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Toxicology Study No. S.00058221-19
Protocol No. 0FMA-92-iv17-03-01 J

Microtox® Toxicity Testing of Energetic Replacement Candidate N-Propylnitroguanidine (PrNQ)

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General Medical: 500C, Specialty; Toxicity Study

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The energetic and toxicological properties of N-propylnitroguanidine (PrNQ) has been assessed as a potential as a replacement for melt castable formulations such as Composition B, which contains trinitrotoluene (TNT) and 1,3,5-hexahydro-1,3,5-trinitrotriazine (RDX), or compositions containing 2,4-dinitroanisole (DNAN). This study evaluated the aquatic toxicity of PrNQ with the Microtox® Acute Toxicity Test System, a bioluminescent bacterial aquatic toxicity test. The data from this study is used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for further development of new munition compounds.

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Microtox Acute Toxicity Testing of Energetic Replacement Candidate N-Propylnitroguanidine (PrNQ)

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Health Effects Division, Toxicology Directorate
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Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations, Part 792, Good Laboratory Practice Standards, except for the following:

The manufacturer conducted the test article characterization (purity) and it is unknown whether the testing was compliant with the above regulation.

1. The non-animal use protocol was approved after initial range-finding testing had been completed for N-Propylnitroguanidine (PrNQ). Other approved protocols were in place and testing was conducted simultaneous to these other protocols. As no toxicity was found in PrNQ at the solubility limit of the test, subsequent testing for effective concentration 50 (EC_{50}) that would have been under the protocol approval was unnecessary.

2. Due to time constraints, the Laboratory Sciences Directorate, Method Development Section (LAB) could not validate the method of analysis prior to the study start in compliance with study protocol and modification requirements. Because of this, the dosing solutions used for all tests were held after being frozen at -80 degrees Celsius until the LAB could validate the method after the study was completed.

No deviations from the aforementioned regulation affected the quality or integrity of the study or the interpretation of the results.

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COMMONLY USED TERMS

AFNOR	Association Française de Normalisation
DA	Department of the Army
DIN	Deutsches Institut für Normung
DMSO	dimethyl sulfoxide
DOD	Department of Defense
DODI	Department of Defense Instruction
EC_{50}	median effect concentration
EPA	U.S. Environmental Protection Agency
ESOH	environmental safety and occupational health
GHS	Global Harmonization System
GLP	Good Laboratory Practice
ISO	International Organization for Standardization
kg	kilogram
L	liter
LD_{50}	median lethal (oral) dose
LOAEL	lowest-observed adverse effect level
µ	micro
µg	micrograms
µL	microliter
mg	milligram
mL	milliliter
min	minute

NOAEL	no-observed adverse effect level
NVN	Nederlandse voornorm
OECD	Organization for Economic Co-operation and Development
RDT&E	research, development, technology, and evaluation
RfD	reference dose
TOX SOP	Toxicology Standing Operating Procedure

TOXICOLOGY STUDY NO. S.0058221-19
MICROTOX® ACUTE TOXICITY TESTING OF ENERGETIC REPLACEMENT CANDIDATE N-PROPYLNITROGUANIDINE (PrNQ)

1 SUMMARY

1.1 Overview

The energetic and toxicological properties of N-propylnitroguanidine (PrNQ) has been assessed as a potential as a replacement for melt castable formulations such as Composition B, which contains trinitrotoluene (TNT) and 1,3,5-hexahydro-1,3,5-trinitrotriazine (RDX), or compositions containing 2,4-dinitroanisole (DNAN). This study evaluated the aquatic toxicity of PrNQ with the Microtox® Acute Toxicity Test System, a bioluminescent bacterial aquatic toxicity test. The data from this study are used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for further development of new munition compounds.

1.2 Purpose

The study provides environmental and occupational health information on new or replacement energetic compounds for military use. This information is critical to the RDT&E of munition formulation alternatives. This study addresses, in part, the ESOH requirements outlined in Army Regulation (AR) 200-1 (DA 2007b); AR 40-5 (DA 2007a); and AR 70-1 (DA 2018); Department of Defense Instruction (DODI) 4715.4 (DOD 2018); and Army Environmental Research and Technology Assessment (AERTA requirement PP-3-02-05 (AERTA 2018) Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces. This program is under the direction of the DOD Strategic Environmental Research and Development Program (SERDP).

