



**US Army Corps
of Engineers®**
Engineer Research and
Development Center



Environmental Quality and Technology (ERDC-EQT) Research Program

Assessing Aquatic Toxicity of Insensitive Munitions (IM) compounds Using Larval Fathead Minnows (*Pimephales promelas*)

Scientific Operating Procedure Series: Characterization of IMX ecotoxicological effects

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

July 2018



The U.S. Army Engineer Research and Development Center (ERDC) solves the nation's toughest engineering and environmental challenges. ERDC develops innovative solutions in civil and military engineering, geospatial sciences, water resources, and environmental sciences for the Army, the Department of Defense, civilian agencies, and our nation's public good. Find out more at www.erdcl.usace.army.mil.

To search for other technical reports published by ERDC, visit the ERDC online library at <http://acwc.sdp.sirsi.net/client/default>.

Assessing Aquatic Toxicity of Insensitive Munitions (IM) compounds Using Larval Fathead Minnows (*Pimephales promelas*)

Scientific Operating Procedure Series: Characterization of IMX ecotoxicological effects

Guilherme R. Lotufo and Mark L. Ballentine

*Environmental Laboratory
U.S. Army Engineer Research and Development Center
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 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) formulations or their single constituents using the larval stage of the freshwater fish *Pimephales promelas* (fathead minnow). The sole endpoint for the acute, 48-hour non-renewal test using *P. promelas* is survival. Exposure is conducted using larval *P. promelas* four to ten days old. The larval fish 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 at $25 \pm 1^\circ\text{C}$ in 300 mL glass beakers containing 200 mL of test solution. For each concentration, four replicate beakers, each containing ten larvae are used. Water quality parameters are measured at the beginning and termination of the test. The tests are only valid when the mean survival for the control treatment is 90% or greater. Instructions and requirements are provided for handling *P. promelas* before and during the toxicity test, preparing aqueous mixtures, initiating and terminating 48-hour tests, maintaining appropriate test conditions, making necessary observations, water quality measurements, assessing the survival endpoint and obtaining samples for chemical analysis.

DISCLAIMER: The contents of this report are not to be used for advertising, publication, or promotional purposes. Citation of trade names does not constitute an official endorsement or approval of the use of such commercial products. All product names and trademarks cited are the property of their respective owners. The findings of this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

DESTROY THIS REPORT WHEN NO LONGER NEEDED. DO NOT RETURN IT TO THE ORIGINATOR.

Contents

Abstract	ii
Preface	iv
Acronyms	v
1 Introduction	1
1.1 Background.....	1
1.2 Scope.....	2
2 Materials	3
3 Procedure	4
3.1 Procedural overview	4
4 Reporting and Analysis	7
4.1 Reporting test results.....	7
4.2 Analysis of results.....	7
4.3 Quality Assurance (QA)/Quality Control (QC) considerations.....	7
References	9
Report Documentation Page	

Preface

This Scientific Operating Procedure (SOP) was developed under the Engineer Research and 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
ACUP	Animal care and use protocol
ANOVA	Analysis of Variance
DNAN	2,4-dinitroanisole
DO	Dissolved Oxygen
EL	Environmental Laboratory
EQT	Environmental Quality and Technology
ERDC	Engineer Research Development Center
IACUC	Institutional Animal Care and Use Committee
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
mL	Milliliter
NTO	2-nitro-1,2,4-triazol-5-one
NQ	1-nitroguanidine
ppt	Parts per thousand
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
SOP	Scientific Operating Procedure
USACE	U.S. Army Corps of Engineers
USEPA	U.S. Environmental Protection Agency

1 Introduction

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 the larvae of the freshwater fish species *Pimephales promelas*, commonly known as fathead minnow, as the test organism.

