The Application of Fish

Masahiro Yamaguchi

Abstract Most heavy metals have toxic effects on aquatic organisms. Therefore, toxicity tests of these metals using living organisms are required to estimate their toxicity. Fish are representative of aquatic organisms and have been used for the toxicity tests. Model species such as fathead minnows (*Pimephales promelas*), zebrafish (*Danio rerio*, formerly referred as *Bracydanio rerio*), medaka (*Oryzias latipes*), and rainbow trout (*Oncorhynchus mykiss*) have been heavily used. However, the intensity of toxicity varies at developmental stages even in the same species. In addition, temperature, pH, hardness, and salinity of test water also affect the toxicity of heavy metals. Thus the influences of these factors should be considered. The toxicity of heavy metals includes both acute and chronic cases. However, not much data exists for the chronic toxicity tests because these toxicity tests require a long time and great effort compared with acute toxicity tests. In the future, an accumulation of chronic toxicity test data and elucidation of the mechanisms for the toxicity expressions will be needed.

1 Introduction

Various kinds of metals, originating from either natural or artificial substances, can contaminate water. In order to evaluate the toxicity of these metals, bioassays are required using aquatic organisms. Some species of algae, crustacean, or fish have been used for the toxicity tests.

Among these organisms, fathead minnows, zebrafish, and medaka, have been widely used as test fish. They have some advantages; (1) they are model animals in various research fields such as developmental biology and neuroscience, and the methods for keeping and breeding have been already established, (2) they are vertebrate and have a similar body plan as humans. Thus the results obtained from the toxicity tests would be applicable to humans to some extent, and

M. Yamaguchi (✉)
National Institute of Technology, Suzuka College, Suzuka, Japan
e-mail: yamaguchi@chem.suzuka-ct.ac.jp

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(3) especially in zebrafish and medaka, genome information has been available. Therefore, we can analyze the gene(s) and/or protein(s) expression pattern(s) in the toxicity tests. In this chapter, the application of fish to the evaluation of metallic toxicity is discussed.

2 Methods for Toxicity Tests Using Fish

Ecotoxicity includes acute and chronic toxicity. Acute toxicity is defined as the toxicity in which the influence appears within a short period after exposure to pollutants. On the other hand, chronic toxicity emerges after long-time exposure to pollutants. Various methods could exist to estimate ecotoxicity. However, the standardized methods should be adopted to compare the independently obtained results. The Organization for Economic Cooperation and Development (OECD) provides guidelines for toxicity tests, and they are the most reliable and widespread protocols. Among the guidelines, some illustrating the tests using fish are TG203, TG210, TG212, TG215, TG229, TG230, TG234, and TG236 [25] (Table 1). Based on these toxicity tests, some parameters such as NOEC (no observed effect concentration; the concentration of a pollutant that will not harm the tested organisms), LOEC (lowest observed effect concentration; lowest concentration of a pollutant that harms the tested organisms), LC_{50} (lethal concentration, 50 %; the concentration of a pollutant that kills half of the tested organism population), and EC_{50} (effective concentration, 50 %; the concentration of a pollutant that affects half of the tested organism population) are determined.

When the influences of heavy metals on aquatic organisms are considered, it is natural to assume they are exposed to a trace amount of substances over a long period of time. Therefore, the parameters obtained from chronic toxicity tests could

Table 1 OECD guidelines concerning fish toxicity tests

	Name of tests	Duration of tests	Main endpoints
TG203	Acute toxicity test	96 h	LC_{50}
TG210	Early-life stage toxicity test	Until free feeding	LOEC, NOEC
TG212	Short-term toxicity test on embryo and sac-fry stages	Until yolk-absorption completed	LOEC, NOEC
TG215	Juvenile growth test	28 days	ECx
TG229	Fish short term reproduction assay	21 days	Egg production
TG230	21-day fish assay	21 days	Vitellogenin and secondary sexual characteristics
TG234	Sexual development test	60 days	Vitellogenin and proportion of males, females, intersex and undifferentiated fish
TG236	Embryo acute toxicity (fet) test	96 h	LC_{50}

