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Toxicology Directorate

Microtox Testing of Thirteen Replacement Candidates for Methylenedianiline
August-September 2018

Prepared by Dr. Emily N. Reinke, Health Effects Division

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The toxicological properties of 13 replacement candidates for 4,4-methylenedianiline (MDA) are under assessment. MDA is a compound used in the preparation of a polymer resin, PMR-15, which is used in the fabrication of high temperature-resistant parts for rockets and similar applications. MDA is mutagenic and is a high priority for replacement with non-mutagenic substitutes. The aquatic toxicity of these 13 candidates was predicted using the Microtox® Acute Toxicity Test System, a bioluminescent bacterial aquatic toxicity test. Data from this study are used to assist in making environmental and health-based decisions regarding the design and selection of formulas and materials for further development.

15. SUBJECT TERMS
4,4-methylenedianiline; MDA; aquatic toxicity

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Microtox Testing of Thirteen Replacement Candidates for Methylenedianiline

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July 2019

Performing Laboratory

U.S. Army Public Health Center
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Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (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 had not been developed 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 frozen (at - 80 degrees C) until methods could be developed and validated by the LAB.

3. For compounds MDA48, MDA53, IDMMDA50, IAMDA51, and IAMMDA52, the protocol modifications were completed after testing was completed. All testing was carried out in accordance with the protocol alongside compounds that did have completed protocol modifications. This occurred due to a lag in development of chemical specific substance profiles needed to complete the modifications, and the need to complete testing while personnel were available.

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

Emily N. Reinke

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Study Director
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6-26-19
Date

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MICROTOX TESTING OF THIRTEEN REPLACEMENT CANDIDATES FOR METHYLENEDIANILINE
August-September 2019

1 SUMMARY

1.1 Overview

The toxicological properties of 13 replacement candidates for 4,4-methylenedianiline (MDA) are under assessment. These candidates include:

- MDA40 [4,4'-methylenebis(2,5-dimethylaniline)],
- MDA42/MDA54 [4,4'-methylenebis(2,6-dimethylaniline)],
- MDA43 [5,5'-(propane-2,2-diyl)bis(2-methoxyaniline)],
- MDA47 [4-(bis(5-aminomethyl)furan-2-yl)methyl)-2-methoxyphenol],
- MDA48 [1,1'-((propane-2,2-diylbis(4,1-phenylene))bix(oxy))bis(2-methylprop-2-en-1-ol)],
- MDA49 [(5,5',5'',5'''-(1,4-phenylenebis(methanetriyl))tetrakis(furan-5,2-diyl))tetramethanamine],
- MDA50 [4-(Bis(5-(aminomethyl)furan-2-yl)methyl)-2,6-dimethoxyphenol],
- MDA52 [4,4'-((3,3'-dimethoxy-5,5'-dimethyl-[1,1'-biphenyl]-2,2'-diyl)bis(oxy))dianiline],
- MDA53 [3,3',5,5'-Tetramethylbenzidine],
- IDMMDA50 [Hexahydrofuro[3,2-b] furan-3,6-diyl bis(2-methacrylate)],
- IAMDA51 [hexahydrofuro[3,2-b] furan-3,6-diyl bis(2-acrylate)], and
- IAMMDA52 [6-(acryloyloxy) hexahydrofuro[3,2-*b*] furan-3-yl methacrylate].

MDA is a compound used in the preparation of a polymer resin, PMR-15, which is used in the fabrication of high temperature-resistant parts for rockets and similar applications. MDA is mutagenic and is a high priority for replacement with non-mutagenic substitutes. As part of the toxicity evaluation of these new replacements, the aquatic toxicity of these 13 candidates was predicted using the Microtox® Acute Toxicity Test System, a bioluminescent bacterial aquatic toxicity test. Data from this study are used to assist in making environmental 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 compounds for military use. This information is critical to the research, development, testing, and evaluation (RDT&E) of new systems. 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]; DA Regulation 70-1 [3]; Department of Defense Instruction (DoDI) 4715.23 [4]; and Army Environmental Research and Technology Assessment (AERTA) requirement PP-3-02-07 [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 plastics, pyrotechnics, propellants, explosives, and incendiaries have been found in soil, air, surface, and groundwater samples; thus creating environmental problems and interfering with training activities. The Microtox test is useful in identifying potential impact of chemical substances on aquatic environments, and may be used in helping establish aquatic discharge limitations.

The DOD is identifying replacements for substances causing environmental and/or occupational risks to health. The purpose of this toxicology study was to evaluate the aquatic toxicity of 13 MDA replacement candidates 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 13 candidates for MDA replacement using the Microtox Acute Toxicity assay. MDA42, MDA47, MDA49, and IAMDA51 were insoluble in DMSO precluding testing in this system. Data were acquired for the remaining compounds:

- MDA48 was relatively harmless (EC_{50} >2,000 milligrams per liter (mg/L)),
- MDA50 and MDA52 were considered practically nontoxic (EC_{50} 100–1,000 mg/L),
- MDA40, MDA42, MDA54, and IDMMDA50 were found to be slightly toxic (EC_{50} 10–100 mg/L), and
- MDA53 and IAMMDA52 were found to be moderately toxic (EC_{50} 1–10 mg/L).

Therefore, MDA40, MDA42, MDA54, and IDMMDA50 are harmful to aquatic life and MDA53 and IAMMDA52 are considered toxic to aquatic life according to Globally Harmonized System (GHS) categorization [6].

1.4 Recommendations

The lack of aquatic toxicity using the luminescent bacteria assay with MDA43, MDA48, MDA50, and MDA52 is encouraging, however, further aquatic testing may be necessary prior to production based upon jurisdictional requirements. There is moderate concern with the toxicity of MDA40, MDA54, and IDMMDA50, including the high water solubility of IDMMDA50. Evaluating the likelihood of environmental releases should be made if these compounds are to be further considered as MDA replacements; this also includes green algae, Daphnia, and fish toxicity testing. Due to the acute aquatic toxicity of MDA53 and IAMMDA52, it is not recommended that these compounds are used as a replacement for MDA. The inability to test MDA42, MDA47, MDA49, and IAMDA51 due to solubility concerns necessitates further analysis

of these compounds to determine if testing is feasible. Additional reporting on mutagenicity and skin sensitization potential of these compounds is currently in progress.

2 REFERENCES

See Appendix A for list of references.

