Chapter 10
Ecotoxicology Methods of Reservoir Water Using Fish

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Abstract This chapter aims at elucidating the use of model fish species in ecotoxicological studies and the methodology of ecotoxicity testing based on fish. Chemicals are released into the reservoir and pose a harmful impact on the biota. Water quality and fish health are associated with each other. Therefore, fish is considered a suitable pollution indicator for monitoring reservoir pollution. To assess the effects of these chemicals, different standardized toxicity methods using fish have been developed by international organizations, which include acute and chronic toxicity tests. Species including zebrafish, rainbow trout, Japanese medaka, and fathead minnow are the most recommended fishes and are selected for a toxicity test according to the contaminant's type and the endpoints to be measured.

Keywords Fish · Aquatic toxicology test · Zebrafish · Acute toxicity · Chronic toxicity

1 Introduction

For decades, the assessment of reservoir ecotoxicological risks and the elucidation of molecular mechanisms of contaminant-induced dysfunctions have remained unambiguous [1]. The formal beginning of ecotoxicology as a separate science was procured in the late 1960s, and reservoir ecotoxicology is proposed in this book. Recently, many studies based on the risks of reservoir ecotoxicology are being conducted including the contamination characteristics, source apportionment, climate change, and potential ecotoxicological effects [2–4]. Environmental toxins profoundly affect the fish population and the health of wildlife and humans. Despite the presence of various species in the aquatic environment, fish is considered a more suitable indicator of water pollution. Fish health is associated with water quality when water contamination affects fish populations by disrupting lifespan, embryonic development, and reproductive health [5]. Toxicological testing using fish has been a common practice in the past, and fish death has been recognized as a general water pollution indicator. Current fish toxicology has moved towards mechanistic and multidisciplinary approaches [5].

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2 Ecotoxicological Importance of Fish and Its Use in Ecotoxicity Testing

Fish is being utilized as sentinel organisms in ecotoxicological studies because of their capability of inhabiting all zones of the aquatic habitat where prevails suitable conditions for their survival, feeding habits, physiological diversity, reproductive strategies, and economic significance [6]. For anthropogenic pollutants, reservoir aquatic environments are acknowledged as the absolute sink, and fish death could be observed as a consequence of the toxic action of the pollutants. Thus, fish is commonly recognized as the substitute for evaluating the deleterious effects of contaminants on reservoir ecosystem health. The first ecosystem disturbances studied using fish were the impacts of mine-tailing effluents as reported by Carpenter [7]. In the middle of the twentieth century, techniques were standardized for acute fish toxicity testing. Toxicological tests were developed and validated to the internationally agreed testing procedures used by laboratories, government, and industry to illustrate prospective hazards of novel and existing contaminants. About 20% of the tests recognized by the OECD (Organization for Economic Co-operation and Development) for evaluating health effects on living systems are conducted using the fish model [8]. In the 1960s, concerns about long-term exposure of organisms to contaminants were raised and flow-through techniques were developed. Various biomarkers and endpoints were developed by studying the effects on early-life stages, reproductive cycles, and complete life cycles. New molecular techniques were developed and research was more concentrated on the detection and understanding of the toxicity mechanism of chemical substances [5]. The practice of using wild fish population indices for the assessment of ecological status in water bodies has also grown since the 2000s [9, 10]. Recently, the assessment of emerging contaminants, such as microplastics, pharmaceuticals, and pesticides, has been performed based on fish acute and chronic toxicity, and lethal and sublethal effects are evaluated using novel approaches of modern molecular biology. The acute and chronic toxicity of emerging chemicals using different fish species are presented in Table 10.1.

