U.S. ARMY PUBLIC HEALTH CENTER
8252 Blackhawk Road, Aberdeen Proving Ground, Maryland 21010-5403

Toxicology Study No. S.0058624-19, January 2020
Protocol No. 0FMA-92-iv17-03-01 K,Q
Toxicology Directorate

Microtox Acute Toxicity Testing of hydrazine replacement candidates: Carbohydrazide (CBZ) and 1-(Ethan-2-ol)-4-amino-1,2,4-triazolium nitrate (HEATN)

Prepared by:
Emily N. Reinke, Health Effects Division

Specialty: 500C, Toxicity Tests

ACKNOWLEDGEMENTS

I would like to acknowledge the support and encouragement provided to this effort by Dr. Robin Nissan of the Department of Defense Strategic Environmental Research and Development Program. I also acknowledge Dr. Valerie Adams and Dr. William Eck for their critical review and comments on this report.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
10/31/2019	Technical Report	September 2018-April 2019

4. TITLE AND SUBTITLE
Microtox Acute Toxicity Testing of the of hydrazine replacement candidates: Carbohydrazide (CBZ) and 1-(Ethan-2-ol)-4-amino-1,2,4-triazolium nitrate (HEATN).

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Emily N. Reinke, Ph.D., D.A.B.T.

5d. PROJECT NUMBER
S.0058624-19

5e. TASK NUMBER

5f. WORK UNIT NUMBER
WP-2400

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Army Public Health Center, Toxicology Portfolio
8252 Blackhawk Road, Aberdeen Proving Ground, MD
21010-5403

8. PERFORMING ORGANIZATION REPORT NUMBER
S.0058624-19

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Strategic Environmental Research and Development Program (SERDP)
4800 Mark Center Drive
Suit 17D03
Alexandria, VA 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)
SERDP

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

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution A: Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The energetic and toxicological properties of carbohydrazide (CBZ) and 1-(Ethan-2-ol)-4-amino-1,2,4-triazolium nitrate (HEATN) have been assessed as potential replacements for propellants in current use, hydrazine. This study evaluated the aquatic toxicity of CBZ and HEATN with the Microtox® Acute Toxicity Test System, a bioluminescent bacterial aquatic toxicity test. 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.

15. SUBJECT TERMS
CARBOHYDRAZIDE; CBZ; 1-(ETHAN-2-OL)-4-AMINO-1,2,4-TRIAZOLIUM NITRATE; HEATN; aquatic toxicity

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Emily N. Reinke
U	U	U	SAR	23	19b. TELEPHONE NUMBER *(Include area code)* 410-436-3980

Sponsor

Strategic Environmental Research and Development Program
4800 Mark Center Drive
Suite 17D03
Alexandria, VA 22350-3605

Study Title

Toxicology Study No. S.0058624-19
Protocol No. 0FMA-92-iv17-03-01 K,Q

Microtox Acute Toxicity Testing of the of hydrazine replacement candidates: Carbohydrazide (CBZ) and 1-(Ethan-2-ol)-4-amino-1,2,4-triazolium nitrate (HEATN)

Authors

Emily N. Reinke, Ph.D., D.A.B.T.

Study Completed

October 2019

Performing Laboratory

U.S. Army Public Health Center
Toxicology Directorate
Health Effects Division
MCHB-PH-HEF
Aberdeen Proving Ground, MD 21010-5403

Laboratory Project ID

Protocol No. 0FMA-92-iv17-03-01 K,Q

Good Laboratory Practice Compliance Statement

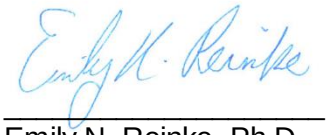
The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. The test article characterization (purity) was conducted by the manufacturer and it is not known whether the testing was done in compliance with the above regulation.

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

3. Due to time constraints, the method of analysis for these compounds could not be validated by the Laboratory Sciences Portfolio (LAB) 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 C until the method could be validated by the LAB 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.



