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Toxicology Study No. S.0043494e-16
Protocol No. 0FMA-92-iv16-06-01F,G,H,I

Microtox Toxicity Testing of the 1,3-dinitro-1,3-diazacyclohexane (DHP), 3,3'-bis(3-nitro-1,2,4-oxadiazoly-4-yl)-5,5'-bis-1,2,4-oxadiazole (LLM-200), methyl trinitropyrazol (MTNP), and 1-methyl-3,5-dinitro-1,2,4-triazole (DNMT)

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Approved for public release; distribution unlimited.

Specialty: 500C, Toxicity Study

APHC FORM 432-E, JAN 18

ACKNOWLEDGEMENTS

The authors would like to acknowledge the support and encouragement provided to this effort by Mr. Erik Hangeland, Director, Environmental Technology Acquisition Program (ETAP), RDECOM, and John LaScala, Ph.D. Co-chair, EQT Pollution Prevention Technology Team (P2TT). I also acknowledge Dr. William Eck for his critical review and comments on this report.

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
22-03-2018	Technical Report	February 2017-March 2018

4. TITLE AND SUBTITLE
Microtox Testing of the Novel Energetics, 1,3-dinitro-1,3-diazacyclohexane (DHP), 3,3'-bis(3-nitro-1,2,4-oxadiazoly-4-yl)-5,5'-bis-1,2,4-oxadiazole (LLM-200), methyl trinitropyrazol (MTNP), and 1-methyl-3,5-dinitro-1,2,4- triazole (DNMT)

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Army Public Health Center, Toxicology Directorate
8252 Black Hawk Road, Building 5158
Aberdeen Proving Ground, MD
21010-5403

8. PERFORMING ORGANIZATION REPORT NUMBER
S.0043494e-16

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research, Development and Engineering Command
Environmental Toxicology Assessment Program
3072 Aberdeen Blvd., Aberdeen Proving Ground, MD
21005

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

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

12. DISTRIBUTION/AVAILABILITY STATEMENT
UNLIMITED

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The energetic and toxicological properties of 1,3-dinitro-1,3-diazacyclohexane (DHP), 3,3'-bis(3-nitro-1,2,4-oxadiazoly-4-yl)-5,5'-bis-1,2,4-oxadiazole (LLM-200), methyl trinitropyrazol (MTNP), and 1-methyl-3,5-dinitro-1,2,4-triazole (DNMT) are being determined to support an evaluation of DHP, LLM-200, MTNP, and DNMT as a replacement for energetics in current use, such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). This study evaluated the aquatic toxicity of DHP, LLM-200, MTNP, and DNMT with a bioluminescent bacterial aquatic toxicity test in order to make environment and health-based decisions regarding the selection of formulas and materials for further development.

15. SUBJECT TERMS
3-dinitro-1,3-diazacyclohexane, DHP, 3,3'-bis(3-nitro-1,2,4-oxadiazoly-4-yl)-5,5'-bis-1,2,4-oxadiazole, LLM-200, methyl trinitropyrazol, MTNP, 1-methyl-3,5-dinitro-1,2,4-triazole, DNMT, in vitro, microtox, 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	30	19b. TELEPHONE NUMBER *(Include area code)* 410-436-2896

Toxicology Study No. S.0043494e-16, March 2018

Sponsor

U.S. Army Research, Development and Engineering Command,
Environmental Technology Acquisition Program
Aberdeen Proving Ground, MD 21005-5201

Study Title

Toxicology Study No. S.0043494e-16
Protocol No. 0FMA-92-iv16-06-01F,G,H,I

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Study Completed

March 2018

Performing Laboratory

Army Public Health Center
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Laboratory Project ID

Protocol No. 0FMA-92-iv16-06-01F,G,H,I

Toxicology Study No. S.0043494e-16, March 2018

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. Due to circumstances beyond the control of the Toxicology Directorate, the method of analysis for these compounds could not be validated by the Laboratory Sciences Directorate (LS) prior to the study start. This is in noncompliance with study protocol and modification requirements. Because of this the dosing solutions used for all tests are currently frozen (at - 80 degrees C) until the methods can be validated by LS. All data analysis is completed on nominal concentrations as a result of this deviation from protocol.

3. Due to calibration error, the balance used for verifying pipette function was flagged as "in need of repair". Four years of weight set verification data logs were reviewed and the balance is operable and functioning properly. The balance was continued in use and weight set verification was performed prior to each day's use. No deviations from the aforementioned regulation affected the quality or integrity of the study or the interpretation of the results.

Emily N. Reinke

Emily N. Reinke, Ph.D., D.A.B.T.
Study Director
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27 March 2018
Date

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Microtox Toxicity Testing of the Novel Energetics, 1,3-dinitro-1,3-diazacyclohexane (DHP), 3,3'-bis(3-nitro-1,2,4-oxadiazoly-4-yl)-5,5'-bis-1,2,4-oxadiazole (LLM-200), methyl trinitropyrazol (MTNP), and 1-methyl-3,5-dinitro-1,2,4-triazole (DNMT)