3 Fish Models

For ecotoxicity testing, fish species are selected based on different factors, such as size, ease of laboratory maintenance, suitability for testing, known sensitivity, available data, and the availability of test procedures and protocols that could be followed [8]. Zebrafish, rainbow trout (*Oncorhynchus mykiss*), Japanese medaka (*Oryzias latipes*), and fathead minnow (*Pimephales promelas*) are the most common species used for ecotoxicological studies. Zebrafish is considered the most popular testing model, because it shares common anatomy and development features, metabolism, as well as physiological and chemical-induced organ responses with

Table 10.1 Acute and chronic fish toxicity studies

Contaminant	Test Organism	Exposure duration	Toxicity	References
Diazinon	*Anabas testudineus*	96 h	Acute (LC 50) 6.55 ppm	[11]
Diazinon	*Channa punctatus*	96 h	Acute (LC 50) 3.09 ppm	[11]
Cadmium, copper, zinc	*Rainbow trout (O. mykiss)*	–	Acute and chronic (LC50 116 (μg/L)	[12]
Permethrin	*Cyprinus carpio*	24 h	Acute (LC 50) 35 μg/L	[13]
Dichlorovinyl Dimethyl phosphate	Zebrafish	24 h	Acute LC 50 39.75 mg/L,	[14]
Methyl parathion	*Catla catla*	96 h	Acute (LC 50) 4.8 ppm	[15]
Cypermethrin	*Colisa fasciatus*	96 h	Acute (LC 50) 0.02 mg/L	[16]
Malathion	*Labeo rohita*	96 h	Acute (LC 50) 15 mg/L	[17]
Endosulfan	*Channa striatus*	96 h	Acute (LC 50) 0.0035 ppm	[18]
Cypermethrin	*Labeo rohita*	96 h	Acute (LC 50) 4.0 μ/L	[19]
Endosulfan	*Labeo rohita*	96 h	Acute (LC 50) 2.15 μg/L	[20]
Dimethoate	*Labeo rohita*	96 h	Acute (LC 50) 24.55 μg/L	[21]
Poly Ethylene	*Pomatoschistus microps*	96 h	Acute: Reduced AChE activity	[22]
Poly Ethylene	Japanese medaka	2 months	Chronic: Histopathological alterations	[23]
Poly Ethylene	Japanese medaka	2 months	Chronic: Altered expression of a gene mediated by the estrogen receptor in the liver	[24]
Poly styrene	Zebrafish	7 days	Uptake and bioconcentration Accumulated in fish gills, liver, and gut	[25]
Chromium	*Channa punctatus*	60 d, 120 d	Chronic: 2.6 mg L^{-1} LDH activity inhibited in liver and kidney.	[26]
3-benzylidene camphor	*Pimephales promelas*	14 d, 21 d	Chronic: VTG LOEC 435, 74 μg L^{-1}	[27]
Oxybenzone UV filter	*Oncorhynchus mykiss*	14 d	Chronic: VTG LOEC 749 μg L^{-1}	[28]
Triclosan	*Oncorhynchus mykiss*	96 d	Chronic: Hatching, Survival No Effect, LOEC 71.3 μg L^{-1}	[29]
Triclosan	*Oryzias latipes*	14 d	Chronic: Hatching LOEC 213 μg L^{-1}	[30]
Triclosan	*Oryzias latipes*	21 d	Chronic Growth, Fecundity, HSI and GSId, VTGe LOEC 200 μg L^{-1}, No Effect,	[30]
Fluoride	Salmon	–	Chronic: 0.5 mg/l Significant disruption of PGC migration	[31]

(continued)

Table 10.1 (continued)