3 AUTHORITY

Military Interdepartmental Purchase Request No. W74RDV33432103. This technical report addresses, in part, the environment, safety and occupational health (ESOH) requirements outlined in DoDI 4715.23 [4]; Department of the Army Regulation (AR) 200-1, Environmental Protection and Enhancement [1]; AR 40-5, Preventive Medicine [2]; AR 70-1, Army Acquisition Policy [3]; and Army Environmental Research and Technology Assessment Requirement PP-3-02-07, Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces [5]. The Strategic Environmental Research and Development Program as part of project WP-2402, Novel Chemistries, conducted this report as an on-going effort for Replacement of Methylenedianiline in Polyimide Composites.

4 BACKGROUND

Current regulations require the assessment of human health and environmental effects 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 environmental, safety, and occupational health issues. The candidates under development for MDA replacement include MDA40, MDA42, MDA43, MDA47, MDA48, MDA49, MDA50, MDA52, MDA53, MDA54, IDMMDA50, IAMDA51, and IAMMDA52.

MDA (4,4'-methylenedianiline) is used in the preparation of a polymer resin known as PMR-15, which is used in the fabrication of high temperature-resistant parts for rockets and similar applications [11]. The use of MDA is problematic due to its high liver toxicity and likely carcinogenicity. It has an occupational exposure limit of 10 parts per billion over the course of an 8-hour workday [12]. The U.S. National Institute for Occupational Safety and Health (NIOSH) lists MDA as a potential occupational carcinogen and the International Agency for Research on Cancer (IARC) classifies it as a possible human carcinogen (Group 2B) [13, 14]. Because of its mutagenic and other toxic properties, MDA is high priority to be replaced by less toxic substitutes.

To support the development of sustainable low toxicity materials, high-throughput methods are needed to assess relative toxicity of new 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, in newly developed materials, the *in vitro* tests are the most suitable and effective screening tools because very limited amounts of test substances are often available. Detecting ESOH effects early in the acquisition process can identify unacceptable replacement compounds.

The Toxicology Directorate (TOX) of the U.S. Army Public Health Center (APHC) uses an *in vitro* test system (Microtox) to estimate acute aquatic toxicity. Microtox is a toxicity testing system that uses a strain of naturally occurring bioluminescent bacteria, *Aliivibrio fischeri* (formerly *Vibrio fischeri;* Note: the supplier of the reagents, Modern Water, and this report refer to this bacteria as *V. fischeri*). The marine bacterial bioluminescence directly relates 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 is an effective screening tool in assessing toxicity of varied chemical compounds compared 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 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 MDA40, MDA42, MDA43, MDA47, MDA48, MDA49, MDA50, MDA52, MDA53, MDA54, IDMMDA50, IAMDA51, and IAMMDA52 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	13 February 2017
Study Start Date	15 February 2018
Experimental Start Date	15 February 2018
Experimental Completion Date	10 September 2018
Study Completion Date	July 2019

5 MATERIALS

5.1 Test Substance

The U.S. Army Research Laboratory at Aberdeen Proving Ground, Maryland completed the synthesis of MDA40 (Chemical Abstracts Service Registry Number (CASRN) 5339-30-0), MDA52/MDA54 (CASRN 4073-98-7), MDA43 (CASRN Unknown), MDA47 (CASRN Unknown), MDA48 (CASRN Unknown), MDA49 (CASRN Unknown), MDA50 (CASRN Unknown), MDA52 (CASRN Unknown), MDA53 (CASRN Unknown), IDMMDA50 (CASRN Unknown), IAMDA51

(CASRN Unknown), and IAMMDA52. Purity analyses for these compounds were not available. Table 2 shows the molecular structures of the compounds.

The Ames assay uses solubility checks to determine initial solubility [15, 16]. At the end of study, the final serial dilutions were frozen and later analyzed by the PHC 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-CSD; the validation data is kept by MDV and is available for review. The contributing scientist analytical report from PHC-MDV-CSD is archived with the study records.

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* (formerly known as *Photobacterium phosphoreum*, NRRL number B-11177). 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 and are described in the Toxicology Standing Operating Procedure (TOX SOP) 037 and study protocol [17, 18].

5.3 Positive Control

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

5.4 Quality Assurance

APHC's policy requires that all experiments and studies conducted by any element of the APHC Toxicology Directorate will be compliant with the applicable Good Laboratory Practice (GLP) Standard guideline [19]. For this study, the test article dictates that the following GLP guideline applies [20]:

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

According to this regulation, and since these results may be used in regulatory decisions involving the EPA, Microtox 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. This statement provides the audit dates and phases as well as when the audit results were 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 [17]. The test kit is designed to estimate the aquatic toxicity of a test material in compliance with the APHC TOX Type Protocol: "Microtox Toxicity Testing System" [18], 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 Toxicity Test

MDA40, MDA42, MDA43, MDA47, MDA48, MDA49, MDA50, MDA52, MDA53, MDA54, IDMMDA50, IAMDA51, and IAMMDA52 were dissolved in DMSO at their solubility limit or the limit of the test (200 milligrams per milliliter (mg/mL)). The solubility of each test article was determined previously in the Ames test [16]; where compounds were found to be insoluble, no further testing was conducted. 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* (10 microliters (µL)) was added to each test concentration, samples were incubated, and luminescence was measured 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 Appendices E through I for final chemical specific ranges). If compounds were found to be non-toxic (EC_{50} at or above solubility/test limit), no further testing was conducted.

6.3 Main 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 eight-dose testing range. Samples were diluted 1:100 into 1 mL diluent, 10 µL of 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 held for analysis by the PHC-MDV-CSD. All test chemical concentrations are expressed as the nominal concentration and will be adjusted following sample analysis. If, upon completion of concentration analysis, values are significantly different from nominal values (> 10 percent) and are not likely to be due to sample degradation in storage, toxicity estimations will be amended.