1 Summary

1.1 Overview

The energetic and toxicological properties of 1,3-dinitro-1,3-diazacyclohexane (DHP), 3,3'-bis(3-nitro-1,2,4-oxadiazoly-4-yl)-5,5'-bis-1,2,4-oxadiazole (LLM-200), methyl trinitropyrazol (MTNP), and 1-methyl-3,5-dinitro-1,2,4-triazole (DNMT) are being determined to support an evaluation of DHP, LLM-200, MTNP, and DNMT as a replacement for energetics in current use, such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). This study evaluated the aquatic toxicity of DHP, LLM-200, MTNP, and DNMT 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, in part, the environmental safety and occupational health (ESOH) requirements outlined in Department of the Army (DA) Regulation 200-1 [1]; DA Regulation 40-5 [2]; and DA Regulation 70-1 [3]; Department of Defense Instruction 4715.4 [4]; and Army Environmental Research and Technology Assessment (AERTA requirement PP-3-02-05 [5], Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces. This program is under the direction of the Army Environmental Quality (EQT) Program, Environmental Technology Acquisition Program (ETAP).

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 they are 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 have been found in soil, air, surface, and groundwater samples, creating environmental problems and interfering with training activities.

The Department of the Army 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 DHP, LLM-200, MTNP, and DNMT using a bioluminescent bacterial assay, and to conduct the assay consistent with Good Laboratory Practice (GLP) Standards.

1.3 Conclusions

This study reports the aquatic toxicity for the new munitions compounds DHP, LLM-200, MTNP, and DNMT via the Microtox Acute Toxicity assay. Results show that DHP was considered slightly toxic, GHS Acute Toxicity Category III (EC_{50}: 88.27 mg/L). LLM-200, MTNP, and DNMT are considered very toxic, GHS Acute Toxicity Category I (EC_{50}: 0.1183 mg/L, 0.0077 mg/L, and 0.094 mg/L respectively). LLM-200, MTNP, and DNMT would be considered extremely toxic hazards for aquatic life, while DHP is considered harmful by OECD and GHS hazard class guidelines [6, 7].

1.4 Recommendations

The acute aquatic toxicity of LLM-200, MTNP and DNMT are of concern and further testing and evaluation should be continued to determine the hazard of environmental releases following use of these test articles particularly relevant for waste water discharges during manufacturing. Additional concerns with MTNP are that it may be a skin sensitizer due to positive results in preliminary skin sensitization testing [8]. DHP is of limited concern with slight toxicity to aquatic life and, despite *in silico* modeling indicating that it is a skin sensitizer, preliminary testing has been negative for sensitization. Further skin sensitization testing is on-going for both MTNP and DHP. The predicted water solubility of both of these compounds (12.9 g/L and 56.5 g/L) may be of concern for their potential environmental contamination, as is the predicted water solubility for DNMT (75.6 g/L). This raises additional concerns based on their high toxicity and relatively high water solubility. Further *in vitro* and *in vivo* testing is warranted for any of these compounds if development is to continue.

2 References

See Appendix A for list of references

3 Authority

Military Interdepartmental Purchase Request No. 10896394. This technical report addresses, in part, the environment, safety and occupational health (ESOH) requirements outlined in Department of Defense Instruction (DODI) 4715.4 [9], Department of the Army Regulation (AR) 200-1, Environmental Protection and Enhancement[1]; AR 40-5, Preventive Medicine [2]; and AR 70-1, Army Acquisition Policy [10]; Department of Defense Instruction 4715.4, Pollution Prevention [9]; and Army Environmental Research and Technology Assessment (AERTA) 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 Strategic Environmental Research and Development Program.

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. When 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. The U.S. Army RDECOM, ETAP has been dedicated to finding replacements for substances known to cause environmental and/or

occupational risks to health or developing less hazardous new explosives. A goal of this program is to investigate these new compounds with operational and/or environment, safety, and occupational health issues. The candidates under development for high density energetics include DHP, LLM-200, MTNP, and DNMT.

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 RDX (1,3,5-hexahydro-1,3,5-trinitrotriazine) and trinitrotoluene (TNT) in warheads has constrained use of training ranges potentially affecting military readiness. Unexploded ordnance and low-order detonations have become sources of ground water contamination and have affected drinking water resources.

The Centers for Disease Control and Prevention (CDC), Agency for Toxic Substances and Disease Registry (ATSDR) has developed an acute oral minimum risk level (MRL) for RDX of 60 micrograms per kilograms per day (µg/kg-day) based on its epileptiform seizure neurotoxicity in humans and rodents [11-14]. The USEPA has derived a chronic reference dose (RfD) of 3 µg/kg-day based prostatic inflammation in rodents. RDX is also classified as a possible carcinogen [15, 16].

TNT is acutely toxic to rats causing ataxia, tremors, and mild convulsions; oral LD_{50} values in laboratory animals range from 660 to 1320 mg/kg. The reference dose (RfD) for subchronic and chronic oral exposures of 0.0005 mg/kg-day is based on a LOAEL of 0.5 mg/kg-day for liver effects in dogs. TNT is classified in weight-of-evidence Group C, possible human carcinogen [17, 18].

The Army Environmental Technology Acquisition Program (ETAP) is dedicated to finding replacements for RDX and TNT that will reduce or eliminate the health risks from occupational and environmental exposure and will reduce adverse ESOH effects; RDX adversely affects the readiness and costs associated with training [19]. To support the development of sustainable, low toxicity materials for use, fast, high-throughput methods are needed to assess relative toxicity of new munition compounds as they are developed. 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 DHP, LLM-200, MTNP, and DNMT are being evaluated as potential replacements for TNT and RDX.

