Experiences with Test Concentration Selection for FSTRA and AMA Testing: What Has Worked and What Has Not Worked

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Abstract

EPA has provided very detailed guidance documents for the Fish Sort-Term Reproduction Assay (FSTRA) and Amphibian Metamorphosis Assay (AMA). This guidance includes recommendations for selecting the three test concentrations required for each assay. For both assays, the highest test concentration should be just below the maximum tolerated concentration (MTC), with either the water solubility limit or 100 mg a.i./L being the limit concentration for testing. The MTC represents a concentration just below the highest achievable concentration that does not result in significant toxicity (<10%) to the test organisms. We have tested approximately six organophosphorus (OP) insecticides and two to three non-OP pesticides that were included on EPA's list 1 for Tier 1 testing. Even among the OP compounds, these test substances have varying physio-chemical properties. For both the FSTRA and AMA studies, the test guidelines recommend a high test concentration of one-third of the fish 96-hour LC50 value as an estimate for the MTC. Preferably, the 96-hour LC50 should be on the same species as the species used in the FSTRA study (fathead minnows) and the AMA study (Xenopus tadpoles). Discussion will focus on our experiences with selecting test concentrations to meet EPA's criteria. Our experiences indicate that using one-third of the 96-hour LC50 as the highest test concentration, even when conducted on the same species and life stage as the animals used for FSTRA or AMA testing, has resulted in unreliable results. Depending on the test compound, using a high concentration of one-third of the LC50 has resulted in a high test concentration that is either too high, resulting in toxicity to the test organisms, or a concentration that is too low. These results will be discussed, and may be related to properties of the test substances. We will also discuss results using subchronic data (fish early life stage) data to estimate an MTC for the FSTRA test, as well as unexpected water solubility issues based on previous data. Discussion will also include experiences with selecting concentrations for the AMA test. Overall, we have obtained the most reliable results for selecting test concentrations by conducting 14 to 15-day range-find studies using the same life stage as organisms tested in the FSTRA and AMA studies.

Introduction

Two ecotoxicology tests, the fish short-term reproduction assay (FSTRA; EPA Guideline 890.1350) and the amphibian metamorphosis assay (AMA; EPA Guideline 890.1100), are included among the 11-test Tier one EDSP battery. Both of these tests are 21-day *in vivo* assays. Sexually mature fathead minnows (*Pimephales promelas*) are used for the FSTRA test, while the African clawed frog (*Xenopus laevis*) is the species used in the AMA test.

The FSTRA focuses on potential effects on reproduction [effects on the hypothalamic-pituitary-gonadal (HPG) axis] of non-target vertebrates, while the AMA focuses on possible developmental effects [the hypothalamic-pituitary-thyroid (HPT) axis] during a portion of a critical phase for amphibians, metamorphosis from tadpoles to juvenile frogs.

Overall, we have tested approximately half a dozen OP compounds and two to three non-OP compounds in the FSTRA and AMA assays. One of the critical challenges in the FSTRA and AMA studies is selecting appropriate test concentrations. The test guidance suggests that 96-hour LC50 data serve as the range-finding data to select the highest test concentration for both the FSTRA and AMA tests. However, our experience using 96-hour LC50 data to select definitive test concentrations has provided unreliable results, so we have switched to conducting much longer range-find testing to select definitive test concentrations.

The consequences of not selecting an appropriate high test concentration for both tests can result in a compromised test concentration, in which possible indications of endocrine effects can be confounded by toxicity.

General Test Design

The FSTRA study consists of three test concentration groups plus a control group. Each test or control group contains four replicates, with six sexually mature fathead minnows (four females and two males, approximately 4.5 to 6 months old) per replicate. Thus, each test concentration contains 24 fish. A pretest using just dilution water (no test compound added) is conducted using extra replicates to ensure that a sufficient number of replicates of actively spawning fish are available for testing. A number of reproduction-related endpoints are evaluated during the tests, including endpoints such as number of spawns, fecundity, and fertilized embryos.

