine, implicated in agricultural drainwater toxicity to waterbirds in California, 2-year-old mallard drakes were fed diets containing supplementation of excess selenium from 1 to 32 ppm for 14 weeks.[58] Selenium accumulated readily in the liver in a dose-dependent manner. Mortality (10%) and histopathological effects, including bile duct hyperplasia and hemosiderin pigmentation of the liver and spleen, occurred at the highest level tested. These histopathological effects were accompanied by elevated plasma-alkaline-phosphatase activity, which is indicative of cholestatic liver injury. Other manifestations of hepatotoxicity included significant dose responses for hepatic oxidized glutathione (GSSG) concentrations and increased ratio of GSSG to reduced glutathione (GSH). Mean hepatic malondialdehyde (a measure of lipid peroxidation) concentration was elevated at the two highest levels tested. A number of these subchronic effects were similar to effects reported in wild waterfowl in a seleniferous location.

Subchronic studies have also been used for comparative purposes among species. For example, the potential hazard of ingestion of lead-contaminated sediment was assessed in mallard ducklings and goslings of Canada geese (*Branta canadensis*) from hatching for 6 weeks.[59–61] At similar dietary concentrations of lead, blood and liver lead concentrations increased almost twice as much in mallards as in geese. Yet when species were compared for responses at similar blood and liver lead concentrations, manifestations of toxicity were greater in geese, as reflected by reduced survival and growth, hematological effects, and hepatic oxidative stress. Hepatic GSH-S-transferase activity was nearly three times higher in geese than in mallards and presumably had a role in the binding of lead to GSH and subsequent biliary excretion. In contrast, mallards showed higher hepatic levels of GSH and activities of GSH peroxidase (GPX) and GSSG reductase (GR). An increase of lipid peroxidation with lead exposure was more evident in geese than mallards. Hepatic GSH was inversely related to hepatic lipid peroxidation — only in mallards and in agreement with the differences observed in GPX and GR activities. This apparent lower resistance to lipid peroxidation

of Canadian geese compared to mallards may explain why geese found dead in the field by lead ingestion often have lower liver lead concentrations than mallards.

A third example of specialized subchronic testing comes from studying the potential of several organophosphorus insecticides to induce delayed neurotoxicity (OPIDN) in mallards.[62] Here mallard hens received up to 270 ppm technical grade EPN in the diet for 90 days. Muscular incoordination, or ataxia, was first observed with 270 ppm in the diet after 16 days, with 90 ppm after 20 days, and with 30 ppm after 38 days; 10 ppm failed to produce ataxia. Brain neurotoxic esterase activity was inhibited by about 70% or more in groups experiencing ataxia. Brain and blood plasma cholinesterases and plasma alkaline phosphatase were significantly inhibited as well. Distinct histopathological manifestations of OPIDN were seen at concentrations as low as 30 ppm, which included demyelination and degeneration of axons of the spinal cord. Additional ducks exposed in a similar manner to leptophos experienced similar behavioral, biochemical, and histopathological alterations. These findings showed that adult mallards were sensitive to OPIDN but somewhat less so than chickens, which have served as the traditional model for screening substances for OPIDN.

4.4 AVIAN CHRONIC TOXICITY TESTS

Most avian chronicity tests are designed with reproduction as the primary endpoint. Such tests permit study of simulated field exposure under controlled conditions with a relevant species and exposure route. The Avian Reproduction Test guideline has been modified from 40 CFR 797.2130 and 797.2150 and OPP 71–4 to be harmonized with OECD guideline 206. The OECD guideline identifies Japanese quail as an acceptable species, but until several technical issues are resolved for this species the United States is listing only mallard and bobwhite as acceptable species for avian reproduction testing. For certain other tests, such as part of the Endocrine Disruptor Screening Program (EDSP, listed in federal register notice, 63 FR 71542), a two-generation test with Japanese quail is allowed. This test includes endocrine-specific endpoints in addition to the conventional fitness endpoints of existing avian reproduction test designs for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants.

Avian reproductive studies are required by the U.S. EPA[63,64] with both waterfowl (mallards) and an upland game species (northern bobwhite) to support the registration of an end-use product that meets one or more of the following criteria: (1) the end-use product is intended for use where birds may be subjected to repeated or continuous exposure to the pesticide or any of its major metabolites or degradation products, especially preceding or during the breeding season; (2) the pesticide or its products may persist in the food at toxic levels; (3) the pesticide or its products accumulate in plant or animal tissues; and (4) the pesticide or its products cause adverse reproductive effects in mammals. The basic study usually required by the U.S. EPA is designed to elucidate reproductive effects of chemicals that persist in potentially toxic amounts in wildlife habitats throughout the reproductive season. Alternative studies have also been used to evaluate reproductive effects from pesticides that have a short life span in nature but may be applied repeatedly during the reproductive season. The basic reproductive study and an example of an altered reproduction study follow.

4.4.1 Reproduction Studies — Basic Protocol

This basic protocol was designed for testing environmentally persistent chemicals and may be required for pesticide registration (Table 4.1). Test concentrations for regulatory purposes are based on measured or calculated residues expected in the diet from the proposed use pattern, including an actual field exposure level and a multiple level, such as ten times the field level. Treatment commences at least 10 weeks in advance of breeding and continues through egg laying. Eggs are collected daily and set at weekly intervals for artificial incubation in the laboratory. Reproductive

endpoints in such studies include number of eggs laid, percent fertility, live 3-week embryos, hatchability, 14-day-old posthatch survivors, and eggshell thickness. Two examples of reproductive studies conducted with mallards include: (1) studies with endrin, a chlorinated hydrocarbon pesticide, and (2) studies with selenium, an agricultural drainwater contaminant. Both contaminants have been associated with avian mortality in the field.

In the early 1980s endrin was found to be quite toxic to birds as the cyclodiene insecticide was used in the western United States. Reproductive effects were suspected mostly in waterfowl. Therefore, studies were conducted in which concentrations of 0, 1, and 3 ppm endrin in dry feed were fed to mallards from late fall through the following breeding season; health and reproduction were monitored.[65] Endrin at 3 ppm increased adult mortality, reduced hatching success, and delayed hatching. In a comparative study screech owls were more sensitive than mallards to reproductive effects of endrin yet accumulated less endrin in eggs and tissues than mallards.[66] Eggshell thickness was not affected in either species.

Another example is from reproductive studies with mallards fed selenium. The purpose was to determine the form of selenium, dietary concentration, and concentration in eggs responsible for high embryonic mortality and teratogenicity in aquatic birds exposed to evaporation ponds receiving agricultural drainwater at the Kesterson Reservoir in central California and other western locations. Another facet of the studies was to examine the interaction of selenium and mercury. In summary, the organic form of selenium, selenomethionine, accumulated to levels in eggs comparable to those found in eggs of wild birds in contaminated locations; sodium selenite and selenocystine did not accumulate readily or produce reproductive impairment similar to selenomethionine.[22] Characteristic malformations of the extremities occurred with selenomethionine, and included deformities of the bill, eyes, brain, and feet identical to those found at Kesterson.[67] Threshold concentrations for reproductive impairment from selenomethionine were 4 to 8 ppm in the diet (dry diet) and >3 ppm (wet weight) in the eggs. Studies of selenium and mercury interaction revealed antagonistic effects in adult mallards, but the two substances were synergistic in their effects on the reproductive process.[68] Individually, selenium and mercury lowered hatching success and survival of ducklings; the combination of mercury and selenium further reduced reproduction. Controls produced an average of 7.6 young per female — females fed 10 ppm selenium produced an average of 2.8 young, females fed 10 ppm mercury produced 1.1 young, and females fed both mercury and selenium produced 0.2 young. Furthermore, teratogenic effects were notably increased for the combined treatment; deformities were recorded in 6.1% of the embryos of controls, 16.4% for those fed methylmercury chloride, 36.2% for those fed selenomethionine, and 73.4% for those fed combined methylmercury chloride and selenomethionine.

4.4.2 Reproduction Studies: An Alternative Protocol

Several protocols have been used for testing comparative labile chemicals such as anticholinesterase pesticides.[63,69–74] Because of use patterns the initial contact with these pesticides may occur at any time during reproduction but usually not in advance of nesting. Therefore, a shorter exposure period is initiated once the test population is in egg production. Northern bobwhite reproductive tests with organophosphorus insecticides have shown significant effects with treatment periods of 8 days,[70] 10 days,[71] and 3 weeks.[69,72–74] A mallard test of methyl parathion in the diet for 8 days resulted in reduced daily egg production, changes in incubation behavior of hens, and decreased number of hatchlings.[75]

Test methods used in the above studies varied substantially and should be further considered if standardization is desired. There are many potential advantages to using short-term exposure tests including: (1) known layers of fertile eggs can be used, hence reducing variability in test data; (2) pretreatment values for each pen can serve as an additional control; and (3) timing of the test

can coincide with maximum egg production. However, the most important advantage may be to mimic actual exposure spikes as they may be encountered in nature.