Pimephales promelas is a suitable test species for the evaluation of the aquatic toxicity of IM compounds because of (1) the extensive use as a toxicity test organism both for standard toxicity testing of effluents (USEPA 2002) and dredged material elutriates (USEPA and USACE 1998), and (2) 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 acute lethal effects resulting from exposure to IM formulations or to their single constituents to early life stage freshwater fish.

1.1 Background

IMs are more stable, and therefore far less susceptible, to inadvertent detonation resulting from accidental stimulus than 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 1-nitroguanidine (NQ), is qualified as a replacement for 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 of eventually contaminate surface and groundwater (Taylor et al. 2015).

1.2 Scope

The scope of this document is to provide a detailed method for conducting acute water-only toxicity testing using larvae of the freshwater fish *Pimephales promelas* to assess the lethal effects of IM formulations or their single constituents.

2 Materials

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

- IM formulations (e.g., IMX-101 and IMX-104), their single constituents (e.g., DNAN, NTO, NQ, RDX), or related compounds
- Four to ten day old *Pimephales promelas* larvae, <24-hour range in age
- *Artemia* sp. nauplii cysts
- 250 or 300 mL 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
- Temperature controlled environmental chamber or water bath set to 25 ± 1 °C
- Separatory flask
- 2 L volumetric flask(s)
- 1 L volumetric flask or 1 L graduated cylinder
- 500 mL graduated cylinder
- 20 L aquaria
- Light table
- Disposable plastic pipettes
- Meters (pH, D.O., temperature, conductivity)
- Ammonia kit (or probe)
- Alkalinity and hardness titration kits

3 Procedure

3.1 Procedural overview

Acute toxicity tests to assess the toxicity of IM compounds employing larval *P. promelas* are conducted as 48-hour static, non-renewal tests at 25 °C. Acute exposures are performed in 250 or 300 mL beakers with a test solution volume of 200 mL. 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 larvae, four to ten days old, are exposed in each beaker, and four replicate beakers are tested per treatment. Fish are fed <24-hour old *Artemia* sp. nauplii prior to test initiation. The measurement endpoint is survival after 48-hour exposure. All animal testing must be conducted using methods and animal care and use protocols (ACUPs) approved by a properly constituted Institutional Animal Care and Use Committee (IACUC) according to DoD Instruction 3216.01 (2010).

1. Secure IACUC approval of ACUP before purchasing animals.
2. Obtain four to ten day old *P. promelas* larvae from a commercial vendor (e.g., Aquatic Biosystems, Fort Collins, Colorado; Aquatic Research Organisms, Hampton, NH) or from in-house cultures. All test organisms must be less than or equal to 24-hour range in age.
3. Acclimate *P. promelas* larvae to laboratory conditions immediately upon receiving in a 20 L aquarium (or aquaria depending on the number and compliance to IACUC standards) containing 25 °C dechlorinated tap water. Acclimate fish in the bag they were shipped in by floating the bag unopened in the water for approximately 1-hour, then replace about 50% of the water in the bag for dechlorinated tap water and add gentle aeration to the water inside the bag. Temperature acclimation should not exceed 3 °C/day until 25 °C is achieved. Combine shipment water containing fish to water in acclimation tank.
4. Feed *P. promelas* larvae *Artemia* sp. nauplii (<24 hours old) concentrate. Make sure nauplii are available upon receiving fish from commercial vendor.
 - a) Obtain *Artemia* sp. nauplii cysts. Place two cysts approximately 5 mL into 1 L of 30 ppt seawater in a 1 L separatory funnel.
 - b) Keep funnel at 30 to 35 °C and aerate vigorously for 24 to 48 hours until cysts hatch.

- c) Place fine mesh net under separatory funnel and open valve. Obtain sufficient *Artemia* sp. nauplii for one feeding event. Rinse lightly with dechlorinated tap water before transferring nauplii into the tank.
- d) After 4 hours, siphon out excess or dead *Artemia* sp. Nauplii from tank. Feed in excess, but use caution as nauplii remaining will decompose potentially impacting water quality.
5. Immediately remove and record fish that are dead or swim abnormally.
 6. Fish should be acclimated for 24 to 48 hours prior to use in testing.
 7. 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 solutions and associated dilution water (dechlorinated tap water).
 8. Using 1 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 3 L of dechlorinated water by mixing until dissolution is complete (this may take multiple days for some compounds). Longer mixing times may be required for bringing IM into solution.