be informative. However, these values are less reliable than those of acute toxicity tests, because the chronic toxicity tests require a long time and lots of effort. Thus the number of tested organisms is small. However, some methods to speculate the chronic toxic values have been developed. For example, the method using the index of ACR (acute to chronic ratios; the ratio between chemical concentrations exerting acute toxicity, such as LC_{50}, versus a chronic toxic value such as NOEC) is useful. AF (application factor) is the inverse of ACR. Kenega [20] reported that the values of ACRs are less than 100 in 86 % of analyzed chemicals. However, Tabata [31] showed that the AF of lead, chromium (VI), and cadmium is 0.003. Therefore, the assumption that ACR is less than 100 could underestimate the risks of these heavy metals.

3 Species and Developmental Stages of the Sample Fish

The OECD guidelines recommend certain species of sample fish. For example, TG203 recommends zebrafish, fathead minnows, carp (*Cyprinus carpio*), medaka, guppy (*Poecilia reticulate*), bluegill (*Lepomis macrochirus*), and rainbow trout as sample fish. These species show different tolerances to heavy metals. For instance, LC_{50} of zinc during 96 h for an acute toxicity test with fathead minnows, bluegill, and zebrafish were 0.551 mg/L [8], 2.86–3.87 mg/L [5], and 25 mg/L [6], respectively, suggesting that the toxicity of zinc differs depending on the species of test fish. From the viewpoint of the protection of aquatic organisms, most susceptible species should be used for toxicity tests. However, a most susceptible species for all pollutants does not exist. Instead, most susceptible species differ depending on the tested substances. Thus, the use of various fish species is preferable to estimate the toxicity for most of the aquatic organisms. In addition to fish, the use of creatures in other kingdoms such as algae and crustacean is also preferable.

The toxicity of heavy metals or chemicals differs depending on the stages of development even in the same species. The developmental process of fish could be divided into embryonic stage (from the fertilization to hatching), pre-larval stage (from the hatching to the absorption of yolk), post-larval stage (from the absorption of yolk to the phase in which the number of fin rays reaches the same with those of an adult fish), juvenile stage (from the phase in which the number of fin rays reaches the same with those of an adult fish to the phase when scale is synthesized), pre-adult stage (from the stage when scale is synthesized to the first maturity), and adult stage (after the first maturity). During embryogenesis, the sensitivity to heavy metals or chemicals is relatively low, because the chorion entraps these substances. Generally, the sensitivity increases after hatching, and again decreases gradually thereafter. Thus, it is possible that the toxicity tests with post-hatching stages, such as TG203 detects metallic toxicity more sensitively than the one with embryonic stages such as TG236. Eaton et al. [11] analyzed cadmium toxicity using seven different fish species and found that larvae were more sensitive than embryos in all species. In addition, McKim et al. [23] examined copper toxicity using eight

different fish species and also found that larval stages were more sensitive than the embryonic stages. However, it was reported that copper was toxic to the same degree in the embryonic stage and larval stage in rainbow trout [28]. This suggests that the embryonic stage is not necessarily an insensitive stage. Furthermore, the pattern of the change in sensitivity to metals is different depending on the type of metals, even in same fish species [28]. Therefore, it would be important to examine the toxicity in various developmental stages.

When the adult fish were used with toxicity tests, the amount of required reagents and wastewater was large. In addition, it was claimed that the application of adult fish to toxicity tests is not preferable ethically, and that toxicity tests with adult fish should be replaced by those with embryos [3].

4 The Quality of the Test Water

4.1 Temperature of Water

Each fish species has a specific water temperature appropriate for development, propagation, and living. TG203 designated a water temperature for each test fish species. A suitable water temperature for most fish is 20–25 °C, but for rainbow trout it is 13–17 °C. Thus more than 25 °C could damage rainbow trout even if harmful substances do not exist.

