ANNAMALAI UNIVERSITY
CAS IN MARINE BIOLOGY
III BFSC- 606 Toxicology
by
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This chapter focuses on aquatic toxicology of fish culture. Aquaculture is the production of aquatic animals in the aquatic environment for human food, replenishing fish stocks and other uses. The water may be a sheltered oceanic bay containing penned organisms, inland ponds or an indoor tank system. The more contained the aquatic rearing system, the more diligent must be monitoring and controls over the artificial ecosystem. The smaller the volume of water and the more contained the system is, the more susceptible it is to water safety issues. Issues in water safety can contribute to outbreaks of infectious diseases. The hobbyist also raises or maintains fish and other aquatic animals/plants in a variety of integrated ecosystems. Fish are also used as public displays by commercial organizations.

Chemical and physical causes of disease in aquatic organisms are generally linked to water and food because these are the primary pathways of toxic substances to animals in the aquatic environment. Noise pollution is being shown to be an important physical agent (NAS, 2016). Intoxication of fish can be acute, sub acute, or chronic. Chemical-linked food safety issues can occur if chemical contamination of edible aquatic organisms occurs. The toxicity of a specific substance can vary between fish species and can change with water temperature, pH, and ion composition (Wlasow et al., 2010). In recirculation systems, waste materials and microbial degradation products can reach toxic levels. Chemical intoxication and other environmental stressors can increase the susceptibility of aquatic organisms to infectious diseases (Morley, 2010). The predisposing causes of infectious disease and larval survival can be overlooked because they may be subtle or unrecognized. The toxicology of the water column differs from that of the sediment, and these variations in the aquatic environment present different issues to various segments of the aquatic environment.

Aquatic toxicology generally involves the measurement of contaminant levels to characterize the hazards imposed on the aquatic environment; however, this field of study also includes information on how those contaminants can affect humans in and around these aquatic environments. The multidisciplinary research that comprises the field of aquatic toxicology has provided a better understanding of the effects anthropogenic activities and

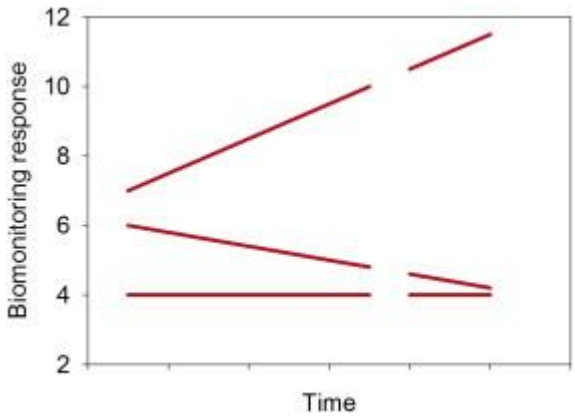
chemical contaminants have on aquatic environments. Additionally, this increase in knowledge has improved the methods utilized and consequently, the confidence in measuring the potential hazards associated with humans exposed to contaminated aquatic environments and organisms (Pritchard 1993). This chapter provides a plethora of resources used in the field of aquatic toxicology. The resources assembled are informative whether one is looking at the pollution generated by anthropogenic activities and the affected aquatic ecosystems or observing how contaminants in water and aquatic organisms affect water quality and the subsequent exposure to humans.

In aquatic toxicology, it is now well-established that the chemical characterization of pollutants exposure is not sufficient and that multidisciplinary approaches coupling chemistry and biology have to be developed to allow linking of the presence of contaminants and their putative toxic impacts. Along with the improvement of molecular biology methods, the development of "omics" technologies is booming worldwide since the early 1990s when these techniques have begun to emerge. During the past 20 years, these techniques have been in turn described as a unique research opportunity to decrypt all the biological mechanisms. In this chapter, the major genomic, proteomic, metabolomic, and fluxomic approaches developed in aquatic ecotoxicology are described and illustrated by studies on fish and mollusks from the recent literature. The advantages and main limitations of these techniques will be discussed. Finally, some important points to be taken into account in future prospects will be discussed (Patrice Gonzalez, Fabien Pierron, in Aquatic Ecotoxicology, 2015).

Modelling Toxicity

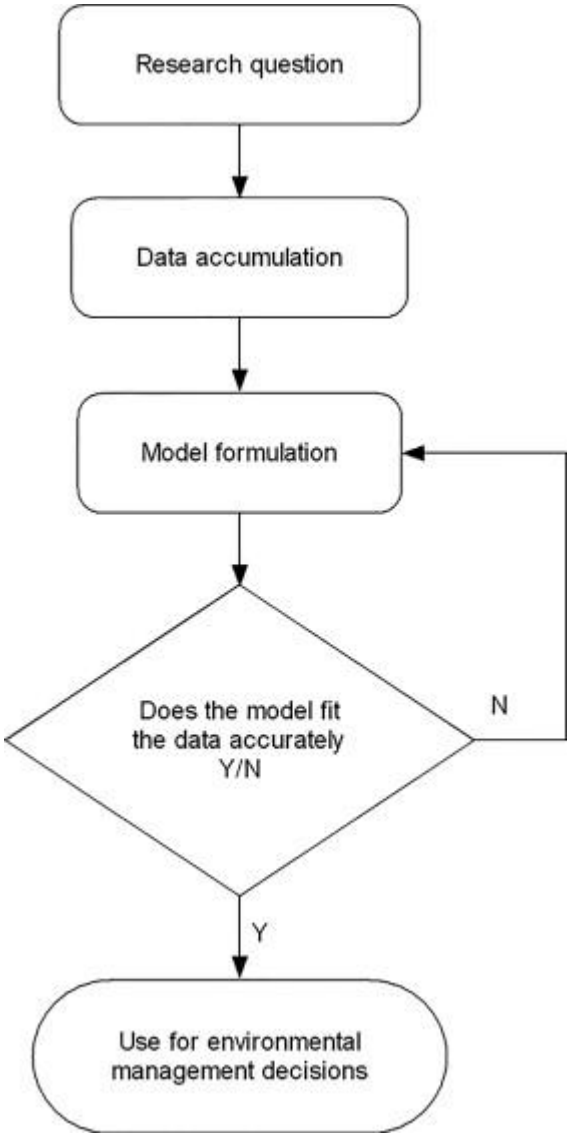
The goal of aquatic toxicology is to predict the effects of contaminants in ecosystems. This should form the basis of policies affecting aquatic systems (see Figure 18.1 for a scheme of how environmental management an toxicological studies should interact). Predictions require that existing observations can be used to generate scenarios about the future. This is the case, although aquatic toxicological studies in the natural environment necessarily report what has already happened (thus being retrospective). This holds also for biomonitoring studies, which rely on it being possible to extrapolate from any observed trend to the future (see Figure 18.2). The utility of retrospective studies is twofold. First, they can indicate how the environment has already been affected. Such information can be required in order to identify the sources of contamination, to require those responsible either to carry out stricter cleaning of their effluent or to pay for the damages incurred. Both necessitate the damaging contamination being pinpointed to a definite source, which requires highly specific exposure

biomarkers. Second, they can be used to prevent similar discharges and effects in other places. This requires the assumption that other environmental factors do not significantly affect the contaminant responses that are observed. Laboratory studies, on the other hand, can use novel contaminants and their mixtures. Thus, possible contaminants can be studied before their appearance in the environment. However, these studies do not include all the contaminants, their interactions, and interactions with natural abiotic and biotic factors. Consequently, one must always assume that the factors studied in the laboratory are decisive for the effects in nature.



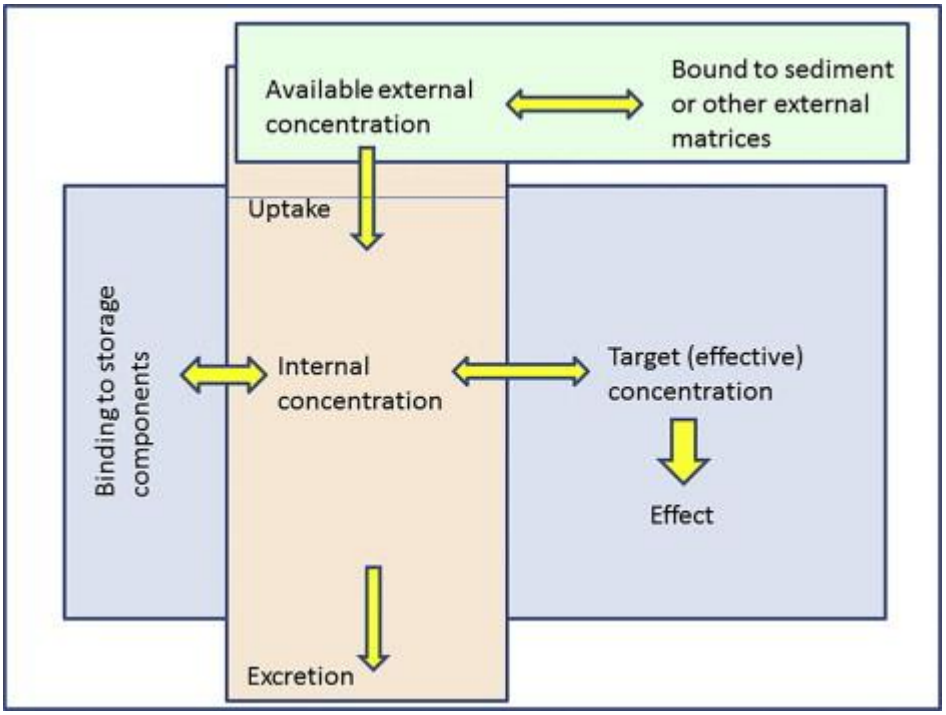
Extrapolation from biomonitoring data to the future supposes that the trends measured (lines before the break) continue in the future (lines after the break).

From the above it is clear that building and using models is necessary for predictive aquatic toxicology. It should be noted that any model is as good (or bad) as its least accurate component. A factor complicating any predictions is that the behavior of chemicals in the aquatic environment is highly complex. Figure 18.4 summarizes the aspects that need to be taken into account when building individual-based models. The toxicants may, further, have different targets (modes of action, MOA) at different concentrations. This heterogeneity has been poorly included in modeling of toxicant effects done so far.

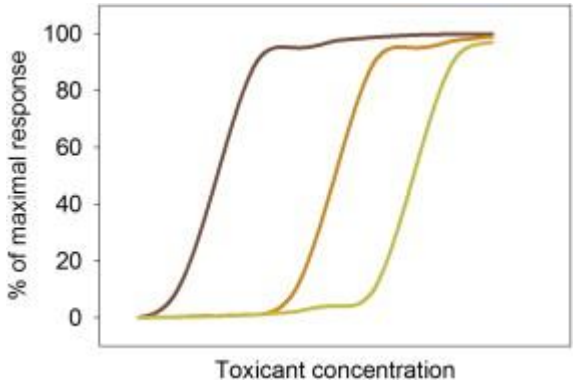


Flowchart of model building

In model building, one has to evaluate whether data are available for all the components needed for the model. If not, a research question and consequent experiment to address the lack of information is required.



In building models about toxicant effects on organisms, one needs to take into account bioavailability (green rectangle), build toxicokinetic models (pink rectangle), and consider the organismic distribution and its influence on toxicant effects (blue rectangle).

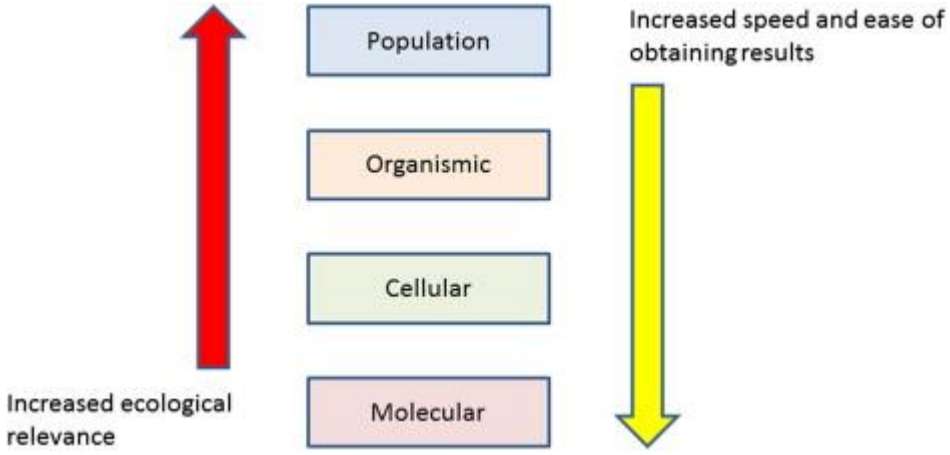


A toxicant may have several modes of action (targets), varying with concentration; different concentration–response profiles are depicted with lines of different colors.

