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Commentary

The weaker points of fish acute toxicity tests and how tests on embryos can solve some issues

Claus Wedekind [a,b,*], Beat von Siebenthal [a,b], Ruth Gingold [b,c]

[a] Department of Ecology and Evolution, University of Lausanne, Biophore, 1015 Lausanne, Switzerland
[b] Division of Conservation Biology, University of Bern, 3012 Bern, Switzerland
[c] CICESE, Biological Oceanography Department, 22860 Ensenada, BC, Mexico

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We list the main weaknesses of fish acute toxicity tests and suggest that multi-factorial embryo tests could solve many of these issues.

Abstract

Fish acute toxicity tests play an important role in environmental risk assessment and hazard classification because they allow for first estimates of the relative toxicity of various chemicals in various species. However, such tests need to be carefully interpreted. Here we shortly summarize the main issues which are linked to the genetics and the condition of the test animals, the standardized test situations, the uncertainty about whether a given test species can be seen as representative to a given fish fauna, the often missing knowledge about possible interaction effects, especially with micropathogens, and statistical problems like small sample sizes and, in some cases, pseudoreplication. We suggest that multi-factorial embryo tests on ecologically relevant species solve many of these issues, and we shortly explain how such tests could be done to avoid the weaker points of fish acute toxicity tests.

Keywords: Fish acute toxicity test; Ecotoxicology; OECD guidelines; Inferential statistics; Representative sampling; Multi-factorial testing; Pseudoreplication; Embryo test

1. Introduction

Synthetic-organic chemicals to be marketed are in many countries required by law to pass a number of tests for environmental risk assessment and hazard classification. The test procedure depends on the properties of the substance, i.e. its ecotoxicological potential, as well as on the expected quantity to be produced (Newman and Strojan, 1998; Newman, 1995; Fent, 2003b; ECETOC, 2003). As part of such mandatory procedures, juvenile or adult fish are tested in ''fish acute toxicity tests'' that are standardized by OECD guidelines (OECD, 1992a). Further tests (e.g. OECD, 1984) are typically based on the results of these first tests. Because acute toxicity tests are used in environmental risk management, it is important to know what kind of inferences are possible with what kind of test designs. The major purpose of the OECD guidelines is to permit a comparison of chemicals with respect to their relative hazard. This requires a maximal standardization of test protocols. However, such a standardization automatically leads to a number of problems when the tests results are used to establish region-specific water quality criteria or to predict the possible influence of a substance on a given fish population. The following list summarizes the main reasons why fish acute toxicity tests only provide first estimates of relative hazards. We also list some more general problems with the existing testing designs. We then recommend testing embryos instead of juveniles or adult fish and discuss the main advantages and disadvantages of these tests.

* Corresponding author. Department of Ecology and Evolution, University of Lausanne, Biophore, 1015 Lausanne, Switzerland. Tel.: +41 21 692 42 50; fax: +41 21 692 42 65.
E-mail address: claus.wedekind@unil.ch (C. Wedekind).

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2. The major weaknesses of fish acute toxicity tests

2.1. Representative species?

The test species that are recommended by the OECD guidelines are rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), guppy (*Poecilia reticulata*), and common carp (*Cyprinus carpio*). This recommendation helps to achieve comparable results and thus saves experiments and resources (i.e. they increase practicability). However, none of the recommended species are native to Western Europe. Hence, they may not be species representative of European aquatic systems and their fish fauna. Moreover, it is not clear whether these species can be seen as representative for the fish fauna even in the regions where they occur. In some cases, species and geography appears to have little or no impact on fish sensitivity, while in other cases species have been found to differ significantly in their susceptibility and tolerance to different chemicals (Emans et al., 1993; Dyer et al., 1997; Versteeg et al., 1999; Fent, 2003b; ECETOC, 2003; Maltby et al., 2005). The data of single species toxicity tests are therefore often seen as "*… highly questionable with respect to accuracy and, in more general terms, to toxicity relevance*" (Braunbeck et al., 2005, page 88). Also, it seems largely accepted that there is no most susceptible species that could be used in a possibly conservative testing approach (Fent, 2003b). The susceptibility and tolerance of most fish populations may therefore be hard to predict from single standard fish acute toxicity tests. Multiple tests on various species are necessary to get an estimate of the range of susceptibilities to chemical substances.

