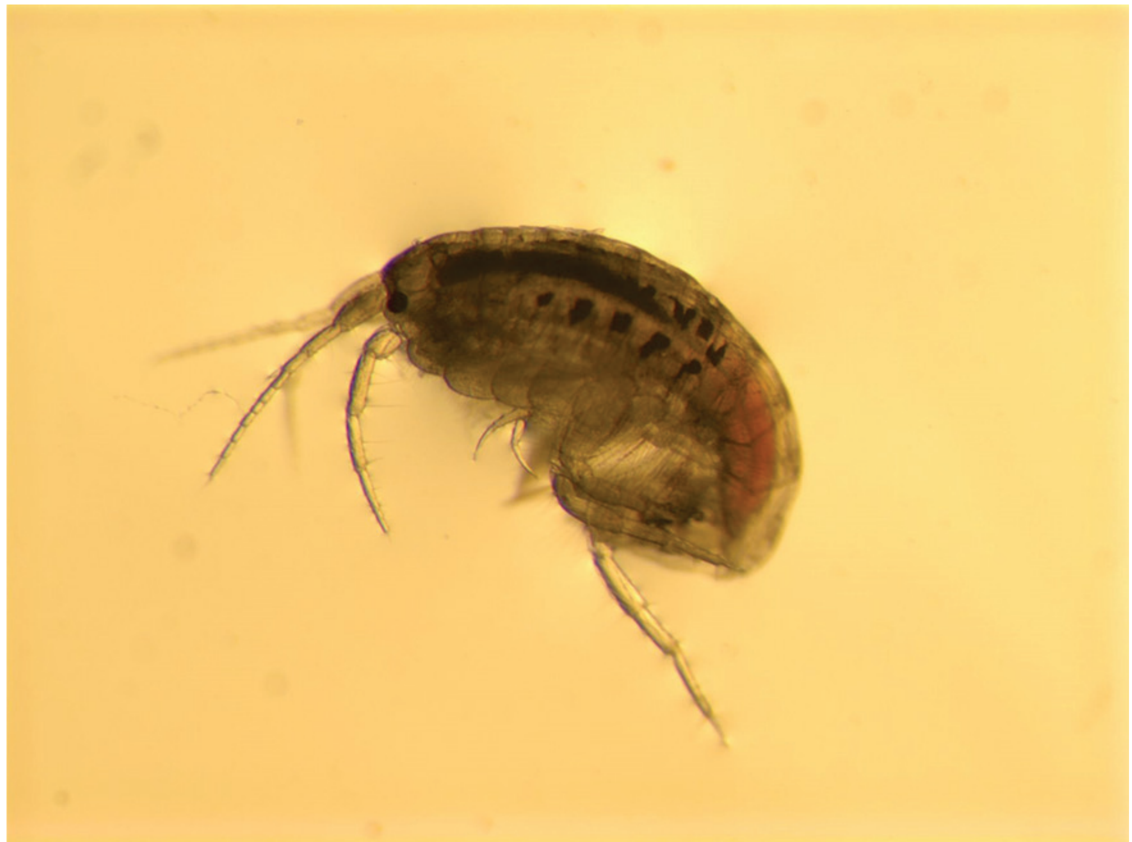
ERDC/EL SR-18-5

Environmental Quality and Technology (EQT) Research Program

Assessing the Aquatic Toxicity of Insensitive Munitions (IM) Compounds Using 10-Day Aqueous Exposures with the Amphipod *Hyalella azteca*

Scientific Operating Procedure Series: Characterization of IMX Ecotoxicological Effects

Guilherme R. Lotufo, Jacob K. Stanley, and Mark L. Ballentine July 2018



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Guilherme R. Lotufo, and Mark L. Ballentine

U.S. Army Engineer Research and Development Center (ERDC)
Environmental Laboratory (EL)
3909 Halls Ferry Road
Vicksburg, MS 39180-6199

Jacob K. Stanley

Stanley Environmental Consulting
Waynesboro, MS 39367

Final Report

Approved for public release; distribution is unlimited.

