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Toxicology Study No. S.0002728b-15, March 2018
Protocol No. 0FMA-92-iv16-06-01A,B,C,D,E
Toxicology Directorate

Microtox Toxicity Testing of the Novel Energetics, 1,4-dinitroglycoluril (DNGU), 3,4-dinitropyrazole (DNP), 1,4,7-trinitrohexahydro-1H-imidazo[4,5-b]pyrazine-2(3H)-one (HK-56), 2,6-diamino-3,5-dinitro-2,3-dihydropyrazine 1-oxide (LLM-105), and 2,4,6-trinitro-3-bromoanisole (TNBA)

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The energetic and toxicological properties of 1,4-dinitroglycoluril (DNGU), 3,4-dinitropyrazole (DNP), 1,4,7-trinitrohexahydro-1H-imidazo[4,5-b]pyrazine-2(3H)-one (HK-56), 2,6-diamino-3,5-dinitro-2,3-dihydropyrazine 1-oxide (LLM-105), and 2,4,6-trinitro-3-bromoanisole (TNBA) were determined in support of their evaluation as potential replacements 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 DNGU, DNP, HK-56, LLM-105, and TNBA with the Microtox® Acute Toxicity Test System.

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Toxicology Study No. S.0002728b-15
Protocol No. 0FMA-92-iv16-06-01A,B,C,D,E

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Authors

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Study Completed

April 2018

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-iv16-06-01A, B, C, D, E

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 time constraints, the method of analysis for these compounds could not be validated by the Laboratory Sciences Directorate (LS) prior to the study start in compliance with study protocol and modification requirements. Because of this the dosing solutions used for all strains were verified after being frozen (at - 80 degrees C) until the method could be validated by the LAB after the study was completed.

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, Ph.D., D.A.B.T.
Study Director
Health Effects Division

18 April 2018
Date

Toxicology Study No. S.0002728b-15, April 2018

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1 Summary

1.1 Overview

The energetic and toxicological properties of 1,4-dinitroglycoluril (DNGU), 3,4-dinitropyrazole (DNP), 1,4,7-trinitrohexahydro-1H-imidazo[4,5-b]pyrazine-2(3H)-one (HK-56), 2,6-diamino-3,5-dinitro-2,3-dihydropyrazine 1-oxide (LLM-105), and 2,4,6-trinitro-3-bromoanisole (TNBA) were determined in support of their evaluation as potential replacements 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 DNGU, DNP, HK-56, LLM-105, and TNBA with the Microtox® Acute Toxicity Test System. 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.

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 (DoDI) 4715.4 [4]; and Army Environmental Research and Technology Assessment 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 Department of Defense 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 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 Defense 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 DNGU, DNP, HK-56, LLM-105, and TNBA using the Microtox Acute Toxicity 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 DNGU, DNP, HK-56, LLM-105 and TNBA via the Microtox Acute Toxicity assay. According to U.S. Environmental Protection Agency (EPA) Hazard Categories, results show that DNGU was not considered toxic, with a maximal concentration of 1,000 milligrams per liter (mg/L), DNP was slightly toxic (EC_{50}: 13.55 mg/L); HK-56 is considered moderately toxic with an EC_{50} of 1.1 mg/L. LLM-105 and TNBA are highly toxic (0.8 and 0.4 mg/L respectively). LLM-105, HK-56, and TNBA would be considered hazards for aquatic life, while DNP and DNGU are not by Organization for Economic Co-operation
and Development (OECD) hazard class guidelines [6].

1.4 Recommendations

The acute aquatic toxicity of LLM-105, HK-56, and TNBA is of concern and further testing and evaluation should be continued to determine if environmental releases are likely following use of these test articles. The limited toxicity of DNGU and DNP is encouraging; however, exposure to DNP can potentially cause skin sensitizing reactions [7, 8], so further analysis of exposure scenarios is recommended. The water solubility of both of these compounds (3 grams per liter (g/L) and 51.93 g/L) may be of concern; however, the lower toxicity may mitigate the issue. LLM-105 and TNBA were also found to potentially act as skin sensitizers based upon additional skin sensitization testing carried out by U.S. Army Public Health Center (APHC) [9]. 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. W74RDV5291790. This technical report addresses, in part, the environment, safety and occupational health (ESOH) requirements outlined in DoDI 4715.4 [4], 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 [3]; DoDI 4715.4, Pollution Prevention [4]; and Army Environmental Research and Technology Assessment Requirement PP-3-02-05, Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces . 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. The candidates under development for high-density energetics include DNGU, DNP, HK-56, LLM-105, and TNBA.

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. In the past, the use of RDX (1,3,5-hexahydro-1,3,5-trinitrotriazine) and other energetics have been 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 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 [10-13]. The EPA 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 [14, 15].

TNT is acutely toxic to rats causing ataxia, tremors, and mild convulsions; oral LD_{50} values range from 660 to 1,320 mg/kg. The 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 [16, 17]. TNT has caused anemia in the workforce at ammunition manufacturing facilities.

The DoD SERDP is dedicated to finding replacements for RDX and TNT that will reduce or eliminate the health risks from environmental and occupational exposure and will reduce adverse ESOH effects; RDX adversely affects the readiness and costs associated with training [18]. 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, while reducing or eliminating animal use for testing. 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 DNGU,

DNP, HK-56, LLM-105, and TNBA are being evaluated as potential replacements for TNT and RDX.