4.5 SINGLE-DOSE AVIAN EMBRYOTOXICITY AND TERATOGENICITY TESTS

Toxic contaminants can enter the natural environment by numerous routes, including air pollution from mobile and stationary sources, pesticide spray applications, or aquatic translocation. All these routes have the potential to contaminate avian eggs, which are well known for susceptibility to direct applications of toxicant.[30,76] Even a parent bird may be the source of contamination. For example, petroleum pollutants carried to the nest on breast feathers, feet, or nesting materials of sea birds caused reduced hatchability of contaminated eggs.[77] Laboratory studies showed that as little as 1 to 10 μL of crude or refined oil topically applied to eggs of various species was embryotoxic or teratogenic. The extent of toxicity was from egg penetration and the aromatic hydrocarbon composition, rather than from blockage of shell pores and interference with oxygen transfer as previously believed.[78,79] Dose-dependent liver necrosis was one of the best indices of embryotoxicity dependent on petroleum composition and stage of development.[80]

A variety of pesticides, including paraquat and parathion, have been shown to be embryotoxic and teratogenic to several avian species. Embryos did not develop normally when eggs were sprayed with or immersed in chemical formulations at concentrations encountered in nature.[76,81]

With this background, routine test methods were developed for embryotoxicity and teratogenicity with mallard eggs. Measurement of embryolethality was modeled after the avian acute toxicity test, that is, the median lethal effect (LD_{50} or LC_{50}) is derived by exposure of eggs or embryos to at least three geometrically arranged treatments. Treatment has been by egg immersion (LC_{50}) or topical application by spraying or pipetting (LD_{50}).

An evaluation of the potential hazard of external exposure of mallard eggs to petroleum, pesticides, and industrial effluents was carried out; tests conducted on more than 70 environmental contaminants indicated that 8 of 30 pesticides tested caused teratogenic effects at exposure levels well below the calculated LC_{50} or LD_{50}.[81] Other measurements of embryotoxicity included presence of subcutaneous edema, blisters, hemorrhages, and stunted growth. Studies with different species (e.g., northern bobwhite) but the same classes of pesticides often lead to similar conclusions.[76] Several studies with insecticides and herbicides are summarized below.

The order of toxicity of commercial formulations was determined for 14 insecticides in aqueous emulsion, as reflected by LC_{50} values in mallard eggs.[81] The LC_{50} values for these insecticides ranged from 30 g/L to greater than 600 g/L, and order of toxicity was endrin > sulprofos > parathion > acephate > lindane > temephos > diazinon > dimethoate > toxaphene > malathion > carbaryl, permethrin, phosmet, and methomyl. However, the order of potential hazard in terms of the highest permissible field level of application in the United States was toxaphene > malathion > endrin = dimethoate > lindane > sulprofos > diazinon > parathion > acephate > temephos > carbaryl, phosmet, methomyl, and permethrin. Apparent differences between the ranking based on toxicity and potential hazard were due to differences in maximum permissible levels of application, which, for example, were extremely high for toxaphene and malathion. Insecticides with LC_{50} values that occurred at approximately ten times the highest permissible field level of application or less included dimethoate, endrin, malathion, and toxaphene. Of these, all were teratogenic but only dimethoate and endrin were teratogenic at levels below the LC_{50}. Subsequent observations have shown that many of these abnormal embryos are unable to hatch, and this would have lowered the calculated LC_{50} if hatching success had been included.

The order of toxicity of herbicides in aqueous emulsion (LC_{50} values) was paraquat = trifluralin > propanil, bromoxynil with MCPA (Bronate®) > methyl-diclofop > prometon > picloram > 2,4,5,-T > amitrole > glyphosate > 2,4,-D > atrazine, dicamba, and dalapon.[81] However, the order of

potential hazard in terms of the highest permissible field level of application in the United States was trifluralin > paraquat > prometon > methyldiclofop = propanil > bromoxynil with MCPA (Bronate®) > picloram > amitrole > 2,4,5,-T > 2,4,-D > glyphosate > atrazine and dicamba. Herbicides with LC_{50} values that occurred at ten times the field level of application or less included bromoxynil with MCPA, methyldiclofop, paraquat, prometon, propanil, and trifluralin.

Embryotoxicity testing has also involved hazard evaluations of aquatic weed control,[82] mosquito control,[83,84] and wildfire control agents.[85] Some examples follow. (1) Diquat dibromide, a bipyridylium compound, is commonly used as an aquatic weed control. Tests with mallard eggs indicated that concentrations of diquat in aqueous solutions as used for aquatic weed control would probably have little impact on mallard embryos. This is, of course, dependent upon the dilution effect of average water depth of the application area. However, concentrations applied above ground to weeds and cattails along ditches could affect the survival and normal development of mallard embryos and presumably other avian species nesting in such habitats.[82]

(2) Golden Bear Oil or GB-1111 (also GB-1313) is a petroleum distillate that is used throughout the United States as a larvicide for mosquito pupae. External application of the product to mallard, bobwhite, and red-winged blackbird eggs reduced hatching success but at different levels of treatment. Hatching was significantly reduced in mallards treated on day 4 or day 11 at three and ten times the maximum field application; the LD_{50} was 1.9 times the maximum field application.[83,84] Hatching success of bobwhite and red-winged blackbirds was only reduced at ten times the maximum field application. Recommended rates of field application of GB-1111 were potentially toxic to mallard embryos, especially under conditions of spray overlap but unlikely to impair the survival or development of bobwhite or red-winged blackbird embryos.

(3) Eggs of nesting birds situated in peripheral areas serving as fire breaks are at risk of being sprayed with fire-control chemicals. Acute toxicity tests were conducted with northern bobwhite quail eggs using different water-based concentrations of Silv-Ex® (S-E), a fire suppressant chemical, and Phos-Chek® G75-F (P-C), a fire-retardant chemical, on day 4 or day 11 of incubation.[85] Mortality appeared higher in most groups exposed on day 11 than on day 4, suggesting that on day 11 the extensive chorioallantoic vascular network permitted greater uptake of chemical. A combination of S-E and P-C was synergistic (202 g/L P-C and 50 g/L S-E) at day 11 of incubation resulting in a large decrease in hatching success. However, lower combined concentrations of S-E (10 g/L or 30 g/L) with 202 g/L of P-C appeared antagonistic. This may be due to S-E, as a surfactant, altering the ability of P-C to penetrate. It was concluded that Phos-Chek G75-F could pose a potential threat to developing galliform species, especially during heavy application in densely wooded areas (the LC_{50} was 220 g/L compared to the standard application concentration of 135 g/L).

Other studies have utilized egg injections as a short cut for initial comparison of relative toxicities of environmental contaminants that could be ingested by birds in nature to an array of avian species.[86–88] For example, the effects of polychlorinated biphenyl (PCB) congeners, PCB 126 and PCB 77, were examined in chicken (*Gallus gallus*), American kestrel, and common tern (*Sterna hirundo*) embryos through hatching following air cell injections on day 4.[87] PCB 126 caused malformations and edema in chickens starting at 0.3 ppb, in kestrels at 2.3 to 23 ppb, but in terns only at levels affecting hatching success (44 ppb). The estimated LD_{50} for PCB 126 in chickens, kestrels, and terns was 0.4, 65 and 104 ppb, respectively, and for PCB 77 it was 2.6 and 316 ppb, respectively, for chickens and kestrels. High concentrations of PCB 126 found in bald eagle eggs in nature are nearly 20-fold higher than the lowest toxic concentration detected in kestrel eggs suggesting potential for toxicity in nature. In contrast, concentrations of PCB 126 causing low-level toxic effects in common tern eggs are comparable to highest levels in common terns and Forster's terns found in nature, suggesting additional involvement of other compounds causing toxicity in areas such as the Great Lakes.

Another example of using egg injections as a short cut for comparing relative embryotoxicities among species involves the evaluation of mercury in the environment, which is especially hazardous

to fish-eating birds since it accumulates in the food chain. Therefore, studies were conducted by injecting methylmercury (dissolved in corn oil) into eggs of several species.[88] When mallard eggs were injected, hatching success was 76% for controls, and 56, 62, 53, 44, and 29% for eggs injected with 0.1, 0.2, 0.4, 0.8, and 1.6 ppm mercury, respectively. With white ibis (*Eudocimus albus*) eggs, hatching success was 62% for controls and 10, 25, and 20% for eggs injected with 0.2, 0.4, and 0.8 ppm mercury, respectively. For tricolored herons success was 60% for controls and 10% for eggs injected with 0.4 ppm mercury. For great egrets (*Casmerodius albus*) success was 60% for controls and 0% for eggs injected with either 0.4 or 1.3 ppm mercury. These results indicate that the embryos of some fish-eating birds may be more sensitive to methylmercury than mallard embryos and that estimates of toxic levels of mercury in eggs, based on reproductive trials with mallards, may have to be re-evaluated.