6.4 Data Analysis

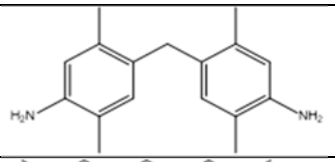
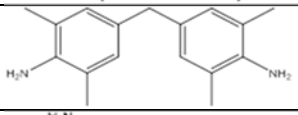
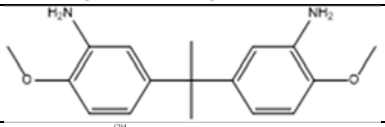
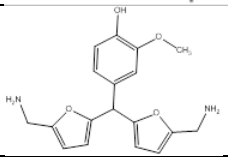
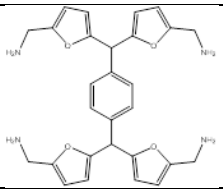
Raw luminescence data were recorded at 5, 15, and 30 minutes by the Microtox analyzer. The EC_{50} values at 5, 15, and 30 minutes were given by the MicrotoxOmni® software and further fitted to the Hill function using GraphPad PRISM version 5.04®. All data (prints and files) were archived.

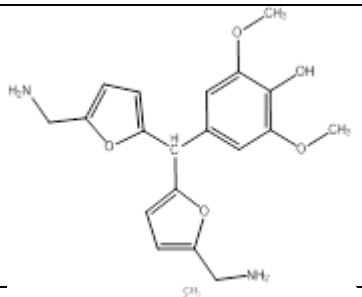
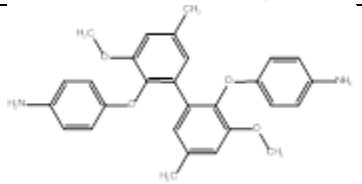
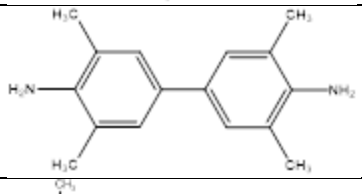
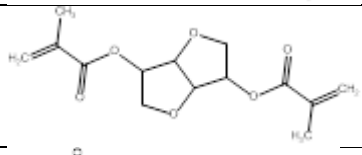
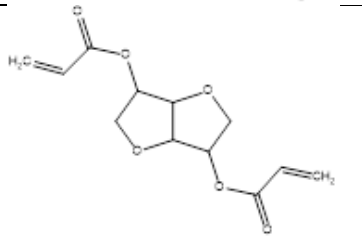
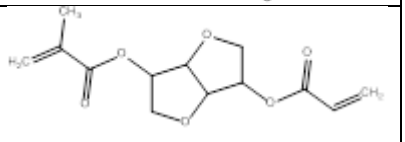
7 RESULTS AND DISCUSSION

7.1 Microtox Test and Risk Assessment

The toxicity of MDA40, MDA43, MDA48, MDA50, MDA52, MDA53, MDA54, IDMMDA50, and IAMMDA52 to marine bacteria, *V. fischeri*, was measured by the Microtox acute toxicity test system at 5, 15, and 30 minutes. MDA42, MDA47, MDA49, and IDMMDA51 were not soluble in DMSO, so no further testing was conducted. MDA48 and MDA52 did not induce toxicity at the limits of the test, so testing was halted at the range-finding stage. For each remaining test compound, three individual experiments were performed in duplicate. Table 2 presents the toxicity data (EC_{50} and the 95% 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. Data were further analyzed using the Hill function performed using GraphPad PRISM version 5.04 and presented in Appendix E through I: 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.

Table 2. Test Compounds and Structures

Chemical Name	Laboratory Designation	Structure
4,4’-methylenebis(2,5-dimethylaniline)	MDA40	
4,4’-methylenebis(2,6-dimethylaniline)	MDA42/MDA54	
5,5'-(propane-2,2-diyl)bis(2-methoxyaniline)	MDA43	
4-(bis(5-aminomethyl)furan-2-yl)methyl)-2-methoxyphenol	MDA47	
1,1'-((propane-2,2-diylbis(4,1-phenylene))bix(oxy))bis(2-methylprop-2-en-1-ol)	MDA48	
(5,5',5'',5'''-(1,4-phenylenebis(methanetriyl))tetrakis(furan-5,2-diyl))tetramethanamine	MDA49	

Chemical Name	Laboratory Designation	Structure
4-(Bis(5-(aminomethyl)furan-2-yl)methyl)-2,6-dimethoxyphenol	MDA50	
4,4'-((3,3'-dimethoxy-5,5'-dimethyl-[1,1'-biphenyl]-2,2'-diyl)bis(oxy))dianiline	MDA52	
3,3',5,5'-Tetramethylbenzidine	MDA53	
hexahydrofuro[3,2-b] furan-3,6-diyl bis(2-methacrylate)	IDMMDA50	
hexahydrofuro[3,2-b] furan-3,6-diyl bis(2-acrylate)	IAMDA51	
6-(acryloyloxy) hexahydrofuro[3,2-*b*] furan-3-yl methacrylate	IAMMDA52	

The short-term response of *V. fischeri* to a variety of compounds has been found to be a reliable and sensitive indicator of aquatic toxicity [21-23]. Thus, the data from Microtox tests are useful screens for assessing toxicity to aquatic organisms. Table 3 shows the aquatic toxicity criteria of the United States Environmental Protection Agency (USEPA), the Organization for Economic Co-operation and Development (OECD), and the GHS to categorize estimated ecotoxicity [6, 24, 25]. Table 4 provides the data and ecotoxicity scale for each compound tested. This evaluation suggests MDA53 and IAMMDA52 are “Moderately Toxic” and toxic to aquatic life; MDA40, MDA43, MDA54, and IDMMDA50 are “Slightly Toxic” and harmful to aquatic life;

MDA50 and MDA52 are “Practically Nontoxic” and are not categorized by GHS; and MDA48 is “Relatively Harmless” (see Table 4).

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

Table 4. 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			
MDA40	49.82 [33.49-74.10]	41.54 [28.01-61.61]	35.41 [22.75-55.12]	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. III
MDA42	Insoluble	Insoluble	Insoluble	—	—	—
MDA43	45.11 [41.21-49.38]	47.86 [44.69-51.24]	51.3 [47.63-55.26]	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. III
MDA47	Insoluble	Insoluble	Insoluble	—	—	—

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			
MDA48	>2000#	>2000#	>2000#	Relatively Harmless	—	—
MDA49	Insoluble	Insoluble	Insoluble	—	—	—
MDA50	217.4† [109.8-430.7]	127.5† [87.7-185.4]	84.28† [66.47-106.9]	Practically Nontoxic	—	—
MDA52	>500#	>500#	>500#	Practically non-toxic	—	—
MDA53	1.34 [0.85-2.115]	1.18 [0.69-2.01]	1.32 [0.86-2.05]	Moderately Toxic	Acute Toxicity II (toxic to aquatic life)	Acute Cat. II
MDA54	39.02 [29.6-51.44]	38.64 [29.36-50.84]	39.37 [29.75-52.1]	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. III
IDMMDA50	11.46 [7.563-17.35]	13.61 [9.085-20.38]	14.89 [10.33-21.46]	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. III
IAMDA51	Insoluble	Insoluble	Insoluble	—	—	—
IAMMDA52	6.77 [4.52-10.12]	6.29 [4.08-9.73]	5.97 [3.97-8.97]	Moderately Toxic	Acute Toxicity II (toxic to aquatic life)	Acute Cat. II

Legend:
USEPA = United States 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.
†The value of the EC_{50} was extrapolated due to solubility/cytotoxicity limits
#The compound was non-toxic at the solubility limit of testing, testing concluded after initial range-finding.