The Toxicology Directorate (TOX) of the APHC has been tasked with providing aquatic acute toxicity data for DNGU, DNP, HK-56, LLM-105, and TNBA 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 luminescent 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, luminescent 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 EC50 (the midpoint of the effective concentration). The Microtox test has been shown to be an effective screening tool in assessing toxicity of varied chemical compounds comparing with other bioassays. The Microtox Acute Toxicity Test has been validated by the industrial, academic, and governmental testing communities and achieved official "Standards Status" in several countries including the following:

- International Organization for Standards (ISO) 11348-3 (United States of America)
- Standard Method 8050 (United States of America)
- Association Française de Normalisation (AFNOR) T90-320 (France)
- German Institute for Standardization (DIN) 38412 (Germany)

This assay is useful in predicting/screening for adverse effects to aquatic organisms.

This report describes the mutagenic effect of DNGU, DNP, HK-56, LLM-105, and TNBA in the Microtox 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	10 April 2017
Experimental Completion Date	23 June 2017
Study Completion Date	9 April 2018

5 Materials

5.1 Test Substance

Synthesis of DNGU (Chemical Abstracts Service Registry Number (CASRN) 55510-04-08), DNP (CASRN 38858-92-3), HK-56 (CASRN unknown), LLM-105 (CASRN 194486-77-6), and TNBA (CASRN unknown) was completed by Holston Army Ammunition Plant, Kingsport, Tennessee. Purity analyses for these compounds were not available. Figure 1 shows the molecular structures of the compounds.

DNGU was soluble at 100 mg/mL, HK-56 and DNP were soluble at 50 mg/mL, LLM-105 was soluble at 10 mg/mL and TNBA was soluble at 1 mg/mL. Initial solubility was determined by solubility checks in the Ames assay [19, 20]. At the end of study, the final serial dilutions were frozen and later analyzed by the APHC Method Development Section Client Services Division (PHC-MDV-CSD) for dose validation. The method of analysis was validated prior to dosing solution verification by the PHC-MDV-LCD; the validation data is kept by MDV and is available for review. The contributing scientist analytical report from PHC-MDV-LCD is archived with the study records.

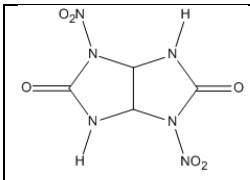	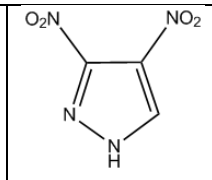	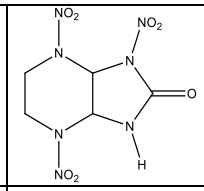	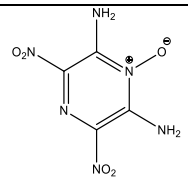	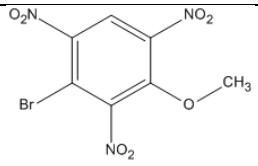
1,4-Dinitroglycoluril (DNGU)	3,4-Dinitropyrazole (DNP)	1,4,7-Trinitrohexahydro-1H-imidazo[4,5-b]pyrazin-2(3H)-one (HK-56)	2,6-Diamino-3,5-dinitro-2,3-dihydropyrazine 1-oxide (LLM-105)	2,4,6-Trinitro-3-bromoanisole (TNBA)

Figure 1. Molecular Structure of the Compounds

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 according to manufacturer instructions as described in the Toxicology Standing Operating Procedure (SOP) 037 and study protocol [21, 22].

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 EC_{50} of 13-26 mg/L at 5 min were qualified for further use.

5.4 Quality Assurance

APHC policy requires that all experiments and studies conducted by any element of the APHC TOX will be compliant with the GLP Standards [23]. For this study, the test article dictates that the following GLP standard applies [24]

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 APHC TOX SOP 037 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 APHC TOX Type Protocol: *"Microtox Toxicity Testing System*" [22], and modifications. The modifications to the protocol are approved and signed by the Study Director and QSARC. 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

DNGU, DNP, HK-56, LLM-105, and TNBA were dissolved in DMSO at their solubility limit. The solubility of each test article was determined previously in the Ames test [20]. 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* 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 EC_{50} from the range finding

determined the final test concentration range (See Appendix E-I for final chemical specific ranges).

6.3 Cytotoxicity Test

Following the range finding, each test article was tested in duplicate on three separate days. On each testing day, test articles were prepared in DMSO at 100x the top dose as determined in the range finding 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. At the end of the study, the samples were analyzed by the PHC-MDV-LCD. The final dilution of the empirical starting stock solution for DNGU would theoretically be, for example, 781.2 µg/mL.