Research, development, testing, training, and use of substances potentially less hazardous to human health and the environment is vital to the readiness of the U.S. military. Safeguarding the health of Soldiers, Civilians, and the environment requires an assessment of alternatives before fielded. Continuous assessments begun early in the RDT&E process can save significant time and effort during RDT&E, as well as over the life cycle of the items developed. Residues of pyrotechnics, propellants, explosives, and incendiaries found in soil, air, surface, and groundwater samples, created environmental problems and interfered with training activities.

The DOD is identifying replacements for substances causing environmental and/or occupational risks to health. This toxicology study examined the aquatic toxicity of PrNQ using a bioluminescent bacterial toxicity assay and conducted the assay consistent with GLP Standard Regulations.

1.3 Conclusions

This study reports the aquatic toxicity for energetic replacement PrNQ via the Microtox® Acute Toxicity assay. Results show that PrNQ was nontoxic at the solubility limit of the test (2000 mg/L). The PrNQ is not considered a hazard for aquatic life following the results of this assay and GHS classifications (UNECE 2015).

1.4 Recommendations

The acute aquatic toxicity of PrNQ was evaluated. Aquatic toxicity does not appear to be a concern based upon the levels at which these compounds were tested. The PrNQ was tested at the concentration limit for the assay; it is considered non-toxic by EPA Hazard classes and is outside the category levels of GHS. Additional aquatic toxicity testing in the Daphnia and fathead minnow would confirm aquatic toxicity predictions.

2 REFERENCES

See Appendix A for list of references.

3 AUTHORITY

Military Interdepartmental Purchase Request No. W74RDV80665511. This technical report addresses, in part, the ESOH requirements outlined in DODI 4715.4, *Pollution Prevention* (DOD 1998); AR 200-1, *Environmental Protection and Enhancement* (DA 2007b); AR 40-5, *Preventive Medicine* (DA 2007a); AR 70-1, *Army Acquisition Policy* (DA 2018); and Army Environmental Research and Technology Assessment Requirement PP-3-02-05, *Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces*. The Strategic Environmental Research and Development Program conducted it as part of an on-going effort.

4 BACKGROUND

Current regulations require the assessment of human health and environmental effects arising from exposure to substances in soil, surface water, and ground water. Applied after an item has been fielded, these assessments can reveal the existence of adverse environmental and human health effects that must be addressed, often at substantial cost. It is more efficient to begin the assessment of exposure, effects, and environmental transport of military-related compounds/substances early in the RDT&E process to avoid unnecessary costs, conserve physical resources, and sustain the health of those potentially exposed. A goal of this program is to investigate these new compounds with operational and/or environment, safety, and occupational health issues. Candidates under development as new energetic melt pour compounds include PrNQ.

National defense requires developing unique energetic compounds to perform specialized mission requirements. These requirements include the sustainable use of these materials in the environment, particularly during training operations. The use of t TNT and DNAN in warheads is a concern due to their ability to contaminate groundwater and, thus, enter into the drinking water

supply. Unexploded ordnance and low-order detonations have become sources of ground water contamination and have affected drinking water resources.

The TNT is acutely toxic to rats causing ataxia, tremors, and mild convulsions; oral LD50 values range from 660 to 1320 mg/kg. The subchronic and chronic oral RfD is 0.5 µg/kg-day based on a LOAEL of 0.5 mg/kg-day for liver effects in dogs. The TNT is classified in weight-of-evidence Group C, possible human carcinogen (Lima et al. 2011; RAIS 2012).

The DNAN is acutely toxic to rats, with oral LD_{50} values around 199 mg/kg in rats, with clinical signs of lethargy, breathing abnormalities, increased salivation, and soft stools (Dodd et al. 2002). A subchronic NOAEL was determined at 5 mg/kg-day, with lethargy, rapid respiration, recumbent posture, and neuromuscular alterations evident in higher dose groups (USAPHC 2012). Similar effects were noted in animals exposed via inhalation. Historically, DNAN has been used as a weight-loss drug, with side effects of developing reversible cataracts noted (Horner 1942).

The SERDP is dedicated to finding replacements for RDX and TNT that will reduce or eliminate ESOH risks and decrease potential impacts on readiness and the costs associated with training (USACHPPM 2007). The energetic and toxicological properties of PrNQ are being evaluated as potential replacements for TNT and DNAN in melt castable formulations. Toxicity tests can be conducted *in vivo* and *in vitro*. *In vitro* methods have the advantage of being relatively inexpensive, high-throughput, and capable of addressing many mechanistic issues at the cellular and molecular level. *In vitro* tests are ideally suitable and effective toxicity screening tools, especially when limited quantities of a compound are available. By identifying ESOH effects early in the acquisition process, unacceptable, or "regrettable," replacement compounds can be identified.