The Toxicology Directorate (TOX) of the U.S. Army Public Health Center (APHC) was tasked with providing toxicity data for DHP, LLM-200, MTNP, and DNMT to determine their 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 a 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 ISO 11348-3 and Standard Method 8050 in the US, AFNOR T90-320 in France, and DIN 38412 (Germany).

This report describes the mutagenic effect of DHP, LLM-200, MTNP, and DNMT in the bioluminescent bacteria acute toxicity assay. Table 1 identifies the critical events and dates of this study.

Table 1. Critical Events

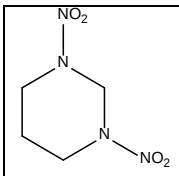
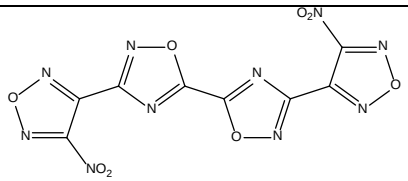
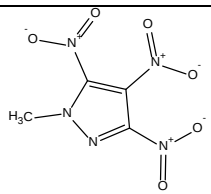
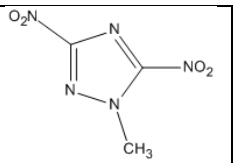
Critical Event	Date of Event
Non-Animal Use Protocol Approved	29 June 2016
Study Start Date	13 February 2017
Experimental Start Date	19 April 2017
Experimental Completion Date	8 December 2017
Study Completion Date	March 2018

5 Materials

5.1 Test Substance

DHP (CASRN: unregistered) was synthesized by Dr. Joseph Banning of the Army Research Lab, Aberdeen Proving Ground, MD. LLM-200, MTNP, and DNMT (CASRN: unregistered) were synthesized by the Holston Army Ammunition Plant, Kingsport, TN. Full purity analyses for these compounds were not provided by the sponsor, however correspondence with the sponsor has indicated that purity is > 99 percent. The molecular structures of the compounds are shown in Figure 1.

DHP, LLM-200, MTNP and DMNT were soluble at 50 mg/ml in DMSO. Initial solubility was determined by solubility checks in the Ames assay [20, 21]. At the end of study, the final serial dilutions were frozen for eventual analysis by the PHC Method Development Section Client Services Division (PHC-MDV-CSD) for dose validation. The method of analysis will be validated prior to dosing solution verification by the PHC-MDV-LCD.

			
1,3-Dinitro-1,3-diazacyclohexane (DHP)	3,3'-Bis(3-nitro-1,2,4-oxadiazoly-4-yl)-5,5'-bis-1,2,4-oxadiazole (LLM-200)	Methyl trinitropyrazol (MTNP)	1-Methyl-3,5-dinitro-1,2,4-triazole (DNMT)

5.2 Test System

The Microtox® Acute Toxicity Test reagent and associated media and solutions were obtained from Modern Water, Inc., New Castle, DE. 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). A list of media, solutions and other necessary test materials with expiration dates and lot numbers is provided in Appendix D. All reagents were stored in according to manufacturer instructions as described in the Toxicology Standing Operating Procedure (TOX SOP) 037 and study protocol [22, 23].

5.3 Positive Control

Phenol is the recommended standard or positive control for the test system. The phenol standard was purchased from Sigma-Aldrich (St. Louis, MO). Each vial of lyophilized *V. fischeri* was tested against the standard following reconstitution. Only vials with a calculated EC50 of 13-26 mg/L at 5 min were qualified for further use.

5.4 Quality Assurance

Army Public Health Center policy requires that all experiments and studies conducted by any element of the APHC Directorate of Toxicology will be compliant with the applicable Good Laboratory Practice (GLP) Standards [24]. For this study, the test policy dictates that the following GLP standard applies [25]:

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 Microtox tests were conducted in compliance with GLP standards and followed the appropriate regulatory testing guidelines.

In compliance with the GLP requirements, the PHC Quality Systems and Regulatory Compliance Office (QSARC) audited critical phases of this study. A Quality Assurance Statement is provided 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. The additional Quality Assurance/GLP requirement of archives location is provided in Appendix C 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 PHC TOX SOP for the Microtox® Acute Toxicity Assay [21]. The test kit is designed to determine the aquatic toxicity of a test material in compliance with the PHC TOX Type Protocol: *"Microtox® Toxicity Testing System*" [20], and modifications. The modifications to the protocol are approved and signed by the Study Director. 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

DHP, LLM-200, MTNP and DMNT were dissolved in DMSO at their solubility limit. The solubility of each test article was determined previously in the Ames test [21]. Samples were serially diluted 1:2 in DMSO and further diluted 1:100 in diluent. A total of 8 concentrations were tested for the range finding. Reconstituted *V. fischeri* was 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 EC50 from the range finding determined the final test concentration range (See Appendix E-H for final chemical specific ranges).

6.3 Cytotoxicity Test

Following the range finding, each test article was tested in duplicate on 3 separate days. On each testing day, test articles were prepared in DMSO at 100x, the top dose as determined in the range finding (100 mg/mL for DHP and 0.05mg/ml for LLM-200, MTNP, and DNMT), and serially diluted 1:2 in DMSO to create an 8-dose testing range. Samples were diluted 1:100 into 1 mL diluent and 10 µL reconstituted *V*. *fischeri* was added to each sample and luminescence measured at 5, 15, and 30 minutes as above. APHC-Client Services Division (CSD)-Method Development Division (MDV)V was unable to analyze the final concentrations of each test article. They have been stored at -80 ^{O}C for analysis at a later date.