7.2 Criteria for Valid Assay

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

8 CONCLUSIONS

This study reports the aquatic toxicity for the 13 candidates for MDA replacement using the Microtox Acute Toxicity assay. MDA42, MDA47, MDA49, and IAMDA51 were insoluble in DMSO precluding testing in this system. Data were acquired for the remaining compounds:

- MDA48 was relatively harmless (EC_{50} >2,000 mg/L),
- MDA50 and MDA52 were considered practically nontoxic (EC_{50} 100–1,000 mg/L),
- MDA40, MDA42, MDA54, and IDMMDA50 were found to be slightly toxic (EC_{50} 10–100 mg/L), and
- MDA53 and IAMMDA52 were found to be moderately toxic (EC_{50} 1–10 mg/L).

Therefore, MDA40, MDA42, MDA54, and IDMMDA50 are harmful to aquatic life and MDA53 and IAMMDA52 are considered toxic to aquatic life according to GHS categorization [6].

9 RECOMMENDATIONS

The lack of aquatic toxicity using the luminescent bacteria assay with MDA43, MDA48, MDA50, and MDA52 is encouraging, and however, no further aquatic testing may be necessary prior to production based upon jurisdictional requirements.There is moderate concern with the toxicity of MDA40, MDA54, and IDMMDA50, including the high water solubility of IDMMDA50. Evaluations should be considered for the probability of environmental releases if these compounds are further considered as MDA replacements; the likelihood would suggest additional toxicity testing with green algae, Daphnia, and fish. Due to the acute aquatic toxicity of MDA53 and IAMMDA52, it is not recommended that these compounds be selected as replacements for MDA. The inability to test MDA42, MDA47, MDA49, and IAMDA51 due to solubility concerns necessitates further analysis of these compounds to determine if testing is feasible. Additional reporting on mutagenicity and skin sensitization potential of these compounds is currently in progress.

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.

Prepared by:

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Directorate Director, Toxicology

APPENDIX A

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Appendix B

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. S.0058642, Protocol No. 0FMA-92-iv17-03-01A,B,C,D,E,G,H,I,J,K,L Microtox Toxicity Testing of MDA Replacements (MDA40, MDA42, MDA43, MDA47, MDA48, MDA49, MDA50, MDA52, MDA53, MDA54, IDMMDA50, IAMDA51, IAMMDA52) the following critical phases were inspected/audited by the Quality Systems Office (QSO):

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	04/4/2018-08/21/2018	04/04/2018-08/21/2018
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	05/13/2019	05/13/2019
Final Study Good Laboratory Practice Standard Report Review	05/13/2019	05/13/2019

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 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

05/13/2019
Date

APPENDIX C

ARCHIVES AND STUDY PERSONNEL

C-1. Archives

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

The Toxicology Directorate will archive records on the test system 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.0002728-15, Protocol No. 0FMA-92-iv17-03-01A,B,C,D,E,F,G,H,I,J,K,L

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., Chief, Health Effects Division (HEF).

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

Technical staff: Tariq Armstead, ORISE Fellow.

Quality Assurance: Michael P. Kefauver, Chemist.

APPENDIX D

MICROTOX TEST REAGENTS

Table D-1. Microtox Test Reagents

Microtox Reagents	Source	Lot #	Date Expiration
Modern Water Microtox Diluent	Modern Water	16C4015	07/2019
Modern Water Microtox Diluent	Modern Water	17E4130	05/2020
Modern Water Microtox Acute Reagent	Modern Water	17H4227	09/2019
Modern Water Microtox Acute Reagent	Modern Water	17C4076	03/2019
Dimethyl sulfoxide	Sigma	RNBG1729	07/2019
Zinc Sulfate	Sigma-Aldrich	SLBC2469V	---
Modern Water Microtox Reconstitution Solution	Modern Water	16D4031	4/2019