The Toxicology Directorate (TOX) of the U.S. Army Public Health Center (APHC) has been tasked with providing aquatic acute toxicity data for PrNQ to determine its potential to negatively affect the environment. The data from these studies will help in making recommendations for continued development and toxicity testing resulting in appropriate exposure guidance.

Microtox® is an acute toxicity testing system that uses a strain of naturally occurring bioluminescent bacteria, *Aliivibrio fischeri* (formerly *Vibrio fischeri* and still referred to as *V. fischeri* by the supplier of the reagents, Modern Water and will be referred to as *V. fischeri* in this report). The marine bacterial bioluminescence is tied directly to cellular respiration, which is fundamental to cellular metabolism and associated life processes. These non-pathogenic, marine, bioluminescent bacteria are sensitive to a broad range of toxicants resulting in a decreased rate of respiration and a corresponding decrease in the rate of luminescence. Reduction of the microorganism's light emission is proportional to the toxicity expressed as EC_{50} (the midpoint of the effective concentration). This test has been shown to be an effective screening tool in assessing toxicity of varied chemical compounds comparing with other bioassays. Comparisons of toxicity results using these methods for a variety of compounds found that *V. fischeri* were, in most cases, more sensitive than other aquatic organisms (Dutka and Kwan 1981; McFeters et al. 1983; Riva et al. 2007). Thus, the results with Microtox® tests are often useful screens in the assessment of relative toxicity to aquatic organisms. The bacterial bioluminescence aquatic toxicity test has been validated by the industrial, academic,

and governmental testing communities; testing achieved official “Standards Status” in several countries including an ASTM Standard (D-5660; withdrawn), ISO 11348-3 and Standard Method 8050 in the United States, AFNOR T90-320 in France, NVN 6516 (withdrawn) in the Netherlands, and DIN 38412 (Germany).

This report describes the toxic effect of PrNQ in the bacterial bioluminescent acute toxicity assay. Table 1 identifies the critical events and dates of this study.

Table 1. Critical Events

Critical Event	Date of Event
Non-Animal Use Protocol Approved	6 September 2018
Study Start Date	20 August 2018
Experimental Start Date	20 August 2018
Experimental Completion Date	21 August 2018
Study Completion Date	October 2019

5 MATERIALS

5.1 Test Substance

Army Research Laboratory, Aberdeen Proving Ground, Maryland completed synthesis of PrNQ (CASRN 35091-64-6). The molecular structure of the compound is in Figure 1.

The PrNQ was soluble at 200 mg/mL in dimethyl sulfoxide (DMSO). Initial solubility was determined by solubility checks in the Ames assay (APHC 2016a, 2017c). At the end of study, the final serial dilutions were frozen and held for analysis by the APHC Method Development Section Client Services Division (APHC-MDV-CSD) for dose validation.

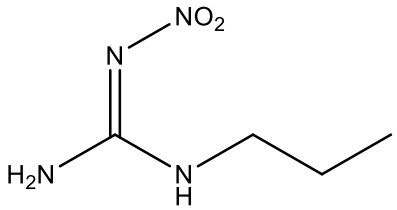

Figure 1. Molecular Structure of N-propylnitroguanidine (PrNQ)

5.2 Test System

The Microtox® Acute Toxicity Test reagent and associated media and solutions were obtained from Modern Water, Inc., New Castle, Delaware. The reagent is a freeze-dried preparation of a specially selected strain of the marine bacterium *V. fischeri* (also known as *A. fischeri*, formerly known as *Photobacterium phosphoreum*, NRRL number B-11177). Appendix D lists media, solution, and other necessary test materials with expiration dates and lot numbers. All reagents

were stored according to manufacturer instructions as described in the Toxicology Standing Operating Procedure (TOX SOP) 037 and study protocol (APHC 2017a, 2017d).

5.3 Positive Control

Zinc sulfate heptahydrate is the recommended standard or positive controls for the test system; and was purchased from Sigma-Aldrich (St. Louis, Missouri). Each vial of lyophilized *V. fischeri* was tested against the standard following reconstitution. Only vials with a calculated EC_{50} of 2-10 mg/L at 15 min for zinc sulfate were qualified further use.