Effects on Organisms

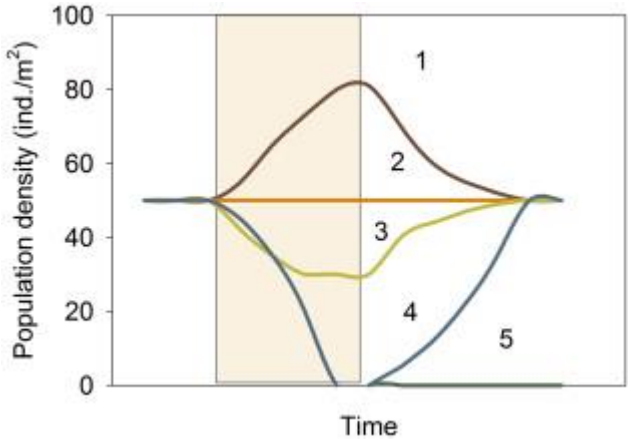
RNA Sequencing

The importance of RNA sequencing in aquatic toxicology stems from two facts. First, when the whole transcriptome is sequenced, one can get information about, for example, how different splice variants (of mRNAs) are affected by contamination. Second, noncoding RNAs (e.g. microRNAs) are important in posttranscriptional regulation of gene expression (they are involved in the regulation of translation of mRNA to proteins or mRNA stability, see section 1.3). If, for example, microRNA abundance is affected by a chemical, protein abundance may change even if the mRNA level is not affected. Consequently, the effect of the toxicant would not be seen in microarray or quantitative PCR measurement, but would be apparent in protein-level measurements or RNA sequencing. The choices of RNA sequencing range from sequencing the whole transcriptome (both coding and noncoding mRNAs) to sequencing only small noncoding RNAs. An increasing number of commercial providers offer RNA sequencing services with prices that are competitive. Thus, increasingly, the analyses can be done as a paid service, enabling the aquatic toxicologist to concentrate on the biological significance of the work. Also, guidelines as to what needs to be taken into account and reported in an RNA sequencing effort are available (Encyclopedia of DNA Elements (ENCODE) guidelines on RNA sequencing). Notably, in mammalian molecular biology, several microRNAs have been tied to specific conditions regulating translation. From the small number of studies available on organisms relevant for aquatic toxicology, it appears that microRNAs may be evolutionarily surprisingly well conserved, which suggests that mammalian findings can be related to even phylogenetically distant organisms. However, the significance of microRNA changes in aquatic toxicology is, as yet, little explored.



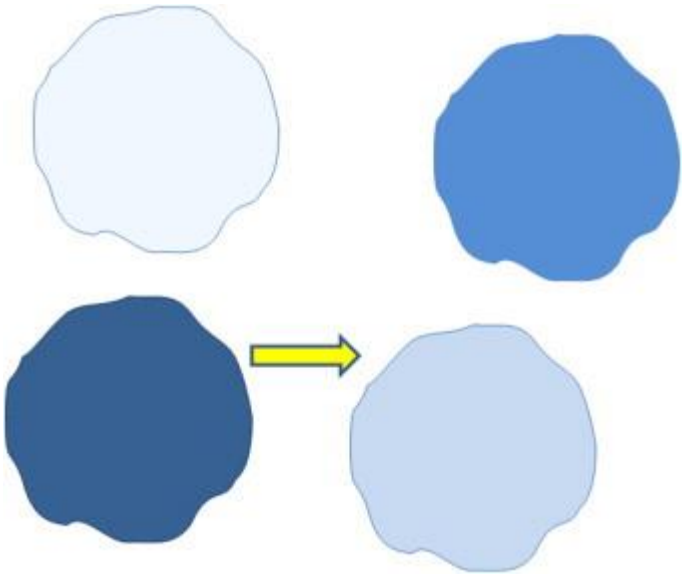
The study types in aquatic toxicology, from the molecular to the population level.

When effects of contamination on a population are considered, it is important to note that if the size and reproduction of the population are not affected, there is no contaminant effect. The reproductive efficiency must be included when natural populations are considered, since the size of a population can remain constant even if the contaminant affects the population, if the decreased reproduction or increased mortality is compensated for by immigration. The efficiency of immigration in replacing lost individuals depends on how migrant an organism is. Thus, it is more likely for pelagic fish than sessile mussels. Now, if only population size were taken to indicate the toxicant effect, an erroneous conclusion could be reached, as with migrant species immigration could replace any lost organisms, whereby toxicity to a sessile animal would be considered higher than to a migrant species even when they are actually the same. Immigration and emigration events can also have an effect on the estimated overall exposure. If organisms are living in a patchy population, and the different patches have different exposure histories, the overall movement of organisms between patches will affect both the apparent exposure and the apparent effects. The effect decreases with a decrease of movement between patches. When organisms are most of the time living as discrete populations, but immigration from one population to another is possible and occasionally occurs, one is talking of metapopulations. The different metapopulations may have drastically different exposure histories. Thus, migration from one metapopulation to another also affects the overall exposure experienced by the organisms.



Possible effects of contamination on population density.

The colored area indicates the period of contaminant exposure. (1) Contaminant exposure increases the population density of a species. The density decreases back to the original level after the exposure is discontinued. Originally the population is stable. With contaminant exposure, the fitness (efficiency of reproduction) of the studied species increases, whereby its density increases to a higher steady-state value. (2) The contaminant exposure does not affect the population density. This can be caused either by the contaminant not affecting the fitness of the species or by the reduced recruitment being replaced by increased immigration. (3) Contaminant exposure causes a decline in the population of species with a recovery upon cessation of the exposure. (4) Exposure to the contaminant causes a local extinction of the species with recovery of population through immigration after the discontinuation of the exposure. (5) The extinction caused by contamination persists in the absence of immigration.

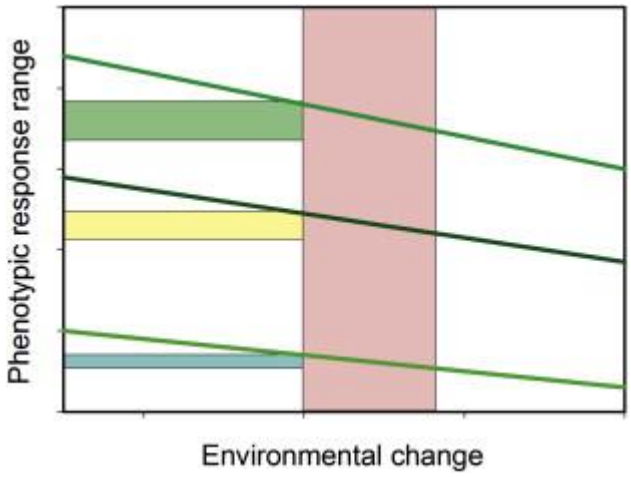


A population consists of four metapopulations, all of which have different contaminant exposures (the darker the color, the more exposure).

An individual that has experienced the most intensive exposure is immigrating to an area with a metapopulation that has experienced less contamination. If a response is measured from such an individual and related to where it is presently living, a faulty conclusion about exposure and response is reached.

If immigration or emigration is not possible in the short term, the population of a species may be affected. To be able to evaluate which alternative results from contaminant exposure, it is imperative that population size/density can be determined. The most accurate, but in most cases impossible, alternative is to count all individuals in the population of a given space. Consequently, the population density is normally determined from smaller samples. For fish, population estimation usually involves initial catching of fish, often with gill nets with varying mesh size. However, electro fishing, trawling, etc., can also be used. The fish are marked and released, and the population size can be estimated from the proportion of marked fish appearing in a new catch (mark–recapture method). In addition to the population size, the mark–recapture method can be used to estimate the age distribution of fish. To obtain an estimation of population densities of plankton species, several samples need to be taken from different parts of the water column (different depths, different locations) so that the sampling takes into account the different environments for any species. Similarly, benthic populations need to be sampled at different locations to cover the densities of organisms at the different bottom types.

The effects of contaminants on populations depend on how plastic the individual phenotypes in the population are. If the individuals of a population show reversible acclimation to toxicant exposure, the overall population response remains small. If, on the other hand, the phenotypic plasticity of individuals in a population is limited, large effects at the population level can be observed. Phenotypic plasticity (described in Figure 16.4) in a population depends on the genetic composition of the population (see also section 16.4). The relationship between environmental change and range of phenotypes produced for a genetic type of organism (the same genotype can produce several phenotypes) is determined by its reaction norm, and genetically distinct reaction norms are likely to occur.



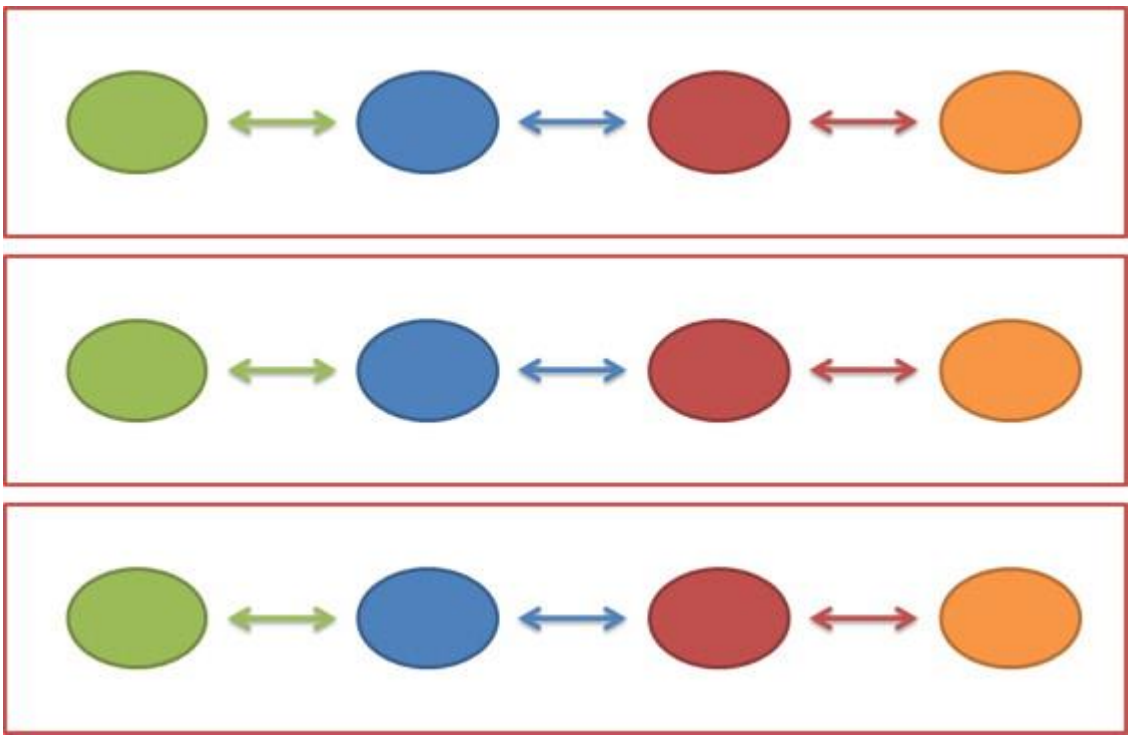
Phenotypic plasticity

Three genotypes are given as differently colored lines. The plausible environmental changes are shown with pink. The proportion of the genotype lines in the pink area gives the reaction norm, which translates to the possible phenotypic responses, given in green, yellow, and blue. The different genotypes are characterized by different ranges of plausible phenotypes.