Contaminant	Test Organism	Exposure duration	Toxicity	References
3-benzylidene camphor	*Pimephales promelas*	14 d	VTG, Reproduction, Gonad Histology LOEC 434.6, 74 μg L^{-1}	[32]
Benzylparaben	*Pimephales promelas*	48 h	Acute LC_{50} 3.3 mg/L	[33]
Isobutylparaben	*Pimephales promelas*	48 h	Acute LC_{50} 6.9 mg/L	[33]

humans. Moreover, other characteristics, such as small size, rapid development, the optical transparency of embryos, low cost, and easy maintenance, make it to be an ideal model species [34]. Zebrafish is also responsive to chemical and genetic screens and has a fully sequenced genome [35]. Further, zebrafish offers in vivo high-throughput assays, which are less costly than rodents. The huge population size of zebrafish provides a prompt assessment of multiple toxicity testing and facilitates the study of molecular mechanisms, and developmental and health effects related to exposure to contaminants across a population of organisms [36]. Thus, the OECD recommends the use of zebrafish as a model organism [34]. Japanese medaka has been used for toxicity testing for over 50 years, which is a small-sized (2–4 cm) freshwater fish and is well characterized as a model species because of being tolerant to wide salinity and temperature ranges. Japanese medaka is also being used as a model in the OECD test guidelines for developmental stages like early-life stages, juveniles, and adults [34].

The fathead minnow is also extensively used as a model species, especially in endocrine disruption studies [1, 37, 38]. It is native to North American and temperate waters and inhabits muddy pools of small rivers and streams. It is also among the three species validated by the OECD for ecotoxicity testing and has a huge toxicological database [39], which is mostly favored for embryotoxicity testing because of its well-known rapid development, transparent chorion, and sensitivity to toxic contaminants. The OECD has validated a test guideline for fathead minnow, zebrafish, and Japanese medaka [34]. Some of the fish species recommended by the European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC) are given in Table 10.2 [40].

Fish species are selected and preferred according to the type of test and type of endpoint to measured, such as for toxicity test of early-life stage smaller species. For example, zebrafish, fathead minnow, and Japanese medaka are favored instead of rainbow trout because of their shorter test duration (30 days *versus* 90 days). Conversely, for longer exposure tests, rainbow trout are preferred to check endpoints [8].

Table 10.2 Recommended fish species for ecotoxicity testing

Recommended species	Recommended test temperature range (°C)	Recommended the total length of test fish (cm)
Zebrafish *Danio rerio* (*Cyprinidae*)	21–25	2.0 ± 1.0
Fathead minnow *Pimephales promelas*	21–25	2.0 ± 1.0
Cyprinus carpio (*Cyprinidae*)	20–24	3.0 ± 1.0
Rice fish *Oryzias latipes* (*Cyprinodontidae*)	21–25	2.0 ± 1.0
Guppy *Poecilia reticulata* (*Poeciliidae*)	21–25	2.0 ± 1.0
Blue gill *Lepomis macrochris* (*Centrarchidae*)	21–25	2.0 ± 1.0
Rainbow trout *Oncorhynchus mykiss* (*Salmonidae*)	13–17	5.0 ± 1.0

4 Fish Reservoir Ecotoxicity Tests

Reservoir toxicity tests aim at determining the level of biological response shown by adverse effects demonstrated by test species after being exposed to reservoir chemicals of concern. These tests are often conducted in controlled laboratory settings where the exposure concentration is of primary concern mainly regarding adverse biological effects associated with chemicals [41]. To enhance comparability, test methods are standardized. Toxicity tests are either conducted in situ or ex situ. Water samples may be collected from the contaminated area of the reservoir or prepared for simulated water after composition analysis [42]. Comprehensive assessments of the growth and reproduction of fish are more anticipated for population, multispecies, and community-level studies where reduction in the growth or the reproduction of particular species could be inferred regarding its ecological importance. For such kinds of reservoir toxicity assessments, standard toxicity tests including early-life stage fish tests are more suitable, compared to the other tests [43]. In reservoir environments, most of the exposures are chronic apart from those caused by accidental spill discharges. The results of acute and chronic tests are extrapolated to fluctuating aquatic environments and could be used to predict the effects of chemicals on the reservoir ecosystem [44].