6.4 Data Analysis

Raw luminescence data were recorded at 5, 15, and 30 minutes by the Microtox analyzer. The EC_{50} values at 5, 15, and 30 minutes were given by the MicrotoxOmni software and further fitted to the Hill function using GraphPad PRISM 5.04® (GraphPad PRISM 5.04® is a registered trademark of GraphPad Software, Inc.). All data (prints and files) were archived.

7 Results and Discussion

7.1 Microtox toxicity and Risk Assessment

Toxicity of DHP, LLM-200, MTNP and DMNT to marine bacteria, *V. fischeri*, was measured by the Microtox acute toxicity test system at 5, 15, and 30 minutes. For each test compound, 3 individual experiments were performed in duplicate. The toxicity data (EC_{50} and the 95% Confidence Interval) and risk assessment are presented in Table 2. Best-fit EC_{50} values for 5, 15, and 30 minutes were calculated in GraphPad Prism version 5.04 using percent effect data calculated by MicrotoxOmni. Data was further analyzed using the Hill function performed using GraphPad Prism version 5.04 and presented in Appendix E-H: Figures - Microtox. The X and Y axis represent log concentrations of the test article and the percentage of the effect bacteria of the control, respectively.

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 [26-28]. Thus, the results with Microtox tests are often useful screens in the assessment of relative toxicity to aquatic organisms. We used the aquatic toxicity criteria of the United States Environmental Protection Agency (USEPA), the Organization for Economic Co-operation and Development (OECD) and the Global Harmonization System (GHS) to categorize the potential ecotoxicity of these new compounds (Table 3) [6, 7, 29]. This evaluation suggests that DHP is “Slightly Toxic” and

potentially harmful to aquatic life, LLM-200 is “Highly Toxic” and potentially very toxic to aquatic life, and MTNP and DNMT are “Extremely Toxic” and very toxic to aquatic life (Table 2).

Table 2. Microtox Toxicity and Risk Assessment

Compound	Microtox EC_{50} (mg/L) [95 percent CI]			Hazard Categories (USEPA 2017)	Hazard Classes (OECD 2001)	Acute Aquatic Toxicity (GHS 2005)
	5 min	15 min*	30 min			
DHP	71.99 [34.78-149.0]	88.27 [50.11-155.5]	93.78 [56.77-154.9]	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. III
LLM-200	0.1362 [0.08616-0.2152]	0.1183 [0.07617-0.1837]	0.09077 [0.05414-0.1522]	Highly Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. I
MTNP	0.1676 [0.1503-0.1870]	0.07703 [0.06925-0.08567]	0.05003 [0.04521-0.05536]	Extremely Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. I
DNMT	0.3264 [0.2760-0.3860]	0.09440 [0.07866-0.1133]	0.03699 [0.02681-0.05104]	Extremely Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. I

USEPA = United States Environmental Protection Agency
OECD = Organization for Economic Co-operation and Development
GHS = Global Harmonization System
mg/L = milligrams per liter
*The value of EC_{50} at 15 min is used for the risk assessment.

Table 3. Ecotoxicity Assessment Scale

LC_{50} or EC_{50} Concentration Range (mg/L)	Hazard Categories (USEPA 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	–	–

OECD = Organization for Economic Co-operation and Development
USEPA = United States Environmental Protection Agency

GHS = Global Harmonization System
mg/L = milligrams per liter

7.2 Criteria for Valid Assay

The phenol positive control must meet specified EC50 criteria as stated in section 5.3 for a test to be considered valid.

8 Conclusions

This study reports the aquatic toxicity for the new munitions compounds DHP, LLM-200, MTNP, and DNMT via the Microtox Acute Toxicity assay. Results show that DHP was considered slightly toxic, Acute Toxicity Category III (EC_{50}: 88.27 mg/L). LLM-200, MTNP, and DNMT are considered very toxic, Acute Toxicity Category I (EC_{50}: 0.1183 mg/L, 0.0077 mg/L, and 0.094 mg/L respectively). LLM-200, MTNP, and DNMT would be considered extremely toxic hazards for aquatic life, while DHP is considered harmful by OECD and GHS hazard class guidelines [6, 7].

9 Recommendations

The acute aquatic toxicity of LLM-200, MTNP and DNMT is of concern and further testing and evaluation should be continued to determine the hazard of environmental releases following the production, manufacturing, and use of these test articles. Additional concerns with MTNP are that it may be a skin sensitizer following positive results in preliminary skin sensitization testing [8]. DHP is of limited concern with slight toxicity to aquatic life and despite *in silico* modeling indicating that it is a skin sensitizer, preliminary testing has been negative for sensitization. Further skin sensitization testing is on-going for both MTNP and DHP. The water solubility of both of these compounds (12.9 g/L and 56.5 g/L) may be of concern, as is the water solubility for DNMT (75.6 g/L). This raises additional concerns based on their high toxicity and relatively high water solubility. Further *in vitro* and *in vivo* testing is warranted for any of these compounds if development is to continue.