Microcosms and Mesocosms

In the simplest case, studies of aquatic toxicology try to assess the mechanisms that toxicants may have at the organismic or cellular level. This is most conveniently done in laboratory exposures, which normally use single organisms and single pure toxicants. Furthermore, the duration of exposure is usually limited. Although this type of experimentation continues to give valuable information, it fails to impart knowledge about any interactions between toxicants or between organisms. Knowledge of both is required for full understanding of how a contaminated ecosystem functions. This is especially important as several of the sub lethal effects of toxicants affect organisms by influencing their interactions with other organisms. Such interactions can be studied experimentally using micro- and mesocosms. These are completely defined entities that enable organismic interactions to be studied experimentally, thus forming a continuum from pure laboratory experiments to ecosystem monitoring. The principles of micro- and mesocosms are described (Fig 5.1). The differences between the two are mainly in size and complexity. Microcosms are typically flasks containing only a couple of species from the different trophic levels, lacking any of the large organisms of the food web. They are used, for example, when interactions between phytoplankton and zooplankton are studied. Mesocosms are more complex systems with several trophic levels. However, even in the most complex mesocosms, the long-living and large organisms (mostly predators) are lacking. Thus, if such organisms play an important role in the function of ecosystem, the information given by a

mesocosm is limited. Another problem is that the equilibrium state reached in the mesocosm may be quite different from the equilibrium state of natural ecosystems, whereby the information obtained about the effects of environmental toxicants may be different from in natural cases. Another problem is that any experimentation with mesocosms has a limited time frame, which may cause toxicant responses to be different from what is observed in natural ecosystems. Difficulties associated with these time limitations are that the community of the mesocosm may not have reached equilibrium and that the responses to a toxicant may be season-dependent, so that mesocosm studies often give results that are different according to the season in which the mesocosm experiment is carried out.



A schematic representation of a micro-/mesocosm

The "cosm" is replicated (triplicated in the figure). The several trophic levels interact. In the figure, the food web consists of phytoplankton (green ovals), zooplankton (blue ovals), primary predators (red ovals, e.g. crustaceans eating zooplankton), and secondary predators (orange ovals, e.g. small fish eating crustaceans). Often, mesocosms are artificial streams, which also include macroscopic green plants.

There are two major alternatives for generating the communities in the micro- and mesocosms. The first is to use organisms from laboratory cultures, to get an exact standardization of the system. The number of seeded organisms will also be exactly defined. This guarantees that the replicates used in the controls and treatment communities are

initially alike. However, the communities will not be like natural communities in the area. In the second alternative, the organisms seeded in the micro-/mesocosm are taken from natural settings. The community they form should then be allowed to reach equilibrium before the treatment starts. Although this alternative is ecologically relevant, it is very difficult or even impossible to define the organisms present in the seeds completely, and the replicates and their equilibrium communities necessarily deviate from each other. Also, the equilibrium reached may be different from the one that would have been reached in the natural ecosystem. Further, the development of equilibrium takes time, which may be the limiting resource in experimentation.

Aquatic toxicology

Aquatic toxicology is the study of the effects of manufactured chemicals and other anthropogenic and natural materials and activities on aquatic organisms at various levels of organization, from subcellular through individual organisms to communities and ecosystems. Aquatic toxicology is a multidisciplinary field which integrates toxicology, aquatic ecology and aquatic chemistry.

This field of study includes freshwater, marine water and sediment environments. Common tests include standardized acute and chronic toxicity tests lasting 24–96 hours (acute test) to 7 days or more (chronic tests). These tests measure endpoints such as survival, growth, reproduction, that are measured at each concentration in a gradient, along with a control test.[2] Typically using selected organisms with ecologically relevant sensitivity to toxicants and a well-established literature background. These organisms can be easily acquired or cultured in lab and are easy to handle.[3]

History

While basic research in toxicology began in multiple countries in the 1800s, it was not until around the 1930s that the use of acute toxicity testing, especially on fish, was established. Over the next two decades, the effects of chemicals and wastes on non-human species became more of a public issue and the era of the *pickle-jar bioassays* began as efforts increased to standardize toxicity testing techniques.[1]

In the United States, the passage of the Federal Water Pollution Control Act of 1947 marked the first comprehensive legislation for the control of water pollution and was followed by

the Federal Water Pollution Control Act in 1956.[4] In 1962, public and governmental interests were renewed, in large part due to the publication of Rachel Carson's *Silent Spring*, and three years later the Water Quality Act of 1965 was passed, which directed states to develop water quality standards.[1] Public awareness, as well as scientific and governmental concern, continued to grow throughout the 1970s and by the end of the decade research had expanded to include hazard evaluation and risk analysis.[1] In the subsequent decades, aquatic toxicology has continued to expand and internationalize so that there is now a strong application of toxicity testing for environmental protection.

Aquatic toxicity tests

Aquatic toxicology tests (assays): toxicity tests are used to provide qualitative and quantitative data on adverse (deleterious) effects on aquatic organisms from a toxicant. Toxicity tests can be used to assess the potential for damage to an aquatic environment and provide a database that can be used to assess the risk associated within a situation for a specific toxicant. Aquatic toxicology tests can be performed in the field or in the laboratory. Field experiments generally refer to multiple species exposure and laboratory experiments generally refer to single species exposure. A dose–response relationship is most commonly used with a sigmoidal curve to quantify the toxic effects at a selected end-point or criteria for effect (i.e. death or other adverse effect to the organism). Concentration is on the x-axis and percent inhibition or response is on the y-axis.[1]

The criteria for effects, or endpoints tested for, can include lethal and sublethal effects (see Toxicological effects).[1]

There are different types of toxicity tests that can be performed on various test species. Different species differ in their susceptibility to chemicals, most likely due to differences in accessibility, metabolic rate, excretion rate, genetic factors, dietary factors, age, sex, health and stress level of the organism. Common standard test species are the fathead minnow (Pimephales promelas), daphnids (*Daphnia magna*, *D. pulex*, *D. pulicaria*, *Ceriodaphnia dubia*), midge (Chironomus tentans, C. ruparius), rainbow trout (Oncorhynchus mykiss), sheepshead minnow (Cyprinodon variegatu)[5], zebra fish (*Danio rerio*)[6], mysids (Mysidopsis), oyster (Crassotreas), scud (Hyalalla Azteca), grass shrimp (Palaemonetes pugio) and mussels (*Mytilus galloprovincialis*).[7] As defined by ASTM, these species are routinely selected on the basis of availability, commercial, recreational, and ecological importance, past successful use, and regulatory use.[1]

A variety of acceptable standardized test methods have been published. Some of the more widely accepted agencies to publish methods are: the American Public Health Association, US Environmental Protection Agency (EPA), ASTM International, International Organization for Standardization, Environment and Climate Change Canada, and Organisation for Economic Co-operation and Development. Standardized tests offer the ability to compare results between laboratories.[1]

There are many kinds of toxicity tests widely accepted in the scientific literature and regulatory agencies. The type of test used depends on many factors: Specific regulatory agency conducting the test, resources available, physical and chemical characteristics of the environment, type of toxicant, test species available, laboratory vs. field testing, end-point selection, and time and resources available to conduct the assays are some of the most common influencing factors on test design.[1]

Exposure systems

Exposure systems are four general techniques the controls and test organisms are exposed to the dealing with treated and diluted water or the test solutions.

- **Static.** A static test exposes the organism in still water. The toxicant is added to the water in order to obtain the correct concentrations to be tested. The control and test organisms are placed in the test solutions and the water is not changed for the entirety of the test.
- **Recirculation.** A recirculation test exposes the organism to the toxicant in a similar manner as the static test, except that the test solutions are pumped through an apparatus (i.e. filter) to maintain water quality, but not reduce the concentration of the toxicant in the water. The water is circulated through the test chamber continuously, similar to an aerated fish tank. This type of test is expensive and it is unclear whether or not the filter or aerator has an effect on the toxicant.
- **Renewal.** A renewal test also exposes the organism to the toxicant in a similar manner as the static test because it is in still water. However, in a renewal test the test solution is renewed periodically (constant intervals) by transferring the organism to a fresh test chamber with the same concentration of toxicant.
- **Flow-through.** A flow-through test exposes the organism to the toxicant with a flow into the test chambers and then out of the test chambers. The once-through flow can either be intermittent or continuous. A stock solution of the correct concentrations of contaminant must be previously prepared. Metering pumps or diluters will control the flow and the

volume of the test solution, and the proper proportions of water and contaminant will be mixed.[1]

Types of tests

Acute tests are short-term exposure tests (hours or days) and generally use lethality as an endpoint. In acute exposures, organisms come into contact with higher doses of the toxicant in a single event or in multiple events over a short period of time and usually produce immediate effects, depending on absorption time of the toxicant. These tests are generally conducted on organisms during a specific time period of the organism's life cycle, and are considered partial life cycle tests. Acute tests are not valid if mortality in the control sample is greater than 10%. Results are reported in EC50, or concentration that will affect fifty percent of the sample size.[1]

Chronic tests are long-term tests (weeks, months years), relative to the test organism's life span (>10% of life span), and generally use sub-lethal endpoints. In chronic exposures, organisms come into contact with low, continuous doses of a toxicant. Chronic exposures may induce effects to acute exposure, but can also result in effects that develop slowly. Chronic tests are generally considered full life cycle tests and cover an entire generation time or reproductive life cycle ("egg to egg"). Chronic tests are not considered valid if mortality in the control sample is greater than 20%. These results are generally reported in NOECs (No observed effects level) and LOECs (Lowest observed effects level).

Early life stage tests are considered as subchronic exposures that are less than a complete reproductive life cycle and include exposure during early, sensitive life stages of an organism. These exposures are also called critical life stage, embryo-larval, or egg-fry tests. Early life stage tests are not considered valid if mortality in the control sample is greater than 30%.[1]

Short-term sublethal tests are used to evaluate the toxicity of effluents to aquatic organisms. These methods are developed by the EPA, and only focus on the most sensitive life stages. Endpoints for these test include changes in growth, reproduction and survival. NOECs, LOECs and EC50s are reported in these tests.

Bioaccumulation tests are toxicity tests that can be used for hydrophobic chemicals that may accumulated in the fatty tissue of aquatic organisms. Toxicants with low solubilities in water generally can be stored in the fatty tissue due to the high lipid content in this tissue. The storage of these toxicants within the organism may lead to cumulative toxicity.

Bioaccumulation tests use bioconcentration factors (BCF) to predict concentrations of hydrophobic contaminants in organisms. The BCF is the ratio of the average concentration of test chemical accumulated in the tissue of the test organism (under steady state conditions) to the average measured concentration in the water.

Freshwater tests and saltwater tests have different standard methods, especially as set by the regulatory agencies. However, these tests generally include a control (negative and/or positive), a geometric dilution series or other appropriate logarithmic dilution series, test chambers and equal numbers of replicates, and a test organism. Exact exposure time and test duration will depend on type of test (acute vs. chronic) and organism type. Temperature, water quality parameters and light will depend on regulator requirements and organism type.[1]

In the US, many wastewater dischargers (e.g., factories, power plants, refineries, mines, municipal sewage treatment plants) are required to conduct periodic **whole effluent toxicity** (WET) tests under the National Pollutant Discharge Elimination System (NPDES) permit program, pursuant to the Clean Water Act. For facilities discharging to freshwater, effluent is used to perform static-acute multi-concentration toxicity tests with *Ceriodaphnia dubia* (water flea) and *Pimephales promelas* (fathead minnow), among other species. The test organisms are exposed for 48 hours under static conditions with five concentrations of the effluent. The major deviation in the short-term chronic effluent toxicity tests and the acute effluent toxicity tests is that the short-term chronic test lasts for seven days and the acute test lasts for 48 hours. For discharges to marine and estuarine waters, the test species used are sheepshead minnow (*Cyprinodon variegatus*), inland silverside (*Menidia beryllina*), *Americamysis bahia*, and purple sea urchin (*Strongylocentrotus purpuratus*).[8][9]

Sediment tests

At some point most chemicals originating from both anthropogenic and natural sources accumulate in sediment. For this reason, sediment toxicity can play a major role in the adverse biological effects seen in aquatic organisms, especially those inhabiting benthic habitats. A recommended approach for sediment testing is to apply the Sediment Quality Triad (SQT) which involves simultaneously examining sediment chemistry, toxicity, and field alterations so that more complete information can be gathered. Collection, handling, and storage of sediment can have an effect on bioavailability and for this reason standard methods have been developed to suit this purpose.[1]

Toxicological effects

Toxicity can be broken down into two broad categories of direct and indirect toxicity. Direct toxicity results from a toxicant acting at the site of action in or on the organism. Indirect toxicity occurs with a change in the physical, chemical, or biological environment.