Emily N. Reinke, Ph.D.
Study Director
Health Effects Division

3 April 2020
Date

TABLE OF CONTENTS

Page

APPENDICES

FIGURE

TABLES

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.0058624-19
MICROTOX ACUTE TOXICITY TESTING OF HYDRAZINE REPLACEMENT CANDIDATES: CARBOHYDRAZIDE (CBZ) AND 1-(ETHAN-2-OL)-4-AMINO-1,2,4-TRIAZOLIUM NITRATE (HEATN)

1 SUMMARY

1.1 Overview

The energetic and toxicological properties of carbohydrazide (CBZ) and 1-(Ethan-2-ol)-4-amino-1,2,4-triazolium nitrate (HEATN) have been assessed as potential replacements for propellants in current use, hydrazine. This study evaluated the aquatic toxicity of CBZ and HEATN with the Microtox® Acute Toxicity Test System, a bioluminescent bacterial aquatic toxicity test. 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 purpose of this study is to provide environmental and occupational health information on new or replacement energetic compounds for military use. This information is critical to the research, development, testing, and evaluation (RDT&E) of munition formulation alternatives. This study addresses the environmental safety and occupational health (ESOH) requirements outlined in Department of the Army (DA) Regulation 200-1 (DA 2007b); DA Regulation 40-5 (DA 2007a); and DA Regulation 70-1 (DA 2003); Department of Defense Instruction (DoDI) 4715.4 (DoDI 1998); 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 Department of Defense (DOD) Strategic Environmental Research and Development Program (SERDP).

Items vital to the readiness of the U.S. military include—

- Research,
- Development,
- Testing,
- Training, and
- Use of substances less hazardous to human health and the environment.

Safeguarding the health of Soldiers, Civilians, and the environment requires an assessment of alternatives before they are fielded. Continuous assessments that begin 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 have been found in soil, air, surface, and groundwater samples, which create environmental problems and interfere with training activities.

The DoD is identifying replacements for substances causing environmental and/or occupational risks to health. The purpose of this toxicology study was to examine the aquatic toxicity of CBZ

and HEATN using a bioluminescent bacterial toxicity assay, and to conduct the assay consistent with Good Laboratory Practice (GLP) Standard Regulations.

1.3 Conclusions

This study reports the aquatic toxicity for the propellant compounds CBZ and HEATN via the Microtox® Acute Toxicity assay. Results show that CBZ was considered practically nontoxic at a maximal soluble concentration of 250 mg/L and HEATN was nontoxic at the concentration limit of the test (2000 mg/L). Neither CBZ nor HEATN are considered to be hazards for aquatic life following the results of this assay and globally harmonized system (GHS) classifications (United Nations Economic Commission for Europe (UNECE) 2015).

1.4 Recommendations

The acute aquatic toxicity of CBZ and HEATN were evaluated. Aquatic toxicity does not appear to be a concern based upon the levels at which these compounds were tested. While CBZ did not exceed the limits of the test, at the limit of its solubility it was considered relatively nontoxic by USEPA Hazard classes and is outside the category levels of GHS. Likewise, with HEATN, which was tested to the limits of the assay, the compound appears to be nontoxic to aquatic life. Additional aquatic toxicity testing in Daphnia and fat-head minnow would support these aquatic toxicity screening data.

2 REFERENCES

See Appendix A for a list of references.

3 AUTHORITY

Military Interdepartmental Purchase Request No. W74RDV41496835. This technical report addresses, in part, the ESOH requirements outlined in Department of Defense Instruction (DoDI 4715.4 (DoDI 1998), Army Regulation (AR) 200-1, Environmental Protection and Enhancement (DA 2007b); AR 40-5, Preventive Medicine (DA 2007a); and AR 70-1, Army Acquisition Policy (DA 2018); DoDI 4715.4, Pollution Prevention (DoD 1998); and Army Environmental Research and Technology Assessment Requirement PP-3-02-05, Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces. It was conducted as part of an on-going effort by SERDP. The Principle Investigator is Ms. Nora Dimas of Aerojet Rocketdyne, Sacramento, California.

4 BACKGROUND

Current regulations require the assessment of human health and environmental effects arising from exposure to substances in soil, surface water, and groundwater. 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 environmental, safety, and occupational health issues. CBZ and HEATN are candidates under development for propellant replacement.