- The validated concentrations of the final serial dilution for DNGU were:
    - Test 1 – 834 µg/mL
    - Test 2 – 870 µg/mL
    - Test 3 – 852 µg/mL
- The validated concentrations of the final serial dilution for DNP were:
    - Test 1 – 34.1 µg/mL
    - Test 2 – 22.2 µg/mL
    - Test 3 – 40.0 µg/mL
- The validated concentrations of the final serial dilution for HK-56 were:
    - Test 1 – 3.3 µg/mL
    - Test 2 – 3.9 µg/mL
    - Test 3 – 3.6 µg/mL
- The validated concentrations of the final serial dilution for LLM-105 were:
    - Test 1 – 4.9 µg/mL
    - Test 2 – 1.8 µg/mL
    - Test 3 – 6.1 µg/mL
    - Test 4 – 9.6 µg/mL
- The validated concentrations of the final serial dilution for TNBA were:
    - Test 1 – 2.1 µg/mL
    - Test 2 – 2.7 µg/mL
    - Test 3 – 2.7 µg/mL

The concentrations of the initial concentrates were back-calculated from these verified concentrations.

6.4 Data Analysis

Raw luminescence data were recorded at 5, 15, and 30 minutes by the Microtox analyzer. The EC50 values at 5, 15, and 30 minutes were given by the MicrotoxOmni software and further fitted to the Hill function using GraphPad PRISM 4®. All data (prints and files) were archived.

7 Results and Discussion

7.1 Microtox Toxicity and Risk Assessment

Toxicity of DNGU, DNP, HK-56, LLM-105, and TNBA to marine bacteria, *V. fischeri*, was measured by the Microtox acute toxicity test system at 5, 15, and 30 minutes. For each test compound,
three individual experiments were performed in duplicate. Table 2 presents the toxicity data (EC_{50} and the 95 percent Confidence Interval) and risk assessment. 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. The tables and figures within Appendices E–I presents data that was further analyzed by performing the Hill function using GraphPad Prism version 5.04. 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 [25-27]. 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 EPA, the OECD and the Global Harmonization System (GHS) to categorize the potential ecotoxicity of these new compounds (Table 3) [6, 28, 29]. This evaluation suggests LLM-105 and TNBA are “Highly Toxic” and potentially very toxic to aquatic life; HK-56 is “Moderately Toxic” and potentially toxic to aquatic life; DNP is “Slightly Toxic” and potentially harmful to aquatic life; and DNGU is “Practically Nontoxic” (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			
DNGU	492.3 [451.0-537.4	273.7 [251.7-297.6]	173.9 [158.1-191.4]	Practically Nontoxic	–	–
DNP	13.6 [12.6-14.8]	10.9 [10.2-11.8]	9.1 [8.3-10.1]	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. III
HK-56	2.6 [2.2-3.0]	1.1 [0.9-1.2]	0.6 [0.5-0.7]	Moderately Toxic	Acute Toxicity II (toxic to aquatic life)	Acute Cat. II
LLM-105	1.1 [1.0-1.2]	0.8 [0.7-0.9]	0.7 [0.6-0.8]	Highly Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. I
TNBA	0.8 [0.4-1.4]	0.4 [0.3-0.5]	0.2 [0.1-0.3]	Highly Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. I

Legend:
USEPA = United States Environmental Protection Agency
OECD = Organization for Economic Co-operation and Development
GHS = Global Harmonization System
mg/L = milligrams per liter

Note:
*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	–	–

Legend:
OECD = Organization for Economic Co-operation and Development
USEPA = United States Environmental Protection Agency
mg/L = milligrams per liter

7.2 Criteria for Valid Assay

The phenol 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 new munitions compounds DNGU, DNP, HK-56, LLM-105 and TNBA via the Microtox Acute Toxicity assay. Results show that DNGU was not considered toxic, with a maximal concentration of 1,000 mg/L., DNP was slightly toxic (EC_{50}: 13.55 mg/L); HK-56 is considered moderately toxic with an EC_{50} of 1.1 mg/L. LLM-105 and TNBA are highly toxic (0.8 and 0.4 mg/L respectively). LLM-105, HK-56, and TNBA would be considered hazards for aquatic life, while DNP and DNGU are not by OECD hazard class guidelines [6].

9 Recommendations

The acute aquatic toxicity of LLM-105, HK-56, and TNBA are of concern and further testing and evaluation should be continued to determine if environmental releases would pose a threat following use of these test articles. Elevated toxicity may cause difficulties in obtaining a wastewater discharge permit if disposal to natural rivers or streams of any portion of the waste liquors is desired. The limited toxicity of DNGU and DNP is encouraging; however, exposure to DNP can potentially cause skin-sensitizing reactions, so further analysis of exposure scenarios is recommended. The water solubility of both of these compounds may be of concern; however,

the lower toxicity may mitigate the issue. LLM-105 and TNBA were also found to potentially act as skin sensitizers based upon additional skin sensitization testing carried out by APHC [9]. This raises additional concerns based on their high toxicity and relatively high water solubility. Further *in vitro* and *in vivo* testing may be warranted for any of these compounds if development is to continue.

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.