Water temperature also could influence metallic toxicity. For instance, the metallic toxicity tends to increase with a rise of water temperature for goldfish (*Carassius auratus*); acute toxicity test of chromium (VI) and zinc for goldfish in 24 h revealed that the rise of water temperature from 5 to 30 °C accompanied a decrease of LC_{50} [30]. It is known that 20–28 °C is suitable for keeping goldfish. Thus metallic toxicity is not necessarily lowered when the temperature of the test water is most suitable for sample fish. Seasonal fluctuation of water temperature is relatively large in temperate zones, and daily fluctuation is also large in a small river. Therefore, the influence of water temperature on metallic toxicity should be considered especially in such places. However, in the case of some fish species such as bluegill and rainbow trout, there is no relationship between water temperature and the toxicity of heavy metals. This suggests that the existence or absence of the relationship depends on the fish species.

4.2 pH

Potential of hydrogen (pH) of natural environmental fresh water such as a river or lake is usually neutral. However, it is known that acid rain decreases pH and eutrophication increases pH. Extremely low and high pH values are not suitable

for aquatic organisms. In addition, it was reported that pH influences metallic toxicity.

When the concentration of H^+ rises, the binding of H^+ to ligands of dissolved organic substances would be accelerated, thus antagonizing the binding of heavy metal ions to dissolved organic substances, resulting in the increase of metallic toxicity because of the rise of the concentration of free heavy metal ions. However, it is not only free ions that are toxic to aquatic organisms. Heavy metals in a natural aquatic environment are found in many different forms such as free ions, complexes, colloids, and absorbed species on the surface of suspended particles, and each form of heavy metal has different toxic effects on aquatic organisms. Because pH influences metal speciation, it has a complex effect on metallic toxicity.

Acute toxicity tests of zinc for rainbow trout and brown trout (*Salmo trutta*) revealed that a rise of pH elicits an increase of toxicity [2, 12]. The difference of speciation for zinc would not account for the difference of toxicity between pH5 and pH7, because most zinc exists as free ions both in pH5 and pH7. It is possible that a rise of pH (i. e., the decrease of H^+ concentration) would reduce antagonistic effects of H^+ on free zinc ions against positive ion-binding sites of sample fish, resulting in the increase of zinc toxicity. On the other hand, the rise of pH from 7 to 9 elicits a moderate increase of zinc toxicity. Most of the zinc exists as $ZnCO_3$ or Zn (OH)x in pH9. Thus the adhesion of such a zinc precipitate to body surfaces or mucus of test fish (rather than the binding of free zinc ions to putative positive-ion binding sites of test fish) would account for the toxicity of zinc in pH9.

In the case of copper, it was also reported that a rise of pH is accompanied by an increase of the toxicity in rainbow trout [15]. On the other hand, acute toxicity tests of aluminum for smallmouth bass (*Micropterus dolomieui*) showed that the rise of pH weakens its toxicity [19]. When pH is elevated, toxic Al^{3+}, $Al(OH)^{2+}$, and $Al(OH)^{4-}$ would decrease, and less toxic hydroxide precipitate would increase. These changes of speciation could explain the weakened toxicity.

As mentioned above, metallic toxicity is largely influenced by pH. However, the effects of pH vary depending on the type of species and the type of metals present. The pH of test water should be controlled carefully with consideration of such effects.

4.3 Hardness

It is well-known that an increase of water hardness decreases metallic toxicity. For instance, LC_{50} of zinc during 96 h of an acute toxicity test with brown trout is 0.14 mg/L when the hardness is 10 mg/L $CaCO_3$, and it rises to 1.0 mg/L when the hardness is 204 mg/L $CaCO_3$ [12]. The same effect of the hardness on the metallic toxicity is also reported in rainbow trout [29]. However, the effect of the hardness differs depending on the types of metals. The increased hardness weakened the

toxicity of cadmium very much, but its effect on the toxicity of lead is relatively small. The effect on the toxicity of copper, zinc, or nickel is intermediate between that of cadmium and lead [4]. In the case of cadmium, the hardness almost does not affect its speciation. Thus a rise in the hardness would cause decreased incorporation of cadmium, resulting in decreased toxicity. It is also possible that some positive ions such as Ca^{2+} or Mg^{2+} antagonize Cd^{2+} by blocking absorption of Cd^{2+} from the gills. The metallic toxicity-reducing effect of Ca^{2+} is greater than that of Mg^{2+} [7]. In addition, it is thought that carbonate (in water) captures metallic ions and composes complexes, resulting in decreased toxicity because the concentration of free metallic ions is reduced [32].