National defense requires the development of unique energetic compounds to perform specialized mission requirements. These requirements also include the sustainable use of these materials in the environment, particularly during training operations. The use of hydrazine as a propellant in liquid-fuel missiles is a concern due to its ability to contaminate groundwater and thus entering the drinking water supply. It is highly miscible in water and is toxic to a wide-range of aquatic species and plants. Unexploded ordnance and low-order detonations have become sources of groundwater contamination and have affected drinking water resources.

The National Institute for Occupational Safety and Health (NIOSH) has a Recommended Exposure Limit for a 2-hour exposure of 0.03 parts per million (ppm) (0.05 mg/m^3), while the Occupational Safety and Health Administration (OSHA) has a Permissible Exposure Limit of 1 ppm (1.3 mg/m^3) to skin (NIOSH 2016). It is also regulated under the Clean Air Act as a Hazardous Air Pollutant. OSHA has an 8-hour time weighted average (TWA) of 1 ppm and the American Conference of Governmental Industrial Hygienists has established an 8-hour TWA threshold limit value of 001 ppm (U.S. Department of Labor 2011; HSDB 2016). Releases of greater than 1 pound of hydrazine are reportable under the Comprehensive Environmental Response, Compensation and Liability Act [Superfund]/Superfund Amendments and Reauthorization Act. It is classified as Group 2B by the International Agency for Research on Cancer.

Hydrazine is acutely toxic to rats; oral LD_{50} values are approximately 60 mg/kg in rats, while inhalation LD_{50} values in rats are approximately 570 ppm (U.S. National Library of Medicine 2013). It is highly caustic to skin and can dissolve hair; it has also been reported to cause allergic contact dermatitis. Hydrazine is highly damaging to the eye, causing burns and hemorrhage upon exposure. It is suspected to be a developmental and reproductive toxicant, affecting fetal weight and viability; however, no major malformations were detected. Extensive exposure to hydrazine is also suspected of causing memory and concentration deficits in technicians, with only partial resolution after exposure was ceased (HSDB 2016).

The DoD SERDP is dedicated to finding replacements for hydrazine that will reduce or eliminate the health risks from environmental exposure and will reduce adverse ESOH effects. Fast, high-throughput methods are needed to assess relative toxicity of new munition compounds as they are developed. This supports the development of sustainable, low toxicity materials for use. 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. Specifically for the newly developed materials, the *in vitro* tests are most suitable and effective screening tools, given that often very limited amounts of test substances are available. By identifying ESOH effects early in the acquisition process, unacceptable replacement compounds can be identified. The energetic and toxicological properties of CBZ and HEATN are being evaluated as potential replacements for hydrazine.

The Toxicology Directorate (TOX) of the U.S. Army Public Health Center (APHC) has been tasked with providing aquatic acute toxicity data for CBZ and HEATN to determine their

potential to affect the environment negatively. 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. The bacterial bioluminescence aquatic toxicity test has been validated by the industrial, academic, and governmental testing communities and achieved official "Standards Status" in several countries including an ASTM International Standard (D-5660; withdrawn), ISO 11348-3, and Standard Method 8050 in the U.S.; AFNOR T90-320 in France; NVN 6516 (withdrawn) in the Netherlands; and DIN 38412 (Germany).

This report describes the toxic effect of CBZ and HEATN 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 (HEATN); 25 April 2019 (CBZ)
Study Start Date	20 August 2018
Experimental Start Date	20 August 2018
Experimental Completion Date	23 April 2019
Study Completion Date	21 October 2019

5 MATERIALS

5.1 Test Substance

Aerojet Rocketdyne (El Segundo, California) completed synthesis of CBZ (Chemical Abstracts Service Registry Number (CASRN) 497-18-7), and the Naval Air Warfare Center in China Lake, California completed synthesis of HEATN (CASRN unknown). Figure 1 shows the molecular structures of the compounds.

CBZ and HEATN approximate maximal solubility in dimethyl sulfoxide (DMSO) was previously determined (APHC 2016a, APHC 2017b). CBZ was soluble at 100 mg/mL and HEATN was soluble at 200 mg/mL in DMSO. At the end of study, the final serial dilutions were frozen and

held for analysis by the APHC Method Development Section Client Services Division for dose validation.

