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Assessing the Sediment Toxicity of Insensitive Munitions (IM) Compounds Using 10-day Whole-Sediment Exposures with Freshwater Invertebrates

Scientific Operating Procedure Series: Characterization of IMX Ecotoxicological Effects

Guilherme R. Lotufo and J. Daniel Farrar

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*U.S. Army Engineer Research and Development Center (ERDC)
Environmental Laboratory (EL)
3909 Halls Ferry Road
Vicksburg, MS 39367*

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Abstract

This document provides a standard method for assessing the sediment toxicity of insensitive munitions (IM) compounds using juveniles of the amphipod *Hyalella azteca* and larvae of the midge *Chironomus dilutus*. The 10-day test is conducted at 23 ± 1 °C in 300 mL glass beakers containing sediment spiked with an IM formulation or a single constituent. Each beaker receives 100 mL of spiked or control sediment, 175 mL of overlying water, and ten organisms. The endpoints are survival and growth. Five replicate beakers per treatment are used. To prevent excessive loss of IM compounds, the overlying water in the beakers should not be replaced during the 10-day exposure period. Water quality parameters are measured at the start, during, and at test termination. The tests are only valid if the mean survival for the control treatment is 70% or greater for *C. dilutus* and 80% or greater for *H. azteca*. Instructions and requirements are provided for handling test organisms before and at the initiation of the toxicity test, initiating and terminating whole-sediment exposures, maintaining appropriate test conditions, making necessary observations and water quality measurements, assessing the survival and growth endpoints, and obtaining samples for chemical analysis.

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Preface

This Scientific Operating Procedure (SOP) was developed under the 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 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 Ivan P. Beckman and the Director was Dr. David W. Pittman.

Acronyms and Abbreviations

Acronym	Meaning
ANOVA	Analysis of Variance
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-101	Mixture of DNAN, NTO and NQ
IMX-104	Mixture of DNAN, NTO, and RDX
L	Liter
LC ₅₀	A statistically estimated concentration that is expected to be lethal to 50% 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
<i>p</i>	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

1.1 Background

The acute 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 *Hyaella azteca* or larvae of the midge *Chironomus dilutus* as the test organisms.

1.2 Objectives

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 nitroguanidine (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 and IM 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).

Hyaella azteca and *Chironomus dilutus* have been extensively used as standard toxicity test organisms for whole-sediment toxicity testing (USEPA 2002; ASTM 2010). Specimens of both species are readily available throughout the year from commercial sources or in-house culturing. The primary objective of this SOP is to describe a whole-sediment standard toxicity testing method for assessing the acute lethal toxicity effects, as well as sublethal effects on growth resulting from exposure to IM formulations or to their single constituents to freshwater invertebrates.

1.3 Scope

This document provides a detailed method for conducting whole-sediment acute toxicity testing using the freshwater amphipod *Hyaella azteca* and the midge *Chironomus dilutus* to assess lethal and sublethal effects of IM formulations or their single constituents. Procedures for amending sediment with an IM formulation or a single IM constituent is provided in Lotufo (2018).

2 Materials

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

- Sediment spiked with an IM formulation (e.g., IMX-101 and IMX-104) or a single constituents (e.g., DNAN, NTO, NQ) per Lotufo (2018) SOP-P-#.
- Juvenile *Hyalella azteca* (approximately 7–8 days old) or larvae of *C. dilutus* (approximately 10 days old).
- Lint free paper towels and or blotting paper.
- Fine tip jewelers forceps.
- 300 mL lipless glass beakers.
- Pre-labeled and pre-cleaned glass jars (e.g., 100 mL) for holding sediment for chemical analysis.
- Pasteur pipettes.
- Squirt bottles.
- Pre-weighed aluminum foil envelopes.
- Dechlorinated tap water, reconstituted synthetic water or suitable surface or well water for holding and testing.
- Temperature controlled environment chamber or water bath set to 23 ± 1 °C.
- Tetramin® and Tetrafin® – commercially available dried fish flakes finely ground and sieved (<500 µm).
- Yeast- cerophyll -trout chow (YCT) prepared according to USEPA (2000).
- 4 and 12 inch diameter culture bowls.
- Hand held click counter.
- 600 µm (#30) and 425 µm (#40) sieves.
- Disposable plastic transfer pipettes.
- Meters (pH, dissolved oxygen (DO), temperature, conductivity).
- Ammonia kit (or probe).
- Alkalinity and hardness titration kits.

3 Procedure

Acute toxicity tests to assess the toxicity of IM compounds using juvenile *H. azteca* or larval *C. dilutus* are conducted as whole-sediment, 10-day tests at 23 °C. The measurement endpoint is survival and growth at termination. Acute exposures are performed in 300 mL beakers with 100 mL of sediment and 175 mL of overlying water. Typically, five or six concentrations of the IM formulation or a single compound, in addition to control sediment, are used as treatments to determine concentration-response relations. Overlying water exchanges are a requirement for sediment toxicity tests using *H. azteca* or larval *C. dilutus* (USEPA 2000).