10 Point of Contact

Dr. Emily N. Reinke, the principal investigator, is the point of contact for this project. She may be reached at DSN 584-3980 or commercial 410-436-3980, email: usarmy.apg.medcom-aphc.mbx.tox-info@mail.mil.

Toxicology Study No.S.0043494e-16, March 2018

Submitted by:

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3/27/18

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3/28/18

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Appendix A

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Appendix B

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. S.0043494e-16, March 2018, Protocol No. 0FMA-92-iv16-06-01F,G,H,I Microtox Testing of the Novel Energetics, 1,3-dinitro-1,3-diazacyclohexane (DHP), 3,3'-bis(3-nitro-1,2,4-oxadiazoly-4-yl)-5,5'-bis-1,2,4-oxadiazole (LLM-200), methyl trinitropyrazol (MTNP), and 1-methyl-3,5-dinitro-1,2,4-triazole (DNMT).

:

Study Specific Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Type Protocol Good Laboratory Practice Standard Review	06/29/2016	06/29/2016
Test Article Specific Type Protocol Modifications Review	02/13/2017	02/13/2017
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	05/02/2017	05/05/2017
Microtox - Data Processing and Raw Data Documentation Procedures	05/02/2017	05/05/2017
Microtox - Compliance with GLP requirements for Test Facility SOPs	05/02/2017	05/05/2017
Microtox - Calibration Verification of Equipment - Balance and Pipettes	05/02/2017	05/05/2017
Microtox Test Study Endpoint Criteria Compliance	05/02/2017	05/02/2017
Study Raw Data Good Laboratory Practice Standard Review	03/16/2018	03/16/2018
Final Study Good Laboratory Practice Standard Report Review	03/16/2018	03/16/2018

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 inspection not specifically related to this study are done monthly or annually in accordance with QA Standard Operating Procedure.*

Michael P. Kefauver

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

03/23/2018
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, PHC, 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.0043494e-16, Protocol No. 0FMA-92-iv16-06-01F,G,H,I

The protocol, raw data, summary data, and the final report pertaining to this study will be physically maintained within Building E-2100, PHC. 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 PHC. Maintenance and calibration logbooks may be found in Room 1026, Building E-2100, PHC, APG, MD, 21010.

Archivist: Lee Crouse

C-2. Personnel

Management: Mark Johnson, Ph.D., D.B.A.T., Portfolio Toxicology Director; Michael J. Quinn, Ph.D., Program Manager, Health Effects Research Program (HERP)

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

Technical staff: Alyssa Sikorski, M.S., ORISE Fellow

Quality Assurance: Michael P. Kefauver, Chemist, Quality Systems and Regulatory Compliance Office.

Appendix D

Microtox® Test Reagents

Table D-1. Microtox Test Reagents

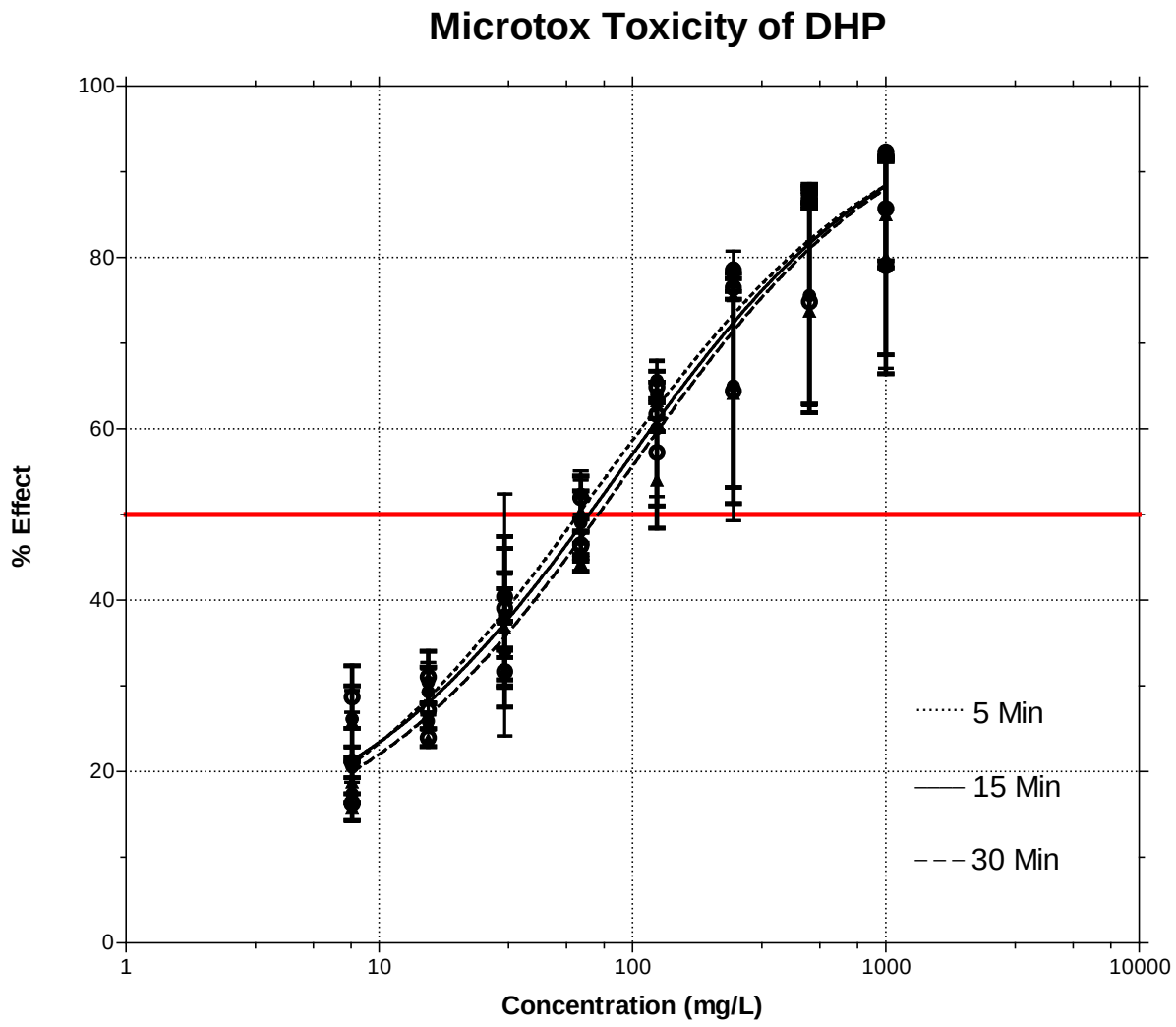
Microtox Reagents	Source	Lot #	Date Expiration
Modern Water Microtox Diluent	Modern Water	14K4141	10/2017
Modern Water Microtox Diluent	Modern Water	16C4015	03/2019
Modern Water Microtox Acute Reagent	Modern Water	15K4119A	10/2017
Modern Water Microtox Acute Reagent	Modern Water	16M4144	12/2018
Modern Water Microtox Acute Reagent	Modern Water	17C4076	03/2019
Dimethyl sulfoxide	Sigma	RNB7475	11/2018
Dimethyl sulfoxide	Sigma	RNBF2710	2/2018
Dimethyl sulfoxide	Sigma	RNBF4251	5/2018
Phenol	Sigma-Aldrich	SHBF1351V	N/A
Modern Water Microtox Reconstitution Solution	Modern Water	16D4031	4/2019