2.2. Representative genetics of the test animals?

For some tests, the fish are laboratory-reared offspring of wild-caught fish; others derive from aquaculture or laboratory populations. Especially in the latter cases, the test fish can potentially be inbred to some degree. If so, they have not only lost some of the genetic variation of their respective wild population, but they are also likely to be on average more homozygous than individuals of the founder population. A number of studies suggest that the genetic effects of the susceptibility to toxins can be strong (Nevo, 2001; Maes et al., 2005). It may often be difficult or impossible to work with random samples of juveniles or adults of natural fish populations, but laboratory-bred fish can usually not be assumed to represent the genetics of natural populations, either in their average response or in the range of their response. As a consequence, results on laboratory-bred fish may not provide reliable quantitative predictions of the influence a substance may have on a given fish population.

2.3. Variability in the wild versus standardization in the laboratory

The standardization procedures recommended by the OECD exclude most of the variation that is expected in the wild. This includes variation in age or nutritional status of the test organisms and a number of chemical and physical test conditions (OECD, 1984, 1992a,b, 1998, 2000). Bioavailability and toxicity of chemical pollutants have often been found to depend on various chemical and physical factors on the one hand (Landis and Yu, 2003; Rand, 1995; Wright and Welbourn, 2002), and to the organisms' genetics, condition, age, and other biological characteristics on the other hand (Duan et al., 2001; Roark et al., 2005; Rocha-Olivares et al., 2004; Lopes et al., 2004; van Straalen and Timmermans, 2002; Maes et al., 2005). It would clearly be difficult to include the natural variability in ecotoxicological testing on juvenile or adult fish. However, in order to learn about the susceptibility and tolerance of a given population in a given environment, the toxicity tests would have to include factors that vary in the wild, i.e. daily and seasonal variation in temperature, feeding regime, pathogen pressure, predators, etc. Even if in some cases variation in the wild does not seem to have a significant influence on fish reaction (Dyer et al., 1997), such results would have to be confirmed for other substances and factors, i.e. the test designs would have to include the environmental conditions that are of relevance for a specific question, in general or for more regional scenarios.

2.4. Interaction effects that can only be seen in multi-factorial experiments

As mentioned above, the bioavailability of chemicals is normally dependent on various biogeochemical and physiological factors (Fent, 2003a). Testing the effects of varying temperature on the toxicity of a substance would only provide information about temperature as a main effect. In the natural habitat, various stress factors often act in parallel and may amplify an individual's susceptibility to a chemical stressor. Multi-factorial experimental designs are therefore required to learn more about these possible interaction effects (for example the possible interaction between temperature variation and various levels of physical disturbance on an organism's tolerance to a chemical substance). Such multi-factorial designs would increase the necessary number of test fish by orders of magnitudes, i.e. they would be difficult if not impossible with juvenile or adult fish.

2.5. The significance of pathogens in ecotoxicology

A number of parasites, especially micropathogens, are known for affecting mainly weakened hosts. Such pathogens are therefore often called 'facultative pathogens' or 'opportunistic pathogens'. Typical examples in fish could be the oomycete *Saprolegnia* sp., or the bacteria *Aeromonas hydrophila*, *Aeromonas sobira*, *Pseudomonas fluorescens*, or *Pseudomonas putida* (Bernet et al., 2001). Chemical stress can increase the susceptibility of fish to micropathogens (Carballo et al., 1995; Austin, 1999; Baker et al., 1983; Dunier, 1996; Arkoosh et al., 1998), and vice versa infections may reduce the tolerance to chemical stress. This has two main consequences for current experimental ecotoxicology. First, as laboratory