4.6 "NEONATAL" TOXICITY TESTING IN ALTRICIAL NESTLING BIRDS

Neonatal mammals are especially susceptible to accumulation and toxicity of many environmental contaminants. With this as a guide, studies with altricial and semi-altricial nestling birds were conducted and have shown that nestlings of different species including American kestrels, European starlings, red-winged blackbirds, herring gulls (*Larus argentatus*), and great egrets are considerably more sensitive to oral ingestion of contaminants, such as heavy metals, herbicides, OP insecticides and PCBs, than are adults of the same species or the young of fully precocial species such as northern bobwhite, mallards, and ring-necked pheasants.[89] Following are examples of the effective use of nestlings of various species in elucidation of contaminant hazard to birds.

4.6.1 American Kestrels

Studies have been conducted with American kestrel nestlings that were orally intubated daily for the first 10 days posthatching. Environmental contaminants administered were lead, herbicides (paraquat, nitrofen, bifenox, and oxyfluorfen), and PCB congeners (Table 4.3). Endpoints have included survival; body, organ, and skeletal growth; and blood and organ biochemistry.

Overt toxicity of metallic lead was manifested by 40% nestling mortality at the dose level of 625 mg/kg, with significant impairment of growth occurring at 125 mg/kg.[90] Since nestling kestrels consume approximately their own body weight in food per day, 625 mg/kg of lead daily can be considered equivalent in daily lead intake to approximately 625 ppm lead in the diet, and 125 mg/kg equivalent to 125 ppm in the diet. Lead ingestion was considerably more toxic to nestlings than previously reported for adults and young precocial species, where LC_{50}s were greater than 5000 ppm. Biochemical and hematological alterations in nestlings were also more severe than those reported in adult kestrels or precocial young birds exposed to lead.[91]

Paraquat ingestion by nestling American kestrels was also more toxic than previously reported for adults and young precocial species.[92] For northern bobwhite, coturnix, ring-necked pheasants, and mallards, the LC_{50}s varied from nearly 1000 ppm to over 4000 ppm after a 5-day feeding trial. In kestrels 60 mg/kg of paraquat resulted in 44% mortality on days 4 to 8 of the experimental treatment period.

Diphenyl ether herbicides, including bifenox, nitrofen, and oxyfluorfen, were examined for developmental toxicity in kestrels. Bifenox was found to be more toxic than reported for precocial species; for ducks and pheasants, the 5-day LC_{50}s for bifenox were reported to be greater than 5000 ppm in the diet.[93] Precocial young also appear to be less sensitive than nestling kestrels to nitrofen ingestion. Bifenox at 500 mg/kg caused 66% mortality in nestling kestrels, whereas 500 mg/kg nitrofen caused 100% mortality within 5 days. Levels of bifenox or nitrofen at 250 mg/kg caused 9% mortality within 7 days. In addition to reduced growth and survival, nitrofen caused some

Table 4.3 "Neonatal" Toxicity Testing of Environmental Contaminants in Nestling Birds

Species	Exposure Method	Chemical	Observation Period	Effects	References
American kestrel	Daily oral	Lead, metallic	Days 1–10	525 mg/kg, high mortality; 125 mg/kg, reduced growth; 25 mg/kg, altered physiology	90, 91
American kestrel	Daily oral	Paraquat	Days 1–10	60 mg/kg, high mortality; 10 to 25 mg/kg, reduced growth, altered physiology	92
American kestrel	Daily oral	Bifenox	Days 1–10	500 mg/kg, high mortality, 250 mg/kg, reduced growth, altered physiology	93
American kestrel	Daily oral	Nitrofen	Days 1–10	500 mg/kg, complete mortality; 250 mg/kg, reduced growth; 50 mg/kg, altered physiology	93
American kestrel	Daily oral	Oxyfluorfen	Days 1–10	500 mg/kg, few effects	93
American kestrel	Daily oral	PCB 126	Days 1–10	50 ug/kg, onset of lymphoid depletion, decreased thyroid content, hepatic necrosis	94
European starling	Single oral (day 5 or 15)	Dicrotophos	24 h postdose	Day 5 LD_{50} = 4.9 mg/kg Day 15 LD_{50} = 9.0 mg/kg, reduced growth, brain cholinesterase	95
European starling	Single oral	Diazinon	24 h postdose	Day 1 LD_{50} = 13 mg/kg fledgling LD_{50} = 145 mg/kg	96, 39
European starling	Single oral	Terbufos	24 h postdose	Day 2 LD_{50} = 2.3 mg/kg fledgling LD_{50} = 61 mg/kg	39
Red-winged blackbird	Single oral	Diazinon	24 h postdose	Day 0–3 LD_{50} = 2.4 mg/kg fledgling LD_{50} = 8.3 mg/kg	39
Red-winged blackbird	Single oral	Terbufos	24 h postdose	Day 0–3 LD_{50} = 0.4 mg/kg fledgling LD_{50} = 3.3 mg/kg	39
Herring gull	Single oral (3–4 weeks old)	Crude oil	For 9 days	0.3 mg/kg reduced growth, altered physiology	97
Herring gull	Single i.p. injection (day 2 or 6)	Lead nitrate	19 days of age or older	100 mg/kg on day 6 was a critical period for disruption of behavior	99, 100
Herring gull	Single i.p. injection (day 2 or 6)	Chromium nitrate	50 days of age	50 mg/kg on day 2 affected growth and 12 of 14 behaviors	101
Herring gull	Single i.p. injection (day 2 or 6)	Manganese acetate	50 days of age	50 mg/kg on day 2 affected growth and 12 of 14 behaviors	101
Black guillemot	Single oral	Crude oil (weathered)	For 22 days	0.1–0.2 mL reduced growth, altered physiology	98
Great egret	Dosed orally from 1 to 14 weeks	Methylmercury chloride	Through 14 weeks of age	0.5 mg/kg affected immune system and behavior; 5 mg/kg caused severe ataxia and neural lesions	103–105

hepatotoxicity, including an increase in liver weight relative to body weight with significant increases in activities of several plasma enzymes. Hepatic tissue GSH peroxidase activity was significantly higher in all nitrofen-treated groups and in the two treated with the highest doses of bifenox (50 mg/kg and 250 mg/kg). Other alterations included an increase in plasma total thyroxine concentration (T4) by nitrofen, which may indicate thyromimetic activity, as suggested for mammals.

The developmental toxicity of the planar PCB 126 was also studied in the posthatching kestrel as a model for the eagle.[94] Nestlings were dosed with 5 μL/g body weight of corn oil (controls) or PCB 126 at concentrations of 50, 250, or 1000 ng/g body weight. Dosing with 50 ng/g of PCB 126 resulted in a hepatic concentration of 156 ng/g wet weight, liver enlargement and mild coagulative necrosis, over tenfold increases in hepatic microsomal ethoxyresorufin-O-dealkylase and benzyloxyresorufin-O-dealkylase, and a fivefold increase in methoxyresorufin-O-dealkylase. At this dose mild to moderate lymphoid depletion of the spleen was apparent, as were decreased follicle size and content of the thyroid. At 250 ng/g concentration of PCB 126 in the liver was 380 ng/g, with increasing multifocal coagulative necrosis, decreased bone growth, decreased spleen weight with lymphocyte depletion of the spleen and bursa, and degenerative lesions of the thyroid. At 1000 ng/g the liver concentration was 1098 ng/g, accompanied by decreased bursa weight, decreased hepatic thiol concentration, and increased plasma enzyme activities (ALT, AST, and LDH-L), in addition to the previous effects. Highly significant positive correlations were noted between liver concentrations of PCB 126 and the ratio of oxidized to reduced glutathione. These findings indicated that nestling kestrels are more susceptible to PCB 126 toxicity than are adults but less sensitive than embryos.

4.6.2 European Starlings and Red-Winged Blackbirds

Acute oral LD_{50}s for European starlings were determined for nestlings from hatching until fledging. The LD_{50} of dicrotophos for free-living 5-day-old nestlings was about half that obtained for 15-day-old nestling and adults.[95] Brain cholinesterase activity was depressed by 74 to 94% in all birds that died, but neither the degree of inhibition nor the baseline cholinesterase activity varied with age. In another study of anticholinesterase pesticides to starlings newly hatched young were 20 times more sensitive to a single dose of diazinon than were fledglings of about 21 days of age.[96] Nestling sensitivity was supported by decreased plasma and brain cholinesterase activities. Acute age-dependent toxicity of terbufos and diazinon was evaluated in nestling starlings and red-winged blackbirds.[39] In brief, for starlings, LD_{50}s for turbufos increased from 2 days of age to 9 days by nearly ninefold and by 26-fold at 19 days and fledging; whereas for diazinon the increases between 2 days and 9 and 19 days were 7- and 11-fold. In comparison, blackbird nestlings were tested at 0 to 3 days and about halfway to fledging (8 to 11 days). The LD_{50} increased during this period by ninefold for turbufos and threefold for diazinon. At both ages the red-winged blackbird was substantially more sensitive to turbufos and diazinon than was the European starling. Baseline cholinesterase activities in both brain and plasma increased with age in both species and may have contributed to the changes in sensitivity between hatching and fledging. It was also noted that fledgling starlings (19 days) were about three to four times more sensitive than adults to turbufos and diazinon.