Toxicology Study No. S.0002728b-15, April 2018

Submitted by:

U.S. Army Public Health Center
Health Effects Division
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Aberdeen Proving Ground, MD 21010-5403
410-436-3980

Prepared by:

18 April 2018

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Biologist
Health Effects Division
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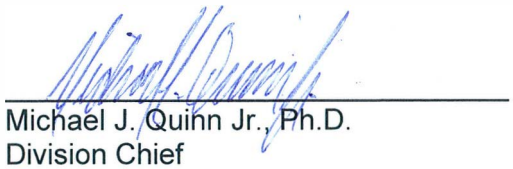
Date

Approved by:



18 April 2018

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Date

18 Apr 2018

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

Date

Appendix A

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Appendix B

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. S.0002728-15, Protocol No. 0FMA-92-iv16-06-01A,B,C,D,E, Microtox Toxicity Testing of the Novel Energetics, 1,4-Dinitroglycoluril (DNGU), 3,4-Dinitropyrazole (DNP), 1,4,7-trinitrohexahydro-1Himidazo[4,5-b]pyrazine-2(3H)-one (HK-56), 2,6-diamino-3,5-dinitro-2,3-dihydropyrazine 1-oxide (LLM-105), and 2,4,6-trinitro-3-bromoanisole (TNBA), the following Good Laboratory Practice Standard Inspections were conducted:

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	01/10/2018	01/10/2018
Final Study Good Laboratory Practice Standard Report Review	01/10/2018	01/10/2018

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
Good Laboratory Practice Standard
Quality Assurance Specialist, QSARC

18 APRIL 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 Portfolio, 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 Portfolio 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.0002728b-15, Protocol No. 0FMA-92-iv16-06-01A,B,C,D,E,

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., Portfolio Toxicology Director; Michael J. Quinn, Ph.D., Program Manager, Health Effects Research Program (HERP).

Study Director: Emily N. Reinke, Ph.D., 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 Acute Reagent	Modern Water	15K4119A	10/2017
Modern Water Microtox Acute Reagent	Modern Water	16M4144	12/2018
Dimethyl sulfoxide	Sigma	RNB7475	11/2018
Dimethyl sulfoxide	Sigma	RNBF2710	2/2018
Dimethyl sulfoxide	Sigma	RNBF4251	5/2018
Phenol	Sigma-Aldrich	SHBF1351V	
Modern Water Microtox Reconstitution Solution	Modern Water	16D4031	4/2019