Lethality is most common effect used in toxicology and used as an endpoint for acute toxicity tests. While conducting chronic toxicity tests sublethal effects are endpoints that are looked at. These endpoints include behavioral, physiological, biochemical, histological changes.[1]There are a number of effects that occur when an organism is simultaneously exposed to two or more toxicants. These effects include additive effects, synergistic effects, potentiation effects, and antagonistic effects. An additive effect occurs when combined effect is equal to a combination or sum of the individual effects. A synergistic effect occurs when the combination of effects is much greater than the two individual effects added together. Potentiation is an effect that occurs when an individual chemical has no effect is added to a toxicant and the combination has a greater effect than just the toxicant alone. Finally, an antagonistic effect occurs when a combination of chemicals has less of an effect than the sum of their individual effects.[1]

Important aquatic toxicology resources

- ASTM International (formerly American Society for Testing and Materials). A consensus-based organization, representing 135 countries, that develops and delivers international voluntary standard methods for aquatic toxicity testing.[10]
- *Standard Methods for the Examination of Water and Wastewater*. A compilation of techniques for water analysis, jointly published by the American Public Health Association (APHA), the American Water Works Association (AWWA), and the Water Environment Federation.[11]
- "Ecotox." A database maintained by EPA that offers single chemical toxicity information for both aquatic and terrestrial purposes.[12]
- Society of Environmental Toxicology and Chemistry (SETAC). A nonprofit, worldwide society working to promote scientific research to further our understanding of environmental stressors, environmental education, and the use of science in environmental policy.[13]
- US EPA publishes guidance manuals outlining aquatic toxicity test procedures.[8][9]

- Organisation for Economic Co-operation and Development (OECD). A forum for governments to work together to promote policies for the betterment of people's social and economic well-being around the world. One way in which they accomplish this is through the development of aquatic toxicity test guidelines.[14]
- Environment and Climate Change Canada. Canada's lead federal agency for environmental protection.[15]

Terminology

- Median Lethal Concentration (LC50) – The chemical concentration that is expected to kill 50% of a group of organisms.
- Median Effective Concentration (EC50) – The chemical concentration that is expected to have one or more specified effects in 50% of a group of organisms.
- Critical Body Residue (CBR) – An approach that routinely examines whole-body chemical concentrations of an exposed organism that is associated with an adverse biological response.
- Baseline toxicity – Refers to narcosis which is a depression in biological activity due to toxicants being present in the organism.
- Biomagnification – The process by which the concentration of a chemical in the tissues of an organism increases as it passes through several levels in the food web.
- Lowest Observed Effect Concentration (LOEC) – The lowest test concentration that has a statistically significant effect over a specified exposure time.
- No Observed Effect Concentration (NOEC) – The highest test concentration for which no effect is observed relative to a control over a specified exposure time.
- Maximum Acceptable Toxicant Concentration (MATC) – An estimated value that represents the highest "no-effect" concentration of a specific substance within the range including the NOEC and LOEC.
- Application Factor (AF) – An empirically derived "safe" concentration of a chemical.
- Biomonitoring – The consistent use of living organisms to analyze environmental changes over time.
- Effluent – Liquid, industrial discharge that usually contain varying chemical toxicants.
- Quantitative Structure-Activity Relationship (QSAR) – A method of modeling the relationship between biological activity and the structure of organic chemicals.

- Mode of Action – A set of common behavioral or physiological signs that represent a type of adverse response.
- Mechanism of Action – The detailed events that take place at the molecular level during an adverse biological response.
- KOW – The octanol-water partition coefficient which represents the ratio of the concentration of octanol to the concentration of chemical in the water.
- Bioconcentration Factor (BCF) – The ratio of the average chemical concentration in the tissues of the organism under steady-state conditions to the average chemical concentration measured in the water to which the organisms are exposed.

Perspectives of Aquatic Toxicology/Aquatic Toxicity Tests

Contents

Chapter One: Aquatic Toxicity Tests

INTRODUCTION

Aquatic species are vital to our planet. Phytoplankton, algal plankton, and kelp are major sources of the planet's oxygen. They absorb and store carbon dioxide, and maintain a

hospitable climate. They also play an important role in the global nitrogen cycle and support aquatic animals such as fish, mollusks, sponges, and corals. Aquatic species help maintain the earth's ecosystem and help preserve its rich biodiversity as well as providing food, medicine, livelihoods, tourism, and recreational opportunities[1].

It is therefore essential to protect the planet's rich and diverse aquatic life, and combat the many threats facing aquatic organisms including climate change, habitat destruction, overfishing, the introduction of invasive species, and chemical pollution[2]. This chapter will focus on chemical pollution. The risks to aquatic life can be minimized and better managed by understanding how chemicals impact it.

There are more than 140,000 man-made chemicals in the environment[3], with the United States alone producing 2000 new chemicals every year[4]. It is conceivable that aquatic species are exposed to many of these chemicals on an acute (short-term) and chronic (long-term) basis, although there is an absence of data to indicate how many of these chemicals are released into various water bodies. Chemical exposure can affect organisms' growth, development, fecundity, behavior, and survival, among other biological processes. Hence, it is important to test chemical toxicity before it is released into the environment in order to determine maximum acceptable toxicant concentrations (see section II of this chapter) and to protect species from potential harm.

Toxicity testing is done to identify the degree to which chemicals can damage living organisms in a controlled environment. It has four major objectives:

a. To obtain toxicity and exposure data for various chemicals

b. To aid in estimating and managing risks posed by various chemicals

c. To aid in setting chemical regulations and environmental standards

d. To classify chemicals based on how toxic they are to various species

The dose makes the poison in toxicology. It is possible to determine safe and unsafe doses, or concentrations, for nearly every chemical. For example, the most toxic substance on earth, the bacteria-produced botulinum toxin, can kill humans with a very small dose, but it can be used safely in Botox[5].

Risk is a function of toxicity and exposure. A chemical can be very toxic, but it will have zero risk to aquatic organisms if it never enters water bodies (i.e., there is no exposure). The maximum allowable concentration for a chemical in the environment is based on the risk it

poses to various species. An acceptable “safe” concentration is usually one that does not harm 95% of the species.

Many questions can be answered by carrying out toxicity tests:

a. At what concentration is a chemical non-toxic to an organism? At what concentration is it toxic?

b. What effects can be observed from short-term and long-term chemical exposure?

c. Which chemicals are the most and least toxic to an organism?

d. Which organisms are the most or least sensitive to a chemical?

e. Are some life stages of an organism more sensitive?

f. Do certain environmental conditions make a chemical more toxic?

g. Is the toxicity of a chemical similar in lab and in the outside environment?

h. What is the effect of a mixture of chemicals?

Aquatic organisms can be exposed to chemicals when effluents and sewage are released into water bodies. Sometimes chemicals inadvertently enter water through oil spill or runoffs from agricultural fields. Chemicals present in the air can be deposited into water bodies either directly (dry deposition) or through rainfall, snowfall, and fog (wet deposition). Some of the chemicals commonly found in water bodies include detergents, fertilizers, pesticides, pharmaceuticals, food and cosmetic preservatives, chemicals used in kitchenware and plastic, and metals[6-8]. Aquatic animals such as fish can take up these chemicals via their gills, absorb them through their integument, and/or ingest them. Aquatic plants that have vascular systems can absorb chemicals through their epidermal surface and/or roots. Plants that are not completely submerged in water can take up chemicals in the air through their stomata.

Chemical properties and type of aquatic species determine how chemicals are taken up, distributed, stored, metabolized, and excreted. Hydrophobic (fat-loving) chemicals are more likely to enter a fish’s body, and warm temperatures typically increase the uptake as the fat become more fluid-like. Smaller, uncharged molecules also cross membranes more easily. Hydrophilic (water-loving) chemicals are more likely to be transported by the circulatory system. On the other hand, hydrophobic chemicals are more likely to bind to molecules and accumulate in fat bodies. While chemical storage is protective in the short term (they are not free to move and act), they can be released later and cause toxicity. This usually happens

when an organism breaks down fat for greater energy needs, i.e., during illness, starvation, or reproduction.

A species' metabolic enzymes often modify a chemical in order to detoxify its effects, but this modification can sometimes make a chemical more toxic. Chemicals with many halogen atoms such as chlorine, and fluorine are often difficult to modify. Many aquatic animals eliminate chemicals through their gills or skin. Further details on chemical biotransformation can be found in the *Biotransformation of Xenobiotics Chapter of this book.*

Chemical exposure can kill or harm aquatic organisms directly through such means as growth reduction, delayed development, decreased fertility, and behavioral changes, or can reduce or eliminate its food supply by killing its prey, or limiting its shelter through habitat destruction. This can lead to increased competition for food and shelter, disrupting the food web, and altering the ecological balance.

BASIC CONCEPTS

Units of concentration

Concentration is used more commonly than dose in aquatic toxicology. This is because water chemical concentration is easier to measure than the amount of chemical taken up by fish through its gills, integument, and mouth. Concentration of a solution is defined as the ratio of solute to solvent, or the ratio of solute to total solution. This can be either expressed as mass of chemical per unit volume (e.g. mg/mL) or the number of moles of chemicals per liter of solution (e.g. mol/L).

Terms like *ppm*, *ppb* and *ppt* are also often used to describe units of concentration:

a. Parts per million (ppm) corresponds to 1 mg of chemical/L of solution. The amount of chemical is six orders of magnitude lesser than the amount of solution. This is like emptying a large soft drink bottle of a chemical into an Olympic-sized swimming pool.

b. Parts per billion (ppb) corresponds to 1 µg of chemical/L of solution. The amount of chemical is nine orders of magnitude lesser than the amount of solution. This is like putting half a teaspoon of a chemical into an Olympic-sized swimming pool.

c. Parts per trillion (ppm) corresponds to 1 ng of chemical/L of solution. The amount of chemical is twelve orders of magnitude lesser than the amount of solution. This is like putting *one-twentieth* of a drop of chemical into an Olympic-sized swimming pool.

Concentration and response

There is a relationship between the chemical concentration to which an organism has been exposed and the resultant nature and degree of harmful effects. However, it is important to note that the chemical concentration that enters an organism is typically higher than the concentration that causes a toxic effect. This could be due to the organism producing enzymes or molecules that break down or bind to the chemical. This reduces the availability of the chemical to the body, and thus a lower concentration binds to the site of action and exerts a toxic effect.

Assumptions in a concentration-response relationship are as follows:

a. It is a cause-and-effect relationship, i.e., the response occurs due to the organism's exposure to a chemical.

b. The response is due to a chemical interacting at the site of action.

c. The concentration of a chemical at the site of action is a function of how much chemical the organism was exposed to.

d. Above the threshold concentration (concentration at which a response can be detected), the magnitude of response is proportional to the amount of chemical interacting at the site of action.

e. The response can be measured and reproduced under similar conditions.

The duration of an organism's chemical exposure is also significant. If a fish is exposed to a low chemical concentration for only one day, it may be unaffected, but if it is exposed to the same concentration for months it may develop cancer, skeletal abnormalities, issues with fertility, etc.

The response to a chemical for any given species usually follows a normal distribution or bell-shaped curve (see Figure 1). This means that some organisms of the same species are very sensitive to a given chemical, some are very resistant, while most are neither very sensitive nor very resistant.