4.6.3 Herring Gulls and Black Guillemots

Nestling birds are quite sensitive to exposure of petroleum hydrocarbons containing an aromatic fraction. For example, a single small oral dose of Kuwait Crude Oil (KCO) or South Louisiana Crude Oil (SLCO) at approximately 0.3 mL/kg body weight caused reduced growth, osmoregulatory impairment, and hypertrophy of hepatic, adrenal, and nasal gland tissue in herring gull nestlings living in a simulated marine environment.[97] Weathered SLCO caused similar effects in black guillemot (*Cepphus grylle*) nestlings in nature.[98] This suggests that ingestion of oil by nestlings could reduce a fledgling's ability to survive at sea.

Another example is a series of studies with herring gull chicks designed to examine the relationship between dose, tissue level, and response to lead.[99,100] These studies showed that lead affected neurobehavioral development at critical periods. Seventy-two 1-day-old herring gull chicks were randomly assigned to six treatment groups to receive a lead nitrate concentration of 100 μg/g at age 2 days or at age 6 days, a similar cumulative dose evenly divided on days 2, 4, and 6, or matched-volume saline injections on the same days. Behavioral tests were performed (some at 2- and others at 5-day intervals) to examine locomotion, balance, righting response, thermoregulation, and visual cliff. Overall, the data showed that the lead-6 group was more affected by the dose than the other groups, suggesting that 6 days of age may be a more critical period than earlier ages for some behaviors.

The same researchers also examined the effects of chromium and manganese on early neurobehavioral development in herring gulls.[101] Each of 36 two-day-old herring gull chicks was randomly assigned to one of three treatment groups to receive chromium nitrate (50 mg/kg), manganese acetate (50 mg/kg), or a control dose of sterile saline solution. Behavioral tests examined food begging, balance, locomotion, righting response, recognition, thermoregulation, and perception. There were significant differences in begging behavior by 5 days postinjection, and there were significant differences in weight gain throughout development until 50 days of age, when the experiment was terminated. Behavioral tests, administered from 18 to 48 days postinjection, indicated differences between control and the exposed groups for time to right themselves; thermoregulation behavior; and performance on a balance beam, inclined plane, actual cliff, and visual cliff. Of the 14 behavioral measures with significant differences, control birds performed best on 12.

4.6.4 Great Egrets

The effects of mercury were studied on captive great egret nestlings, which were either maintained as controls or were dosed from 8 days of age for 13 weeks with 0.5 or 5 mg methylmercury chloride/kg wet weight in fish.[102] Low-dosed birds were given methylmercury at concentrations comparable to exposure of wild birds in the Florida Everglades. Subacute toxicity was indicated for birds dosed with 5 mg/kg after 9 weeks. Growing feather concentrations of mercury were closely correlated with cumulative mercury consumed per unit of body weight. After 8 weeks of exposure, appetite and weight index (weight/bill length) declined significantly in the high-dose group, with the same response noted a week later in the low-dose group.

Other effects indicative of mercury toxicity in low-dosed birds included lower packed cell volumes, dingy feathers, increased lymphocytic cuffing in a skin test, increased bone marrow cellularity, decreased bursal wall thickness, decreased thymic lobule size, fewer lymphoid aggregates in lung, increased perivascular edema in lung, and decreased phagocytized carbon in lung.[103] High-dosed birds became severely ataxic and had severe hematologic, neurologic, and histologic changes. The most severe lesions were in immune- and nervous-system tissues. Manifestations of oxidative stress and elevated plasma enzyme activities were also apparent.[104] Comparison of responses in captive and wild birds indicated that sublethal effects of mercury occurred at lower levels in captive than in wild birds. This may be due to the reduced sources of variation characteristic of the highly controlled laboratory study. Conversely, thresholds for more severe changes (death, disease) occurred at lower concentrations in wild birds than in captive birds, possibly because wild birds were exposed to multiple stressors. Thus, caution should be used in applying lowest observed effect levels between captive and wild studies.

As an integral part of the above study, behavioral effects, including activity levels, maintenance behavior, and hunting behavior, were measured.[105] Mercury affected activity and maintenance behavior. Birds dosed with 5 mg/kg in fish (as above) became severely ataxic (lost muscle coordination) and were euthanized by 12 weeks of age. The low-dosed birds exhibited less tendency to hunt fish. Therefore, even at 0.5 mg/kg mercury concentration in the food there were significant effects of methylmercury on activity, tendency to seek shade, and motivation to hunt prey.

4.7 AVIAN TERRESTRIAL FIELD STUDIES

In the United States the EPA no longer requires terrestrial field studies for pesticide registration except under extremely compelling circumstances; however, protocols are available for limited testing or if the requirements are reinstated (Table 4.1). In place of the field study the U.S. EPA's ecological risk-assessment process uses laboratory toxicity data in quotient indices to characterize risks to wildlife. However, these data sometimes fall short of predicting actual field effects.[106,107] For example, laboratory and field results of the toxicity of azinphos-methyl were compared with northern bobwhite.[106] Chick survival, brain cholinesterase activity, and growth in the field exposure study were significantly different from equivalent exposures in the laboratory, and temporal patterns of effects differed between field and laboratory. It was concluded that the effects observed in the field differed from that predicted by risk quotients because the quotient method did not consider alternate routes of exposure, behavioral responses, influence of spatial and temporal environmental variability, or indirect effects.

Avian die-offs due to anticholinesterase insecticide exposure include incidents varying from small-scale poisonings of only a few birds in a barnyard or on a golf course to massive die-offs with at least several hundred colonial breeding birds or migrants.[15] The dimension of this problem is indicated by a review of raptor mortality in the United States, the United Kingdom, and Canada for the period 1985 to 1995; of 520 incidents evaluated, the largest number of poisonings were attributed to anticholinesterase pesticides.[18] Anticholinesterase poisonings have also involved many other species, ranging in size and diversity from American robins and warblers (*Vermivora* spp.) to Canada geese and great blue herons (*Ardea herodias)*.[29] Most of these were highly visible situations, and undoubtedly there are a multitude of undetected incidents for every one discovered. In a recent review on factors influencing estimation of pesticide-related wildlife mortality, it was concluded that most effects on wildlife are not observed, much of observed mortality is not reported, and the actual number of affected animals per mortality event typically exceeds the number recovered.[107] Therefore, larger-than-expected net losses could occur because of such cumulative events, but the real impact on bird populations is not known. Secondary poisoning of predatory birds and other carnivorous animals consuming prey poisoned by anticholinesterase pesticides is also an important contributing factor to wildlife mortality that had been generally disregarded prior to 1980.[45,108–110]

4.7.1 Prerequisites for Testing

In assessing the need for required terrestrial field studies under FIFRA, the U.S. EPA considers the following prerequisites: (1) environmental concentration of the substance, which must exceed the lowest-effect level eliciting a biological response; (2) chemical properties of the pesticide (e.g., persistence, toxic metabolites and degradates, retention on food); (3) intended use pattern (areas and species likely to be exposed, treatment intervals); (4) margin between lowest-effects level and estimated environmental concentration; and (5) dose-response slopes in laboratory studies.[34,111] Field studies are designed to evaluate survival and reproductive success of nontarget wildlife species under actual conditions of use of the pesticide. Potential outcomes of pesticides in field tests can include (1) direct poisoning by ingestion, dermal, or inhalation exposure; (2) sublethal effects indirectly causing death by reducing resistance to natural environmental stresses such as disease, weather, or predators; (3) altered behavior such as abandonment of nests or young and change in parental care; (4) habitat alteration that results in reduced food resources or greater vulnerability to predators; and (5) reduced productivity.

4.7.2 Types of Field Studies

Field studies consist of two types: (1) *screening field studies* to evaluate whether impacts are occurring and (2) *definitive field studies* to estimate the magnitude of the impact.[111] Generally,

species representative of areas where pesticide applications occur are utilized. In most instances screening studies monitor for overt signs of toxicity such as mortality or aberrant behavior and changes in biochemical and histological indicators of toxicity. Carcass searches, radiotelemetry, depression of cholinesterase in the event of anticholinesterase pesticide exposure, residue analysis, behavioral observations, and population parameters may all be components of screening field studies.

With definitive field studies, mark-recapture techniques and radiotelemetry are often used to monitor survival and behavior. Extensive monitoring of reproduction and survival of dependent young is often required. Active nests are periodically monitored at the study site for number of eggs laid, hatched, young fledged, and nest abandonment. Sometimes artificial nest structures are provided to increase nest sites. Incubation behavior and parental care are sometimes monitored. A further indicator of reproductive effects may include comparison of young-adult ratios between treated and untreated plots. A number of pesticide–related field studies using different resident avian species are summarized below.