Using the suggested treatments, the 100% treatment and dechlorinated tap water will be mixed in the proportions contained in Table 1. 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%	1,000	0
50%	500	500
25%	250	750
12.5%	125	875
6%	62	938
Total	1,937	1,937

9. Transfer a small volume of each treatment (e.g., 40 mL) into a 50 mL beaker for initial water quality parameters (i.e., pH, conductivity, dissolved oxygen, temperature, hardness, and alkalinity).
10. Obtain the necessary amount of each concentration (e.g., 10-20 mL in pre-labeled scintillation vials) 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.
11. Prepare the toxicity test exposure vessels. Each treatment, including the control, will have four replicates. Transfer approximately 200 mL of each treatment solution into each 250 or 300 mL beaker labeled with the assigned concentration and the test replicate designation (i.e., A, B, C, and D). Beakers are placed in a water bath or environmental chamber at 25 °C under wide-spectrum fluorescent lights set for a 16-hour:8-hour light:dark cycle.
12. Feed fish prior to use in the experiment (a minimum of 2 hours prior to transfer to exposure chambers). Increase fish concentration in the holding vessel by carefully siphoning out excess water through fine mesh net, if necessary.
13. Load ten larval fish into each replicate test beaker while a minimizing the amount of transferred water, this minimizes the potential dilution of the test concentrations. Use a plastic pipette with the tip cut off (to increase bore size) to gently transfer fish into test beakers. Injured or dropped fish cannot be used in testing.
14. Loosely cover all beakers to minimize evaporation (e.g., with Plexiglass acrylic sheet or watch glass covers).
15. Test maintenance
 - a) All water quality parameters are taken at the start and final time points with temperature and dissolved oxygen (DO) also taken on day 1.
 - b) Remove dead fish daily. Fish are considered dead if they do not respond to gentle prodding with a blunt probe.
 - c) Record survival at day 1 and at experiment termination.
16. Terminate the exposure after 48 hours.
 - a) Record and assess the number of live fish in each test replicate.
 - b) Measure water quality parameters (i.e., pH, dissolved oxygen and temperature) in each test beaker. Ammonia, hardness and conductivity should be measured in at least one replicate per concentration.

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. Activities to record include exposure termination, water quality parameters, test organism survival at day 1, and test organism survival at test termination.

4.2 Analysis of results

Analyze the data using analysis of variance (ANOVA) with a Dunnett's post hoc test (one-tailed) for comparison 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 the significance of differences in survival between IM treatments and their respective control.

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. Summary statistics are expressed as the regression-based LC_x, which is the concentration at which there is an x% effect (reduction) of the survival endpoint. For example, an LC₅₀ signifies a 50% reduction in survival relative to the control. Maximum likelihood-probit analysis should be used to estimate the LC₅₀, LC₂₀ and the LC₁₀ values and their associated 95% confidence limits. Alternatively, LC₅₀ values should be calculated using the Trimmed Spearman-Kärber analyses when the maximum likelihood-probit analysis fails to yield 95% confidence limits.

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

1. Acceptability of test results
 - a) Mean survival in the control must be at least 90% for a successful test.
 - b) Water temperature must not deviate more than 3 °C during testing.