5.4 Quality Assurance

The APHC policy requires that all experiments and studies conducted by any element of the APHC TOX will be compliant with the applicable GLP Standard guideline (APHC 2016b). For this study, the test article dictates that the following GLP guideline applies (CFR 1989):

> *Code of Federal Regulations (CFR), Title 40: Protection of Environment, Part 792-Good Laboratory Practice Standards.*

According to this policy and that these results may be used in regulatory decisions involving the EPA, these assays were conducted in compliance with GLP standards and followed the appropriate regulatory testing guidelines.

In compliance with the GLP requirements, the APHC Quality Systems Office audited critical phases of this study. A Quality Assurance Statement in Appendix B, which provides the dates of these audits along with the audited phases and the dates that the results of the audits, were reported to Management and the Study Director. Appendix C provides additional Quality Assurance/GLP requirement of archives as well as the names of personnel contributing to the performance of this study.

6 METHODS

6.1 Experimental Design

The experimental design and general procedures of this study were conducted under the APHC TOX SOP for the Microtox® Acute Toxicity Assay (APHC 2017b). The test kit is designed to determine the aquatic toxicity of a test material in compliance with the APHC TOX Type Protocol: *"Microtox® Toxicity Testing System"* (APHC 2017e) and modifications. The Study Director approves and signs modifications to the protocol. The electronic and hard copy versions of the protocol modifications are saved and archived with the protocol and the raw data.

6.2 Range Finding

The PrNQ was dissolved in DMSO at 200 mg/L, the concentration test limit of the assay. Samples were serially diluted 1:2 in DMSO and further diluted 1:100 in diluent. Eight concentrations were tested for the range finding. Reconstituted *V. fischeri* were added to each

test concentration (10 µL) and samples were incubated and tested for luminescence at 5, 15, and 30 minutes using the Microtox® Model 500 Analyzer (Modern Water, Inc.). The EC_{50} from the range finding determined the final test concentration range.

6.3 Cytotoxicity Test

In instances where the range-finding test does not produce an EC_{50}, the cytotoxicity test is used to verify the range-finding data using the methodology described in section 6.2.

6.4 Data Analysis

Raw luminescence data were recorded at 5, 15, and 30 min by the Microtox® analyzer. As no EC_{50} was found, no further analysis was necessary.

7 RESULTS AND DISCUSSION

7.1 Microtox Acute Toxicity and Risk Assessment

Toxicity of PrNQ to marine bacteria, *V. fischeri*, was measured by the Microtox® acute toxicity test system at 5, 15, and 30 min. No cytotoxicity was observed in the range-finding test up to the concentration test limit at any of the three time points. The cytotoxicity test was used to confirm the lack of toxicity to PrNQ. Table 2 presents the toxicity data and hazard assessment. Without a dose response, the EC_{50} cannot be calculated. The aquatic toxicity criteria from the EPA, the OECD and the GHS is shown in Table 3. Using these toxicity ranges, PrNQ is categorized as "relatively harmless;" PrNQ is outside the level of concern for OECD and GHS.

We used the aquatic toxicity criteria to categorize the potential ecotoxicity of these new compounds (Table 3) (EPA 2017; OECD 2001; UNCED 2005). This evaluation suggests PrNQ is "relatively harmless."

Table 2. Microtox® Acute Toxicity and Risk Assessment

Compound	Microtox EC_{50} (mg/L) [95 % CI]			EPA Hazard Categories	OECD Hazard Classes	GHS Acute Aquatic Toxicity
	5 min	15 min (used for risk assessment)	30 min			
PrNQ†	>2000 mg/L	>2000 mg/L	>2000 mg/L	Relatively harmless	–	–

Notes:
The value of EC_{50} at 15 min is used for the hazard assessment.
†PrNQ was not toxic at the solubility limit of the test.

Table 3. Ecotoxicity Assessment Scale

LC_{50} or EC_{50} Concentration Range (mg/L)	Hazard Categories (EPA 2017)	Hazard Classes (OECD 2001)	Acute Aquatic Toxicity (GHS 2005)
< 0.01	Super Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. 1
0.01 to 0.1	Extremely Toxic		
0.1 to 1	Highly Toxic		
1 to 10	Moderately Toxic	Acute Toxicity II (toxic to aquatic life)	Acute Cat. 2
10 to 100	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. 3
100 to 1000	Practically Nontoxic	—	—
> 1000	Relatively Harmless	—	—

7.2 Criteria for Valid Assay

The zinc sulfate positive control must meet specified EC_{50} criteria as stated in section 5.3 for a test to be considered valid.