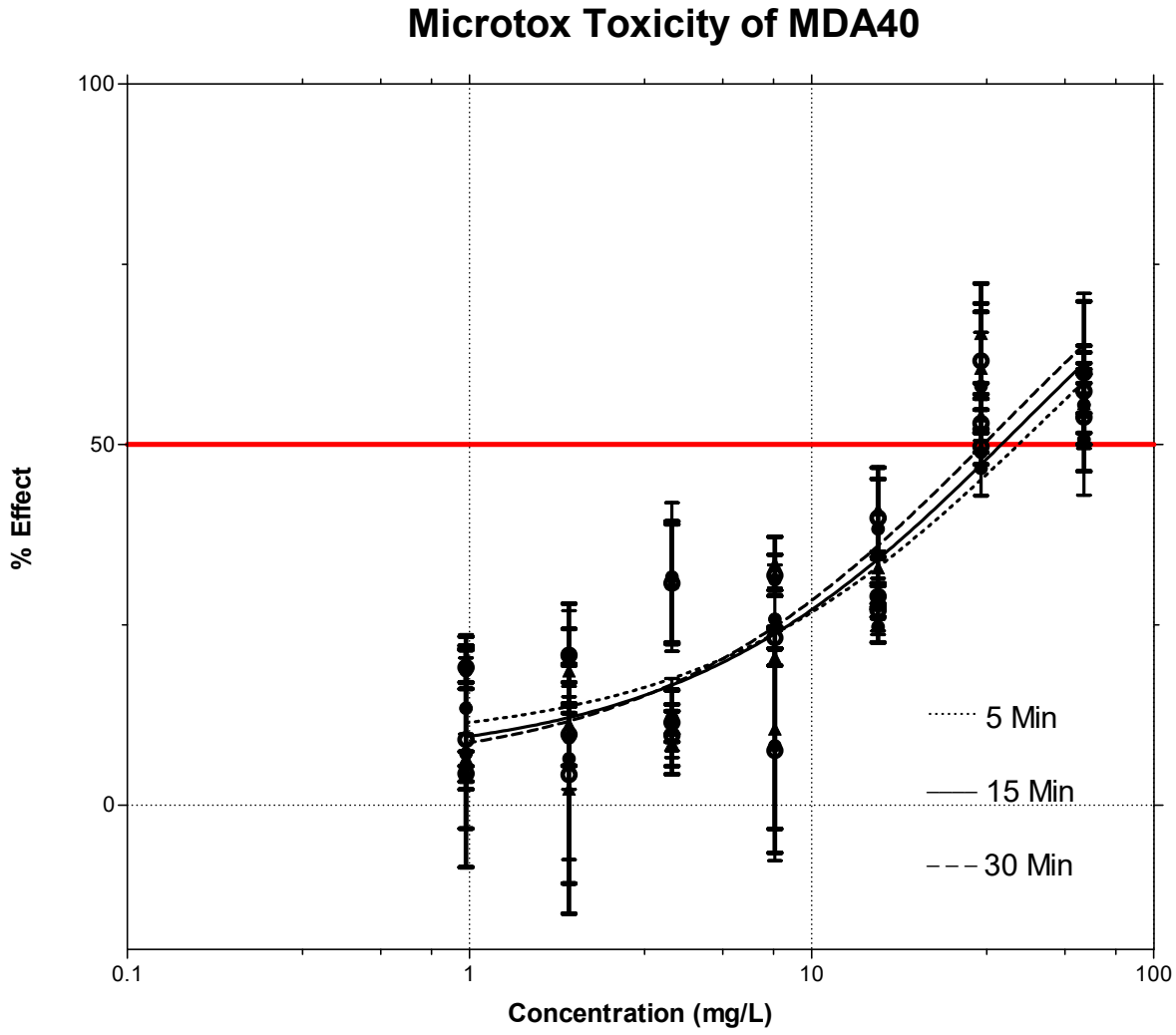
APPENDIX E

MDA40 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*
0.0781			
0.1563			
0.3125			
0.625			
1.25			
2.50			
5.00			
10.0			

Note:
*Working concentrations were not available at the time of the report due to no available methods for concentration analysis by LAB.

MDA40 EC_{50} (mg/L; 95% CI)		
5 minute	15 minute	30 minute
49.82 [33.49-74.10]	41.54 [28.01-61.61]	35.41 [22.75-55.12]


Microtox Toxicity of MDA40
100
50
0
% Effect
0.1
1
10
100
Concentration (mg/L)
5 Min
15 Min
30 Min

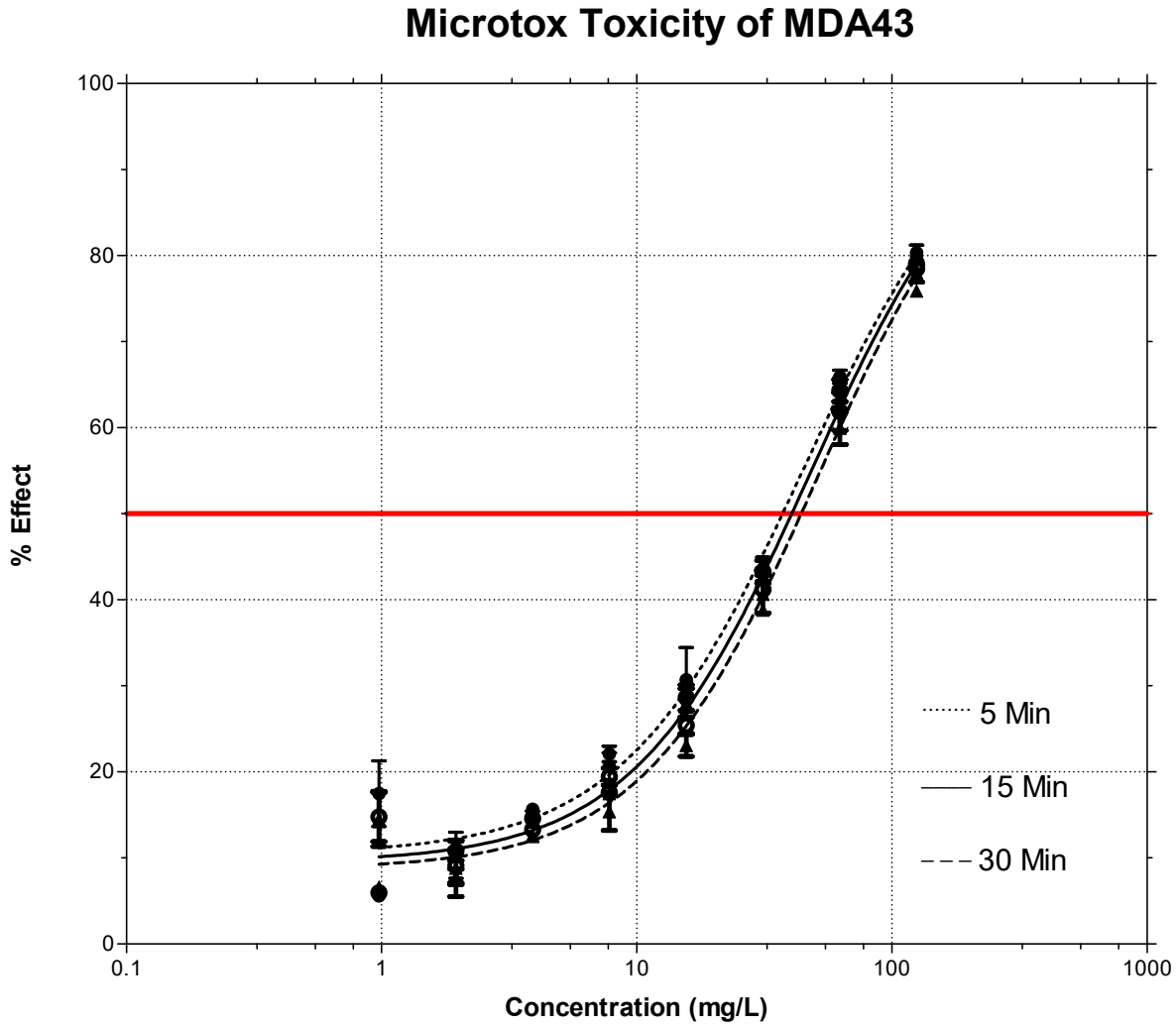
APPENDIX F

MDA43 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*
0.1			
0.2			
0.39			
0.78			
1.56			
3.13			
6.25			
12.5			

Note:
*Working concentrations were not available at the time of the report due to no available methods for concentration analysis by LAB.