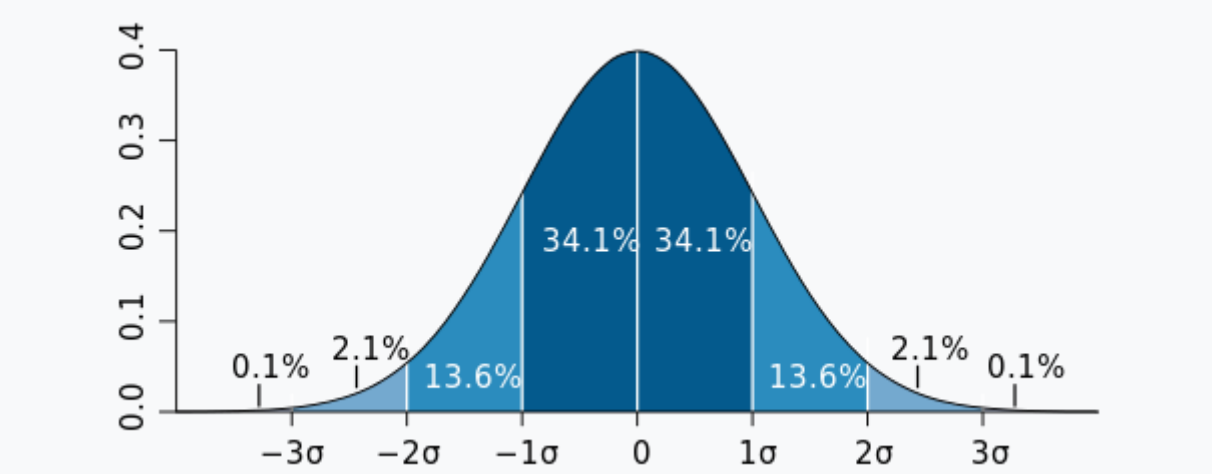


Figure 1: A normal distribution curve. The response of the majority (68%) of organisms in a population will be within one standard deviation from the mean (response). Image created by M. W. Toews [CC BY 2.5 (https://creativecommons.org/licenses/by/2.5)

Creating a toxicity curve

A toxicity curve or a concentration-response curve is a graph which plots the results obtained from a traditional concentration-response toxicity test. The toxicity test should ideally fulfill the following criteria:

a. There should be five different concentrations of a chemical plus a negative control.

b. The concentrations should be equally spaced.

c. The concentrations should cause a range of effects, from 0% effect to 100% effect.

d. The study should be replicated at least three times.

The toxicity curve generated from such an experimental design should have the following features in most cases (see Figure 2):

a. The x-axis should be the concentration and the y-axis should be the response.

b. The curve should be sigmoidal in shape above the threshold dose.

c. The average cumulative response from the three replicated studies should be plotted with the 95% confidence intervals.

The slope of a generated toxicity curve can indicate several things. A very steep slope indicates that small increases in concentration caused large increases in response; a flat slope indicates that large increases in concentrations caused small increases in response. The curve

can also be used to estimate a concentration that causes a particular response (for example, a 10% response). Typically, the concentration which causes a 50% response is calculated, as it has the least variability/noise.

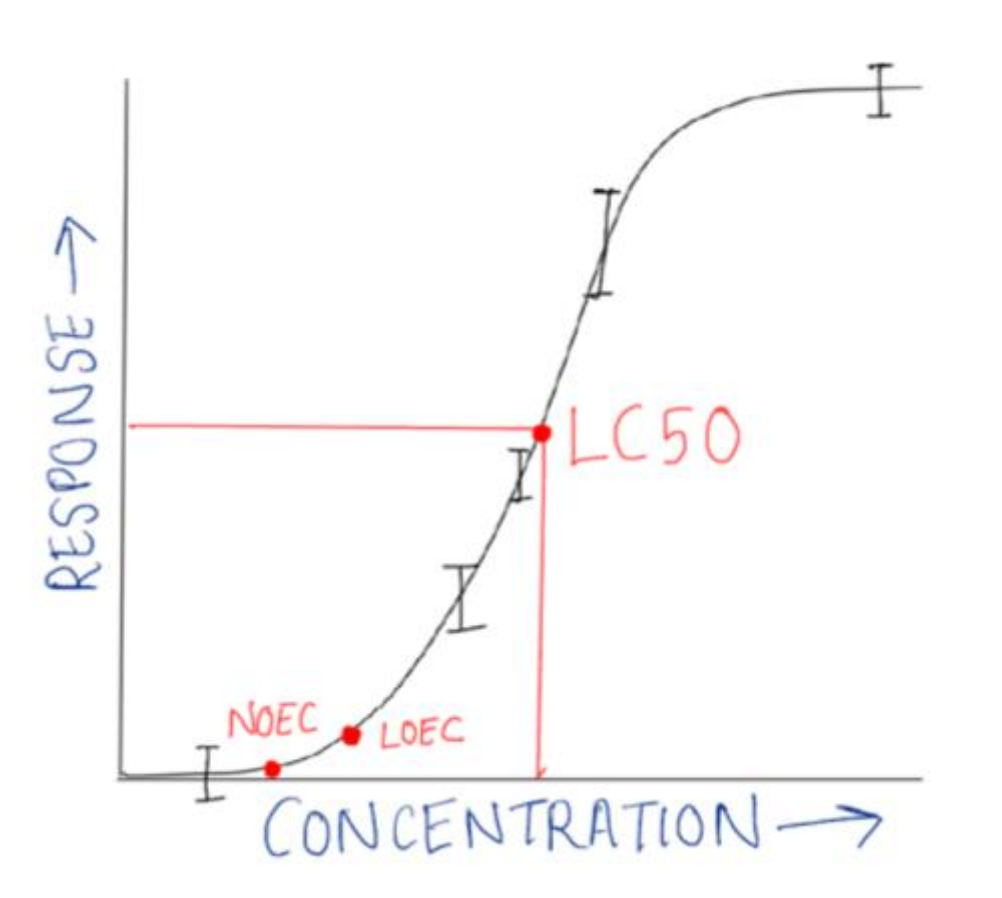


Figure 2: A standard concentration-response curve. This response is observed for non-essential chemicals. The LC50, NOEC, LOEC and 95% confidence intervals for the different concentrations have been plotted.

A non-standard toxicity curve is observed for essential chemicals such as water, oxygen, and vitamins. There is an optimal range for these chemicals: a very low concentration causes deficiency and death, and a very high concentration causes toxicity and death (see Figure 3). Another kind of non-standard toxicity curve is seen with chemicals found in such materials as plastics. These chemicals have different modes of action at different concentrations: they can disrupt hormones at low concentrations but not at high concentrations[9]. Some toxicity curves can be bimodal if males and females have different toxicity thresholds to a chemical.

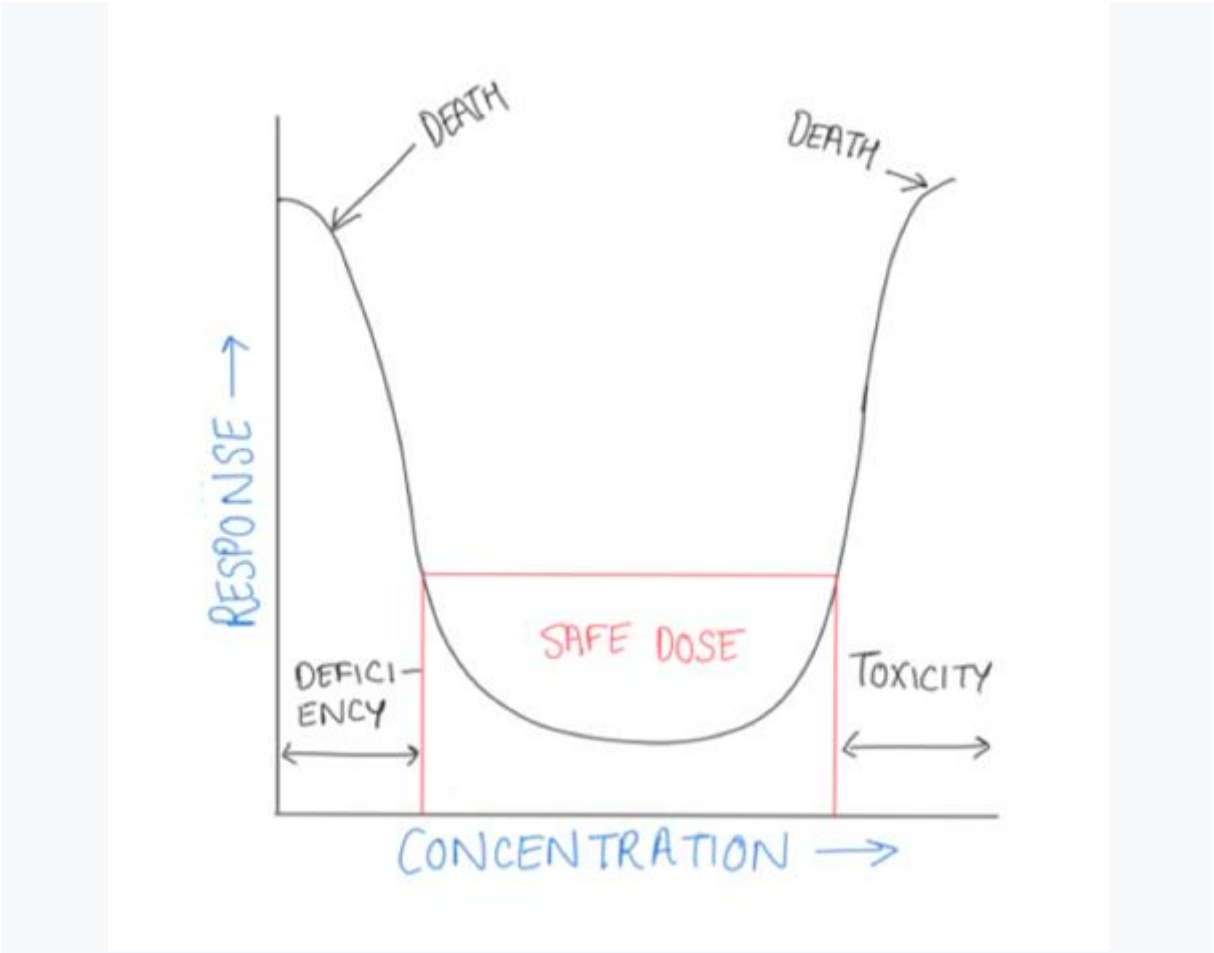


Figure 3: A non-standard concentration-response curve. This response is observed for essential chemicals.

Measures of toxicity

Toxicity is commonly measured in toxicological studies as follows:

Lethal Concentration 50 (LC50): Concentration at which 50% of the organisms in a population are killed following chemical exposure.

Effective Concentration 50 (EC50): Concentration at which 50% of the organisms in a population are affected following chemical exposure. The effects observed could be reduced growth, delayed development, etc.

Inhibitory Concentration 50 (IC50): Concentration at which 50% of organisms in a population are inhibited following chemical exposure. The chemical could have inhibited a specific biological or biochemical function.

Often LC/EC/IC 10 (harm to 10% of population) and LC/EC/IC 90 (harm to 90% of population) are also calculated.

No Observed Effect Concentration (NOEC): Highest chemical concentration that does not cause a toxic effect in the treated population.

Lowest Observed Effect Concentration (LOEC): Lowest chemical concentration that causes a toxic effect in the treated population.

Maximum Acceptable Toxicant Concentration (MATCC): Concentration between NOEC and LOEC, or is a geometric mean of the two. It is calculated for chronic studies only.