experiments on fish are rarely (or never) done under sterile conditions, the effect of an experimentally induced stress factor can be influenced by non-detected infections. Fish exposed to chemical pollutants and subsequently challenged by an experimental infection can suffer from mortalities that are twice as high or higher compared to fish facing the same infection in the control group. The range of these interaction effects depends on the type of pollutants or pathogens as well as on the concentration of the pollutants and pathogens and their exposure time to the test fish (Hetrick et al., 1979; Arkoosh et al., 2001; Clifford et al., 2005). Thus, non-detected infections can lead to non-replicable results. Confounding effects of undetected micropathogens can occur within few hours, even at a relatively low incubation temperature of 8 °C (Wedekind, 2002). Second, we have to assume that fish in the wild are constantly in contact with all sorts of micropathogens, and many of these pathogens could become relevant if their host suffers from any kind of stress. Therefore, even if laboratory tests could be done under sterile conditions, they would probably underestimate the impact that a chemical stressor could have in the wild. Using non-sterile conditions in the laboratory does not fully solve this problem, because it is difficult to compare the average effect of pathogens in the wild with something of an average effect of uncontrolled infections in the laboratory. Much research is still necessary before we understand the significance of pathogens in ecotoxicology.

2.6. Pseudoreplication

The OECD guideline no. 203 (OECD, 1992a) gives recommendations about the design of acute toxicity tests. According to this guideline, at least seven fish per tank need to be tested in at least five different concentrations of the test substance, additional to the necessary control(s). All fish that are tested in the same concentration can be (and often are) kept in the same tank each. If tests follow the minimal version that is recommended in these guidelines, they are done in five tanks with seven fish each, with each tank receiving another concentration of the test substance. Such an experimental design will lead to pseudoreplication if fish are taken as replicates instead of tanks ($n = 5$) and could therefore lead to wrong conclusions (Hurlbert, 1984). For a frequency or variance analysis, for example, 35 fish distributed to five tanks cannot be regarded as 35 independent replicates because possible tank effects could confound the results. Other OECD guidelines may lead to analogous statistical problems.

2.7. Small sample size

Even if fish were kept singly or in small groups, i.e. in an experimental design that is based on sound independent replications, local animal protection laws and resource constraints normally lead to rather small overall sample sizes. Estimates of means and variances cannot be robust, limiting the power of the test to detect differences that may exist. Moreover, multi-factorial experimental designs that would be necessary to estimate the main effects and the interaction effects between various chemical, physical, and biological factors (see above) would not be feasible because they would require a greatly increased sample size. Former studies demonstrated the significance of such interaction effects. Fish exposed to a carbamate pesticide experienced, for example, 2–9 times higher mortalities at high water temperatures than when exposed to the same concentration of the pesticide at low water temperatures (Altinok et al., 2006). Thus, the toxicity of a substance in the wild should be estimated in experiments that consider the most important interactions that may play a role in a typical habitat.

3. Tests on embryos as an alternative

Tests on fish embryos can potentially solve many of the problems listed above. Many questions that are currently studied with juvenile or adult fish, e.g. the relative toxicity of two chemicals, or the specific toxicity of a chemical within a given ecosystem, could also be studied on embryos with the appropriate test design (e.g. Aydin and Koprucu, 2005). Moreover, a broader range of species could be tested. Gamete donors could be taken from natural populations of various species (Wedekind et al., 2001; Wedekind and Müller, 2005), including most fish that occur in the habitats for which the test substance is potentially relevant. Sampling natural populations would also allow to estimate the effects of genetic variation on the toxicity of a substance and vice versa. Estimates of the evolutionary long-term effects of exposure to chemicals would then be possible. If breeders are used in fully factorial breeding designs where gametes of a number of females and males are used to produce all possible sibships (Wedekind et al., 2001), one can directly estimate the additive and non-additive genetic variation and the maternal environmental effects on fish susceptibility or tolerance to various chemical substances. This design has been called ‘‘nested half-sib design’’ or ‘‘North-Carolina II design’’ (Lynch and Walsh, 1998). So far, it has mainly been used in crop science, but the availability of dozens or hundreds of eggs per female, and the fact that gametes can usually be gained by simple methods and used for external and very controlled fertilization allows for such powerful experimental designs in fish (Wedekind et al., 2001). No test can be omnipotent, i.e. include all possible factors that may affect the toxicity of a substance. However, tests on embryos would allow to include a larger number of important factors into routine testing.

