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Assessing the Chronic and Sublethal Aquatic Toxicity of Insensitive Munitions (IM) Compounds Using Aqueous Exposures with the Amphipod *Hyalella azteca*

Scientific Operating Procedure Series: Characterization of IMX ecotoxicological effects

Guilherme R. Lotufo and J. Daniel Farrar

July 2018

Environmental Laboratory

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Abstract

This document provides a standard method for assessing the aquatic toxicity of insensitive munitions (IM) compounds using the freshwater amphipod *Hyalella azteca*. The endpoints for the static-renewal test are survival, growth and offspring production. The chronic exposure is conducted for 35 or 42 days at 23 ± 1 °C in 300 mL glass beakers containing 200 mL of test solution. Juvenile amphipods are exposed to decreasing concentrations of IM compounds (e.g., 100%, 50%, 25%, 12.5%, and 6% of the highest concentration) and to a negative control. For each concentration, eight replicate beakers, each containing ten *H. azteca* are used. Test solutions are renewed three times a week. Water quality parameters are measured at the start, during, and at test termination. The tests are only valid if the mean survival, growth, and reproduction for the control treatment are 80%, 0.35 mg dry weight/individual, and three young/female, respectively, or greater. Instructions and requirements are provided for handling *H. azteca* before and during the toxicity test, preparing aqueous mixtures, initiating and terminating chronic tests, maintaining appropriate test conditions, making necessary observations and water quality measurements, assessing the survival, growth and reproductive endpoints, and obtaining samples for chemical analysis.

Contents

Preface

This Scientific Operating Procedure (SOP) was developed under the U.S. Army Engineer Research Development Center - Environmental Quality and Technology (ERDC-EQT) Research Program project 398708 titled "Environmental Fate in the Life Cycle Analysis of New Materials." The technical monitor was Dr. Elizabeth A. Ferguson.

The work was coordinated by the Environmental Processes and Risk Branch of the Environmental Processes Division (CEERD-EPR) at the U.S. Army Engineer Research and Development Center – Environmental Laboratory (ERDC-EL). Dr. William M. Nelson was Branch Chief, CEERD-EPR, Mr. Warren P. Lorentz was the Division Chief, CEERD-EP, and Dr. Elizabeth A. Ferguson (CEERD-ENJ) was the Technical Director for Military Environmental Engineering and Sciences. The Deputy Director of ERDC-EL was Dr. Jack E. Davis and the Director was Dr. Ilker R. Adiguzel.

The Commander of ERDC was COL Bryan S. Green and the Director was Dr. David W. Pittman.

Acronyms

Acronym	Meaning
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
DNAN	2,4-dinitroanisole
DO	Dissolved Oxygen
DoD	Department of Defense
EL	Environmental Laboratory
EPR	Environmental Process Division
EQT	Environmental Quality and Technology
ERDC	Engineer Research Development Center
IC	Inhibition Concentration
IM	Insensitive Munitions
IMX	Insensitive Munitions eXplosive
IMX-101	Mixture of DNAN, NTO and NQ
IMX-104	Mixture of DNAN, NTO, and RDX
L	Liter
LC_{xx}	A statistically estimated concentration that is expected to be lethal to XX % of a group of organisms under specified conditions
LOEC	Lowest-Observed-Effect-Concentration
mL	Milliliter
NOEC	No-Effect-Concentration
NTO	2-nitro-1,2,4-triazol-5-one
NQ	1-nitroguanidine
p	Percent Effect
ppt	Parts per thousand
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
SOP	Scientific Operating Procedure
TNT	2,4,6-trinitrotoluene
USACE	U.S. Army Corps of Engineers
USEPA	U.S. Environmental Protection Agency
YCT	Yeast-Cerophyll-Trout Chow

1 Introduction

The chronic toxicity testing method described herein for assessing the hazard of insensitive munitions (IM) was developed to provide a scientific operating procedure (SOP) to generate consistent and accurate data using juveniles of the freshwater amphipod *Hyalella azteca* as the test organism.