Most of the time, the interaction between a fish and a contaminant under laboratory conditions is mostly more important. However, it is equally important to narrate the effect of chemicals on the fish population in reservoir ecosystems. The status of the fish population at the different concentration levels of contaminant is assessed in field surveys and results are then compared with data acquired from laboratory tests. Due to the fish being mostly exposed to multiple contaminants at a time, a combined toxicity assay in the laboratory is desirable. Besides, *in situ* experiments with caged fish in the reservoir are encouraged to conduct, which may provide more real data on reservoir toxicology [45].

Table 10.3 List of standard fish toxicity tests

Guideline designation	Organization	Title
203	OECD	Fish acute toxicity test
204	OECD	Fish prolonged toxicity test
210	OECD	Fish early-life stage toxicity test
212	OECD	Fish short-term toxicity test: embryo and sac-fry stage
215	OECD	Fish juvenile growth test
229	OECD	Fish, short-term reproduction assay
230	OECD	21-day fish assay
234	OECD	Fish, sexual development test
236	OECD	Fish, embryo acute toxicity test
850.1075	US EPA	Fish acute toxicity test, freshwater and marine
850.1085	US EPA	Fish acute toxicity mitigated by humic acid
850.14	US EPA	Fish early-stage toxicity test

Because of concerns about resource management and the release of chemicals from industries and factories into surface water bodies, aquatic toxicity tests were developed by world-renowned organizations on environmental protection like the US Environmental Protection Agency (US EPA) and OECD. Fish tests mostly relied on species, such as fathead minnow, zebrafish, and the cold-water rainbow trout [46]. A list of the standard fish toxicity test recommended by the OECD and US EPA [5, 46] is presented in Table 10.3.

4.1 Acute Toxicity Tests

When chemicals are released into the environment, they will find their way to enter lakes, rivers, and reservoirs. The EPA data require that fish acute toxicity tests should be typically conducted in three different fish species, including a cold-water freshwater species, a warmwater freshwater species, and a marine/estuarine species. Usually, an acute toxicity test is designed to check the safe concentration of pollutants, which gives a measure of acute lethality [45]. Despite being less ethically accepted as compared to tests with plants and invertebrates, fish acute toxicity commonly required ethics approval authorized by the official animal care and use committee [35, 47]. According to the EU Directive on animal protection utilized for scientific purposes, death as the endpoint should be avoided and substituted by some early endpoints [48]. The OECD guidelines for the testing of chemicals provide a helpful tool for assessing the potential effects of chemicals on human health and the environment. The OECD test guideline of fish acute toxicity tests (OECD TG 203) was published on July 17, 1992, in which fishes are exposed to the test substance for 96 hours under static or semi-static conditions [49]. Fish are exposed to five different concentrations of test chemicals for 96 h and deaths are documented at 24, 48, 72, and 96 h, respectively. Moreover, EC50 is determined to evaluate the

concentration of the chemicals that gives a half-maximal response using a log-logistic model [48]. Recommended species are bluegill sunfish, common carp, zebrafish, fathead minnow, Japanese medaka, guppy, and rainbow trout.

4.1.1 Fish Larvae Test for Acute Toxicity

Rainbow trout acute toxicity test is 96 h static assays in which solutions are not renewed. Fish larvae are exposed to test solutions and are aerated at a rate of 6.5 ml/min at 15 °C and a photoperiod of 16 h light: 8 h dark is maintained. In each test tank, ten fish larvae are retained. Fish are not fed during the experiment and even not 16 h before the test. After exposure for 96 h, numbers of survived fish larvae from each test concentration are counted and mortality is determined [50].

Fathead minnow toxicity test is conducted using a semi-static assay during which solutions are renewed after exposure for the first 48 h. Three replicates are prepared for each test concentration. Ten 4–6 days-old larvae are introduced into each tank with different concentrations. After 24 h of exposure to the test solution, fish larvae are transferred to a clean water container. Then, for the 72 h test, containers are placed in a controlled incubator at 25 °C with a 16 h light: 8 h dark photoperiod. To remove metabolic waste containers are renewed with fresh water. Finally, after exposure for 96 h, survived larvae are recorded from each concentration, and mortalities are observed [50].