4.7.2.1 Sage Grouse (*Centrocercus urophasianus*)

Avian studies have focused on a series of die-offs of sage grouse associated with increased agricultural applications of organophosphorus insecticides to irrigated meadows and cropland.[112] Sage grouse in Idaho were radio-tagged, and their brain cholinesterase activity was monitored over several seasons; nearly 20% of the birds died or were seriously affected by organophosphorus insecticides. Mortality was higher among juvenile grouse than adults. Since these studies deal with a species that is both vulnerable and nonmigratory (which provides ease of tracking), there is high probability that such studies will further elucidate the impact of pesticide usage on population dynamics. At present, sage grouse in several parts of the western United States are being considered for listing under the endangered Species Act.

4.7.2.2 Prairie Pothole Waterfowl Studies

The prairie potholes region of the northern plains of the United States and Canada provides breeding habitat for more than 50% of North American waterfowl production. Drainage of prairie wetlands for agricultural purposes has been intense; only 35% of the original wetland area remains.[113] The potential for agricultural chemicals to enter the remaining wetlands and impact wildlife is substantial. Many of the most widely used organophosphorus and carbamate insecticides within the region have been found to be highly toxic to aquatic animals. Both direct (mortality and toxicity) and indirect (loss of invertebrate food items) effects were greater than expected when parathion or methyl parathion was applied by aircraft according to label instructions and county agent recommendations.[113–115] The use of tank mixture combinations of insecticides and of herbicides during spraying further contributes to the toxicity. Of 16 widely used insecticides in North Dakota, nine were implicated in wildlife mortality. In addition, 13 of these were either highly toxic to aquatic invertebrates or vertebrates. Reduced availability of aquatic invertebrates as food for ducklings and egg-laying females affected waterfowl productivity.

4.7.2.3 Passerine Studies

Aerial spraying for western budworms (*Choristoneura occidentalis)* with relatively low volume trichlorfon and carbaryl in Montana forests did not alter the success of nests with eggs or young birds of various species.[116] However, application rates for these two insecticides were lower than normal. In another study the effects of pesticides on mourning doves (*Zenaida macroura*) nesting in orchards in southern Illinois were evaluated.[117] The pesticides usually consisted of a combination of an insecticide and a fungicide and were sprayed at intervals of approximately 10 days from March through September. Most adult doves incubating eggs left during the actual spraying appli-

cation but all returned within 30 min. Nearly 90% of the nests that contained eggs during spraying were unsuccessful, with most failures occurring four or more days postspray, suggesting direct embryotoxicity from contact and penetration of pesticides on eggs.

Reproductive effects were assessed for multiple and varied organophosphorus and carbamate operational exposures on avian productivity.[118] Nest, egg, and nestling daily survival rates (DSRs) were determined for northern mockingbirds (*Mimus polyglottos*), brown thrashers (*Toxostoma rufum*), and northern cardinals (*Cardinalis cardinalis*) nesting along edges of pecan orchards and row crops in southern Georgia (United States). Egg and nestling DSRs for all species combined were negatively correlated with exposure. Nestling growth was reduced with increasing exposure. Brain cholinesterase activities were age-dependent and substantiated adult, but not nestling, exposure. The authors concluded that increasing exposure to operational pesticide use may reduce songbird productivity.

Insecticide hazard to breeding birds in Christmas tree plantations in Quebec were assessed by examining potential deleterious effects of three insecticides (i.e., dimethoate, diazinon, and insecticidal soap) on American robins and song sparrows (*Melospiza melodia*).[119] Cases of complete or partial mortality were recorded in nests. Abandonment of nests and egg infertility were ruled out as possible causes of mortality. No mortality was recorded for broods exposed to the insecticidal soap. The cases of total mortality observed in broods of both species exposed to dimethoate were similar to those recorded for control nests (18 and 25% vs. 14 and 21%, respectively). However, among robin and sparrow nestlings exposed to diazinon, about twice as many cases of total mortality (31 and 38%) were recorded than for the control nests. The authors concluded that American robin eggs are sensitive to diazinon and dimethoate, particularly when spraying is carried out early in the incubation stage. However, for song sparrows, it is mainly the nestlings that succumb after diazinon is sprayed on them or when dimethoate applications are made during incubation.

Organophosphorus insecticides were studied through examination of cholinesterase activity in tree swallows (*Tachycineta bicolor*) and eastern bluebirds (*Sialia sialis*) in apple orchards in Ontario, Canada treated with azinphos-methyl, diazinon, phosalone, or phosmet.[120] In nestlings, brain cholinesterase activities obtained postspray often fell below predicted activities calculated from control siblings. This trend was especially apparent in the younger nestlings, less than 6 days old. However, for bluebirds, the rate of increase of brain cholinesterase with age in nestlings from treated sites was lower than in nestlings from control sites. Results of depressed cholinesterase levels in tree swallows and eastern bluebirds inhabiting apple orchards were consistent with those in avian species in other orchard monitoring studies. However, there was no indication that organophosphorus exposure due to agricultural spraying in apple orchards adversely affected the survival of the birds monitored. Reproduction of tree swallows and eastern bluebirds was further assessed in pesticide-sprayed apple orchards in southern Ontario to evaluate egg fertility, clutch size, egg and chick survival, and pesticide exposure.[121] In this study of cavity-nesting birds reproduction was compared for nest boxes in sprayed and nonsprayed apple orchards from 1988 through 1994. There was a significant increase in unhatched eggs in bluebirds, as organochlorine levels increased in eggs. There were significant associations between toxicity scores of current-use pesticides and at least one avian reproductive parameter in every year of the study, but the reduction in reproductive rates associated with pesticides did not exceed 14%, for either species, in any year. Reduced reproduction occurred in six years for tree swallows, but for bluebirds this occurred in only four years.

Increased spray drift was suspected to be a factor of potential concern experienced by nestling and adult great tits (*Parus major*).[122] Nest boxes were placed in hedgerows bordering fields sprayed with pesticides (pirimicarb or dimethoate). One hedge was sprayed directly with pirimicarb to simulate maximum drift effect. Two hedges were left untreated to serve as control areas. Significant inhibition of blood plasma cholinesterase activity was detected within 24 h in adult birds exposed to drift of dimethoate and in adult birds from the hedge sprayed directly with pirimicarb. Inhibition of nestling plasma cholinesterase activity was found in all treated hedges after 24 h. A tendency toward reductions in weight gain, though not significant, was found in nestlings both between 0

to 24 h and 24 to 48 h after treatment in all the treated hedges compared to nestlings from the control hedge.

Studies with European starlings have provided a valuable avian model for field testing because starlings readily occupy artificial nest boxes installed in test spray fields.[95,123] Nest boxes provide a large synchronous breeding population of a passerine species at treatment sites. Starlings consume soil invertebrates, which come in direct contact with pesticides in the soil. Using this technique, the effects of application of methyl parathion at 1.4 kg active ingredient (a.i.)/ha. to a cultivated field were examined, and it was determined that there was depression of brain cholinesterase in adults and nestlings, and the number of nestlings fledging from treated fields was reduced compared with the control field.[124] In a similar study with the same insecticide control birds had over 50% successful nests, whereas those in a field treated with 2.5 kg a.i./ha. of methylparathion had only a 20% success rate; fledglings were found to be more susceptible to postfledgling mortality when nestlings from each field were radio-collared and followed for 2 weeks after leaving the nest.[96]

4.8 AVIAN BEHAVIORAL TOXICITY TESTING

Behavioral aberrations in wildlife can be manifested at one or two orders of magnitude below lethal levels and therefore can be regarded as sensitive toxic-response indicators.[29] In the laboratory subtle alterations in behavior have been associated with exposure to toxic substances. Peakall[125] reviewed behavioral responses of birds to pesticides and other contaminants and concluded that certain operant tests are relatively simple and reproducible, but that other more complex tests, such as breeding behavior and prey capture, are probably more relevant to survival in the wild. In field studies the presence of interacting environmental factors, such as ambient temperature and weather conditions, complicate establishing a cause-and-effect relationship. The best-documented field cases of behavioral aberrations in wildlife include exposure of birds to anticholinesterase insecticides and of fish-eating birds in the lower Great Lakes to organochlorines. Both of these classes of chemicals have affected reproduction by causing decreased nest attentiveness.[126–128]

Laboratory and pen studies have documented changes in mallard hen and brood behaviors in response to anticholinesterase pesticides. Methyl parathion caused broods to mostly preen and loaf on land, while control broods primarily fed and swam in open water.[129] Methyl parathion also affected incubation behavior, causing nest abandonment and decreased nest attentiveness.[70] Several other studies have demonstrated increased vulnerability of birds, including sharp-tailed grouse (*Tympanuchus phasianellus*), northern bobwhite, and European starlings, to predation following organophosphorus insecticide exposure and field release[130,131] or increased susceptibility to experimental predation by a domestic cat.[132] Evidence has also been developed indicating that migratory orientation may be affected in captured migratory white-throated sparrows (*Zonotrichia albicollis*) exposed to dietary acephate for 14 days.[133] Adult sparrows did not establish a preferred orientation, but juveniles displayed a seasonally correct southward migratory orientation. It was hypothesized that acephate produced aberrant migratory behavior by affecting the memory of the migratory route and wintering ground.