2. Test organism
 - a) Secure IACUC approval of vertebrate animal care and use protocols before acquiring animals.
 - b) Separate test organism culturing/holding and toxicity testing areas are to be used.
 - c) 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 *P. promelas* is KCl. Reagent grade KCl is weighed and completely dissolved into dechlorinated tap water. Six KCl concentrations (0, 0.17, 0.34, 0.68, 1.35, and 2.70 g KCl/L) are prepared and placed in 250 mL beakers (three replicates per concentration). Ten larval fathead minnows are placed in each replicate. The endpoint measured is survival after a 96-hour exposure.
3. Water quality
 - a) Add aeration if the DO concentration falls below 4.0 mg/L (bubbling rate \leq 100 bubbles/min).
 - b) Instruments used for measuring chemical and physical parameters (pH, DO, temperature, alkalinity, and hardness) must be calibrated each day prior to use.
 - c) All data should be recorded on appropriate bench sheets, dated, and initialed.

References

- ASTM International. 2014. *Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians*. ASTM E 729-96. West Conshohocken, PA: ASTM International.
- Department of Defense (DoD). 2010. Use of Animals in DoD Programs. DoDI 3216.01. 13 September 2010.
- Fung, Virgil, David Price, Ed LeClaire, Jacob Morris, Neil Tucker, and Alberto Carrillo. 2010. Further Development and Optimization of IM Ingredients at Holston Army Ammunition Plant. In *2010 Insensitive Munitions and Energetic Materials Technology Symposium*, 11–14 October, Munich, Germany.
- Gray, Nancy. 2008. Insensitive Munitions - New Explosives on the Horizon. *Army Acquisition Logistics Technology Magazine*. January-March:34–35.
- Lee K. E. , W. A. Balas-Hummers, A. R. Di Stasio, C. H. Patel, J. Samuels, B. D. Roos, and V. Fung. 2010. Qualification Testing of the Insensitive TNT Replacement Explosive IMX-101. In *2010 Insensitive Munitions and Energetic Materials Technology Symposium*, 11–14 October, Munich, Germany.
- Taylor, Susan, Eileen Park, Katherine Bullion, Katerina Dontsova. 2015. Dissolution of three insensitive munitions formulations. *Chemosphere* 119:342–348.
- U.S. Environmental Protection Agency (USEPA). 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. EPA-821-R-02-012. Washington, DC: USEPA.
- U.S. Environmental Protection Agency and U.S. Army Corps of Engineers (USEPA/USACE). 1998. *Evaluation of Dredged Material Proposed for Discharge in Waters of the U.S. – Inland Testing Manual*. EPA-823-B-98-004. Washington, DC: Office of Water, Office of Science and Technology. Department of the Army, United States Army Corps of Engineers Operations, Construction, and Readiness Division.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) July 2018		2. REPORT TYPE Final report		3. DATES COVERED (From - To)	
4. TITLE AND SUBTITLE Assessing Aquatic Toxicity of Insensitive Munitions (IM) compounds Using Larval Fathead Minnows (<i>Pimephales promelas</i>)				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-18-4	
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) formulations or their single constituents using the larval stage of the freshwater fish <i>Pimephales promelas</i> (fathead minnow). The sole endpoint for the acute, 48-hour non-renewal test using <i>P. promelas</i> is survival. Exposure is conducted using larval <i>P. promelas</i> four to ten days old. The larval fish 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 at 25 ± 1°C in 300 mL glass beakers containing 200 mL of test solution. For each concentration, four replicate beakers, each containing ten larvae are used. Water quality parameters are measured at the beginning and termination of the test. The tests are only valid when the mean survival for the control treatment is 90% or greater. Instructions and requirements are provided for handling <i>P. promelas</i> before and during the toxicity test, preparing aqueous mixtures, initiating and terminating 48-hour tests, maintaining appropriate test conditions, making necessary observations, water quality measurements, assessing the survival endpoint and obtaining samples for chemical analysis.					
15. SUBJECT TERMS Explosives, Military--Toxicity testing Fathead minnow Water--Toxicology Aquatic organisms					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT UNCLASSIFIED	b. ABSTRACT UNCLASSIFIED	c. THIS PAGE UNCLASSIFIED			19b. TELEPHONE NUMBER (include area code)