The AMA study also consists of three test concentrations plus a control group, with four replicates of 20 tadpoles each. At initiation, the tadpoles are at Nieuwkoop and Faber (NF) developmental stage 51. Five tadpoles from each replicate are randomly selected from each replicate for evaluation at an interim time point (day 7), while the remainder are evaluated after 21 days of exposure for a number of developmental endpoints. Endpoints include hind limb length, snout-to-vent length, developmental stage, and body weight. At test termination, thyroid histological examinations are also conducted on five stage-matched (if possible) tadpoles from each replicate.

Test Materials and Toxicity to Aquatic Organisms

The compounds that we have tested are all compounds from EPA's list 1 for EDSP testing. These compounds are primarily organophosphorus (OP) insecticides, but we have also tested a couple of non-OP compounds that are best described as neutral organics.

Although the majority of compounds that we have tested are OP compounds, they have varying structures and physiochemical properties. Some have aliphatic R3 leaving groups, while others have aromatic R3 leaving groups. Reported water solubility values for the compounds that we tested range from approximately 120-150 µg/L to >10 g/L. When available, bioconcentration potential, expressed as log Kow values, ranged from <1 to approximately 2.5 to 2.8.

The OP compounds that we tested also display a wide range of acute toxicity to fish. Some compounds had a wealth of acute toxicity data available on a number of different fish species, while other compounds had only the standard guideline acute fish toxicity data. Some of these compounds display high acute toxicity to fish, being classified as highly toxic (LC50 <1 mg/L) or very highly toxic (LC50 <100 µg/L) on an acute basis while others are classified as either moderately toxic (LC50 between 1 and 10 mg/L) or slightly toxic (LC50 between 10 and 100 mg/L) to fish on an acute basis.

Some of the individual OP compounds tested displayed a wide range in acute toxicity to fish. One compound displayed much higher acute toxicity to bluegill and trout than to fathead minnows (more than an order of magnitude), but unexpectedly, displayed very high toxicity to sheepshead minnows. Another OP compound displayed moderate toxicity to two species of freshwater fish (LC50 values between 5 and 10 mg/L), but displayed only slight toxicity to sheepshead minnows (LC50 between 80 and 90 mg/L).

A majority of the OP compounds had fish early life stage (ELS) toxicity data available, and one OP compound had data available from a partial full life stage study (approximately 80 days of exposure).

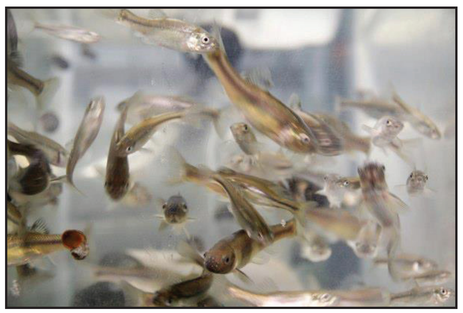
One of the neutral organic compounds that we tested had low reported water solubility, but had a reported acute toxicity value for fish that was well above the reported water solubility value.

Guidance for Definitive Test Concentration Selection

The key to selecting test concentrations for the FSTRA and AMA studies is selection of the highest test concentration. Lower test concentrations that we have used have generally ranged from three-fold to five-fold lower than the next higher concentration, although for one compound we used a larger separation between test concentrations.

The guidance provided by EPA in the FSTRA guideline publication indicates that the highest test concentration in the FSTRA study cannot result in increased mortality or overt morbitity at the highest test concentration. The guidance recommends that either one-third of the 96-hour LC50 value, the water solubility limit, or 100 mg/L (ppm) serve as the high test concentration. If an acute toxicity test is conducted as a range-finder, the guidance recommends conducting the 96-hour test using minnows of the same developmental stage (sexually mature adults) for testing.

For the AMA study, the EPA guidance indicates that the highest test concentration should be the water solubility limit, the maximum tolerated concentration (MTC), or 100 mg/L. The MTC is defined at the highest concentration that results in <10% mortality, or approximately one-third of the LC50 value. To estimate the MTC, the guidance recommends either conducting a 96-hour LC50 test with tadpoles, or relying on acute toxicity data for other amphibian species or fish acute toxicity data.