Two laboratory tests of natural behavior that may be performed on the same subject consist of response of newly hatched precocial birds to the maternal call (measured time a chick takes to approach a recorded call) and avoidance of a fright stimulus. Both tests are conducted in partitioned runways, permitting several chicks to be tested simultaneously with recorded responses. These tests showed that mallard ducklings from parents fed as little as 0.5 ppm methylmercury were less responsive to maternal calls and more responsive to a fright stimulus than were controls.[134] In another study opposite responses were observed for ducklings whose parents were fed 3 ppm DDE.[135] In a test of a carbamate insecticide, decreased duckling approach-response behavior was decreased following exposure to carbofuran-sprayed vegetation.[136]

Tests of learning ability based on operant conditioning appear to be sensitive indicators of toxicant-induced effects in birds. Recent hatchlings are fed low dietary concentrations of toxicant for several months and are then trained via hunger motivation to peck a lighted key in the conditioning box for a food reward. After the correct pattern is learned, the pattern is reversed or changed, and the ability to adjust is measured. This technique has been applied to test responses at dietary concentrations of less than 1.0 ppm endrin, 10 ppm toxaphene, and up to 100 ppm paraquat in northern bobwhite.[137,138] Learning impairment was caused by endrin and toxaphene, but not by paraquat.

4.8.1 Time-Activity Budgets

Time-activity budgets are important aspects of behavioral ecology that aid in understanding habitat utilization and energy consumption. Such measurements were used effectively in evaluation of the hazards to mallard ducklings of toxic drainwater components and lead-contaminated sediments. In one study environmentally reasonable concentrations of either boron or arsenic in the diet affected the activity schedules of developing ducklings, including increased time at rest and selection of supplementary warmth, with less time in alert behavior and in the water compared to controls.[139] In another study the incidence and duration of ten behaviors (resting, standing, moving, drinking, dabbling, feeding, pecking, preening, bathing, and swimming) were recorded. Consumption of diets containing 12 or 24% lead-contaminated sediment (3449 ug/g lead) affected the proportion of time spent swimming but did not affect any of the other recorded behaviors.[140] However, there were signs of impaired balance and mobility and effects on the brain due to lead accumulation (e.g., oxidative stress, altered metabolites, and decreased brain weights). [141]

The behavior of captive great egret nestlings was evaluated in a study of methylmercury.[105] Birds were randomly divided into a control group and groups that received 0.5 or 5 mg methylmercury chloride per kg of food between 12 and 105 days of age. Activity levels, maintenance behavior, and foraging efficiency were studied. During the postfledging period there were no differences between low-dosed and control birds in time required to capture live fish in pools or in efficiency of capture. However, the methylmercury affected their activity, tendency to seek shade, and motivation to hunt prey. Alterations in the allocation of energy in developing ducklings and egret chicks as seen above would have obvious drawbacks in the natural environment including avoidance of predators and foraging strategies. Birds dosed with 5 mg/kg became severely ataxic and were euthanized by 12 weeks of age.

4.8.2 Critical Periods of Development

The temporal effects of lead were evaluated on developing herring gull chicks.[99,100] In these studies, 1-day-old gull chicks were placed into treatment groups to receive a lead acetate dose on day 2 (50 or 100 μg/g), on day 6 (100 μg/g), or on day 12 (50 or 100 μg/g); controls received saline injections on the same days. Behavioral tests were performed at 2- to 5-day intervals to examine locomotion, balance, righting response, thermoregulation, and visual cliff. Flight behavior was examined at fledging. Righting response and balance were disrupted immediately after exposure, regardless of the timing of exposure. Thermoregulatory, visual cliff, and individual recognition behavior were more affected by exposure at 2 to 6 days, and there was little effect with exposure at 12 days. Overall, the data showed that treatment at 6 days of age may be a more critical period than earlier ages for some behaviors. However, chicks treated with single doses of lead acetate solution (100 mg/kg) at day 2 experienced disrupted sibling recognition through fledging at 26 days of age. In nature lead-impaired chicks might be unable to use siblings as a cue to find their nests and could experience higher mortality from territorial adults and chicks as well as from cannibalistic adults. Feathers of some roseate terns (*Sterna dougallii*), herring gulls, and black

skimmers (*Rynchops niger*) contain lead in concentrations that have been experimentally correlated with behavioral impairment and growth retardation.

4.8.3 Food Discrimination and Feeding Behavior

Behavior studies have demonstrated the effects of contaminants on food discrimination.[142–146] Procedures for evaluating the potential ability of birds to avoid chemically contaminated food have been published.[144–146] The discrimination threshold is defined as the dietary concentration above which test animals will decrease the proportion of treated food they consume if alternative untreated foods are available. The field conditions under which wildlife species could utilize the ability to detect and avoid toxic foods are largely unknown. However, when alternative food choices exist, vulnerability to poisoning in northern bobwhite chicks by organophosphorus and carbamate insecticides can be reduced by the number and relative abundance of choices as well as by the bird's ability to detect the chemical.[146]

Low-grade exposure to organophosphorus insecticides may produce long-term changes in bird feeding behavior.[147] This was demonstrated through tests for conditioned taste aversion in a series of field experiments in which independent replicates were large numbers of breeding territories of red-winged blackbirds. Birds freely consumed untreated insect prey offered to them in control territories, but those in treated territories consumed up to three meals of prey tainted with parathion and then avoided offered prey in the treated territories long after parathion tainted prey were no longer present. This long-term change in feeding behavior was produced by organophosphorus in amounts insufficient to induce signs of toxicity or to depress brain cholinesterase activity. The effect was long-term because, unlike noxious repellency, conditioned taste aversion induced by trivial amounts of parathion denied birds the opportunity to discriminate between tainted and untainted prey. Although birds may be spared repeated exposure to the toxic substance, continued avoidance of untainted prey disrupts foraging, endangers breeding efficiency, and reduces predation upon pest insects.

Other behavioral studies have been conducted for assessing and reducing avian risk from granular pesticides. The extant information regarding bird response to grit and granule characteristics (i.e., granule carrier type, color, size, shape, and surface texture) and pesticide load per granule has been summarized.[52] When the efficacy of eight taste repellents for deterring the consumption of granular insecticides in northern bobwhite was evaluated, the most effective were d-pulegone and quinine hydrochloride.[53] The authors concluded that treating pesticide granules with a potent taste repellent, such as d-pulegone, is a promising approach to reduce the risk of their ingestion by birds.

4.9 MAMMALIAN WILDLIFE TOXICITY TESTING

Fewer laboratory studies have been conducted with mammalian than avian wildlife, most likely due to the fact that the human health effects literature and agricultural nutrition literature is abundant with studies conducted with laboratory rodents as well as other species of domestic mammals that are viewed by some as surrogate species for mammalian wildlife.

Toxicity testing with mammalian wildlife for regulatory purposes has been limited in scope and requirement compared to avian wildlife toxicity testing (Table 4.1). Much of the U.S. EPA's FIFRA mammalian toxicity data is derived from routine studies with the laboratory rat (*Rattus norvegicus*) for pesticide registration. However, if the margin of safety appears small, or if the likelihood is high that specific mammals of concern will be exposed, then additional and more ecologically relevant tests may be required. This next level of testing requires a dietary LC_{50} study or acute oral LD_{50} study with a nonendangered and representative species that is likely to be exposed (e.g., microtine rodent). Occasionally, other reproductive and secondary toxicity tests are mandated.

The U.S. EPA has been encouraged to expand its required mammalian studies to include a wild herbivore test species (a microtine rodent such as the meadow vole, *Microtus pennsylvanicus*), an omnivore (the deer mouse, *Peromyscus maniculatus*), and a carnivore (the mink, *Mustela vison*, or european ferret, *Mustela putorius furo*), as recommended by Ringer.[148] The mink may be a preferred carnivore because it (1) is indigenous to North America, (2) can be reared in the laboratory, (3) has a large biological database, and (4) is among, if not is, the mammalian species most sensitive to PCBs, PBBs, hexachlorobenzene, TCDD, and aflatoxins.[149–155] However, it has been argued that the mink is no more sensitive than laboratory rodents to many other chemicals including DDT, dieldrin, and *o*-cresol.[148,155–157] The use of wild mammals has also been proposed for setting water quality criteria for those species that consume aquatic life including the mink as a piscivorous species, the northern short-tailed shrew (*Blarina brevicauda*) as an insectivorous consumer of aquatic invertebrates, and the deer mouse as an omnivorous species.[158]