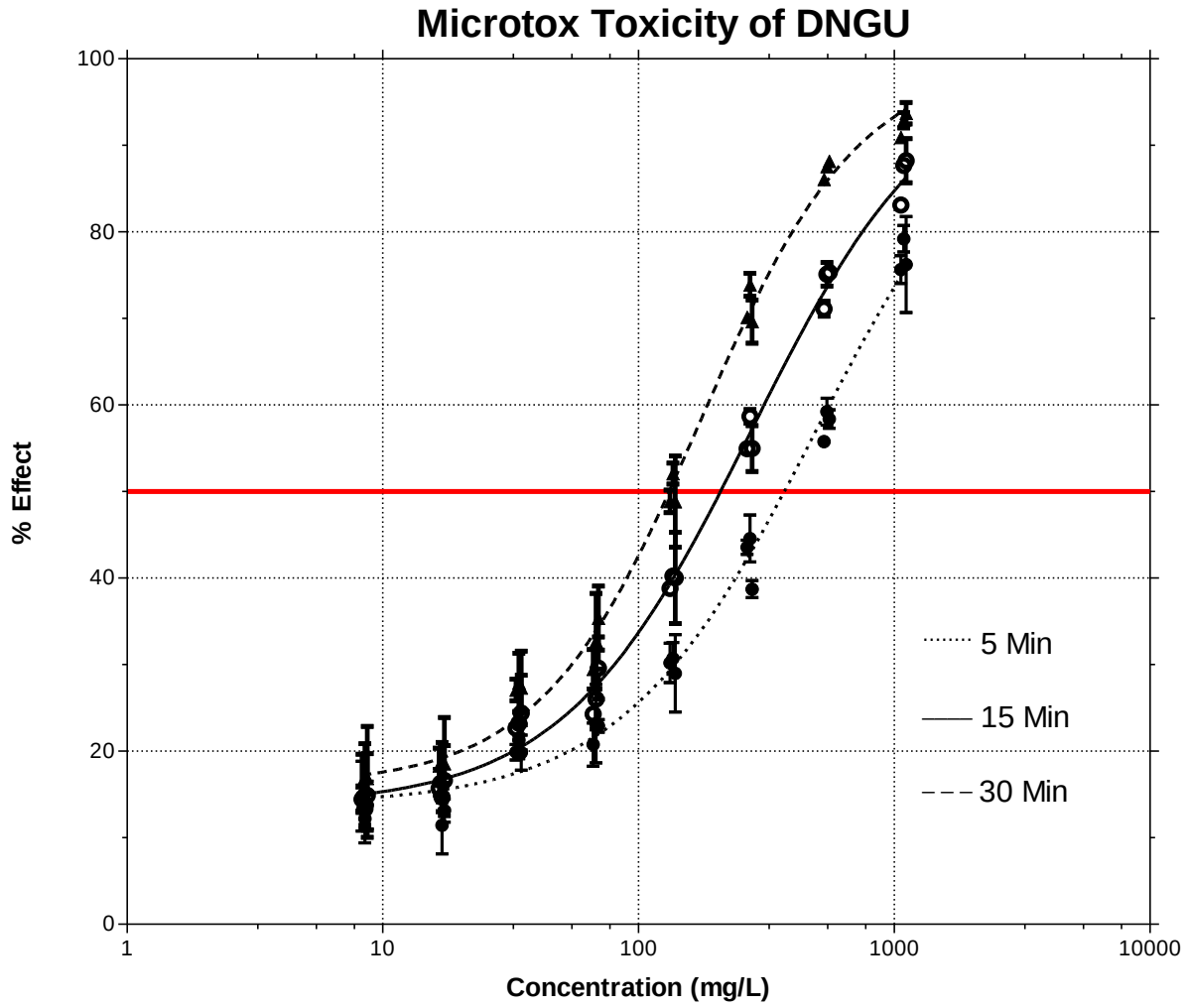
Appendix E

DNGU Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration (mg/mL; 100x test concentration)		
	Test 1	Test 2	Test 3
0.781	0.834*	0.870*	0.852*
1.563	1.668	1.740	1.704
3.125	3.336	3.480	3.408
6.250	6.672	6.960	6.818
12.50	13.344	13.920	13.623
25.00	26.688	27.840	27.264
50.00	53.376	55.680	54.528
100.00	106.752	111.360	109.056

Note:
*****Working concentration measured by MDV, final corrected concentrations were calculated from lowest measured working concentration.

DNGU EC50 (mg/L; 95% CI)		
5 minute	15 minute	30 minute
492.3 (451.0 to 537.4)	273.3 (251.7 to 297.6)	173.9 (158.1 to 191.4)


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

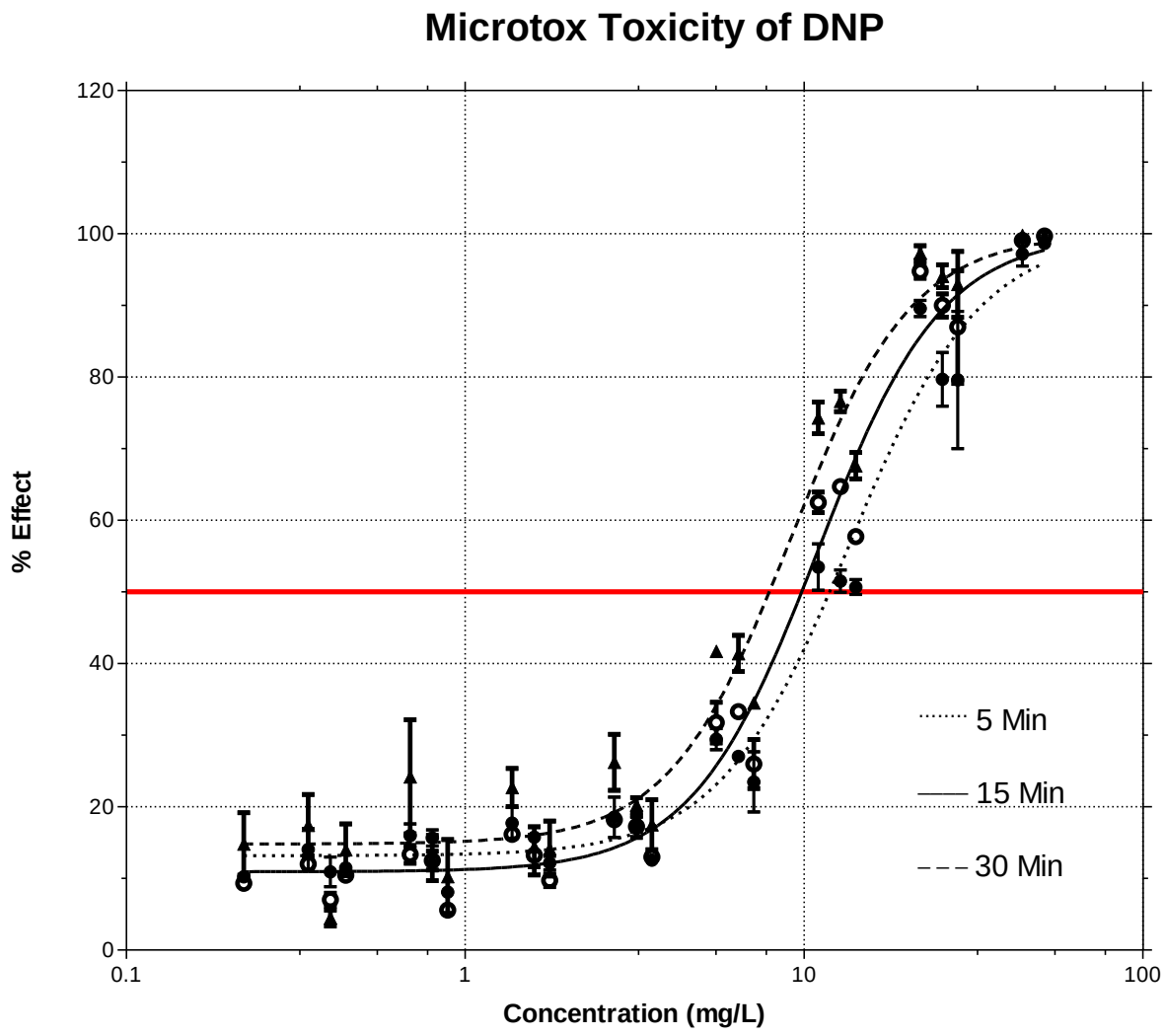
Appendix F

DNP Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration (mg/mL; 100x test concentration)		
	Test 1	**Test 2**	**Test 3**
3.906	3.44*	2.22*	4.00*
7.812	6.88	4.44	8.00
15.624	13.76	8.88	16.00
31.248	27.52	17.76	32.00
62.496	55.04	35.52	64.00
124.992	110.08	71.04	128.00
249.984	220.16	142.08	256.00
499.968	440.32	284.16	512.00

Note:
*****Working concentration measured by MDV, final corrected concentrations were calculated from lowest measured working concentration.