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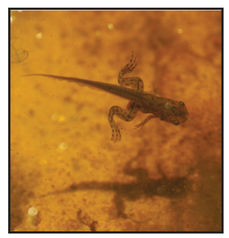
Results and Discussion

The first compound that we tested, one of the OPs, had both the greatest amount of fish toxicity data, including data from a partial life cycle study, and proved to be the most challenging for EDSP testing. For the FSTRA test, we considered acute toxicity data on fathead minnows, fish early life stage data, and data from the partial full lifecycle study for selecting test concentrations. The fish early life stage data were not helpful because the ELS study was conducted on rainbow trout, which are much more sensitive to this test compound than fathead minnows. The fathead minnow acute toxicity data indicated that the high test concentration should be approximately 3 to 4 ppm; to be conservative we initially selected a high concentration of approximately 2.5 ppm. This concentration resulted in significant mortality after approximately 6-8 days of exposure.

We then considered the fathead partial lifecycle data, which suggested that the high concentration should be in the range of 1.5 to 2.0 ppm. Based on these data, we selected a high concentration of approximately 1.5 ppm. Results of this testing also resulted in significant mortality after approximately 12 days of exposure.

We ended up conducting the test with a high concentration of approximately 1.0 ppm. Although no mortality occurred, there were fish that displayed signs of sublethal toxicity at the high test concentration. This compound has a log Kow value between 2 and 3, and therefore some bioconcentration may have occurred during the study that resulted in sublethal toxicity to the fish.



For the AMA study, which was conducted much later than the FSTRA study, we conducted an acute toxicity study with *Xenopus* tadpoles, plus a longer-duration range-finder. Results of this study indicated no adverse effects on the tapoles for any endpoint. Although we may not have exactly reached an MTC, based on the range-finder results, the next highest concentration in the concentration progression scheme would have resulted in overt toxicity to the tadpoles.

Testing with another OP compound, one that displays high water solubility and a very low log Kow (<1) resulted in no signs of cumulative toxicity over the course of either the FSTRA or AMA studies. This compound displayed a large range in acute toxicity values for fish, with LC50 values ranging from <10 mg/L to around 100 mg/L for different species. In both FSTRA and AMA studies, the highest test concentration was 100 mg/L.

Water solubility data for one of the neutral organic compounds indicated the maximum test concentration should be approximately 0.15 mg/L. The available fish acute toxicity data for this compound was >10X the reported water solubility (testing used a co-solvent). In our FSTRA testing, we could not achieve the reported water solubility, and ended up using a high test concentration, based on water solubility, between approximately one-half and two-thirds of the reported water solubility for the compound. Results indicated some possible toxicity at the high test concentration. In retrospect, the log Kow for this compound is >3.

Recommendations for Selecting FSTRA and AMA Test Concentrations

- Selecting test concentrations for the FSTRA and AMA tests based on acute toxicity data, even when available for the same test species, has resulted in unreliable results in the FSTRA and AMA tests.
- Relying on previously conducted acute toxicity testing on the fathead minnow results in unreliable results for the FSTRA and AMA studies (standard acute toxicity testing is conducted on juvenile fish, not sexually mature fish).
- Relying on previously conducted chronic or subchronic toxicity testing with fathead minnows has not resulted in satisfactory test concentrations for the FSTRA study, particularly if the previous testing was conducted at a different laboratory and on a different batch of fish.
- In the majority of cases that we have tested, tadpoles have been less sensitive than fish to the test compounds. Therefore, data on fish are not a good basis for selecting test concentrations for the AMA test.
- Achieving an MTC in the AMA test is challenging, particularly if the high concentration is based on acute toxicity data. Using additional information, such as the dose-response slope, may be helpful, but other factors besides toxicity should also be considered.
- The most reliable range-finding studies for the FSTRA and AMA tests have been longer-term (at least 14-15 days) range-finders conducted using the same life stages as are used in the definitive FSTRA and AMA tests.
- Along with considering toxicity data for the FSTRA and AMA tests, the bioaccumulation potential of a test compound should be considered when selecting test concentrations. Our experience has been that compounds with a higher bioaccumulation potential have resulted in more toxicity over the course of these studies.