Three published protocols describe guidelines for testing mink and European ferrets for conducting dietary LC_{50} and reproductive toxicity tests and for assessing the primary vs. secondary toxicity of test substances.[156] For the LC_{50} test, the dietary exposure period is 28 days, over which signs of toxicity and mortality are recorded. The reproductive protocol is designed to evaluate dietary exposure prior to and during the breeding period and through gestation and lactation. The main endpoints include adult survival, oogenesis and spermatogenesis, reproductive indices, embryo and fetal development, and offspring growth and survival. The third protocol compares the dietary toxicity and lethality for the parent compound of a test substance (primary toxicity test) with the same substance fed at identical concentrations but contained in animal tissue (prey) contaminated by previous exposure (secondary toxicity test). Secondary toxicity testing of Aroclor 1254 revealed enhanced toxicity of the metabolized form in mink.[159] PCB levels in wild mink on Lake Ontario are similar to those reported to cause reproductive problems in controlled-feeding studies, and correlations between organochlorine levels in fish and levels in mink and otter (*Lutra canadensis*) are apparent.[149–160]

In a comparative toxicity study of potential mammalian models, LD_{50} and 5-day LC_{50} tests were conducted in the same laboratory. The laboratory mouse (*Mus musculus*), the meadow vole, and the white-footed mouse (*Peromyscus leucopus*) were equally sensitive to the organophosphorus insecticide acephate.[161] A database of tests conducted at a single laboratory has been developed for acute oral toxicities of 933 chemicals to deer mice and house mice.[162] These were first-line screening tests for discovery of potential economic poisons for use in invertebrate pest control; the authors did not test for, nor offer any opinion on, which species was the best model for regulatory purposes. In one effort to reconcile the need for wild mammal testing the U.S. EPA sponsored a study comparing oral LD_{50} and 30-day dietary LC_{50} tests with four members of the genus *Microtus*.[163] The results were compared with published values for standard tests of laboratory rats and mice. Though the comparison was limited to ten widely different pesticides, the authors concluded that laboratory rodents were generally more sensitive than voles to the compounds tested.[163]

Factors such as food and habitat preference may affect routes and degree of exposure in the field, thereby rendering some species of wild rodents ecologically more vulnerable to certain contaminants. In studies with microtine rodents aversion to carbofuran-treated feed was associated with delays in the time to first breeding, whereas a female-biased sex ratio in offspring of breeding pairs receiving paraquat in the diet was apparent.[164] White-footed mice exposed to PCBs in the diet at 10 ppm through the second generation exhibited poor reproductive success in comparison with second generation controls and the parental generation. PCB-treated young were significantly smaller at 4, 8, and 12 weeks of age.[165] Other studies have suggested inhibition of reproduction and changes in liver, spleen, adrenal, and testis function at a PCB-contaminated field site for this species.[166] Experimental feeding studies with lead have revealed mortality and impaired postnatal growth in young bank voles (*Clethrionomys glareolus*) when their mothers received lead-contaminated food after giving birth.[167] Though wild mammals have not been used extensively in laboratory studies with environmental contaminants, they have been used as monitors of environmental contaminants in nature, especially in studies of metals, organics, or radionuclides.[168–170]

In other situations studies have been conducted with unique species of wild mammals with varied sensitivities to certain classes of contaminants.[171] A review of the recent book *Ecotoxicology of Wild Mammals* by Shore and Rattner[171] clearly indicates the need for more controlled comparative laboratory studies with unique wild mammals. For example, much of the ecotoxicological research of contaminant effects on insectivorous mammals has focused on terrestrial shrews, but little is known about contaminant effects on water shrews, moles, or hedgehogs.[172] Fish-eating marine mammals, including seals, occupy high trophic levels in the aquatic food chain and accumulate high levels of contaminants including polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans. Such chemicals have been found to be immunotoxic at low doses in studies with laboratory mammals and mink. Recent associations have been established between such contaminants and effects on the immune system, e.g., increased incidence of disease was noted for certain free-ranging seal population; laboratory studies with captive harbor seals have confirmed these findings.[173] On the basis of these and other studies it was concluded that complex mixtures of environmental contaminants may represent a real immunotoxic risk to free-ranging marine mammals in many areas of Europe and North America.[173]

4.10 AMPHIBIAN AND REPTILE TOXICITY TESTING

Over the past decade widespread population declines of amphibians have been documented in North America, Europe, Australia, and Central and South America. Population declines in eastern Europe, Asia, and Africa have also been suggested but are not as well documented.[174–177] Contaminants may be involved with amphibian population declines including their possible interaction with other factors, as discussed in Chapter 40 of this book and in a thorough review of the literature as provided in the recent book, *Ecotoxicology of Amphibians and Reptiles,* by Sparling, Linder, and Bishop.[177] In the past it was presumed that tests conducted on fish, birds, and mammals would be sufficiently conservative to protect amphibians and reptiles. This concept can no longer be supported. Comparative toxicities of organic compounds and metals between amphibians and fish have been summarized for a standard embryo larval test (exposure from fertilization through 4 days posthatching).[178]

Fish species commonly used in toxicity tests, such as the rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), and largemouth bass (*Micropterus salmoides*), were used for comparisons that were standardized as much as possible including comparable life stages, water chemistry, and durations. These tests included 28 species of native amphibians from the families of Ambystomatidae, Microhylidae, Hylidae, Ranidae, and Bufonidae and the African clawed frog (*Xenopus laevis*). Median lethal toxicity values for metals in amphibians varied by 100-fold. In all, 50 metals and inorganics as well as 13 organic compounds were tested, for a total of 694 comparisons of amphibians and fish. In summary, amphibians had lower LC_{50} values than fish in: (1) 64% of all the tests, (2) 74% of the comparisons among the 15 most sensitive amphibian species and fishes, (3) 80% of the comparisons involving amphibians and warm-water fishes, (4) 66% of the 13 most metal-sensitive amphibians and rainbow trout, and (5) 74% of all amphibian species vs. the fathead minnow. The overall conclusions were that there was great variation among amphibian species in their sensitivity to metal and organic contaminants, that amphibians generally were more sensitive than fish, and that water quality criteria established for fish may not be protective of amphibians.

The U.S. EPA drafted a guideline (draft revised FIFRA Guidelines Document-Subdivision E, March 1988) with several acceptable protocols for preregistration testing of chemicals for acute lethal toxicity to amphibians and reptiles.[179,180] The provisional species of choice are frog tadpoles (*Rana spp.*) and adult green anole lizards (*Anolis carolinensis*). There is also a U.S. EPA testing guideline for a "tadpole/sediment subchronic toxicity test" under aquatic testing guidelines of the series "850-Ecological Effects Test Guidelines." This guideline is used to develop data on the subchronic toxicity of chemical substances and mixtures of chemicals sorbed to natural sediments to bullfrog tadpoles. Here, test chambers are filled with appropriate volumes of dilution water (control) or

appropriate amounts of contaminated natural sediments and dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. This toxicity test may be performed by either of two methods: (1) dosing the tadpoles orally with a sediment/test substance slurry and maintaining tadpoles in test chambers with only clean dilution water for 30 days or (2) maintaining tadpoles in test chambers containing contaminated sediments and allowing tadpoles to ingest the contaminated sediments. Concentration-response curves and LC_{50}, EC_{50}, LOEC, and NOEC values for the test substance are developed from survival and growth responses. Any abnormal behavior (e.g., erratic swimming, loss of reflex, increased excitability, lethargy) and any changes in appearance or physiology such as discoloration (e.g., reddened leg, excessive mucus production, opaque eyes, curved spine, or hemorrhaging) are also evaluated.

As mentioned in Section 4.1 of this chapter, the U.S. EPA is implementing an Endocrine Disruptor Screening Program that includes several amphibian-based ecotoxicological screens and tests for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. These include a "frog metamorphosis assay" and an "amphibian life cycle reproductive toxicity study with endocrine endpoints."

A strategy for protection of herpetofauna has been developed.[181] Rather inclusive recommendations include: (1) research should examine the relative sensitivity of major groups of amphibians and reptiles to major classes of environmental contaminants to detect possible inherent (taxonomic) variability in response; (2) chemicals with selective toxicity should be examined first, permitting comparison of data from aquatic and terrestrial tests; (3) a variety of *in vitro* procedures, such as effects in embryos or larvae, may reduce cost and time; and (4) laboratory investigation should provide a guide, but should not obviate the need, for well-designed field tests and postregistration vigilance by field biologists.

A summary of acute toxicity to amphibians of more than 200 different contaminants and field studies of over 50 contaminants indicated that neither test species nor protocols were standardized.[182] A searchable database — RATL (Reptile and Amphibian Toxicology Literature, http://www.cws-scf.ec.gc.ca/nwrc/ratl/index_e.htm) — for published ecotoxicological data from studies with amphibians and reptiles has been created by the Canadian Wildlife Service. Currently, there are approximately 2000 references in this database for approximately 6200 contaminant-related studies, divided almost equally between reptiles and amphibians. Approximately 650 different species are included in the database.