DNP EC50 (mg/L; 95% CI)		
5 minute	**15 minute**	**30 minute**
13.62 (12.58 to14.75)	10.92 (10.15 to 11.75)	9.129 (8.296 to 10.05)


Microtox Toxicity of DNP
% Effect
Concentration (mg/L)
0
20
40
60
80
100
120
0.1
1
10
100
5 Min
15 Min
30 Min

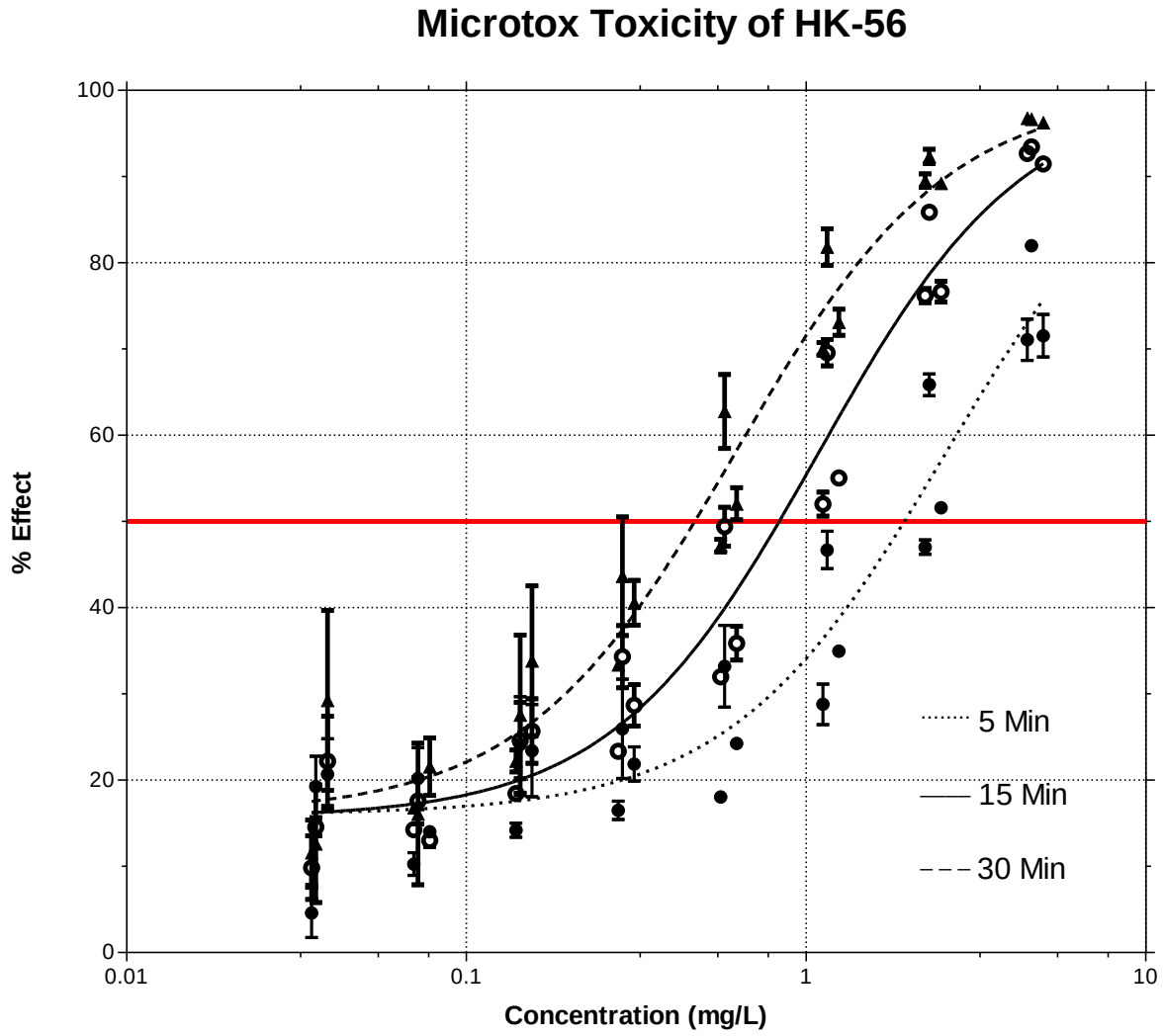
Appendix G

HK-56 Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration (mg/mL; 100x test concentration)		
	Test 1	Test 2	Test 3
0.39	0.35*	0.39*	0.36*
0.78	0.70	0.78	0.72
1.56	1.40	1.56	1.44
3.12	2.80	3.12	2.88
6.24	5.60	6.24	5.76
12.48	11.20	12.48	11.52
24.96	22.40	24.96	23.04
49.92	44.80	49.92	46.08

Note:
*****Working concentration measured by MDV, final corrected concentrations were calculated from lowest measured working concentration.