Appendix E

DHP Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)
0.78125
1.5625
3.125
6.25
12.5
25
50
100

DHP EC50 (mg/L; 95% CI)		
5 minute	**15 minute**	**30 minute**
71.99 [34.78- 149.0]	88.27 [50.11- 155.5]	93.78 [56.77- 154.9]

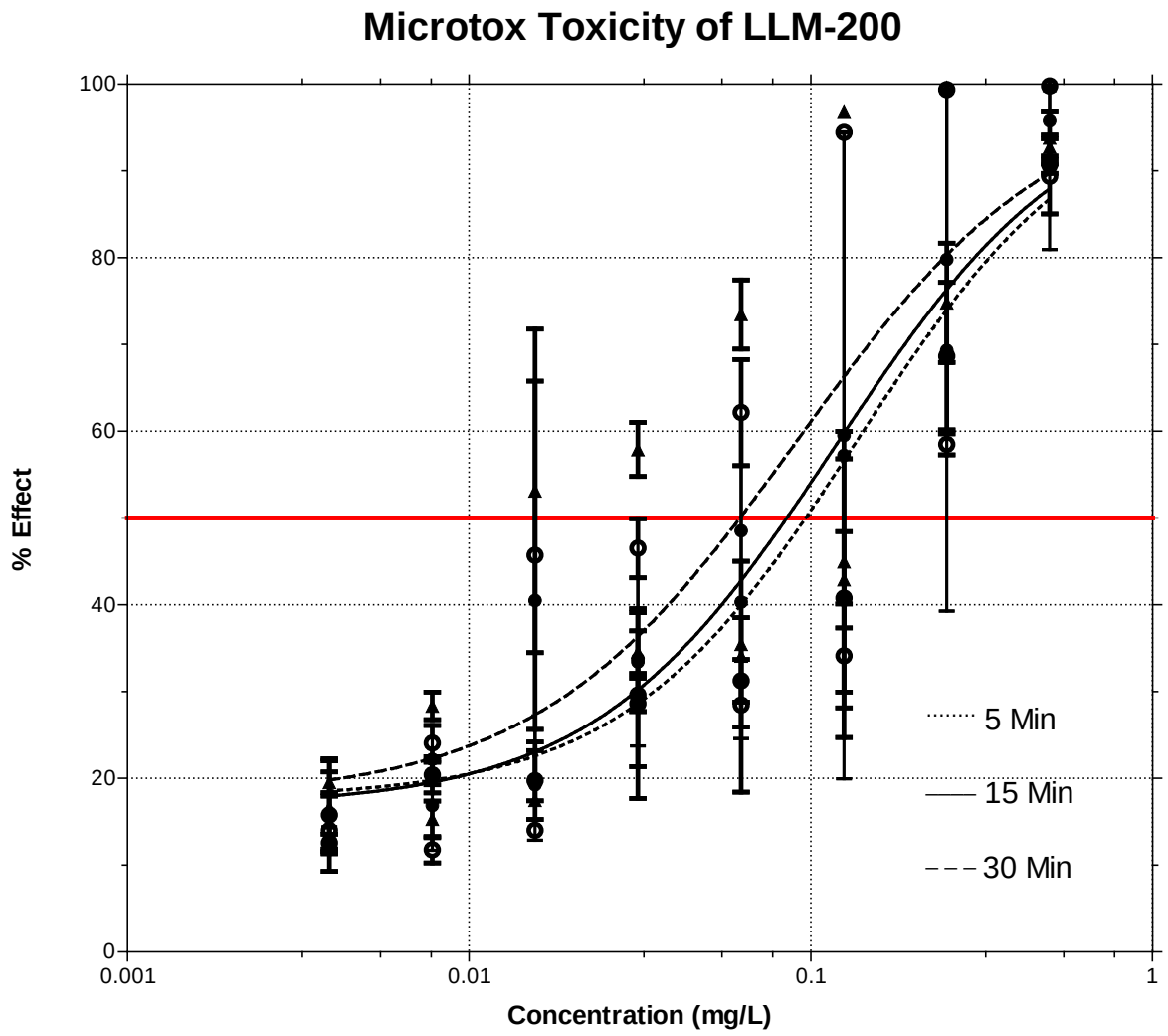

Microtox Toxicity of DHP
% Effect
Concentration (mg/L)
0
20
40
60
80
100
1
10
100
1000
10000
5 Min
15 Min
30 Min

Appendix F