The hardness in a natural fresh water environment varies depending on the regions in the world. In general, the hardness is high in Europe and North America, and low in Japan. However, it varies in each river, lake, or pond, even in the same country. Therefore, the metallic toxicity should also vary in each aquatic environment.

4.4 Salinity

Seawater contains 3.5 % salt. Some fish species living in brackish water can survive in wide ranges of salinity (euryhaline species). However, most saltwater and freshwater fish cannot survive in fresh water and seawater, respectively. Thus an excess or a lack of salinity itself causes lethal damage to test fish, and salinity of test water should be controlled carefully according to the species of test fish. Currently there is no model species of saltwater fish designated as a recommended species by OECD guidelines. In order to estimate metallic toxicity in seawater, it is necessary to establish a saltwater species suitable for toxicity tests. Recently some studies used Java medaka (*Olyzias javanicus*) as test fish in blackish and seawater [9, 21].

Some reports showed that the rise of salinity decreases metallic toxicity. However, there are not so many reports investigating the relationship between the salinity and the metallic toxicity in fish. We conducted acute toxicity tests of zinc with medaka larvae and found that sodium chloride and sodium dihydrogenphosphate decrease the toxicity of zinc [26]. As in the case of Ca^{2+} and Mg^{2+}, it is possible that Na^{+} antagonized Zn^{2+} by inhibiting the intake of zinc from the gills. However, the effect of sodium dihydrogenphosphate is more intense as compared to that of sodium chloride. Therefore, it is possible that $H_2PO_4^{-}$, HPO_4^{2-}, or PO_4^{3-} forms complexes or suspended particles with zinc, which are less toxic than free zinc ions. We also reported that sodium dihydrogenphosphate decreases the toxicity of lithium to medaka larvae in a dose-dependent manner [17]. However, we should keep in mind that medaka is relatively tolerant to salt water.

5 Mechanisms of Action for the Toxicity of Heavy Metals

Most heavy metals are essential trace elements that are required for organisms, but are also toxic substances. Heavy metals such as iron, zinc, copper, manganese, molybdenum, selenium, chromium, and cobalt are known as essential trace elements for animals, and they function by binding to specific proteins in an animal's body. For example, iron is incorporated in hemoglobin, and zinc is an essential component of transcription factors having a zinc-finger domain. Lack of these essential trace elements causes physiological damage such as anemia caused by a deficiency of iron. On the other hand, in general, some types of heavy metals such as cadmium and lead are thought not to be required for organisms. However, these "unessential elements" have similar physical and chemical characteristics to the essential elements. Therefore, they could be taken into the body by the same channels through which essential elements are absorbed.

The mechanisms for acute toxicity of heavy metals against aquatic organisms are still unclear. One possible explanation is that there are positive ion-binding sites (called "biotic ligands") on the surface of the gills, and heavy metal ions compete with other positive ions (such as H^+ and Ca^{2+}) for binding to the biotic ligands. Thus heavy metals would inhibit absorption of such essential ions and cause severe damage. This model is named the Biotic Ligand Model (BLM) [22, 27]. According to the BLM, a decrease of metallic toxicity by an increase of hardness (of the water) could be explained by the decline of binding of heavy metals to the biotic ligands which is caused by an increase of Ca^{2+} concentration. Furthermore, it could be hypothesized that the amount and/or ratio of biotic ligands which is occupied by heavy metals determines the degree of toxicity. This hypothesis has not been verified yet, but there is a report supporting it mathematically [18].