TOXIC EFFECTS OF CHEMICALS

Chemicals can exert toxic effects on organisms through various mechanisms:

a. Binding: Chemicals can bind to molecules on the surface of cells and disrupt communication between cells. If an organism's cells do not communicate with each other they will not function normally. Chemicals can also bind to enzymes and prevent them from carrying out essential activities such as digestion and metabolism, as well as bind to DNA and change the amount of proteins produced. A correct number of proteins are needed to build cells, produce hormones, and maintain immunity.

b. Bioaccumulation: This occurs when an organism takes up a chemical faster than it eliminates it. In bioaccumulation the chemical is taken up by contact, respiration, and ingestion. The term bioconcentration is used when the chemical is taken up through contact and respiration only. A chemical accumulation in living tissue can poison tissue, and subsequently, organs.

c. Interaction: Two or more chemicals in an organism can interact with one another. Usually, the combined chemical effect will equal the sum of their individual effects (1 + 2 = 3). This is called an additive effect and can be observed when aspirin and acetaminophen are taken together. They both act in a similar manner and their combined effect is comparable to taking two doses of one drug. Another kind of interaction occurs when the combined effect of two chemicals are greater than the sum of their individual effects (1 + 2 = 5), and can happen if chemical A increases the activity of chemical B. This is called the synergistic or potentiation effect and can be observed when acetaminophen and alcohol are taken together. Both are broken down by the liver and their combined presence taxes the organ, making it more vulnerable to failure. The final kind of interaction occurs when the combined effect of two chemicals is less than the sum of their individual effects (1 + 2 = 1). This occurs if

chemical A hinders the activity of chemical B, and is called an antagonistic effect. A classic example of this effect is anti-venom drugs canceling the effects of a snake bite.

The intracellular effects of a chemical can also be described with the help of a toxicity pathway. It is a sequence of events, starting with a chemical entering an organism. A proportion of the chemical reaches the target tissue and interacts with it: for example, by binding to cell receptors on the tissue. This causes a perturbation, or disturbance, in normal cell function. If the cell starts to alter and the organism corrects the change in time, there will not be a problem; however, if the organism does not alter and effect change, the cell will be permanently altered/injured[10] (see Figure 4).

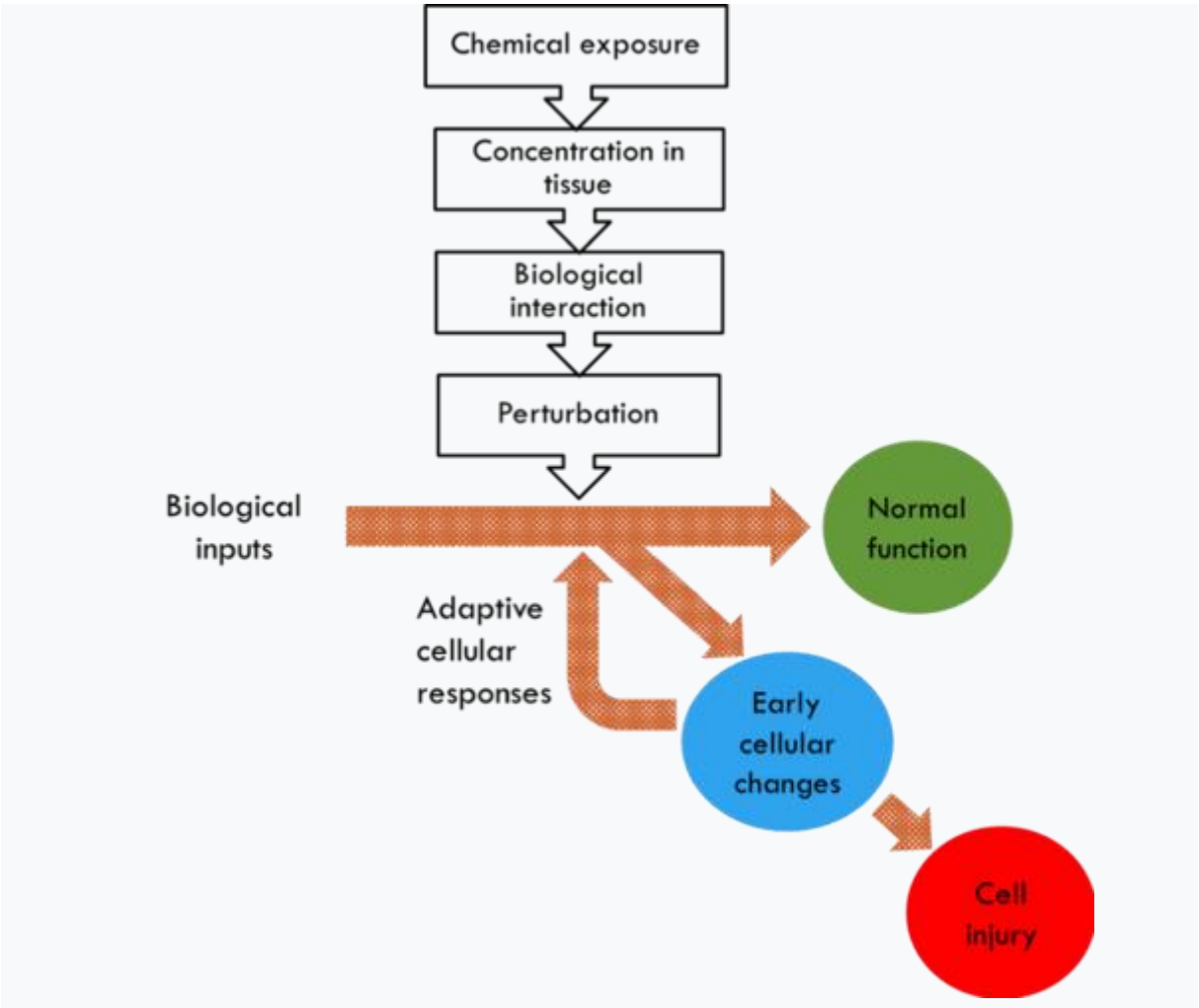


Figure 4: A toxicity pathway depicting how chemical exposure could lead to cell injury. Adapted from U.S. National Library of Medicine

Adverse Outcome Pathways (AOPs) encompass toxicity pathways and beyond. That is, they include chemical-molecular interactions and cellular changes, and determine how this leads

to changes in organs, organisms, and populations. In Figure 5 an AOP has been drawn for a male fish exposed to a chemical that activates the estrogen receptor. At the cellular stage this could lead to the transcription and production of abnormal proteins. These proteins could cause both ovaries and testis to develop, altering the secondary sex characteristics of the fish and impairing its fertility. The sex ratio could become skewed if many males in a population become feminized and infertile[11].

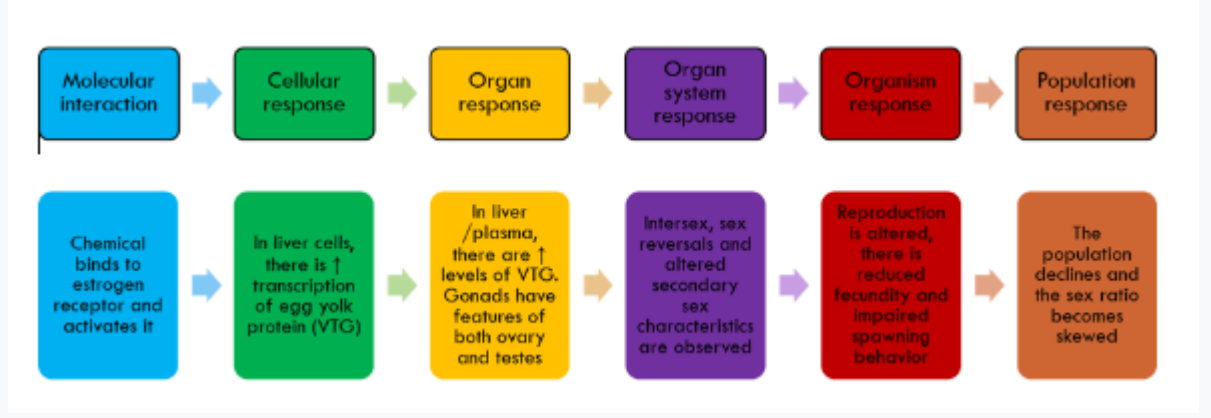


Figure 5: An Adverse Outcome Pathway (AOP) for male fish exposed to a chemical that activates the estrogen receptor. Adapted from Browne et al., 2017

Table 1 shows how toxicity endpoints could manifest in various aquatic organisms. The length of chemical exposure (short-term vs. long-term) often determines which toxicity endpoints should be measured. For example, reproductive problems and tumors are only observed with long-term exposures.

Table 1: Toxicity endpoints for different aquatic species following exposure to chemicals

SPECIES	TOXICITY ENDPOINTS COMMONLY MEASURED
Annelids	Growth, fecundity, bioaccumulation
Algae	Growth, biomass, coloration
Plants	Growth, length, yield

Insects	Mortality, immobility, development, fecundity, emergence, sex ratio
Mollusks	Mortality, growth, fecundity, bioconcentration
Crustaceans	Mortality, growth, immobility, fecundity
Amphibians	Mortality, growth, development, length, histopathology, metamorphosis, reproductive maturity
Fish	Mortality, no heartbeat, loss of swimming equilibrium, developmental deformities, length, yolk coagulation, growth, skeletal abnormalities, tumors, reproductive maturation, fecundity, histopathology, egg hatching, behavior, etc.

TYPES OF TOXICITY TESTS

Aquatic toxicity tests can be divided into categories as described below and summarized in Figure 6.

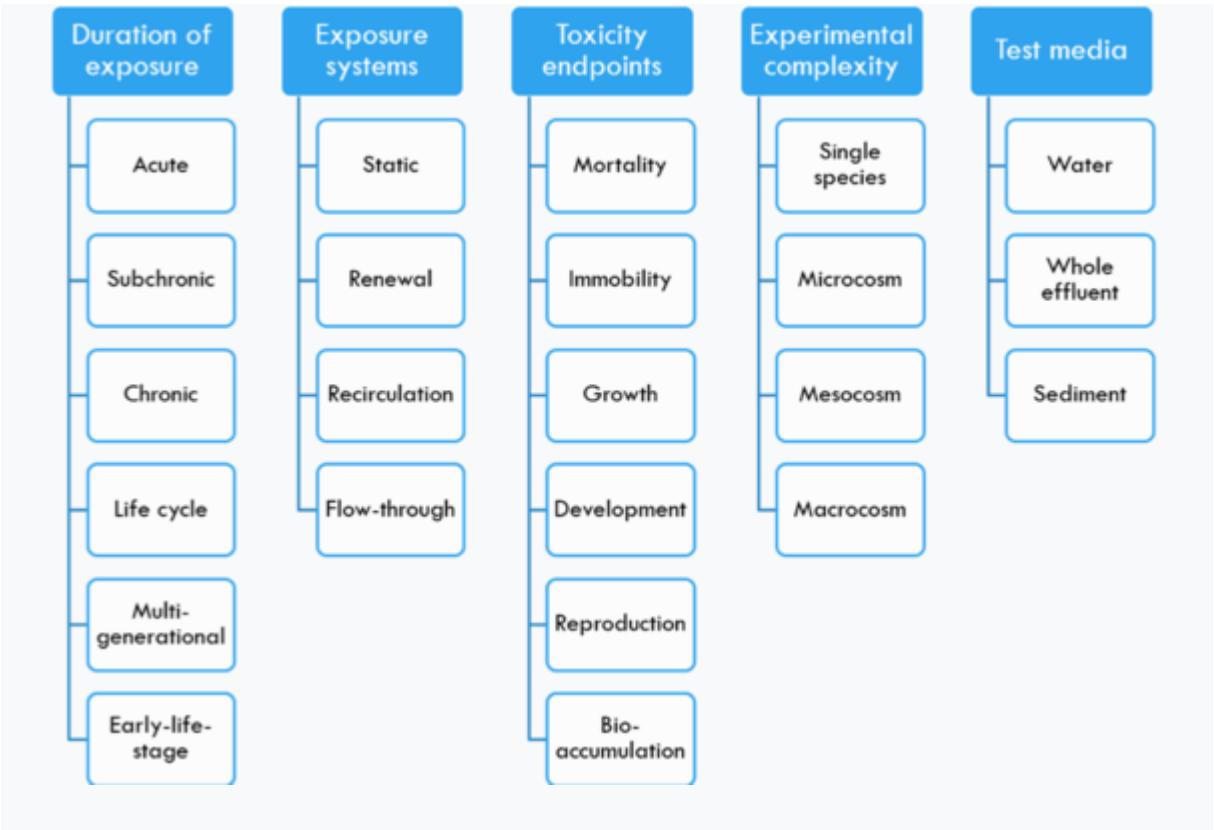


Figure 6: Aquatic toxicity tests can be grouped into categories

Based on duration of chemical exposure

a. Acute: Short-term tests. For fish the tests are 24-96h long; but, for microalgae or bacteria, a 96h test could represent a chronic, life cycle, or multigenerational test. The test is commonly carried out to check chemical lethality.

b. Subchronic: Prolonged acute tests. For fish the tests are typically anywhere between 28 days to 3 months long. The test is usually done to determine if a chemical impacts the growth (body mass) of a species.

c. Chronic: Lasts for at least 10% of the tested species' lifespan. For invertebrates, 21-day chronic studies are common. The test typically lasts longer than 6 months for fish. It is often conducted to see if a chemical causes reproductive and developmental effects.

d. Life cycle: Lasts through an organism's entire life cycle. This can be from egg to sexual maturity, or from egg to egg. This test is done to check if a chemical causes developmental or reproductive effects, as with chronic studies.

e. Multigenerational: Carried out on two or more consecutive generations (parents and offspring). It is usually performed to examine if offspring are affected by parental exposure to chemicals.

f. Early-life-stage: Done on embryos or larval stages. Different life stages of an organism can exhibit different sensitivities to a chemical, with early-life stages often more susceptible.