However, this practice has been shown to rapidly deplete explosives and rated compounds, including IM compounds, from exposure vessels¹ (Lotufo et al. 2001; Conder et al. 2004). Most of the mass of NTO and NQ was present in the water column rather than associated with the sediment and porewater during 10-day exposures to spiked sediment¹. To prevent excessive loss of IM compounds from the sediment-overlying water exposure system, the overlying water in the beakers should be not replaced during the 10-day exposure period. Using static exposures (no renewal of the overlying water), mean 10-day survival in control beakers was >90% for *H. azteca* and >75% *C. dilutus* and water quality met acceptability criteria for exposures to sediment spiked with IM formulations and their single constituents¹). Because ammonia may build up in the porewater and in the overlying water during the exposure period, the sediment selected for assessing the toxicity of IM compounds in sediment exposures should have a low concentration of ammonia so that the concentration of unionized ammonia (as ammoniacal nitrogen) in the overlying water of exposure beakers does not exceed 0.2 mg/L during 10-day tests.

In addition, the use of sediments that cause relatively low degradation of parent compounds is preferable. Procedures for amending the selected sediment with an IM formulation or a single IM constituent are provided in Lotufo (2018). Ten, young age amphipods (approximately 7 – 8 days old) or second- to third-instar larvae (approximately 10 days old) are exposed in each beaker, and five replicate beakers are tested per

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¹ Lotufo, Guilherme R., Chappell Pornsawan, and Michael G. Jung (In Preparation) Toxicity of insensitive munitions formulations and single constituents to freshwater invertebrates in exposure to spiked sediment. Environmental Toxicology and Chemistry.

treatment. Amphipods are fed daily a formulation of YCT and Tetramin® fish food flakes. Midge larvae are fed Tetramin® fish food flakes only.

1. Obtain 7–to 8–day old *H. azteca* (within a 1–to 2–day range in age) or second - to third-instar (about 10–day-post hatch) *C. dilutus* larvae from a commercial vendor (e.g., Aquatic Biosystems, Fort Collins, CO; Aquatic Research Organisms, Hampton, NH). Larval midges can also be reared from egg masses obtained from a commercial vendor per instructions provided in USEPA (2000). Alternatively, *H. azteca* 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. Amphipods should be held and fed at a rate similar to the mass cultures for at least 2 days before the start of a test to eliminate animals injured during handling.
2. *H. azteca* and *C. dilutus* should be cultured and tested at 23 °C. If the culturing temperature is different from 23 °C, or if organisms 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 to 2 hours has been used successfully (USEPA 2000).
3. Water used as overlying water should be natural water (e.g., well water or dechlorinated tap water) or reconstituted water (water used in culturing and testing animals prepared with deionized water and reagent grade chemicals). The control water used for the *H. azteca* test should have >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 (Ivey et al. 2016).
4. Acclimate *H. azteca* to control dechlorinated tap water spiked with NaCl and/or NaBr for two days before the start of the exposures.
5. Prepare the toxicity test exposure vessels. For each treatment, including the control, a total of six beakers will be used. Five replicate beakers are used for toxicity assessment and one additional beaker is used for determining sediment chemistry at exposure termination. Transfer approximately 100 mL of each spiked-sediment treatment (Lotufo 2018) into each 300 mL beaker labeled with the concentration and the test replicate designation (i.e., A, B, C, D, and E). Add 175 mL of control water. 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 and receive trickle flow aeration supplied through a glass pipette at a rate

- of approximately 100 bubbles per minute. Maintain exposure beakers under those conditions for approximately 24 hours before adding test organisms. Measure hardness, alkalinity, conductivity, and ammonia from the overlying water of at least one beaker from each treatment. The selected sediment should yield acceptable levels of ammonia in the overlying water in the exposure beakers (Lotufo 2018). However, if the concentration of unionized ammonia (as ammoniacal nitrogen, $\text{NH}_3\text{-N}$) at experiment initiation exceeds 0.2 mg/L, periodic renewal of the overlaying water is recommended (Environment Canada 2013).
6. At the time exposure beakers are set up, transfer spiked sediment to pre-cleaned glass jars (e.g., 125 mL) for holding sediment for chemical analysis. Keep jars at 4 °C in the dark until analysis is conducted. Consult the analytical laboratory for volume requirement and holding times.
  7. Initial dry weight of *H. azteca* should be determined by transferring eight groups of ten 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.
  8. Load ten amphipods or larval midges into each replicate test beaker while minimizing the amount of transferred water to prevent dilution of the test concentrations. Test organisms should be handled as little as possible. Using a plastic transfer pipette with its tip cut off, gently transfer test organisms into test beakers below the air-water interface. Injured or dropped organisms cannot be used in testing. Approximately one-hour following the addition of test organisms, chambers should be observed for injured and non-burrowing individuals. Replace any injured, unhealthy, or individuals that failed to burrow if the response does not appear to be specific to the particular sediment treatment. Floating organisms should be submerged using a dropping pipette.
  9. Loosely cover all beakers to minimize evaporation (e.g., with Plexiglass with holes for fitting aeration tubing).
  10. Test maintenance.
    - a. Temperature and DO measurements should be taken daily for all beakers from each treatment.