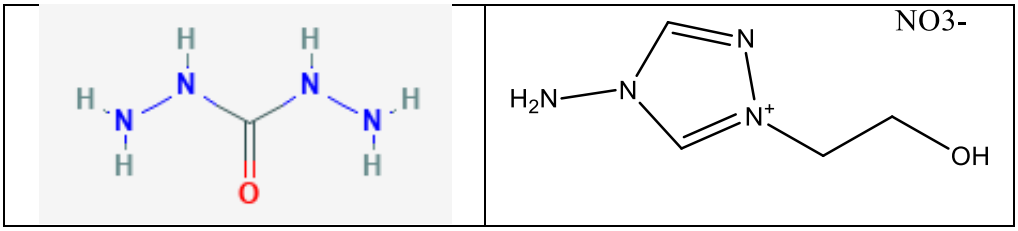


Figure 1. Molecular Structure of CBZ and HEATN
Left panel: Carbohydrazide (CBZ)
Right panel: 1-(Ethan-2-ol)-4-amino-1,2,4-triazolium nitrate (HEATN)

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*. Appendix D provides a list of media, solutions, 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, APHC 2017c).

5.3 Positive Control

Phenol or zinc sulfate heptahydrate are the recommended standards or positive controls for the test system. Both standards were 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 13–26 mg/L at 5 min for phenol or 2–10 mg/L at 15 min for zinc sulfate were qualified further use.

5.4 Quality Assurance

APHC policy requires that all experiments and studies conducted by any element of the APHC Directorate of Toxicology 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 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. Appendix B provides a Quality Assurance Statement that shows audit dates, phases, and dates reported to Management and the Study Director. Appendix C provides the additional Quality Assurance/GLP requirement of archives location 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 2017a). 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 2017c), and modifications. The Study Director approves and signs the 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

CBZ and HEATN were dissolved in DMSO at their solubility limit (25 mg/mL and 200 mg/L, respectively). The solubility of each test article was determined previously in the Ames test (APHC 2017b). Samples were serially diluted 1:2 in DMSO and further diluted 1:100 in diluent. A total of eight concentrations were tested for the range finding. Reconstituted *V. fischeri* were added to each test concentration (10 microliters (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 EC_{50} is not defined in the range finding test, the cytotoxicity test is used verify these data using the same method as described in paragraph 6.2.

6.4 Data Analysis

Raw luminescence data were recorded at 5, 15, and 30 minutes by the Microtox® analyzer. Since no EC_{50} was found for either compound, no further analysis is necessary.

7 RESULTS AND DISCUSSION

7.1 Microtox® Acute Toxicity and Risk Assessment

Toxicity of CBZ and HEATN to marine bacteria, *V. fischeri*, was measured by the Microtox® acute toxicity test system at 5, 15, and 30 minutes. The main test was used to confirm the lack of toxicity to CBZ and HEATN. A confirmation test was completed to replicate the range-finding data. Table 2 presents the toxicity data and risk assessment. Best-fit EC_{50} values for 5, 15, and 30 minutes were not calculated as it was not possible from the produced data.

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). Therefore, the results with Microtox® tests are often useful screens in the assessment of relative toxicity to aquatic organisms. The aquatic toxicity criteria were used from the United States Environmental Protection Agency (EPA), the Organisation for Economic Co-operation and Development (OECD) and the GHS to categorize the potential ecotoxicity of these new compounds (Table 3) (EPA 2017; OECD 2001; United Nations Conference on Environment and Development (UNCED) 2005). This evaluation suggests CBZ is "practically nontoxic" and HEATN is "relatively harmless."