Based on exposure systems

a. Static: Organisms are placed in still water containing a chemical (or in control water). The water is not changed during the test. This system is widely used, but for studies longer than 24h they may not accurately represent chemical effects. This is because the concentration of the chemical may change over time and toxic effects may be produced from a build-up of metabolic byproducts released by the organisms.

b. Renewal: Similar to static, with the test conducted in still water. However, in this test the water containing the chemical (or control water) is regularly changed during the test, usually every 24h, ensuring that the chemical concentration remains stable and that organisms are exposed to clean and fresh water daily.

c. Recirculation: Also similar to static, but the water containing the chemical (or control water) is filtered. This ensures that the water quality does not deteriorate over time. However, filters can add uncertainties to the study as the filter media may interact with the test chemical.

d. Flow-through: Water containing the chemical (or control water) constantly flows in and out of the system, maintaining a high quality flow where the influent and effluent never mix. Pumps control the flow of water and dilutors ensure that the right concentration of chemical is delivered. Flow-through systems mimic the natural flow of water, and though expensive, are regularly used for this reason.

Based on toxicity endpoints

These tests are done to determine if a chemical could cause one or more toxicity endpoints in a test organism. The endpoints frequently analyzed include mortality, growth, development, reproduction, immobilization, respiration, endocrine effects, and chemical bioaccumulation. Most standardized studies have been developed with these endpoints in mind.

Based on experimental complexity

a. Single species: Tests are conducted on a single species in a lab. They are simple and inexpensive to conduct, and constitute the most common type of test. They are often carried out in a flask, beaker, or some other glass container.

b. Microcosm: Tests conducted on two or more species in an artificial and controlled system. They represent a simplified ecosystem. Microcosms should contain less than 1000 liters of water and can be done indoors (e.g. fish tank) or outdoors (e.g. small ponds).

c. Mesocosms: Tests conducted on multiple species placed in experimental water enclosures. Mesocosms represent a complex ecosystem and mimic natural conditions. The volume of water in the system must exceed 1000 liters, and the test is usually done outdoors. More details on this testing method can be found in the *Mesocosm Chapter* of this book.

d. Macrocosms: Tests conducted in lakes and on whole aquatic ecosystems. They are the most realistic, but they are very difficult and expensive to conduct. Canada has 58 experimental lakes that are designated for macrocosm studies only.

Based on test media

a. Water: Water is spiked with a single chemical or a chemical mixture, and aquatic organisms are exposed to it. The vast majority of aquatic toxicity tests are done on water. The

toxicity endpoints of these organisms are compared to organisms exposed to control (non-spiked) water.

b. Whole effluent: Samples of effluents are tested by exposing aquatic organisms to them. It is important to assure that as wastewater effluents are discharged into water bodies they will not harm aquatic organisms--as prescribed by the Clean Water Act. Toxicity endpoints are measured and compared to that of organisms exposed to control water.

c. Sediment: Determines if sediments contain concentrated toxic chemicals that will harm organisms. Sediments are the ultimate repository for many chemicals that enter water bodies. Benthic species such as worms, crabs, clams, and lobsters live in or on sediments. Benthic organisms are exposed to contaminated or spiked sediments in sediment toxicity tests, and their toxicity endpoints are compared to organisms that are exposed to control sediments.

It is important to understand that the five different kinds of aquatic tests mentioned above are not independent of one another; this is just one way to classify them. For example, an acute toxicity test can be done on a single species using the static exposure system. The endpoint could be mortality, and the test could be done to check for the presence of toxic contaminants in whole effluents.

Both the Organization for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency (USEPA) publish guidelines for conducting various kinds of aquatic toxicity tests. These have been summarized in Table 2, along with a link to each test guideline.

Table 2: The different aquatic toxicity test guidelines published by OECD and EPA

TEST NUMBER AND NAME	DURATION OF EXPOSURE	MAJOR ENDPOINTS MEASURED	LINK TO GUIDELINE
Test No. 225: Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment	Subchronic	Growth, fecundity	OECD 225
Test No. 315: Bioaccumulation in Sediment-dwelling Benthic Oligochaetes	Subchronic	Bioaccumulation	OECD 315
Test No. 221: Lemna sp. Growth	Subchronic	Growth, yield	OECD 221

Inhibition Test			
Test No. 239: Water-Sediment Myriophyllum Spicatum Toxicity Test	Subchronic	Growth, length, yield	OECD 239
Test No. 238: Sediment-Free Myriophyllum Spicatum Toxicity Test	Subchronic	Growth, length, yield	OECD 238
Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test	Chronic	Growth, biomass, coloration	OECD 201
Test No. 218: Sediment-Water Chironomid Toxicity Using Spiked Sediment	Chronic	Development, emergence	OECD 218
Test No. 219: Sediment-Water Chironomid Toxicity Using Spiked Water	Chronic	Development, emergence	OECD 219
Test No. 233: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment	Life cycle	Emergence, sex ratio, fecundity, mortality, development	OECD 233
Test No. 235: Chironomus sp., Acute Immobilisation Test	Acute	Immobility	OECD 235
Test No. 242: Potamopyrgus antipodarum Reproduction Test	Subchronic	Mortality, fecundity	OECD 242
Test No. 243: Lymnaea stagnalis Reproduction Test	Subchronic	Mortality, fecundity	OECD 243
850.1025: Oyster Acute Toxicity Test (Shell Deposition)	Acute	Growth	EPA 1025
850.1055: Bivalve Acute Toxicity Test (Embryo-Larval)	Acute	Count of embryos and larvae	EPA 1055
850.1710: Oyster	Subchronic	Bioconcentration	EPA 1710

Bioconcentration Factor			
Test No. 202: Daphnia sp. Acute Immobilisation Test	Acute	Immobility	OECD 202
Test No. 211: Daphnia magna Reproduction Test	Chronic	Fecundity	OECD 211
850.1300: Daphnid chronic toxicity test	Chronic	Mortality, growth, fecundity	EPA 1300
850.1035: Mysid Acute Toxicity Test	Acute	Mortality	EPA 1035
850.1020: Gammarid Amphipod Acute Toxicity Test	Acute	Mortality	EPA 1020
850.1045: Penaeid Acute Toxicity Test	Acute	Mortality	EPA 1045
Test No. 231: Amphibian Metamorphosis Assay	Subchronic	Growth, mortality, development, length, histopathology	OECD 231
Test No. 241: The Larval Amphibian Growth and Development Assay (LAGDA)	Early-life-stage	Development, metamorphosis, mortality, growth, reproductive maturity	OECD 241
Test No. 203: Fish, Acute Toxicity Test	Acute	Mortality	OECD 203
Test No. 210: Fish, Early-life Stage Toxicity Test	Early-life-stage	Growth, length, hatching, appearance & behavior, mortality	OECD 210
Test No. 212: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages	Early-life-stage	Hatching, mortality, behavior, appearance	OECD 212
Test No. 215: Fish, Juvenile Growth Test	Subchronic	Behavior, appearance, growth	OECD 215

Test No. 229: Fish Short Term Reproduction Assay	Subchronic	Fecundity, yolk protein, sex characteristics	OECD 229
Test No. 230: 21-day Fish Assay	Subchronic	Yolk protein, secondary sex characteristics	OECD 230
Test No. 234: Fish Sexual Development Test	Subchronic	Yolk protein, sex ratio	OECD 234
Test No. 236: Fish Embryo Acute Toxicity (FET) Test	Acute	Mortality, yolk coagulation, heartbeat	OECD 236
Test No. 240: Medaka Extended One Generation Reproduction Test (MEOGRT)	Multi-generational	Mortality, growth, development, sex, fecundity, yolk protein	OECD 240
Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure	Subchronic	Bioaccumulation	OECD 305

DESIGNING A TOXICITY EXPERIMENT

The results of a toxicity experiment depend largely on how it was designed. A good study design can ensure that the results obtained are valid, applicable, and reproducible. Below are the major criteria used to ensure good aquatic toxicity test design, but it is important to note that not all tests can satisfy all of the outlined criteria due to the specific nature of various toxicity tests.

a. It should be widely accepted by the general scientific community.

b. It should be standardized (i.e., carried out according to defined protocols) and the results must be replicable in different laboratories.

c. It should be easy to perform and economical.

d. The test species selected must be a well-known model organism (see below).

e. The test should cover a range of concentrations, and at least some of these concentrations should be found in the environment.

f. The duration of chemical exposure must be realistic and manageable (for example, some sharks can live for hundreds of years and it is not possible to expose them to a chemical throughout their lifespan).

g. The test should be statistically sound and robust.

h. The data obtained can be used to estimate risk.

i. The test should be sensitive enough to detect and measure the toxic effects under investigation.

j. The test should be able to predict effects to species outside the lab (i.e., in the environment) and also predict potential effects to similar species.

Major standardized aquatic tests were discussed in the above section, with protocols spelled out by the OECD and USEPA. The International Organization for Standardization (ISO) and the American Society for Testing and Materials (ASTM), which is now an international organization, have also published a few aquatic test protocols and can be found on their websites (https://www.iso.org/home.html and https://www.astm.org/ respectively).

The easy to administer and economical nature of standardized tests enables them to be routinely performed. This precludes macrocosm and mesocosm tests which are very complex and difficult to perform. While all the standardized toxicity tests mentioned in Table 2 were for single species, the USEPA and OECD have published guidelines for indoor microcosms (USEPA 1900) and outdoor microcosms/mesocosms (OECD draft guidance).

The appropriate selection of test species is critical in toxicity testing. While more details on this can be found in the *Model Species in Aquatic Toxicology Chapter*, most toxicity tests are carried out on model species which have the following characteristics: a. High sensitivity to various chemicals. b. Easily available and abundant.

c. Easy to rear and culture in the lab and allow for various types of toxicity testing.

d. High survival rate in the lab under normal conditions.

e. Extensive and available knowledge of the organism (information on their biology, physiology, genetics, and behavior).

There is no single aquatic species used in tests that can provide answers to all questions or evaluate all chemical impacts on an ecosystem. It is therefore imperative to test several species from different classes: from algae to invertebrates to fish. Also, it is important to test

different life stages in a species as every stage has a different sensitivity and a unique response. The environment in which the organism lives should also be taken into consideration (freshwater/marine and warm/cold water).

Under the United States Endangered Species Act (ESA) it is necessary to ensure that chemicals released into the environment do not harm endangered or threatened species. Since the employment of endangered and threatened species in toxicity tests is discouraged, toxicity values obtained from the most sensitive test species are often used to estimate their risk to a chemical. Also, the acceptable risk for these species is often 10-fold lower than that of more abundant species[12].