On the other hand, acute toxicity tests with rainbow trout revealed that the sensitivity for cadmium decreases gradually from fertilization to hatching, but for zinc it is unvaried and for copper it increases in the same developmental stages [28]. BLM cannot explain this phenomenon unless it is assumed that there are specific developmental stages at which specific species of heavy metal ions preferentially bind to the biotic ligands. In addition, it should be mentioned that not only free ions are toxic to aquatic organisms. As mentioned above, the toxicity of zinc is strengthened in alkaline test water, even if the ratio of precipitates such as $ZnCO_3$ and/or Zn(OH)x increases and that of free ions decrease drastically. According to BLM, heavy metals cause a deficiency of essential ions like Ca^{2+} by inhibiting their absorption, and this accounts for lethal damage. However, calcium is stored in bone vertebrae and released by bone resorption when the plasma concentration of Ca^{2+} is reduced. Thus the lack of calcium would not necessarily induce acute toxicity. On the other hand, it is known that heavy metal ions can interact with proteins of various species by binding to the thiol group of cysteine residues, and inhibiting their functions. Thus the behavior of heavy metals in the body, as well as their permeability at the gills, would affect the toxicity.

In order to reveal the mechanisms for the toxicity of heavy metals, pathological and biochemical analyses of the tissues are required. In addition, analyses of swimming behaviors of test fish are also helpful to inspect how the toxicity of heavy metals is expressed. Drummond [10] analyzed swimming ability, response to stimuli, and respiration patterns of fathead minnows, and classified the action of toxic substances into four groups; narcosis, respiratory uncoupler, respiration irritant, and acetylcholinesterase inhibitor [10]. He did not analyze heavy metals, but the methods of his analyses are possibly applicable for evaluating the mode of action of heavy metals.

The mechanisms for chronic toxicity would be somewhat different from those of acute toxicity. The lack of calcium mentioned above could have a chronic, rather than an acute effect. When we consider the action of chronic toxicity of heavy metals, their narcotic or respiration uncoupler effects would be less important. Rather, the amount of accumulated heavy metals over a long period of time, the sites where they accumulated, and their speciation are important. However, there are relatively few reports investigating these points.

6 Future Prospects

6.1 Acute Toxicity Test

A large number of acute toxicity tests have been conducted (about various kinds of metals) with a lot of species of aquatic organisms, and there is an enormous stock of data. These data are very useful to conserve aquatic organisms. Most of these tests adopt survival as the endpoint. However, if the swimming ability of fish is damaged in a natural environment, then they cannot feed and/or escape from their predators, and would die even if they could survive during a relatively short test period for an acute toxicity test. Therefore, they could be regarded as dead "ecologically". The use of this "ecological death" as an endpoint could allow for more sensitive toxicity tests. Recently some studies reported acute toxicity tests using deficiency of behaviors as endpoints.

On the other hand, finer toxicity tests would be allowed if the symptoms of severe damage could be caught before the test fish fall into a critical situation. Nowadays some toxicity tests adopting cellular or molecular events as endpoints are performed. For example, it was reported that the amount and activity of the enzyme cytochrome P450 is upregulated in copper sulfate-treated silver carp (*Hypophthalmichthys molitrix*) [13]. It was also reported that secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland is activated in heavy metal-exposed fish, resulting in the acceleration of cortisol secretion from the internal gland [1]. Thus quantification and monitoring of plasma ACTH and/or cortisol concentration would be helpful to grasp the symptoms of damage to the test fish.

Heat shock protein (HSP) is another useful marker and the amount of HSP70 in the gills increases in heavy metal-exposed larvae of rainbow trout [33]. Thus it can be said that various molecules could be used as a worthwhile marker to analyze metallic toxicity, although some molecules or genes show opposite responses to different toxic substances. Thus their molecular characteristics and functions should be investigated precisely. Recently the data of genome and proteome have become available easily (especially for zebrafish and medaka) and their use could contribute to establishing more sensitive toxicity tests. In order to analyze gene and protein expression, Northern blotting, RT-PCR and in situ hybridization, and Western blotting and immunohistochemistry have been carried out, respectively as conventional methods. Nowadays however, the transgenic fish can be produced easily in which the gene coding green fluorescent protein under the control of a specific gene promoter is introduced. Using such transgenic fish, the gene expression pattern can be visualized in living fish. Thus it provides a good system to analyze the gene expression and the degree of disorder in the same individuals.