HK-56 EC50 (mg/L; 95% CI)		
5 minute	15 minute	30 minute
2.584 (2.215 to 3.014)	1.082 (0.9474 to 1.236)	0.6204 (0.5196 to 0.7407)


Microtox Toxicity of HK-56
% Effect
Concentration (mg/L)
0
20
40
60
80
100
0.01
0.1
1
10
5 Min
15 Min
30 Min

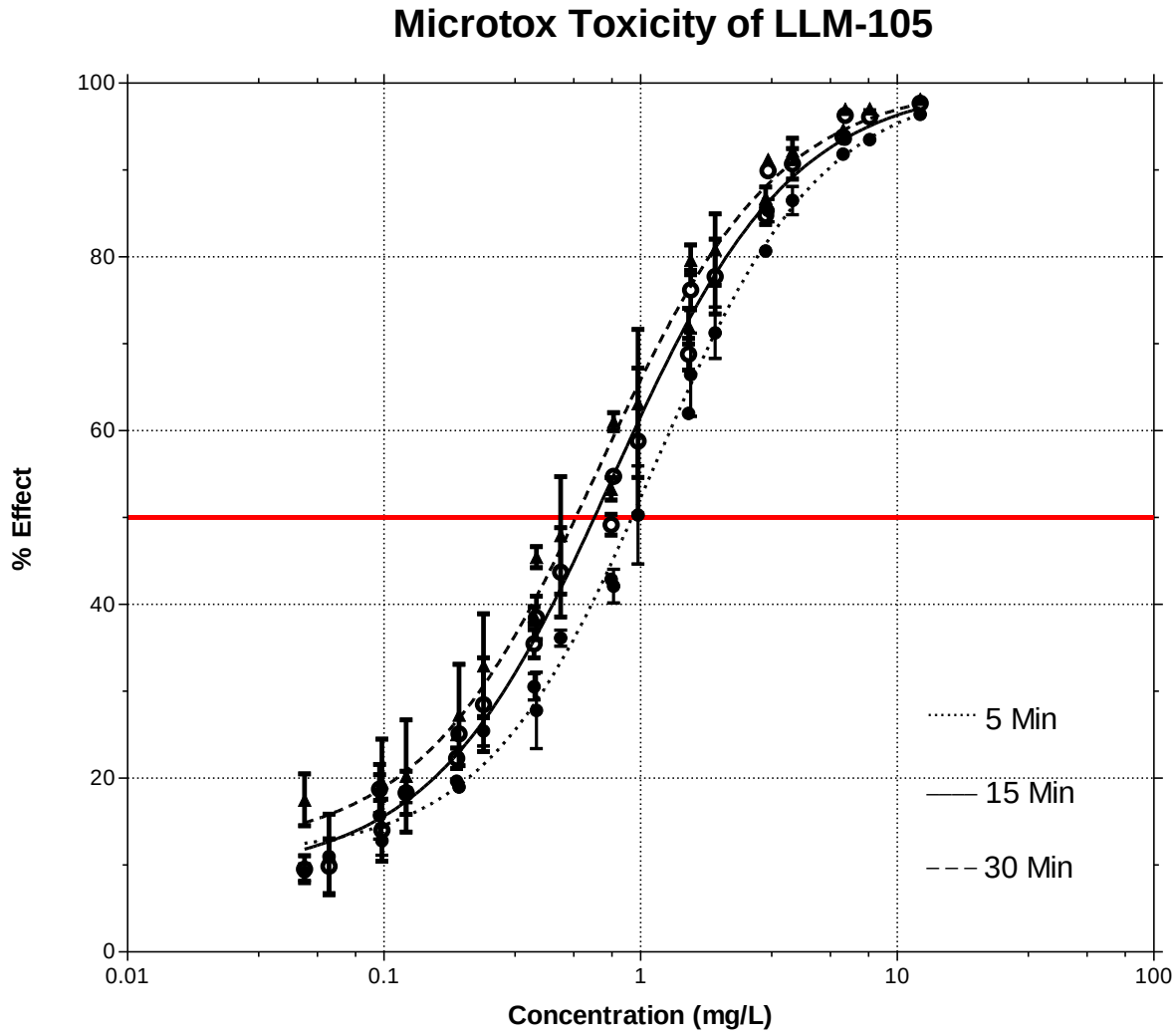
Appendix H

LLM-105 Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration (mg/mL; 100x test concentration)			
	Test 1	Test 2	Test 3	Test 4
0.977	0.49*	0.18*	0.61*	0.96
1.954	0.98	0.36	1.22	1.92
3.908	1.96	0.72	2.44	3.84
7.816	3.92	1.44	4.88	7.68
15.632	7.84	2.88	9.76	15.36
31.264	15.68	5.76	19.52	30.72
62.528	31.36	11.52	39.04	61.44
125.056	62.72	23.04	78.08	122.88

Note:
*****Working concentration measured by MDV, final corrected concentrations were calculated from lowest measured working concentration.