MDA43 EC_{50} (mg/L; 95% CI)		
5 minute	15 minute	30 minute
45.11 [41.21-49.38]	47.86 [44.69-51.24]	51.3 [47.63-55.26]


Microtox Toxicity of MDA43
% Effect
Concentration (mg/L)
5 Min
15 Min
30 Min
0
20
40
60
80
100
0.1
1
10
100
1000

APPENDIX G

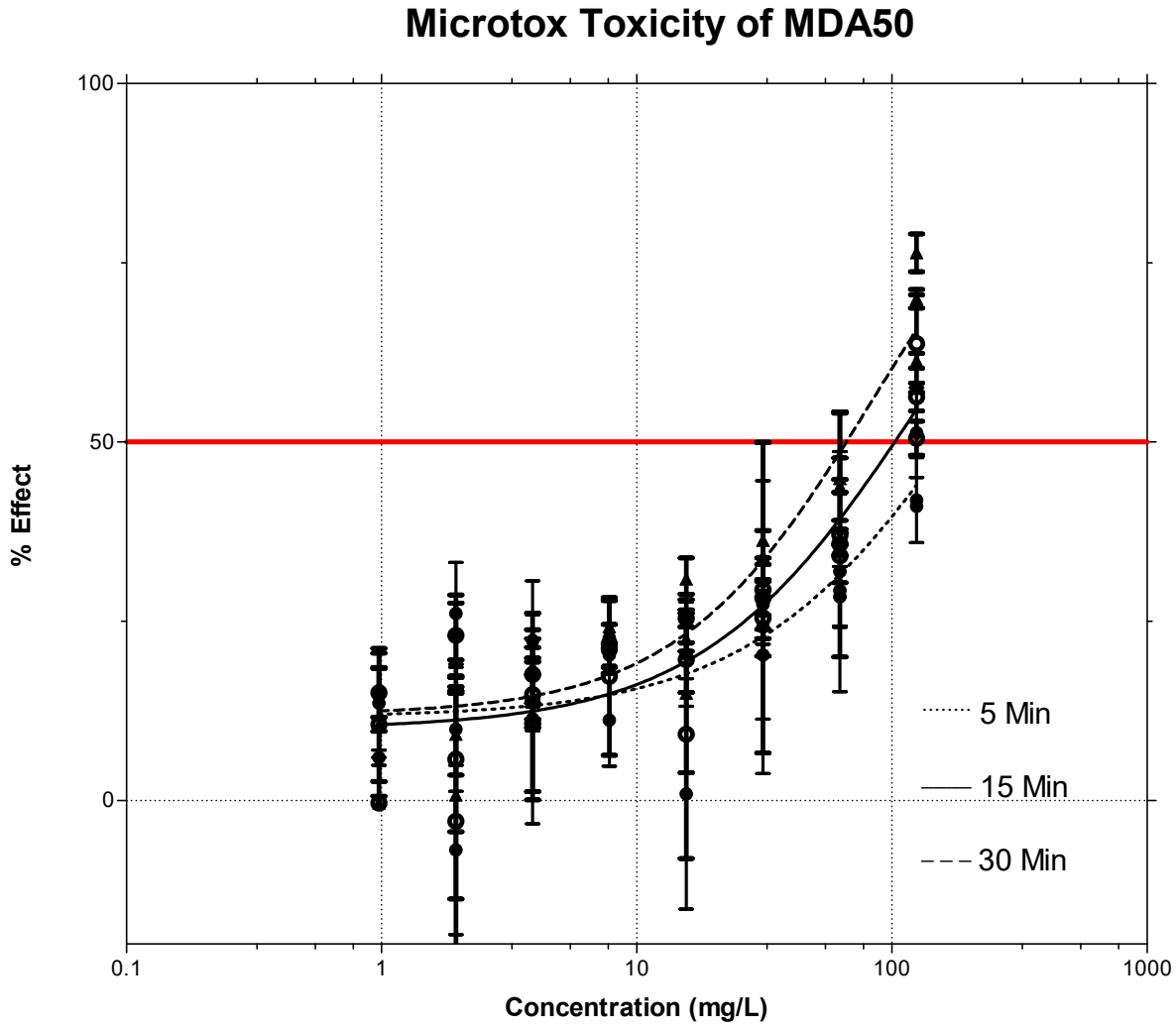
MDA50 MICROTOX TEST DATA TABLES AND CALCULATIONS

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration (mg/mL; 100x test concentration)*		
	Test 1*	Test 2*	Test 3*
0.098			
0.195			
0.391			
0.782			
1.56			
3.13			
6.25			
12.5			

Note:
*Working concentrations were not available at the time of the report due to no available methods for concentration analysis by LAB.

MDA50 EC_{50}† (mg/L; 95% CI)		
5 minute	15 minute	30 minute
217.4 [109.8-430.7]	127.5 [87.7-185.4]	84.28 [66.47-106.9]

Note:
†The value of the EC_{50} was extrapolated due to solubility/cytotoxicity limits


Microtox Toxicity of MDA50
100
50
0
% Effect
0.1
1
10
100
1000
Concentration (mg/L)
5 Min
15 Min
30 Min