Obviously, fish embryo tests can be run with large sample sizes, i.e. with many statistically independent replicates that are tested not only in multi-factorial breeding designs but also in designs that are balanced and multi-factorial with respect to a range of biogeochemical and physiological factors. Hence, factors that might be relevant in the wild could be integrated into the test protocols (e.g. pathogen challenges, temperature fluctuations, physical disturbance, etc.) and tested against the genetic and maternal factors that can be determined in a North-Carolina II design, or genetic factors only as determined in within-family analyses (Wedekind et al., 2004). The conditions under which the eggs can be raised can be very repeatable, for example eggs distributed to Petri dishes

(Wedekind and Müller, 2004; Wedekind et al., 2001) or to 24-well cell culture plates (von Siebenthal et al., unpublished data; Jacob et al., unpublished data) and raised in climate chambers. The egg rearing-conditions can be controlled from right after fertilization, i.e. various potential sources of random error that may be relevant when working with juvenile or adult fish can be excluded in studies on fish embryos. Among these factors that do not apply to embryos are variation in feeding regime, dominance interactions within groups of fish, etc. Hence, the repeatability of embryo tests is expected to be higher than the repeatability of tests on juveniles or adults. This should improve inter-laboratory comparisons.

Fishermen or fishery managers often catch fish during spawning season to collect gametes for artificial fertilization and rearing of embryos in fish hatcheries and subsequent release of the larvae or fry into the wild again. In the case of potentially more fragile populations (e.g. *Thymallus thymallus* or *Salmo trutta*), the spawners are usually anaesthetized before stripping of their gametes and reintroduced into the wild afterwards. In Switzerland, for example, 0.5–1 billion fish of many different species are produced this way and raised in hatcheries every year (www.umwelt-schweiz.ch). With the necessary permission from the local authorities, samples of embryos of various species could certainly be drawn and used for research with no or little impact on the natural populations.

4. Conclusion

The standardized fish acute toxicity tests that are proposed by the OECD guideline allow to get first estimates of the relative toxicity of various chemicals in various species. However, for management purposes on a more regional scale, there are a number of reasons why results of fish acute toxicity tests need to be very carefully extrapolated to the field. These reasons are linked to the genetics and the condition of the test animals, the standardized test situations, the uncertainty about whether a given test species can be representative to a given fish fauna, the often missing knowledge about possible interaction effects, especially with micropathogens, and statistical problems like pseudoreplication and small sample sizes. Most of these problems could each for themselves already cause a lack of repeatability of single toxicity tests. When we need to predict the effects of a chemical to a given environment, fish acute toxicity tests may under- or overestimate the effects. The magnitude of the overall error, i.e. the error that results from all the above listed experimental problems, is still unclear. As far as we know, nobody ever determined the range of this potential error. It could be few percent or several orders of magnitude. A few percent may often not matter much, but orders of magnitude would obviously lead to very misleading results. Unfortunately, most of the problems we listed are difficult to address with juvenile or adult fish. Testing fish embryos may, however, provide a promising alternative test procedure that typically does not even represent an animal test in legal terms, as long as the embryo has not hatched (which usually takes up to several days or weeks in, for example, many cyprinids, and up to several months in, for example, most salmonids). The recent developments in the literature (e.g. Nagel, 2002; Strmac et al., 2002) significantly amend previous suggestions about the use of fish embryos in toxicity tests (OECD, 1992b, 1998) and have the potential of turning embryo toxicity tests into a real and cost-effective alternative that may partly replace the more problematic fish acute toxicity tests. This may eventually influence future hazard assessment (for current assessment plans see, for example, the regulatory framework proposed by the EU commission concerning the registration, evaluation, authorisation and restriction of chemicals (REACH; http://ec.europa.eu)). However, embryo toxicity tests have their limitations in the detection of chronic/latent effects, or when the embryonic chorion is able to protect against a chemical substance (Hutchinson et al., 1998). Thus, life cycle tests including juveniles and adults will be necessary to study chronic/latent effects, and the relative sensibility of embryos, larvae, juveniles and adults would have to be worked out to a useful degree.

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