4.1.2 Fish Embryo Test (FET) for Acute Toxicity

The fish embryo test (FET) is a potential animal alternative for the acute fish toxicity (AFT) test. The OECD test guideline for fish embryo acute toxicity test was published on July 26, 2013 (OECD 2013). In the beginning, this test was intended to ascertain the acute toxicity of chemicals at fish embryonic stages and was designed to replace the fish acute toxicity test later [35]. Because the early-life stages like embryogenesis are the sensitive period of the life cycle, the study of adverse impacts of chemicals on developmental processes is more discrete. Moreover, embryos are not anticipated under animal welfare regulations and could be utilized as screening tools [34].

Taking zebrafish as an example, the fertilized zebrafish eggs are exposed to the test chemical for 96 h with five different concentrations. After every 24 h observations, the indicators of acute toxicity are measured, which include the thickness of fertilized eggs, the absence of somite formation, the lack of heartbeat, and the non-detachment of the tail bud from the yolk sac. After the completion of the test, acute toxicity is determined based on the presence of any four of these observations, and LC50 is calculated. The test report should contain important information on physicochemical properties, such as pH, temperature, water hardness, the concentrations of the chemical being tested, and the conductivity [35, 51].

4.2 Chronic Toxicity Tests

Commonly, chronic toxicity tests are conducted to evaluate the adverse effects of contaminated medium, such as water, soil, or sediment under long-term exposure [46]. In 1956, a chronic exposure test was conducted by Olson and Foster to assess the toxicity of sodium dichromate to successive life stages (eggs, fry, and early juvenile) stages of salmonids [43]. During the exposure process, at least 10% of the test species remain alive after a complete life span. In chronic tests, survival is monitored and sublethal effects, such as reproductive success and growth, are observed. Statistical endpoints that are taken into account include no-observable (NOEC) and the lowest observable effect concentration (LOEC), which shows the maximum concentration of test substance that does not show any effect on the responses of test species under observation, and the minimum concentration of test chemical that shows the substantial effect on the response parameter compared to the control, respectively [52]. During life cycle tests, fish's younger developmental stages have constantly been shown as more sensitive than others. Short-duration tests using early developmental periods could also predict chronic toxicity. Chronic toxicity tests are considered more sensitive, compared to acute tests, because toxicity actions emphasize no adverse effects levels [46]. The conditions for chronic tests are different for different species. The selection of test species depends upon the consideration of whether the desired endpoints could easily be measured using the selected species or not. When the critical endpoints are the secondary sexual characters, the species nominated for testing should be fathead minnow or Japanese medaka instead of zebrafish. However, while using the endpoints such as fecundity, egg hatchability, and body size, zebrafish are preferred [8].

4.2.1 Full-Life Cycle Tests

Full-life cycle tests using fish were first carried out by Mount and Stephen [53]. Toxic effects were assessed for at least one generation under continuous exposure to chemicals [43]. Life cycle tests are carried out for a year or more to reveal more information, compared to the other tests, because hidden effects of contaminants could be exposed. They can provide evidence of not only fecundity and progeny, but also growth rate and disease resistance. The downside of long-term life cycle tests is that only a limited number of species could be investigated, because these tests are time-taking and few contaminants could be assessed under limited exposure conditions [45]. They are carried out using rapidly growing and small-sized warmwater fish, such as zebrafish and fathead minnow [54]. For the life cycle toxicity test, freshwater fish like fathead minnow or zebrafish is cultured in the presence of the test chemicals from one stage of life to the other (whole life cycle) till the same stage of the next generation F_1. The concentration of the test substance in the water is administered periodically during the experiment. By the end of the

experiment, the reproductive, behavioral, pathological, and physiological effects are assessed, and egg numbers, spawning ratio, fertility, and fecundity are recorded [55].