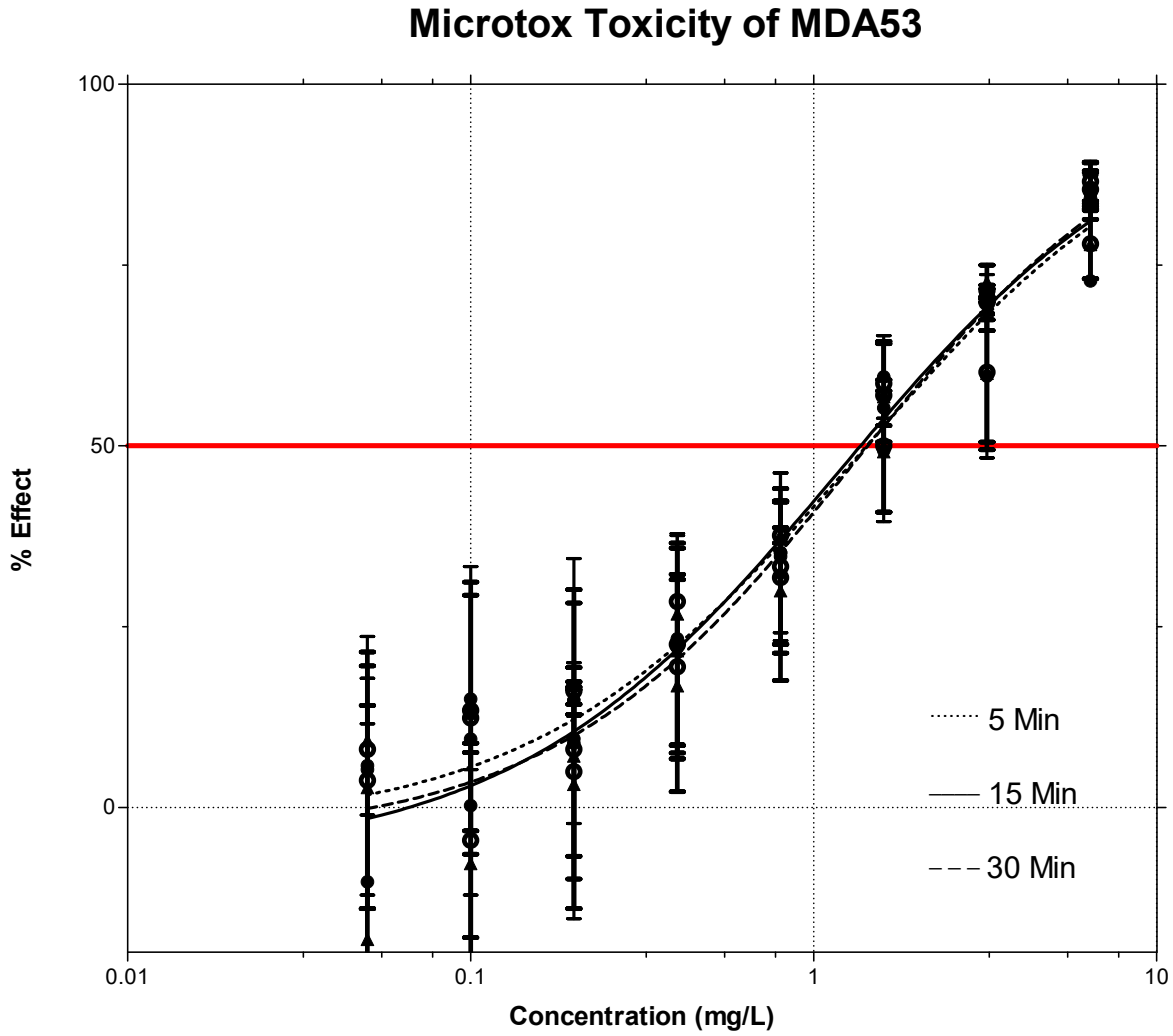
APPENDIX H

MDA53 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*
0.49			
0.98			
1.95			
3.91			
7.81			
15.63			
31.25			
62.5			

Note:
*Working concentrations were not available at the time of the report due to no available methods for concentration analysis by LAB.

MDA53 EC_{50} (mg/L; 95% CI)		
5 minute	15 minute	30 minute
1.34 [0.85-2.115]	1.18 [0.69-2.01]	1.32 [0.86-2.05]


Microtox Toxicity of MDA53
100
50
0
% Effect
0.01
0.1
1
10
Concentration (mg/L)
5 Min
15 Min
30 Min