8 CONCLUSIONS

This study reports the aquatic toxicity for the energetic replacement PrNQ via the Microtox® Acute Toxicity assay. Results show that PrNQ was non-toxic at the solubility limit of the test (2000 mg/L). The PrNQ is not considered to be a hazard for aquatic life following the results of this assay GHS classifications (UNECE 2015).

9 RECOMMENDATIONS

The acute aquatic toxicity of PrNQ was evaluated. Aquatic toxicity does not appear to be a concern based upon the levels at which these compounds were tested. The PrNQ was tested at the concentration limit of the assay and is considered non-toxic by EPA Hazard classes and is outside the category levels of GHS. Additional aquatic toxicity testing in Daphnia and fathead minnow would confirm aquatic toxicity predictions.

10 POINT OF CONTACT

Dr. Emily N. Reinke, the Study Director, is the point of contact for this project. She may be reached at DSN 584-3980 or commercial 410-436-3980.

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APPENDIX A

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Appendix B

QUALITY ASSURANCE STATEMENT

FOR: Toxicology Study No. S.0058221-19, Protocol No. 0FMA-92-iv17-03-01 J; Microtox Toxicity Testing of the Energetic Replacement N-propylnitroguanidine (PrNQ) the following critical phases were inspected/audited Quality Systems and Regulatory Compliance Office (QSARC):

Study Specific Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Type Protocol Good Laboratory Practice Standard Review	03/01/2018	03/01/2018
Test Article Specific Type Protocol Modification Review	04/25/2019	04/25/2019
Analytical Chemistry Support – QA review of Dosing Solution Concentration Verification	12/06/2016	12/06/2016
Microtox - Reagent and Test System Storage and Labeling requirements	05/02/2018	05/05/2018
Microtox - Data Processing and Raw Data Documentation Procedures	05/02/2018	05/05/2018
Microtox - Compliance with GLP requirements for Test Facility SOPs	05/02/2018	05/05/2018
Microtox - Calibration Verification of Equipment - Balance and Pipettes	05/02/2018	05/05/2018
Microtox Test Study Endpoint Criteria Compliance	10/28/2018	10/28/2018
Study Raw Data Good Laboratory Practice Standard Review	10/29/2019	10/29/2019
Final Study Good Laboratory Practice Standard Report Review	10/29/2019	10/29/2019

Note 1: *All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.*

Note 2: *This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and of the procedures followed*

Note 3: *In addition to the study specific critical phase inspections listed here, general facility and process based inspections not specifically related to this study are done monthly and are also listed here in accordance with QA Standard Operating Procedure.*

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Michael P. Kefauver
Good Laboratory Practice Standard
Quality Assurance Specialist, QSARC

02/06/2020
Date

APPENDIX C

Archives and Study Personnel

C-1. Archives

All raw data, documentation, records, protocols, contributing scientist reports, and a copy of the final report generated as a result of this study will be archived in the storage facilities of the Toxicology Directorate, APHC, for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

Records on the test system will be archived by the Toxicology Directorate for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

The present study used the Toxicology Study No. S.0058221-19, Protocol No. 0FMA-92-iv17-03-01 J

The protocol, raw data, summary data, and the final report pertaining to this study will be physically maintained within Building E-2100, APHC. These data may be scanned to a computer disk. Scanned study files will be stored electronically with the study data in the archive.

Archived SOPs can be found in the Master Control database at APHC. Maintenance and calibration logbooks may be found in Room 1026, Building E-2100, APHC, APG, MD, 21010.

Archivist: Martha Thompson

C-2. Personnel

Management: Mark Johnson, Ph.D., D.A.B.T., Director Toxicology Directorate; Michael J. Quinn, Ph.D., Division Chief, Health Effects Division (HEF)

Study Director: Emily N. Reinke, Ph.D., D.A.B.T. Biologist, HEF.

Technical staff: Ms. Taryn Brown, ORISE Fellow

Quality Assurance: Michael P. Kefauver, Chemist, Quality Systems Office.

APPENDIX D

Microtox Test Reagents

Table D-1. Microtox Test Reagents

Microtox Reagents	Source	Lot #	Date Expiration
Modern Water Microtox Diluent	Modern Water	17E4130	05/2020
Modern Water Microtox Acute Reagent	Modern Water	17C4076	03/2019
Dimethyl sulfoxide	Sigma-Aldrich	RNBG1729	07/2019
Zinc Sulfate Heptahydrate	Sigma-Aldrich	SLBC2469V	N/A
Modern Water Microtox Reconstitution Solution	Modern Water	16D4031	4/2019