LLM-200 Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)
0.000391
0.000781
0.001563
0.003125
0.00625
0.0125
0.025
0.05

LLM-200 EC50 (mg/L; 95% CI)		
5 minute	**15 minute**	**30 minute**
0.1362 [0.08616- 0.2152]	0.1183 [0.07617- 0.1837]	0.09077 [0.05414- 0.1522]

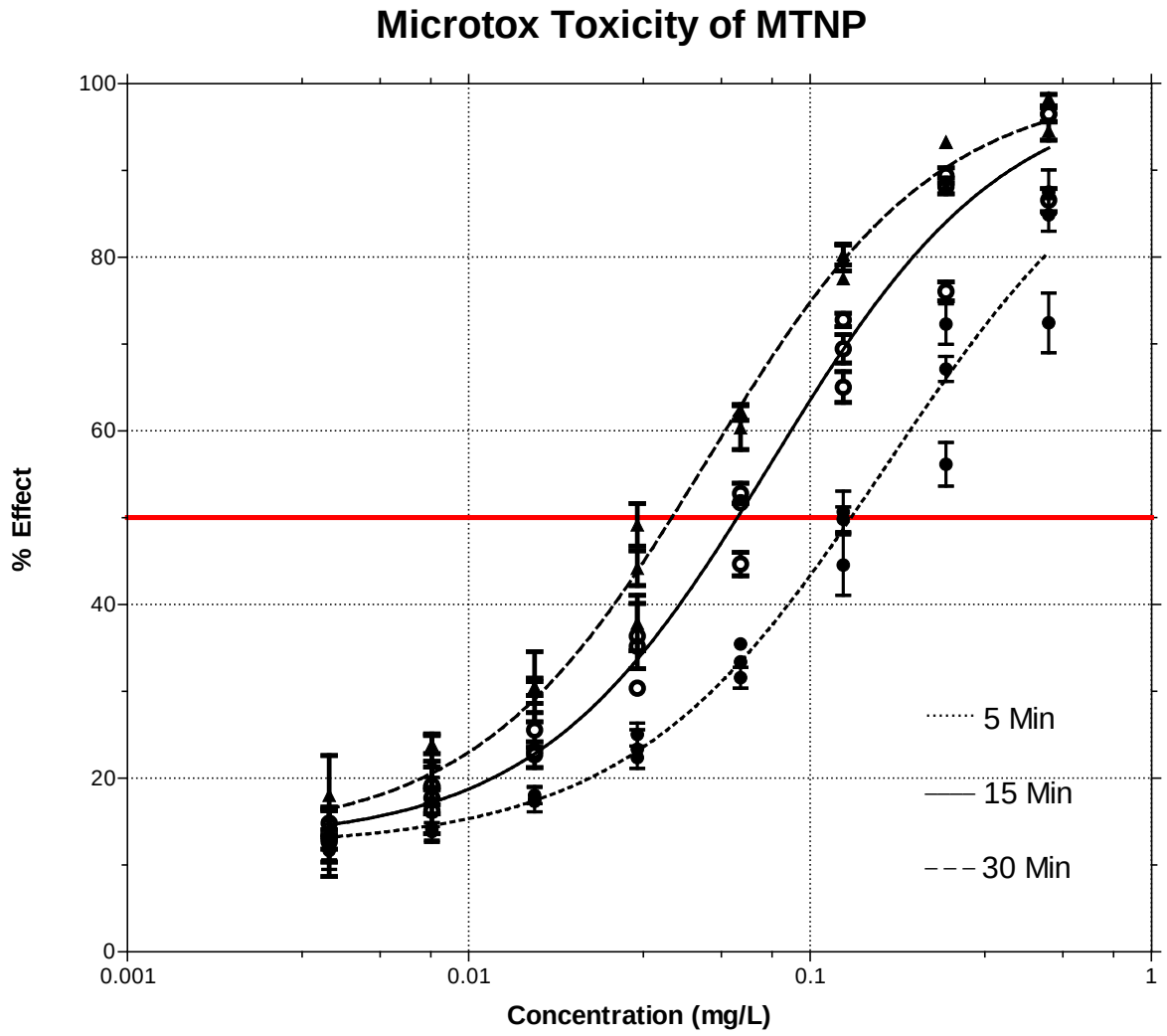

Microtox Toxicity of LLM-200
% Effect
100
80
60
40
20
0
0.001
0.01
0.1
1
Concentration (mg/L)
5 Min
15 Min
30 Min