- b. Aeration should be increased if DO concentration falls below 2.5 mg/L in a replicate beaker.
    - c. Feed *H. azteca* a combination of YCT and Tetramin®. Feed *C. dilutus* Tetrafin®.
  - A slurry of ground Tetramin® or Tetrafin® mixed in dilution test water should be fed using a single channel pipette. Typically a daily ration is provided using a slurry volume of ≤ 1 mL.
  - Feed 1 mL/day of YCT every day to *H. azteca* in each testing beaker.
  - Feed 0.25 mg/day of Tetramin® during days 0-6 followed by 0.5 mg/day of Tetramin® for the remainder of the test to each testing beaker of *H. azteca*.
  - Feed 6 mg/day Tetrafin® to each testing beaker of *C. dilutus*.
  - d. The use static exposure is preferable (i.e., no replacement of the overlying water). If opting for renewal of the overlying water during the 10-day exposure, replace approximately 50% of the volume with control water at the desired periodicity (e.g., every other day). If opting for static exposure, replace evaporated water in each beaker with deionized water.
11. Terminate the exposure after 10 days.
  - a. Measure water quality parameters. DO and temperature are measured for each test beaker. Ammonia, hardness, pH, alkalinity, and conductivity should be measured in at least one replicate per treatment.
  - b. Following removal of the overlying water by pouring or siphoning, sample sediment from the additional beaker into a pre-cleaned glass jars (e.g., 100 mL) for holding sediment for chemical analysis as described in step 6) above.
  - c. Gently pour the content of the remaining beakers through a 425 μm (#40) sieve to remove surviving amphipods or midges for survival and growth determinations. Any of the surviving amphipods or larvae in the water column or on the surface of the sediment can be pipetted from the beaker

before sieving the sediment. The sediment in each beaker should be sieved in two separate aliquots (i.e., most of the organisms will probably be found in the surface aliquot. Using a squirt bottle, transfer the all the material retained on the sieve to a large 12 in. glass culture bowl or crystalizing dish. Enumerate live and dead organisms by placing bowl over a light table and removing the living animals to a separate 4 inch glass culture bowl. Immobile organisms isolated from the sediment surface or from sieved material should be considered dead. Pupae or pupae casings recovered from *C. dilutus* replicates should be included in the survival count but pupae should not be included in the weight measurements for determining growth.

12. Measure final organismal dry weight for assessing growth. Growth is expressed as the mean individual dry weight at exposure termination per replicate (USEPA 2000). Growth can also be determined using body length measurement (USEPA 2000). Dry weight is measured for *H. azteca* and ash-free dry weight is measured for *C. dilutus*.
  - a. Dry weight of *H. azteca* is determined by transferring all surviving amphipods from a replicate (after rinsing to remove sand and other particles and removing excess water on a lint free paper towel or blotting paper) to a pre-weighed aluminum pan 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.
  - b. Ash-free dry weight of *C. dilutus* is determined by first measuring dry weight as described above and then ashing the larvae in the pan by placing in a muffle furnace at 550°C for two hours. The pan with the ashed larvae is then allowed to cool and reweighed. The tissue mass of the larvae is determined as the difference between the weight of the dried larvae, plus pan, and the weight of the ashed larvae, plus pan.

4 Reporting and Analysis

4.1 Reporting test results

Bench sheets should be used to record daily activities from the time test organism are received from the commercial vendor or obtained from in-house cultures to exposure termination. Information to record includes water quality parameters, daily behavioral observations, and test organism survival and growth at test 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 and growth IM treatment data to the respective control treatment 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 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-Kärber analyses when the maximum likelihood-probit analysis fails to yield 95% confidence limits. Summary statistics for the growth endpoint should be derived using the Linear Interpolation Method, which 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 ASTM (2010).

4.3 Quality Assurance/Quality Control considerations

4.3.1 Acceptability of test results

- Mean survival in the control must be at least 80 percent for *H. azteca* and 70% *C. dilutus* for a successful test.
- Average mass of *C. dilutus* in the control sediment at 10 days must be at least 0.6 mg dry weight (or 0.48 ash-free dry weight).
- Growth of *H. azteca* should be measurable in the control at the end of the 10-day test (i.e., average final dry wt. relative to the average dry weight. of organisms at the start of the 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 or vendor laboratory control charts. The suggested reference toxicants for *H. azteca* and *C. dilutus* is KCl and NaCl, respectively. Reagent grade KCl and NaCl are 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) and six NaCl concentrations (0, 1.25, 2.5, 5, 10 and 20 g NaCl/L) are prepared and placed in 250 mL beakers (three replicates per concentration). Ten test organisms (7–8 days old *H. azteca* or approximately 10 days old *C. dilutus*) 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.
- DO concentration should be maintained at above 2.5 mg/L.
- Instruments used for measuring chemical and physical parameters (pH, DO, temperature, alkalinity, and hardness) must be calibrated daily prior to use.
- All data should be recorded on appropriate bench sheets, dated, and initialed.

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