Table 2. Microtox Acute Toxicity and Risk Assessment

Compound	Microtox EC_{50} (mg/L) [95% CI]			Hazard Categories (EPA 2017)	Hazard Classes (OECD 2001)	Acute Aquatic Toxicity (GHS 2005)
	5 min	15 min*	30 min			
CBZ#	>250 mg/L	>250 mg/L	>250 mg/L	Practically nontoxic	—	—
HEATN†	>2000 mg/L	>2000 mg/L	>2000 mg/L	Relatively harmless	—	—

Legend:
EPA = U.S. Environmental Protection Agency
OECD = Organization for Economic Co-operation and Development
GHS = Global Harmonization System
mg/L = milligrams per liter
Notes:
*The value of EC_{50} at 15 min is used for the risk assessment.
#CBZ was not toxic at the solubility limit of the compound.
†HEATN 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. I
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. II
10 to 100	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. III
100 to 1000	Practically Nontoxic	–	–
> 1000	Relatively Harmless	–	–

Legend:
OECD = Organization for Economic Co-operation and Development
EPA = U.S. Environmental Protection Agency
GHS = Global Harmonization System
mg/L = milligrams per liter

7.2 Criteria for Valid Assay

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

8 CONCLUSIONS

This study reports the aquatic toxicity for the propellant compounds CBZ and HEATN via the Microtox® Acute Toxicity assay. Results show that CBZ was considered practically nontoxic at 250 mg/L, which was the highest aqueous concentration tested due to poor solubility. HEATN was nontoxic at the assay solubility limit (2000 mg/L). Neither CBZ nor HEATN are considered hazards for aquatic life following the results of this assay and GHS classifications (UNECE 2015).

9 RECOMMENDATIONS

The acute aquatic toxicity of CBZ and HEATN were evaluated. Aquatic toxicity should not be a concern based upon the data from the Microtox® Assay. While CBZ did not exceed the limits of the test, at the limit of its solubility, CBZ was considered relatively nontoxic by EPA Hazard classes and is outside the category levels of GHS. Likewise with HEATN, which was tested to the limits of the assay, the compound appears to be nontoxic to aquatic life. Additional aquatic toxicity testing in Daphnia and fat-head 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.

Submitted by:
U.S. Army Public Health Center
8252 Blackhawk Road
Health Effects Division
MCHB-PH-HEF
Aberdeen Proving Ground, MD 21010-5403
410-436-3980

Prepared by:

REINKE.EMILY.NICOLE.1447560620 Digitally signed by REINKE.EMILY.NICOLE.1447560620 Date: 2020.04.06 12:46:17 -04'00'

Emily N. Reinke, Ph.D. Date
Biologist
U.S. Army Public Health Center (APHC)
Health Effects Division

Approved by:

QUINN.MICHAEL.J.JR.1282372092 Digitally signed by QUINN.MICHAEL.J.JR.1282372092 Date: 2020.04.06 12:55:26 -04'00'

Michael J. Quinn Jr., Ph.D. Date
Division Chief
Health Effects Division
U.S. Army Public Health Center (APHC)

JOHNSON.MARK.STEVEN.1229380717 Digitally signed by JOHNSON.MARK.STEVEN.1229380717 Date: 2020.04.06 12:35:20 -04'00'

Mark S. Johnson, Ph.D., D.A.B.T. Date
Directorate Director, Toxicology
U.S. Army Public Health Center (APHC)

APPENDIX A

REFERENCES

Army Environmental Command. 2018. Army Environmental Requirements and Technology Assessments (AERTA), Requirement PP-3-02-07. Prepared by: Aberdeen Proving Ground, Maryland.

U.S. Army Public Health Center (APHC). 2017a. TOX SOP 037, Operation and Maintenance of the Microtox Analyzer. Prepared by: Toxicology Directorate, Aberdeen Proving Ground, Maryland.

APHC. 2017b. TOX SOP 068, Xenometrix AMES Test Kit. Prepared by: Toxicology Directorate, Aberdeen Proving Ground, Maryland.

APHC. 2017c. Type Protocol Report No. 0FMA-92-iv-17-03-01. Microtox Toxicity Testing System. Prepared by: Toxicology Directorate, Aberdeen Proving Ground, Maryland.

APHC. 2016a. Type Protocol Report No. 70-iv16-03-01. The Ames Test for Mutagenicity. Prepared by: Toxicology Directorate, Aberdeen Proving Ground, Maryland.

APHC. 2016b. Director's Policy Memorandum No. 70-1, Good Laboratory Practice Policy. Army Public Health Center, 5158 Blackhawk Road, Aberdeen Proving Ground, MD 21010.