Appendix G

MTNP Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)
0.000391
0.000781
0.001563
0.003125
0.00625
0.0125
0.025
0.05

MTNP EC50 (mg/L; 95% CI)		
5 minute	**15 minute**	**30 minute**
0.1676 [0.1503- 0.1870]	0.07703 [0.06925- 0.08567]	0.05003 [0.04521- 0.05536]

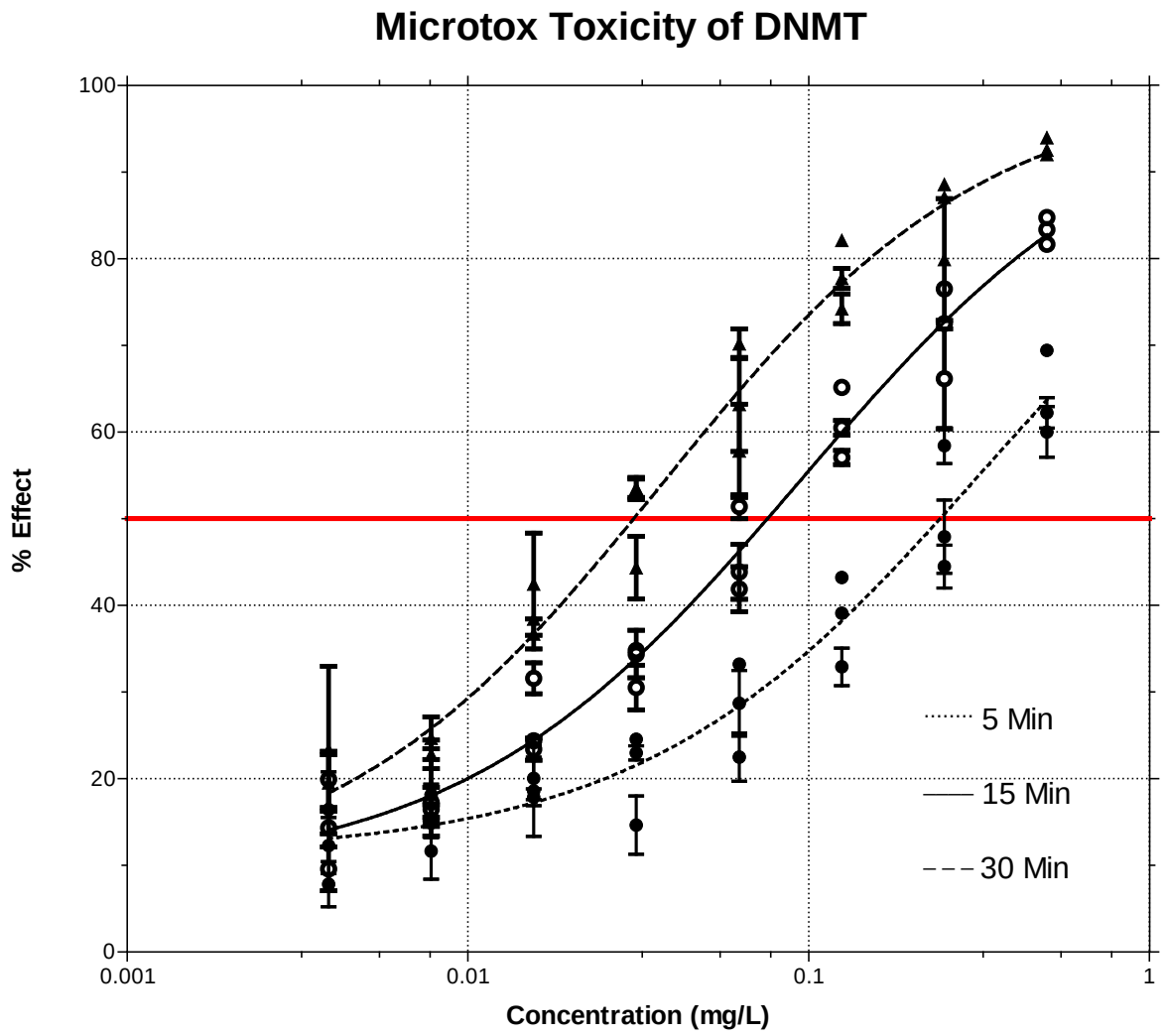

Microtox Toxicity of MTNP
% Effect
Concentration (mg/L)
100
80
60
40
20
0
0.001
0.01
0.1
1
5 Min
15 Min
30 Min

Appendix H

DNMT Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)
0.000391
0.000781
0.001563
0.003125
0.00625
0.0125
0.025
0.05

DNMT EC50 (mg/L; 95% CI)		
5 minute	**15 minute**	**30 minute**
0.3264 [0.2760- 0.3860]	0.09440 [0.07866- 0.1133]	0.03699 [0.02681- 0.05104]


Microtox Toxicity of DNMT
% Effect
Concentration (mg/L)
100
80
60
40
20
0
0.001
0.01
0.1
1
5 Min
15 Min
30 Min