Deformities in tadpoles have been studied as a possible sensitive indicator of environmental contaminants; multiple causes and types of deformities in amphibians have been reviewed.[183] Caged tadpoles in water receiving runoff or spray drift from agricultural fields has been used to identify potential hazard.[184] A standard test (FETAX, Frog Embryo Teratogenesis Assay: *Xenopus*) has been developed with embryos of the clawed frog (*Xenopus laevis*) as an assay for teratogenicity of chemicals and mixtures of contaminants.[185] Under the auspices of the ASTM, a comprehensive guideline for FETAX was published in 1991 and updated in 1998 ["Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-*Xenopus (FETAX)*," Annual Book of ASTM Standards, Designation E143998]. The similarity of response to dieldrin between *Xenopus laevis* and two ranid species in a study conducted by the U.S. EPA supports the utility of this species.[186] Results with nine combinations of developmental toxicants have indicated that FETAX is useful for hazard assessment of mixture toxicity and of sediment extracts.[187]

Short-term toxicity tests with *Xenopus laevis* and *Rana pipiens* were conducted with a 96-hour modified FETAX to assess paraquat toxicity.[188] The commercial formulation was three times as acutely toxic as the technical-grade chemical, and the 96-hour LC_{50} of either form at least sixfold lower for *Rana pipiens* than *Xenopus laevis*. In another comparative study the embryotoxicity of the nonionic surfactant nonylphenol ethoxylate was determined in *Xenopus laevis* and the Australian frogs *Litoria adelaidensis* and *Crinia insignifera* using the FETAX protocol.[189] Growth inhibition as assessed by embryo length was the most sensitive indicator of effect in all three species. *Xenopus laevis* was the most sensitive of the three species and the only species that displayed indisputable

terata. Integrated field and laboratory studies have been used for evaluating amphibian responses in wetlands impacted by mining activities in the western United States, where FETAX was conducted in the lab and *in situ* in the field using the bullfrog.[190]

For reptiles, a considerable portion of the published toxicological research has focused on turtles, especially snapping turtles (*Chelydra serpentina*) and sea turtles. [191] Focus on these groups may be justified in part because snapping turtles are large, long-lived omnivores that live intimately with aquatic sediments and thus are considered excellent bioindicators of wetland conditions, whereas many species of sea turtles are rare or endangered. Snapping turtles have been used as monitors of environmental contaminants including use in tidal wetlands, freshwater ponds, rivers, and lakes.[192–194] Contaminant-related DNA damage was detected in snapping turtles and in sliders (*Pseudemys scripta*).[192,194] Higher rates of deformities and unhatched eggs were related to PCB exposure of snapping turtle in highly contaminated areas of the Great Lakes.[193,195] Reptiles in general, and snakes and lizards in particular, are important although often neglected components of terrestrial and aquatic ecosystems and should be included in studies of environmental contamination.[196]

Accumulation and effects of environmental contaminants on snakes and lizards have been comprehensively reviewed.[197, 198] Since all snakes are secondary, tertiary, and top predators, they are especially subject to the bioaccumulation of environmental contaminants. Their unique life histories make their roles in food webs diverse and important, and they are crucial to the proper functioning of many ecological processes. Lizards may also be excellent bioindicators of contamination.[198] Lizards are a significant part of many ecosystems as well as an important link in many food chains. There are large gaps in data for many environmental contaminants on lizards. Ecotoxicological studies on a wide variety of lizard species are needed; both laboratory and field studies would provide useful information. Because the majority of lizards are insectivores, studies of the effects and accumulation of pesticides are essential. Furthermore, many species are listed as threatened or endangered in the United States.

Reptiles, including turtles and alligators, have been studied for evidence of contaminant-related endocrine disruption, as discussed in Chapter 39 of this book and elsewhere.[195,199]

4.11 SUMMARY

Wildlife toxicology is the study of potentially harmful effects of toxic agents on wild animals. Wildlife toxicology endeavors to predict the effects of toxic agents on nontarget wildlife species and, ultimately, populations in natural environments. Avian toxicity testing protocols were first utilized by the U.S. Fish and Wildlife Service as a consequence of wildlife losses in the 1950s due to the increased use of DDT and other pesticides. The first testing protocols focused on single-dose acute oral toxicities with lethality as the major endpoint. Further protocol development resulted in subacute 5-day dietary tests. These, along with the single acute oral dose tests, are currently required by the U.S. Environmental Protection Agency in support of pesticide registration. The avian subchronic dietary toxicity test was developed as an extension of the subacute test as a precursor to full-scale reproductive studies, but it is not routinely required for regulatory purposes. Subchronic testing has been applied to compare the sublethal effects of different forms of mercury, to study hepatotoxicity of organic selenium, to comparatively study contaminated sediment ingestion, and to study delayed neurotoxicity of certain organophosphorus insecticides. Avian chronic toxicity tests are designed with reproduction as the primary endpoint and are required for both waterfowl and upland gamebirds during chemical registration. Persistent chemicals, such as chlorinated hydrocarbons, require relatively long-term exposures (at least 10 weeks) in advance of breeding, whereas shorter-term exposures may be utilized for less persistent chemicals such as organophosphorus insecticides. The U.S. EPA is presently implementing an Endocrine Disruptor Screening Program (EDSP), which includes an avian two-generation test with Japanese quail with endocrine-specific endpoints in addition to conventional reproduction endpoints.

Single-dose avian embryotoxicity and teratogenicity tests were developed in part to assess the potential contaminant hazard of external exposure of bird eggs; tests with multiple species and chemicals have revealed differential toxicities of a spectrum of chemicals and sensitivities among species. Recent focus has included hazard evaluations of aquatic weed control, mosquito control, and wildfire control agents. Developmental toxicity testing has also focused on the vulnerability of "neonatal" nestling birds, including kestrels, starlings, red-winged blackbirds, great egrets, and gulls, to oral ingestion of environmental contaminants.

Avian terrestrial field studies are basically of two types: (1) screening studies to ascertain whether impacts are occurring, and (2) definitive studies to estimate the magnitude. These studies often require extensive monitoring of reproductive success and survival of young wherein active nests are periodically examined and mark-recapture techniques as well as radiotelemetry are used. Sometimes, artificial nest structures are provided in the vicinity of test-spray fields to increase nest density, and hence experimental sample size, for species such as American kestrels and European starlings. Avian terrestrial field studies have been successfully undertaken using diverse species, including northern bobwhite, sage grouse, waterfowl, and at least five different passerine species, during and following applications of agricultural pesticides.

Behavioral aberrations in wildlife can be manifested at one or two orders of magnitude below lethal levels of environmental contaminants. In birds, changes in nest attentiveness, brood behavior, and increased vulnerability to predation have been documented in field and pen studies. Response time to maternal call, avoidance of fright stimulus, and tests of operant learning ability as well as time-activity budgets, effects at critical periods of development, and altered food discrimination and feeding behavior have been successfully applied to laboratory studies.

Laboratory studies with environmental contaminants and mammalian wildlife have been limited compared to avian studies. This may be due to the fact that the human health effects and agricultural nutrition literature is abundant with studies conducted with laboratory rodents as well as other species of domestic mammals that are viewed as surrogate species for mammalian wildlife. Mammalian toxicity data of the U.S. EPA FIFRA has consisted largely of laboratory rat data for pesticide registration, whereas wildlife testing requires a dietary LC_{50} or acute oral LD_{50} study with a nonendangered representative species likely to be exposed, quite often a microtine rodent. Previous recommendations have included use of an omnivore, such as the deer mouse, and a carnivore, the mink, for which there is a large biological database for both laboratory and field studies. The use of wild mammals has also been proposed for setting water quality criteria for those species that consume aquatic life, including the mink as a piscivorous species, the northern short-tailed shrew as an insectivorous consumer of aquatic invertebrates, and the deer mouse as an omnivorous species. There are many unique species of wild mammals with varied sensitivities to certain classes of contaminants; controlled comparative laboratory studies are needed for many of these species.

Worldwide concern over declining populations of amphibians and reptiles has revealed that numerous taxa of amphibians and reptiles are endangered or threatened. Comparative toxicities of organic compounds and metals indicate a wide variation in sensitivity among amphibian species. Amphibians are generally more sensitive than fish, suggesting that water quality criteria established for fish may not be protective of amphibians. The U.S. EPA is developing guidelines for preregistration testing under FIFRA for acute lethal toxicity to amphibians and reptiles. Also, the U.S. EPA is implementing an Endocrine Disruptor Screening Program (EDSP), which includes several amphibian-based ecotoxicological screens and tests for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. These include a "frog metamorphosis assay" and an "amphibian life cycle reproductive toxicity study with endocrine endpoints."

Reptiles, which are critical components of many food chains, are often neglected in studies of terrestrial and aquatic contamination. A considerable portion of the toxicological research on reptiles has focused on turtles, especially snapping turtles and sea turtles. Snapping turtles live intimately with aquatic sediments and are considered excellent bioindicators of wetland conditions. Turtles and alligators are being studied for evidence of contaminant-related endocrine disruption. All snakes

are secondary, tertiary, and top predators and are susceptible to the bioaccumulation of environmental contaminants. Lizards provide important links in many food chains and are perhaps more influenced by contamination than previously believed.

ACKNOWLEDGMENTS

Reviews of the content of this manuscript provided by E. F. Hill, P. H. Albers, and L. Touart are gratefully acknowledged.

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