Prepared for Headquarters, U.S. Army Corps of Engineers
Washington, DC 20314-1000

Under Project 398708, "Environmental Fate in the Life Cycle Analysis of New Materials"

Abstract

This scientific operating procedure (SOP) provides a standard method for assessing the aquatic toxicity of insensitive munitions (IM) compounds using the freshwater amphipod *Hyalella azteca*. The endpoints for the 10-day static-renewal test are survival and growth. The 10-day test is conducted at 23 ± 1 °C in 300 mL glass beakers containing 200 mL of test solution. Juvenile amphipods are exposed to a decreasing concentration series 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 during the test. Water quality parameters are measured at the start, daily during the exposure period and at test termination. The tests are only valid if the mean survival for the control treatment is 80% or greater. Instructions and requirements are provided for handling *H. azteca* before and during the toxicity test, preparing aqueous mixtures, initiating and terminating 10-d tests, 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 and Development Center (ERDC) Environmental Quality and Technology (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 (CEERD-EMJ) 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 and Abbreviations

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
QA	Quality Assurance
QC	Quality Control
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
U.S. EPA	U.S. Environmental Protection Agency
YCT	Yeast-Cerophyll-Trout chow

1 Introduction

The sub-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 the young 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 Insensitive Munitions eXplosive (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 insensitive munitions 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 to eventually contaminate surface and groundwater (Taylor et al. 2015).

Hyalella azteca has been extensively used as a standard toxicity test organism for sediment toxicity testing (U.S. Environmental Protection Agency 2000; ASTM 2010), more recently for water-only standard toxicity testing (Environment Canada 2013; Ivey et al. 2016), and is readily available throughout the year from commercial sources or in-house culturing.

1.2 Objective

The primary objective of this SOP is to describe a standard toxicity testing method for assessing sub-chronic lethal effects and sublethal effects on growth resulting from exposure to IM formulations, or to their single constituents to freshwater invertebrates.

1.3 Scope

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

2 Materials

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)
- Magnetic stir bars and stir plate
- Juvenile *Hyalella azteca* (approximately 7–8 days old)
- Lint free paper towels and or blotting paper
- Fine tip jewelers forceps
- 300 mL lipless glass beakers
- Pre-labeled scintillation vials or similar for holding water samples for the chemical analysis of IM compounds
- Pre-labeled scintillation vials
- Pasteur pipettes
- Preweighed aluminum foil envelopes
- 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)
- Temperature controlled environment chamber or water bath set to 23± 1 °C
- Light table
- 1000 µL single channel pipette with plastic tips
- 2 L volumetric flask(s)
- 1 L volumetric flask or 1000 mL graduated cylinder
- Magnetic stir bars and stir plate
- 500 mL graduated cylinder
- Disposable plastic transfer pipettes
- 10 and 30 diameter crystallizing dishes or culture bowls
- Hand held click counter
- Meters (pH, dissolved oxygen [DO], temperature, conductivity)
- Ammonia kit (or probe)
- Alkalinity and hardness titration kits

3 Procedure

Sub-chronic toxicity tests to assess the toxicity of IM compounds using juvenile amphipod *H. azteca* are conducted as 10-day static-renewal tests at 23 °C. Exposures are performed in 300 mL beakers containing 200 mL of test solution 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 a daily formulation of yeast-cereal leaves-trout (YCT) chow and finely ground Tetramin® fish food flakes. The measurement endpoint is survival and growth after a 10-day exposure.

1. 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. 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. Amphipods should be held and fed at a rate similar to the mass cultures for at least 2 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 to 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 with the addition of bromide 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 mixing for three to four days using a magnetic stir bar and stir plate. Longer mixing times may be required to bring all IM into solution.

Using the suggested treatments, the 100% treatment and dilution water (e.g., dechlorinated tap water) will be mixed in the proportions below. Use graduated cylinders or volumetric flasks 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**

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, DO, temperature, hardness, and alkalinity).

7. 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 and in the dark until analysis is conducted. Consult the analytical laboratory for holding times.
8. 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 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-hour:8-hour, light:dark cycle.
9. Initial dry weight of *H. azteca* should be determined by transferring with fine tip jewelers forceps, ten groups of ten amphipods, to pre-weighed

aluminum foil envelopes. Excess water should first be removed using a Pasteur 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.

10. Load ten *H. azteca* into each replicate test beakers with a minimum amount of water as 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 to increase bore size gently transfer *H. azteca* into test beakers below the air-water interface. Injured or dropped organisms cannot be used in testing.
11. Loosely cover all beakers to minimize evaporation (e.g., with Plexiglas or watch glass covers).
12. Feed *H. azteca* a combination of YCT and finely ground Tetramin®.