1.1 Background

IMs are more stable and therefore, far less susceptible to inadvertent detonation resulting from accidental stimulus than are traditional munitions (Gray 2008). Presently, the IM formulation IMX-101, a mixture of 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazol-5-one (NTO), and nitroguandine (NQ), is qualified as a replacement for 2,4,6-trinitrotoluene (TNT) in artillery rounds (Lee et al. 2010). The IM formulation IMX-104, a mixture of DNAN, NTO, and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), is qualified as a replacement for Composition B in artillery rounds (Fung et al. 2010). Conventional munitions and IMs are manufactured at ammunition facilities, which typically discharge treated wastewater into the environment. Munitions utilized in live fire can result in the scattering of their explosive fill onto the soil surface of training ranges where it is expected to undergo weathering, dissolution, and transport, with the potential of eventually contaminating surface and groundwater (Taylor et al. 2015).

Hyalella azteca is a suitable test species for the evaluation of the aquatic toxicity of IM compounds because of its extensive use as a standard toxicity test organism for chronic sediment toxicity testing by the U.S. Environmental Protection Agency (USEPA) and the American Society for Testing and Materials (ASTM) (USEPA 2000; ASTM 2010), more recently for water-only standard chronic toxicity testing (Environment Canada 2013; Ivey et al. 2016), and because they are readily available throughout the year from commercial sources or in-house culturing. The primary objective of this SOP is to describe a standard toxicity testing method for assessing chronic lethal effects, as well as sublethal effects on growth and reproduction resulting from exposure to IM formulations or to their single constituents to freshwater invertebrates.

1.2 Scope

The scope of this document is to provide a detailed method for conducting long-term, water-only toxicity testing using the freshwater amphipod *Hyalella azteca* to assess chronic lethal and sublethal effects of IM formulations or their constituents.

2 Materials and Apparatus

This protocol utilizes the following materials, reagents, and equipment:

- IM formulations (e.g., IMX-101 and IMX-104) or their single constituents (e.g., DNAN, NTO, NQ, RDX)
- Juvenile *Hyalella azteca* (approximately 12–13 days old for 35-day exposure and 7–8 days old for 42-day exposures)
- 300 mL lipless glass beakers
- Pre-labeled scintillation vials or similar for holding water samples for the chemical analysis of IM compounds
- Pasteur pipettes
- Dechlorinated tap water, reconstituted synthetic water, or suitable surface or well water for holding and testing
- Reagent grade NaCl and or NaBr
- Silica sand preferably <0.5mm particles (e.g., Granusil 4030)
- Tetramin® – commercially available dried fish flakes, finely ground and sieved (<500 µm)
- Yeast-Cerophyll-Trout Chow (YCT) prepared according to USEPA (2000)
- 1000 µL single channel pipette with plastic tips
- Temperature controlled environment chamber or water bath set to 23± 1 °C
- Aluminum foil envelopes (pre-weighed)
- Lint free paper towels or blotting paper
- Magnetic stir bars and stir plate
- Light table
- 1 L volumetric flask or 1000 mL graduated cylinder
- 500 mL graduated cylinder
- 10 and 30 cm diameter crystallizing dishes or culture bowls
- 70% ethanol with rose bengal stain (0.25g/L)
- 1 L glass jars with screw on lids
- Hand held click counter
- 250 µm sieve
- Disposable plastic transfer pipettes
- Meters (pH, D.O., temperature, conductivity)
- Ammonia kit (or probe)
- Alkalinity and hardness titration kits

3 Procedure

The chronic toxicity tests to assess the toxicity of IM compounds using juvenile amphipod *H. azteca* are conducted as static-renewal tests at 23 °C. The exposure duration is 35 or 42 days. Although the exposure duration for the method described in Ivey et al. (2016) and Soucek et al. (2016) is 42 days, successful assessment of reproduction was obtained when conducting 35-day exposures to IM formulations and single constituents using older test organisms. Chronic exposures are performed in 300 mL beakers with a test solution volume of 200 mL and 5 mL of silica sand to serve as substrate. Typically, a series of five or six concentrations of an IM formulation or a single compound, in addition to control water, are used as treatments. Ten juvenile amphipods are exposed in each beaker, and eight replicate beakers are tested per treatment. Amphipods are fed formulation of YCT and ground Tetramin® fish food flakes daily. The measurement endpoint are survival, growth, and offspring production during the chronic exposure.