LLM-105 EC50 (mg/L; 95% CI)		
5 minute	**15 minute**	**30 minute**
1.120 (1.036 to 1.210)	0.7736 (0.6863 to 0.8720)	0.6919 (0.5957 to 0.8037)


Microtox Toxicity of LLM-105
% Effect
Concentration (mg/L)
0
20
40
60
80
100
0.01
0.1
1
10
100
5 Min
15 Min
30 Min

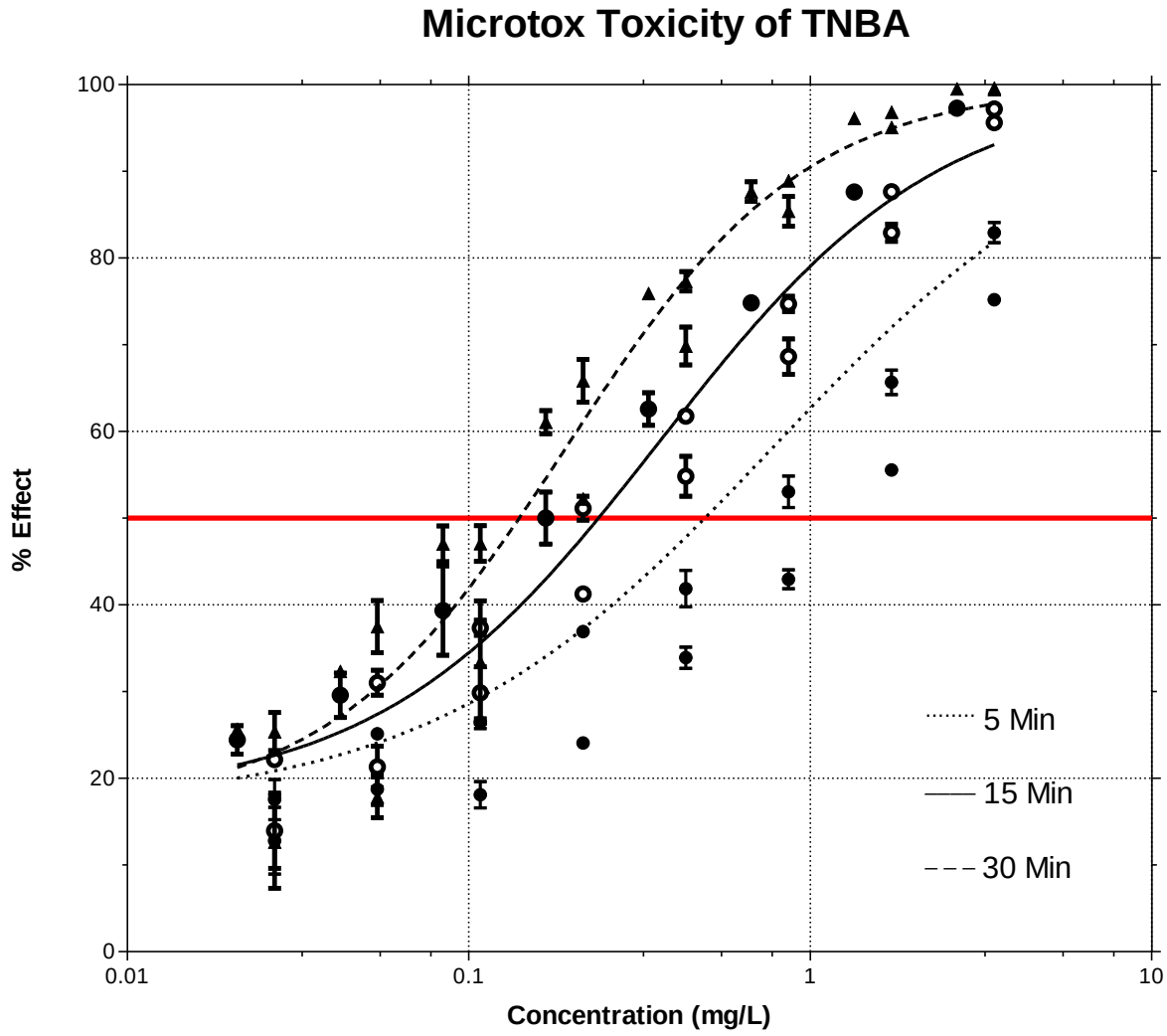
Appendix I

TNBA Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration (mg/mL; 100x test concentration)		
	Test 1	Test 2	Test 3
0.39	0.21*	0.27*	0.27*
0.78	0.42	0.54	0.54
1.56	0.84	1.08	1.08
3.12	1.68	2.16	2.16
6.24	3.36	4.32	4.32
12.48	6.72	8.64	8.64
24.96	13.44	17.28	17.28
49.92	26.88	34.56	34.56

Note:
*****Working concentration measured by MDV, final corrected concentrations were calculated from lowest measured working concentration.

TNBA EC50 (mg/L; 95% CI)		
5 minute	15 minute	30 minute
0.7819 (0.4483 to 1.364)	0.3621 (0.2870 to 0.4569)	0.1347 (0.1484 to 0.2505)


Microtox Toxicity of TNBA
% Effect
Concentration (mg/L)
100
80
60
40
20
0
0.01
0.1
1
10
5 Min
15 Min
30 Min