6.2 Chronic Toxicity Test

Environmental standards should be set based on the values of chronic toxicity tests. This is because most toxicity from heavy metals in natural water environments is chronic, rather than acute. However, there is relatively little data for chronic toxicity tests, and the results of the chronic tests are also somewhat unreliable. This is because these tests require a considerable amount of time and effort, and their results tend to be different depending on the conditions of each test. Therefore there is a need to accumulate data for chronic toxicity tests of heavy metals with various fish species. However, there are several problems to be solved such as establishing methods to evaluate chronic toxicity.

At present, OECD provides some guidelines for chronic toxicity tests dealing with fish such as TG229, TG230, and TG234. However, it still remains controversial as to whether a dozen days, in which test fish are exposed to toxic substances as defined in these guidelines, are enough for being the “long-term” test. Also there is the question of what should be set as the endpoint. Norris et al. [24] compared plasma concentration of ACTH and cortisol in brown trout collected from a site contaminated with cadmium and zinc with those from an uncontaminated site in the same river. They found that there are no significant differences between the two groups. But they also found that when fish are subjected to a continuous confinement stressor in 5-gal buckets, the rise of plasma cortisol level delays in fish residing at a contaminated site significantly, even if ACTH secretion is elevated initially compared with the control fish. Also the plasma cortisol level declines during the test period compared with the control fish. These data suggest that the response to confinement stress by the hypothalamus-pituitary-internal gland axis in fish chronically exposed to cadmium and zinc is depressed and these fish could not sustain stress responses. The concentration of cadmium in the contaminated site of

this study was 1.3 μg/L, less than 1/1000 of LC_{50} in the 48 h acute toxicity test with rainbow trout embryos. This suggests that if we set an appropriate endpoint (for example, the sensitivity against confinement stress) the toxicity caused by a trace amount of heavy metal could be detected. In order to conserve aquatic organisms, the methods concerning their exposure with lower concentrations for a longer amount of time should be established as well as the current OECD guidelines.

6.3 Mechanisms of Action for the Toxicity of Heavy Metals

It is not fully understood how the toxicity of heavy metals is expressed. Heavy metals disrupt transports of ions across the gills or other body surfaces, resulting in an abnormal composition of ions. On the other hand, heavy metals incorporated into the body would bind to various proteins and hamper their functions. It is considered that metallic toxicity is caused by these combined effects. Thus in order to understand how the toxicity is expressed, it is required to reveal the system by which various species of ions are transported into and out of bodies and cells. However, the isolation and functional analysis of ion channels and ion pumps on the surface of fish gills have not been entirely carried out. In 2003, type 3 Na^+/H^+ exchanger (NHE3) was firstly identified in chloride cells of Osorezan dace (*Tribolodon hakonensis*) living in extremely acidic Lake Osorezan in Japan [14]. It has since been isolated in some other fish species, and in 2014, it was reported that NHE3 in zebrafish gill mediates Na^+ absorption from ion-poor fresh water by its Na^+/H^+ and Na^+/NH_4^+ exchange activities [16]. Integration of the theory of environmental toxicology like BLM with the knowledge about physiological and biochemical functions of biotic ligands will be the challenge for the future.

The composition and behaviors of ions in saltwater fish are totally different from those in freshwater fish because saltwater fish are always exposed to an inflow of ions into their bodies. The toxicity tests using saltwater fish species are required to consider the conservation of aquatic organisms in the marine area. However, a model saltwater fish species has not been established as yet. It will be crucial to collect the data of toxicity tests using saltwater fish species.

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