1. If conducting the chronic exposure for 42 days, obtain 7–8-day old *H. azteca* (within a 1–2-day range in age) from a commercial vendor (e.g., Aquatic Biosystems, Fort Collins, CO; Aquatic Research Organisms, Hampton, NH) or from an in-house culture. If conducting the exposure for 35 days, obtain 12–13-day old amphipods. Organisms of approximately 7-8 days of age can be obtained from mixed-age in-house cultured animals that pass through a 600 µm (#30) sieve and are retained on a 425 µm (#40) sieve. If opting for the 35-day exposure, amphipods are held and fed at a rate similar to the mass cultures for 5 days prior to commencing a test.
2. *H. azteca* should be cultured and tested at 23 +/- 1 °C. If culturing at a different temperature or if organism are received from a commercial vendor, temperature acclimation can be achieved by exposing organisms to a gradual change in temperature. A change in temperature of 1° C every 1–2 hours has been used successfully (USEPA 2000).
3. Water used for creating test solutions and for use as control should be natural water (e.g., well water or dechlorinated tap water) or reconstituted water with >0.02 mg Br/L and >15 mg Cl/L. If a laboratory's control water does not meet both of these concentrations, the laboratory should spike their control water with NaCl and/or NaBr to reach these minima.
4. Acclimate amphipods to control water spiked with NaCl and/or NaBr for 2 days before the start of the exposures.

5. Establish the target concentrations of the IM formulation or single compounds being tested based on previous information or on range-finding tests. Suggested treatments are 100%, 50%, 25%, 12.5%, and 6% of the highest concentration and a control treatment made up of the same water used for IM test solutions and associated dilution water (i.e., dechlorinated tap water with the required concentration of NaCl and NaBr).
6. Using 2 L as the target volume for each treatment, prepare a sufficient volume of the highest concentration to prepare all treatments by dilution with dechlorinated tap water. Prepare the 100% concentration IM solution by dissolving desired IM formulation or single compound into 4 L of dechlorinated water and mix for three to four days at room temperature using a magnetic stir bar and stir plate. Longer mixing times may be required for bringing all IM into solution.

Using the suggested treatments, the 100% treatment and dechlorinated tap water will be mixed according to Table 1. Use graduated cylinders for measuring accurate volumes.

Table 1. Mixing portions to prepare IM exposure solutions.

	Target volume	
	IM stock (mL)	Dilution water (mL)
100%	2,000	0
50%	1000	1,000
25%	500	1,500
12.50%	250	1,750
6%	125	1,875
Total	**3,875**	**6,125**

7. Transfer a small volume of each treatment (e.g., 80 mL) into a 100 mL beaker for initial water quality parameters (i.e., pH, conductivity, dissolved oxygen (DO), temperature, hardness, and alkalinity).
8. Obtain the necessary amount of each concentration (e.g., 10–20 mL) for the chemical analysis of IM compounds. Maintain the samples at 4 °C until analysis is conducted. Consult the analytical laboratory for holding times.
9. Prepare the toxicity test exposure vessels. Each treatment, including the control, will have eight replicates. Add 5 mL of silica sand as substrate to each 300 mL beaker. Transfer approximately 200 mL of each treatment solution into each beaker labeled with the assigned concentration and the

test replicate designation (i.e., A, B, C, D, E, F, G, H). Beakers are placed in a water bath or environmental chamber at 23 °C under wide-spectrum fluorescent lights set for a 16 h:8 h light:dark cycle.

10. Initial dry weight of *H. azteca* should be determined by transferring ten groups of eight amphipods to pre-weighed aluminum foil envelopes. Excess water should first be removed using a pipette or by blotting with a lint free paper towel or blotting paper and then the aluminum foil envelope and the amphipods should be dried for 24 hours at 60 °C. The weight of the envelope + amphipods should be measured on a balance to the nearest 0.01 mg. The average dry weight of the individual *H. azteca* at test initiation should be calculated from these measurements.
11. Load ten *H. azteca* into each replicate test beakers using as little amount of transferred water as possible in order to minimize dilution of the test concentrations. Test organisms should be handled as little as possible. Using a plastic transfer pipette with tip cut off, gently transfer *H. azteca* into test beakers below the air-water interface. Injured or dropped organisms cannot be used in testing.
12. Loosely cover all beakers to minimize evaporation (e.g., with Plexiglas or watch glass covers).
13. Feed *H. azteca* a combination of YCT and finely ground Tetramin®.

    a) 1 mL/day of YCT should be fed to *H. azteca* every day in each testing beaker.

    b) 1 mg/day of Tetramin® during days 0–7, 1.5 mg/day during days 8–14, 2 mg/day during days 15–21, and 2.5 mg/day for the remainder of the test should be feed to each testing beaker of *H. azteca*. A slurry of ground Tetramin® mixed in dilution test water should be fed using a single channel pipette. Caution should be taken to use a volume ≤ 1 mL to prevent dilution of the test solutions.