4.2.2 Partial-Life Cycle Tests

Before 1970, it was observed that when carrying out full-life cycle tests with numerous species after exposure to various chemicals, greater sensitivity has been shown by the early developmental stages, such as embryo, larvae, and juvenile stage, compared to the adult life stages [56, 57]. Consequently, in the mid-1970s, embryo-larval stages (30- to 60-day post-hatch) were proposed as a replacement for full-life cycle tests to lessen the time and cost [43]. embryo-larval stage tests intend to define the lethal and sublethal effects of a chemical on embryonic development, hatching, and larval growth are assessed [54]. According to the OECD guideline 210, these tests are recommended as a suitable and sensitive method for toxicity evaluation of chemicals [54]. In such tests, the embryo-larval stage of fish is exposed to three to five concentrations of the test chemicals under flow-through or semi-static conditions. Lethal and sublethal effects are evaluated and the lowest observed effect concentration (LOEC) is determined. The concentrations of the test chemicals are measured at regular intervals [58].

4.3 Bioconcentration and Bioaccumulation Tests

The uptake of pollutants from the external environment (usually water) is referred to as bioconcentration, and bioaccumulation is the absorption of a contaminant in biological tissues. In these tests, organisms are exposed to sublethal concentrations of the chemical, and their residues in the tissue of exposed organisms are evaluated until a steady state is achieved. Fish is usually used for such studies, because it is consumed by humans. Besides, soil invertebrates are also being assessed by fish for chemical uptake [52]. For fish, bioaccumulation and bioconcentration studies are carried out under flow-through and semi-static conditions. The test is divided into two phases: phase 1 is the uptake phase (exposure), which lasts normally 28 to a maximum of 60 days. During this phase, four fish of one species are exposed to at least two concentrations of the test chemical in separate groups. The second phase is the post-exposure or depuration phase, and fish are transferred into a medium devoid of the test chemical. Besides the two test concentrations, a control group without exposure to test chemicals is also performed in parallel. The concentration of the test chemical is monitored in fish in both phases of the test. Physicochemical parameters like pH, TOC, dissolved oxygen, salinity, total hardness, and temperature are also measured inside the test containers during the test. The lipid content is determined and the bioconcentration factor (BCF) at apparent steady state and the kinetic bioconcentration factor (BCFK) are calculated. Bioconcentration is expressed as a ratio of lipid content *versus* the whole bodyweight of fish [59].

5 Limitations of Fish Ecotoxicity Testing

Chronic fish toxicity tests are considered more sensitive than acute tests for the reason that the estimation of toxicity emphasizes endpoints other than survival, which can define better the no adverse effects levels. Moreover, chronic tests also provide a sound measure of responses for a population in the field. However, acute toxicity tests are regarded as fewer sensitive measures of toxic conditions, compared to chronic tests. Notably, chronic tests might not identify all sublethal effects [60]. Among chronic tests, the life cycle test is considered superior, but there is a limitation on time, space, and type of species that can be used. Other tests guarantee only a partial understanding of the impact of pollution on the fish's survival ability [8]. FET is considered a robust test and used as an alternative to the OECD 203 fish acute test [60].

6 Conclusions

Fish has been used as a sentinel organism for reservoir ecotoxicological testing. Various standardized tests have been designed according to contamination type and condition for evaluating the impacts of water-borne chemicals on fish. Standard toxicological tests are performed for acute lethality, fish embryo acute toxicity test, and chronic toxicity tests (full-life cycle toxicity tests). FET for acute toxicity. Fish bioaccumulation and bioconcentration tests are important because they reflect the reservoir ecotoxicology through the food chain. Reservoir toxicological studies also prefer to use small-size freshwater fish species like zebrafish, Japanese medaka, and fathead minnow. To enhance the predictive value and the extrapolation of acquired data at the ecosystem level, biochemical and molecular tools that can characterize the mode of action of chemicals should also be developed.

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