Toxicity tests should not be conducted without first identifying and including one or more available Estimated Environmental Concentrations (EEC) or Actual Environmental Concentration (AEC) of a chemical. EECs are the estimated concentrations of a chemical in an environment and are usually derived through computer modeling or simple predictions. These models were developed with data obtained from laboratory and environmental studies; two such models are available on the USEPA website[13]. The Pesticide in Water Calculator (PWC) is used to estimate pesticide concentrations in water bodies that result from pesticide applications to land. The KABAM (KOW (based) Aquatic BioAccumulation Model) is used to estimate potential bioaccumulation of hydrophobic carbon-based pesticides in freshwater aquatic food webs. AECs on the other hand, are based solely on empirical data. They can be measured by taking a water sample from a natural water body or a tissue sample from a wild aquatic organism, making it possible with the help of analytical instruments to find the concentration/dose of chemical in the samples. Various methods and instruments are required to extract and analyze different chemicals.

Estimating the risk to a species from an acute exposure to a chemical, particularly a pesticide, constitutes a Tier I study—a simple laboratory study where worse-case estimates are used to calculate the Risk Quotient (RQ). RQ is defined as the exposure concentration divided by the toxicity concentration. The toxicity concentration is the LC50 or EC50 for an acute exposure. The exposure concentration is the peak concentration of the pesticide in water bodies. If the RQ does not exceed 0.5 for aquatic animals (that is, the exposure concentration is half the toxicity concentration) and 1.0 for aquatic plants (that is, the exposure concentration does not exceed the toxicity concentration), the risk is considered acceptable[12].

A Tier II study is conducted if the Tier I RQ is exceeded. Here, the species is subchronically or chronically exposed to a pesticide. The toxicity concentration is the NOEC and the exposure concentration is the average pesticide concentration over 21-60 days in water bodies. If the RQ does not exceed 1.0 (for both aquatic animals and plants), the risk is considered acceptable; a Tier III study is carried out if it is exceeded. These investigations are chronic, life cycle, or multigenerational in nature, and can be done in the laboratory or outside (i.e., microcosm studies). Tier IV are mesocosm or macrocosm studies involving multiple species. Both Tiers III and IV usually involve a comparison of the toxicity endpoints of the pesticide-exposed populations to that of the unexposed populations. The endpoints analyzed are biomass, diversity, species richness, etc. Figure 7 summarizes the risk assessment process[14.] Additional information can be found in the *Risk Assessment Chapter*.

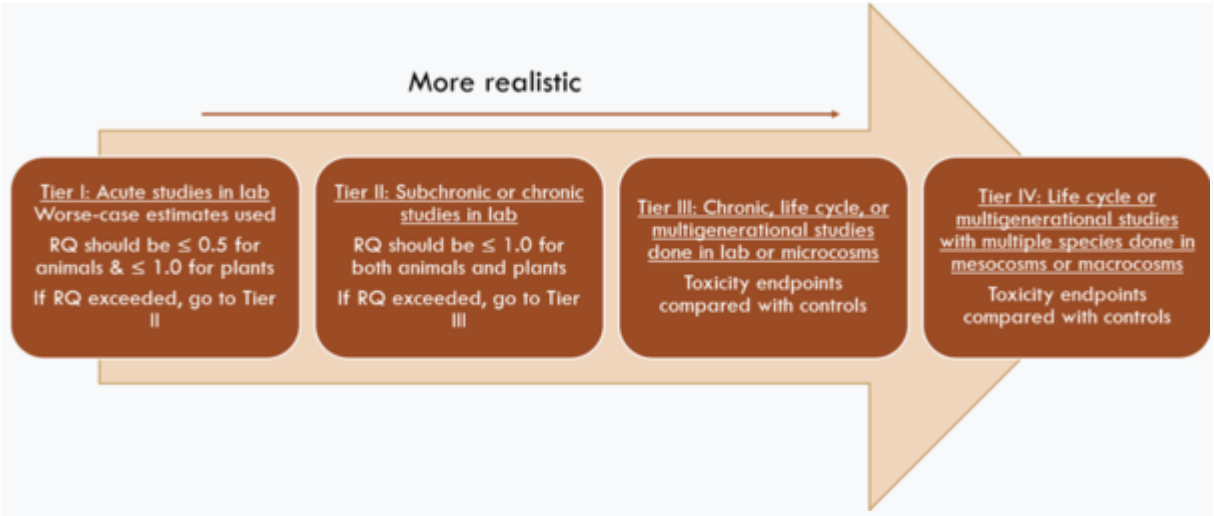


Figure 7: Tiered testing for assessing risk to aquatic organisms

The design of a study depends largely on the question(s) being asked. Consideration must also be given to the physiochemical properties of the chemical being tested, its mode of action, its pattern of use, the environmental conditions, and the characteristics of the test organism. The following is a brief overview:

a. Study objective/question: An aquatic toxicity study could be done for various reasons. It could be done to test the quality of a sediment or effluent, to register a field-applied pesticide, to understand the long-term impacts of a chemical on a community, or for routine monitoring purposes. The objective determines the type and number of tests needed.

b. Physiochemical properties of a chemical: Prior to toxicity testing it is important to collect information on the chemical's structural formula, purity, stability in water and light, partition coefficient, vapor pressure, biodegradability, etc. because its property can influence how it moves, persists, and distributes. For example, chemicals which have poor solubility in water are more likely to accumulate in aquatic organisms or bind to sediment. However, in a lab they may instead bind to the plastic or glass container housing the test organism. Therefore, special steps need to be taken to ensure that the fate of a lab chemical mimics the fate of a chemical in the environment. Similarly, it is not worthwhile to conduct long-term toxicity studies in the lab for chemicals which degrade rapidly in the environment.

c. Mode of a chemical action: Most chemicals found in water bodies have a specific mode of action, i.e., they bind to a specific target site and produce specific downstream effects. Knowledge of a chemical's mode of action will help decide which endpoints are the most important to measure during and after a toxicity test.

d. Pattern of chemical use (or discharge): A species can be exposed to a chemical only if there is an **overlap in space and time.** This means that the species must be present at a location where the chemical is found *and* present at a time the chemical is present in the environment. Some chemicals are only intermittently used and found in the environment. Conducting life cycle or multigenerational studies for such chemicals will not provide much usable information.

e. Environmental conditions: The environment can affect the toxicity and availability of a chemical. High temperatures can more effectively dissolve chemicals, and this might increase the amount of chemical an organism is exposed to. They can also break down chemicals, which can decrease or increase a chemical's toxicity. Other factors can influence toxicity such as percentage of dissolved oxygen, salinity, nutrient level, moisture level, and microbial community. This makes it important to mimic the natural conditions of a test organism in the lab.

f. Characteristics of the test organism: If the objective of the study is to assess sediment toxicity, then organisms that dwell in or near the bottom of a water body should be tested rather than organisms that dwell near the top, unless a major disturbance of sediment is expected (e.g. dredging). It is necessary to choose species that can coexist together if the objective is to study how a community of aquatic species is harmed by a given chemical.

While specific guidelines for various aquatic toxicity tests were linked in Table 2, the following are general guidelines that apply to all tests:

a. Laboratory toxicity tests should be replicated at least thrice under similar conditions to ensure results are reproducible. Mesocosm tests should be replicated at least twice, while there are no requirements for macrocosm tests (due to their complexity and scale).

b. All toxicity tests should have a negative control. The control should have the same conditions and constituents as the treatment group, minus the chemical that is being tested. If the chemical is dissolved in a solvent prior to its introduction into the water, then the negative control should also contain the solvent. This is done to remove any potential effects of the solvent (though solvents should be tested before the study to ensure they are non-toxic).

c. While most toxicity tests are not required to have a positive control, it is encouraged. A positive control is a substance that is known to produce a defined toxic effect in the test organism. It is used to determine if the health and sensitivity of the test organisms have changed over the course of the study. Also, it can help validate data across different labs and help assess reproducibility of the results.

d. No less than three organisms must be treated for every concentration that is used (including control). However, the minimum sample size often depends on the study type and objective. If too few organisms are treated, it is possible to miss significant differences that might exist between treatments and controls. If too many organisms are treated (more than what is necessary), it would cause ethical issues and lead to wasted time and resources. Therefore, a power analysis is often carried out to find the minimum number of organisms needing treatment to study a particular effect.

e. All organisms used in a test must be homogenous (unless specifically instructed otherwise in the test guidelines). They should be of similar age, life stage, body mass, size, etc. They should all be healthy (sick organisms might be more sensitive to the effects of a chemical) and must have followed similar growth patterns prior to chemical exposure.

f. Randomization of controls and treatments should be done to account for non-chemical effects. For example, if all control organisms are placed on the top shelf where the light source is the brightest, they may grow differently than treatment organisms placed on the bottom shelf due to dissimilar light intensity. The difference could be mistakenly attributed to the chemical in the absence of random placement.

g. Conditions such as temperature, light, oxygen concentration, and hardness of water should be maintained throughout the test environmental to avoid impacting a chemical's toxicity and/or availability (i.e., exposure).

h. The concentration of a chemical must be measured and maintained throughout the test. Ideally, the chemical concentration must be analyzed in the water, food, sediment, and in test organism tissues: however, most test guidelines only require that chemical concentration be measured in water.

CONCLUSIONS[edit]

While laboratory toxicity tests greatly help in understanding a chemical's effects, the observed effects will not necessarily be the same in the natural environment. This is due to several laboratory test limitations: a. In nature, there are seasonal fluctuations, temperature changes, diverse microbial communities, etc. that are not captured in lab studies and which can greatly impact results.

b. Organisms in nature are exposed to multiple chemicals and stressors simultaneously. These chemicals and stressors can interact and produce unexpected results. The majority of lab toxicity tests are conducted with only one chemical and under conditions that are favorable to the test organism.

c. In nature, organisms may not be continually exposed to chemicals. In the lab however, long-term studies are carried out where organisms are continuously exposed to the chemical throughout their life cycle. Also, the concentration and availability of the chemical may vary in the environment while they remain constant in the lab.

d. Most lab studies involve spiking the chemical in water and exposing organisms to it. In nature, organisms are also exposed to chemicals through food and sediment.

e. Different physical habitats of ecosystems as well as the genetic structure of populations can also influence toxicity. Most lab studies are carried out on single species, ignoring species-environment, and chemical-environment interactions.

f. Lab studies are done on healthy organisms that are very similar to one another (due to frequent inbreeding). Organisms in nature are more genetically and physiologically diverse.

g. The results from surrogate species tested in the lab are extrapolated to other species since it is not possible to test the thousands of aquatic species present in nature. This adds considerable uncertainty to the results.

The first known aquatic toxicity test was conducted by Aristotle in the 4th century BC, when he exposed midge fly larvae to Athens' effluent streams to monitor their survival and behavior. The field has progressed considerably since then, especially in the last century. Since 1899, many environmental protection laws have been introduced around the globe, and several of these laws require the regulation of chemicals prior to its registration and introduction into the environment. This has led to the development and standardization of toxicity tests. The aquatic toxicity test guidelines that are currently being used were developed within the last 27 years. However, these guidelines are not set in stone. A greater understanding of the world and man-made chemicals has led to the revision of old guidelines and the addition of new ones. In addition, separate test guidelines have been written in recent years to accommodate new chemicals with unique properties.

To better comply with increasingly stringent regulatory requirements and chemical testing while raising ethical standards, organizations such as the European Center for the Validation of Alternative Methods (ECVAM), AltTox, National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), Center for Alternatives to Animal Testing (CAAT), etc., are focused on finding alternatives to testing chemical toxicity in animals.

A seminal report was released in 2007 by the National Academy of Sciences titled *Toxicity Testing in the 21st Century: A Vision and a Strategy*[15]. This report recommended that toxicologists move away from using vertebrates in toxicity tests and move toward the use of non-vertebrates and cell lines. Today it is possible to recreate organs on a chip and test chemicals on it[16]. The report also suggested using computer and mathematical models (examples include Quantitative Structure Activity Relationships, Read-Across[17], Species Sensitivity Distribution[18], Physiologically Based Pharmacokinetic modeling[19]) and knowledge-based pathways (examples include Toxicity Pathways and Adverse Outcome Pathways) to predict the toxicity of chemicals and to extrapolate results from cell lines to whole organisms and ecosystems. These technologies are yet to be implemented on a large scale, and it will probably take several decades for this to be realized.

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