14. Test maintenance

    a) Temperature and DO should be measured daily in at least one beaker from each treatment. Hardness, alkalinity, conductivity, pH, and ammonia in each treatment should be measured weekly from at least one beaker per treatment prior to renewal of the test solution.

b) Water sampling for chemical analysis should be taken prior to the start of the test, at each complete test solution replacement, and before the experiment breakdown at exposure termination. Water from before the water renewal and in exposure beakers following renewal should be placed into individually pre-labeled scintillation (or similar) vials and kept at 4 °C in the dark until analysis is conducted. Consult the analytical laboratory for holding times.

c) Test solution replacement should be performed three times a week (e.g., Monday, Wednesday, Friday). The test solution should be completely replaced at a minimum of approximately every seven days (i.e., generally on days 7, 14, 21, and 28), with the option of partial replacements used for the other weekly replacements. For complete water replacement, exchange the test solution by carefully transferring the entire content of a beaker to a 12 inch diameter glass culture bowl and transferring surviving adult amphipods to a different exposure beaker prepared as described in step 9. Remove offspring in the bowl by placing it over a light table and picking the neonates out with a plastic transfer pipette, transferring them to a separate 4 inch culture bowl while counting on a hand held counter. Alternatively, the contents of the culture bowl can be transferred to a labeled, 1 L glass jar with lid, and a concentration of 70% ethanol with rose bengal stain is added. The volume of ethanol added should double the initial volume of the jar. The offspring can then be later enumerated by sieving the contents of the jars through a 250 µm sieve (after 48 hours of preservation), rinsing with clean dilution water and transferring to a 12 inch culture bowl. Place the culture bowl over a light table and remove and count the neonates which will be pink in color. Offspring should be expected on days 21 and 28, but may be present earlier. One jar per replicate should be used at water exchange events and at experiment termination. The number of surviving and dead amphipods should be noted at each test solution replacement event. Remove and discard dead amphipods. Partial test solution exchange is conducted by transferring 2/3 of the test solution to a culturing bowl, adding 170 mL of newly prepared test solution to the beaker, and returning any test organism removed, including neonates, to the beaker.

d) Aeration should not be used unless DO concentration falls below 2.5 mg/L in any replicate beaker, in which case, aeration should be done in all replicates for the duration of the test.

15. Terminate the exposure after 35 days or 42 days.

a) Measure water quality parameters. DO and temperature are measured for each test beaker. Ammonia, pH, hardness, alkalinity, and conductivity should be measured in at least one replicate per treatment.

b) Transfer the full contents of each beaker to a 12 inch glass culture bowl or crystalizing dish. Enumerate surviving and dead amphipods. Determine the number of adult females by counting the adult males (mature male amphipods will have an enlarged second gnathopod) and assuming all other adults are females. The number of females is used to determine number of young/female/beaker. Enumerate offspring or transfer to 70% ethanol/rose bengal solution as described above and enumerate at a later time.

c) Measure final organismal dry weight for assessing growth. Growth is typically expressed as the mean individual dry weight at exposure termination per replicate (USEPA 2000). Growth can al-so be determined using body length measurement (USEPA 2000). Dry weight of amphipods is determined by transferring all surviving amphipods from a replicate (after rinsing to remove sand and other particles) to a pre-weighed aluminum foil envelope, drying these samples for 24 hours at 60 °C, and weighing the pan and dried amphipods on a balance to the nearest 0.01 mg. Average dry weight of individual amphipods in each replicate is calculated from these data.

4 Reporting and Analysis

4.1 Reporting test results

Data sheets or a notebook should be used to record daily activities from the time test organisms are received from the commercial vendor or obtained from in-house cultures to exposure termination. Activities to record include water quality parameters, test organism survival at each water replacement event and at test termination, and number of adult males and females, surviving adult biomass and number of young present at experiment termination.