Code of Federal Regulations (CFR). 1989. *Title 40 CFR Part 792, Good Laboratory Practice Standards.* U.S. National Archives and Records Administration, Washington, D.C. https://www.ecfr.gov/cgi-bin/text-idx?SID=0c18a6b8f003ddaa77b657413911b656&mc=true&node=pt40.35.792&rgn=div5

Department of the Army (DA). 2007a. AR 40-5. *Preventive Medicine*. http://www.apd.army.mil/pdffiles/r40_5.pdf.

DA. 2007b. AR 200-1, *Environmental Protection and Enhancement*. http://www.apd.army.mil/pdffiles/r200_1.pdf.

DA. 2018. Army Regulation 70-1, *Army Acquisition Policy*. http://www.apd.army.mil/pdffiles/r70_1.pdf.

Department of Defense (DoD) 1998. Department of Defense Instruction (DoDi) 4715.4, Pollution Prevention. Prepared by: http://www.dtic.mil/whs/directives/corres/pdf/471504p.pdf.

Dutka, BJ, and KK Kwan. 1981. Comparison of three microbial toxicity screening tests with the Microtox test. *Bull Environ Contam Toxicol* 27 (6):753–7.

EPA. 2017. Technical Overview of Ecological Risk Assessment, Analysis phase: Ecological effects characterization, ecotoxicity categories for terrestrial and aquatic organisms.

Prepared by: Office of Prevention, Pesticides, and Toxic Substances, U.S. Environmental Protection Agency.

McFeters, GA, PJ Bond, SB Olson, and YT Tchan. 1983. A comparison of microbial bioassays for the detection of aquatic toxicants. *Water Research* 17 (12):1757–1762.

National Institute for Occupational Safety and Health. 2016. *NIOSH Pocket guide to chemical hazards*. U.S. Government Printing Office. Washington, D.C.

OECD. 2001. Harmonized Integrated Classification System for Human and Environmental Hazards of Chemical Substances and Mixtures. Prepared by:

Riva, MC, J Ribo, G Gilbert, and P Alanon. 2007. Acute toxicity of leather processing effluents on Vibrio fisheri and Brachydanio rerio. *Afinidad*. 528:182-188.

U.S. Department of Labor. 2011. *Permissible Exposure Limits – Annotated Tables.* Regulations (Standards - 29 CFR) Part 1910 Occupational Safety and Health Standards Subpart Z. Occupational Safety and Health Administration. Washington, D.C. https://www.osha.gov/dsg/annotated-pels/

U.S. National Library of Medicine. 2016. *Hydrazine*: TOXNET, HSDB Bethesda, Maryland. Date accessed: 12 August 2016. http://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+hsdb:@term+@DOCNO+544

U.S. National Library of Medicine. 2013. ChemIDplus Lite, Bethesda, Maryland. [cited multiple dates, 2013–2018, 2018]. Available from http://chem.sis.nlm.nih.gov/chemidplus/chemidlite.jsp.

UNECE. 2015. *The Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*. New York and Geneva. http://www.osha.gov/dsg/hazcom/ghs.html

Appendix B

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. S.0058624-19, Protocol No. 0FMA-92-iv17-03-01 K,Q Microtox Toxicity Testing of the Novel Propellants: Carbohydrazide (CBZ) and 1-(Ethan-2-ol)-4-amino-1,2,4-triazolium nitrate (HEATN) 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 Modifications Reviews	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.*

KEFAUVER.MICHAEL.P.1229209678 Digitally signed by KEFAUVER.MICHAEL.P.1229209678 Date: 2020.02.06 14:23:44 -05'00'

02/06/2020

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

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 Portfolio, APHC, for a minimum of 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 Portfolio for a minimum of 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.0002728-15, Protocol No. 0FMA-92-iv17-03-01 K,Q.

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

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
Modern Water Microtox Acute Reagent	Modern Water	17H4227	09/2019
Dimethyl sulfoxide	Sigma-Aldrich	RNBG1729	07/2019
Zinc Sulfate Heptahydrate	Sigma-Aldrich	SLBC2469V	N/A
Phenol	Sigma-Aldrich	BCBW8224	N/A
Modern Water Microtox Reconstitution Solution	Modern Water	16D4031	4/2019