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

    b) 0.25 mg/day of Tetramin® during days 0–6 followed by 0.5 mg/day of Tetramin® 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.

13. Test maintenance

    a) Temperature and DO should be measured daily in at least one beaker from each treatment.

    b) Water sampling for chemical analysis should be taken prior to the start of the test, before the first and third water renewal, and before breakdown on day ten. Water from before the water renewal and in-water (water 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) Complete water replacement should be performed every two to three days (or more frequently to maintain constant exposure concentration) by carefully transferring the contents of each test beaker including test solution and sand substrate to a 12 inch diameter glass culture bowls, and transferring the live amphipods to a new exposure beaker prepared as described in step 9. The number of surviving and dead amphipods should be noted at each water replacement event. Remove and discard dead amphipods.

d) 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.

14. Terminate the exposure after 10 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 by placing bowl over a light table and removing the living animals to a separate 4 inch glass culture bowl while recording numbers on a hand held click counter.

15. 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 also 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 and removing excess water on a lint free paper towel or blotting paper) to a pre-weighed aluminum foil envelope; drying these samples for 24 hours at 60°C, and weighing the envelope 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 organism are received from the commercial vendor or obtained from in-house cultures to exposure termination, water quality parameters, and test organism survival at each water replacement event and at test termination.

4.2 Analysis of results

Conduct an analysis of variance (ANOVA) with a Dunnett's post hoc test (one-tailed) for pair-wise comparison of the survival and growth in IM treatments to the 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–Karber 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 ICp value (IC = Inhibition Concentration; where p = percent effect). The procedure was designed for general

applicability in the analysis of sublethal data from 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 *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

1. Acceptability of test results

    a) Mean survival in the control must be at least 80 percent for a successful test and growth of test organisms 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 wt. of organisms at the start of the test).
    b) 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.

2. Test organisms

    a) Separate test organism culturing and toxicity testing areas are to be used.
    b) 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 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 day old) are placed in each replicate. The endpoint measured is survival after a 96-hour exposure.

3. Water quality

   a) 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.
   b) Add aeration if DO concentration falls below 2.5 mg/L.
   c) Instruments used for measuring chemical and physical parameters (pH, DO, conductivity, and temperature) must be calibrated prior to each day.
   d) All data should be recorded on appropriate data sheets, dated, and initialed.

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REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE *(DD-MM-YYYY)*
July 2018

2. REPORT TYPE
Final report

3. DATES COVERED *(From - To)*

4. TITLE AND SUBTITLE

Assessing the Aquatic Toxicity of Insensitive Munitions (IM) Compounds Using 10-day Aqueous Exposures with the Amphipod *Hyalella Azteca*

Scientific Operating Procedure Series: Characterization of IMX Ecotoxicological Effects

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Guilherme R. Lotufo, Jacob K. Stanley, and Mark L. Ballentine

5d. PROJECT NUMBER
398708

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

U.S. Army Engineer Research and Development Center, Environmental Laboratory
3909 Halls Ferry Road, Vicksburg, MS 39180-6199

8. PERFORMING ORGANIZATION REPORT NUMBER

ERDC/EL SR-18-5

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Headquarters, U.S. Army Corps of Engineers
Washington, DC 20314-1000

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This scientific operating procedure (SOP) provides a standard method for assessing the aquatic toxicity of insensitive munitions (IM) compounds using the freshwater amphipod *Hyalella azteca*. The endpoints for the 10-day static-renewal test are survival and growth. The 10-day test is conducted at 23 ± 1 °C in 300 mL glass beakers containing 200 mL of test solution. Juvenile amphipods are exposed to a decreasing concentration series 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 during the test. Water quality parameters are measured at the start, daily during the exposure period and at test termination. The tests are only valid if the mean survival for the control treatment is 80% or greater. Instructions and requirements are provided for handling *H. azteca* before and during the toxicity test, preparing aqueous mixtures, initiating and terminating 10-d tests, maintaining appropriate test conditions, making necessary observations and water quality measurements, assessing the survival and growth endpoints and obtaining samples for chemical analysis.

15. SUBJECT TERMS

Explosives, Military--Toxicity testing
Water--Toxicology
Aquatic organisms
Hyalella Azteca

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED		19	