4.2 Analysis of results

Conduct an analysis of variance (ANOVA) with a Dunnett's post hoc test (one-tailed) for comparison of the survival, growth and offspring production in IM treatments to the respective control treatment data for each experiment. When assumptions of parametric ANOVA are not met, (i.e., normality [Shapiro-Wilk's test] and homogeneity [Bartlett's Test]), conduct the non-parametric Kruskal-Wallis ANOVA on ranks with a Steel's Many-one rank post hoc test. This statistical analysis will determine if differences in survival, growth and reproduction between IM treatments and their respective control are significant and the assignment of treatments as the hypothesis-based no-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) for each test.

The toxicity of contaminants should also be expressed using summary statistics intended to provide useful information that is predictive of potential effects on the exposed population or ecosystem. For the survival endpoint, summary statistics are expressed as the regression-based LC_x, which is the concentration at which there is an x% effect (reduction) at the survival endpoint. For example, an LC_{50} signifies a 50% reduction in survival relative to the control. Maximum likelihood-probit analysis should be used to estimate the LC_{50}, LC_{20} and the LC_{10} values and their associated 95% confidence limits. Alternatively, LC_{50} values should be calculated using the Trimmed Spearman–Karber analyses when the maximum likelihood-probit analysis fails to yield 95% confidence limits.

Summary statistics for the growth and reproduction endpoints should be derived using the Linear Interpolation Method, this calculates a toxicant

concentration that causes a given percent reduction (e.g., 25%, 50%) in the endpoint of interest and is reported as an IC_p value (IC = Inhibition Concentration where p = percent effect). The procedure was designed for general applicability in the analysis of data from chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

Detailed guidance to conduct the analyses described above are provided in the *Standard Test Method for Measuring the Toxicity of Sediment Associated Contaminants with Freshwater Invertebrates* (ASTM 2010).

4.3 Quality Assurance (QA)/Quality Control (QC) considerations

4.3.1 Acceptability of test results

- Mean survival, final biomass, and reproduction for the control treatment must be 80%, 0.35 mg dry weight/individual and three young/female, respectively, or greater, for a successful test.
- Hardness, alkalinity, and ammonia in the overlying water typically should not vary by more than 50% during the test, and DO should be maintained above 2.5 mg/L in the overlying water.

4.3.2 Test organism

- Separate test organism culturing and toxicity testing areas are to be used.
- Reference toxicant tests should be conducted on each batch of test organisms used in testing to assess test organism sensitivity relative to historic information recorded in in-house laboratory or vendor control charts. The suggested reference toxicants for *H. azteca* is KCl. Reagent grade KCl is weighed and completely dissolved into dechlorinated tap water. Six KCl concentrations (0, 0.0625, 0.125, 0.25, 0.5, and 1.0 g KCl/L) are prepared and placed in 250 mL beakers (three replicates per concentration). Ten *H. azteca* (7–8 days old) are placed in each replicate. The endpoint measured is survival after a 96-hour exposure.

4.3.3 Water quality

- The daily mean test temperature must be within ± 1 °C of 23 °C. The instantaneous temperature must always be within ± 3 °C of 23 °C.

- Add aeration if DO concentration falls below 2.5 mg/L by bubbling air thorough a Pasteur pipette near the surface of the water in the beakers at a rate not to exceed 100 bubbles/minute.
- Instruments used for measuring chemical and physical parameters (pH, DO, conductivity and temperature) must be calibrated each day prior to measurement.
- All data should be recorded on appropriate bench sheets, dated, and initialed.

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14. ABSTRACT

This document provides a standard method for assessing the aquatic toxicity of insensitive munitions (IM) compounds using the freshwater amphipod *Hyalella azteca*. The endpoints for the static-renewal test are survival, growth and offspring production. The chronic exposure is conducted for 35 or 42 days at 23 ± 1 °C in 300 mL glass beakers containing 200 mL of test solution. Juvenile amphipods are exposed to decreasing concentrations of IM compounds (e.g., 100%, 50%, 25%, 12.5%, and 6% of the highest concentration) and to a negative control. For each concentration, eight replicate beakers, each containing ten *H. azteca* are used. Test solutions are renewed three times a week. Water quality parameters are measured at the start, during, and at test termination. The tests are only valid if the mean survival, growth, and reproduction for the control treatment are 80%, 0.35 mg dry weight/individual, and three young/female, respectively, or greater. Instructions and requirements are provided for handling *H. azteca* before and during the toxicity test, preparing aqueous mixtures, initiating and terminating chronic tests, maintaining appropriate test conditions, making necessary observations and water quality measurements, assessing the survival, growth and reproductive endpoints, and obtaining samples for chemical analysis.

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