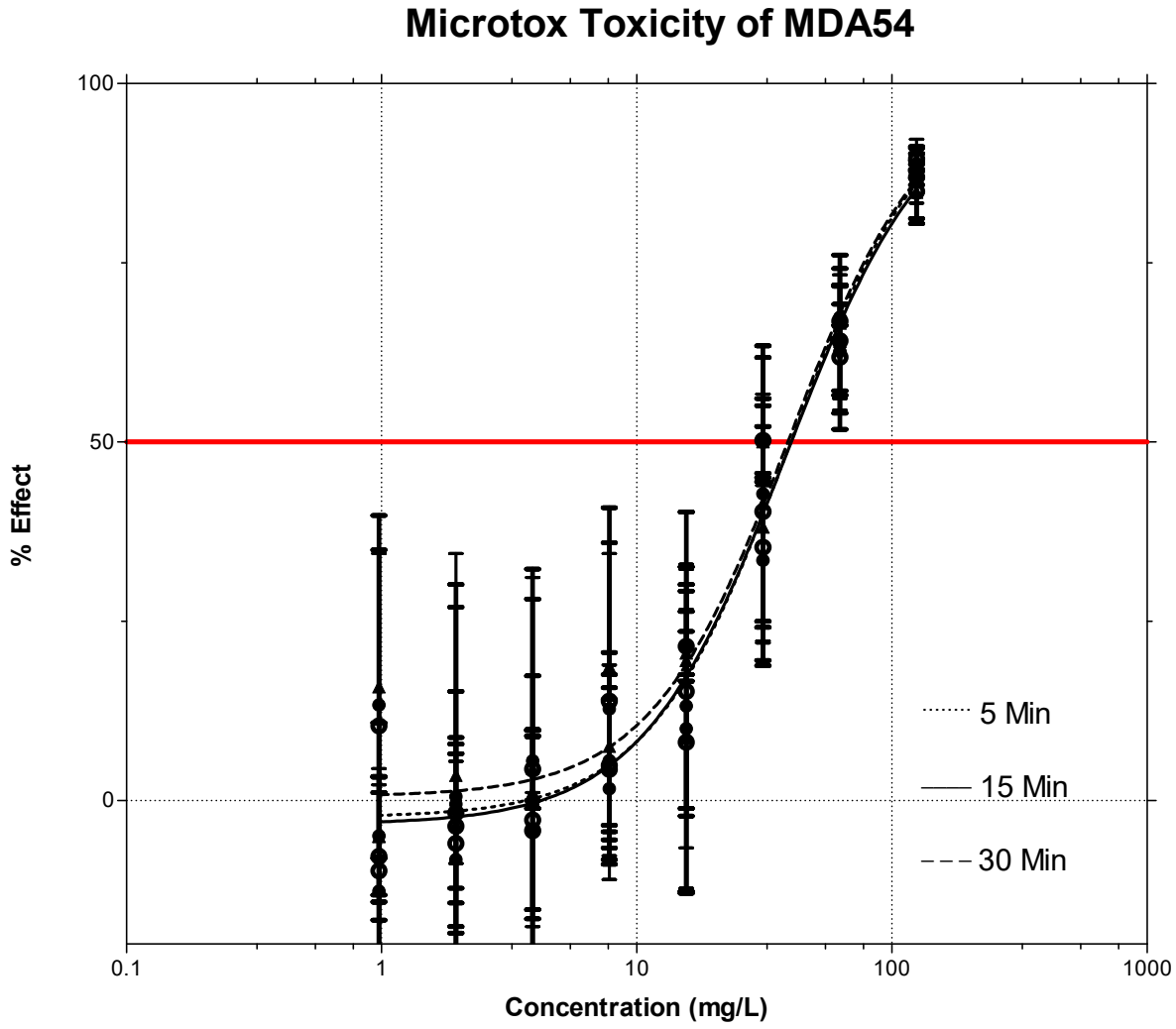
APPENDIX I

MDA54 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*
0.1			
0.2			
0.39			
0.78			
1.56			
3.13			
6.25			
12.5			

Note:
*Working concentrations were not available at the time of the report due to no available methods for concentration analysis by LAB.

MDA54 EC_{50} (mg/L; 95% CI)		
5 minute	15 minute	30 minute
39.02 [29.6-51.44]	38.64 [29.36-50.84]	39.37 [29.75-52.1]


Microtox Toxicity of MDA54
100
50
0
% Effect
0.1
1
10
100
1000
Concentration (mg/L)
5 Min
15 Min
30 Min

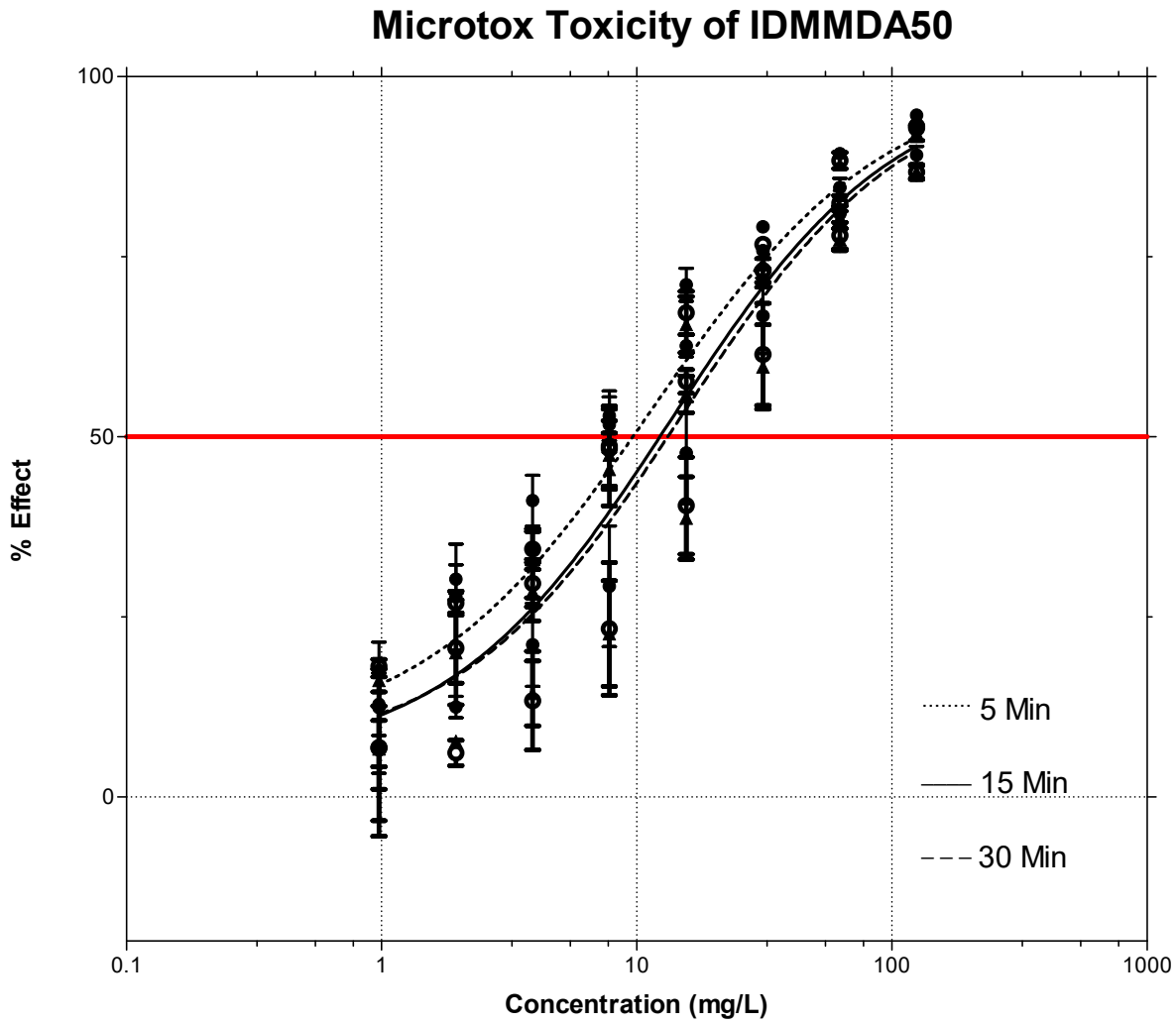
APPENDIX J

IDMMDA50 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*
0.098			
0.195			
0.391			
0.781			
1.56			
3.123			
6.25			
12.5			

Note:
*Working concentrations were not available at the time of the report due to no available methods for concentration analysis by LAB.

IDMMDA50 EC_{50} (mg/L; 95% CI)		
5 minute	15 minute	30 minute
11.46 [7.56-17.35]	13.61 [9.09-20.38]	14.89 [10.33-21.46]


Microtox Toxicity of IDMMDA50
100
50
0
% Effect
0.1
1
10
100
1000
Concentration (mg/L)
5 Min
15 Min
30 Min

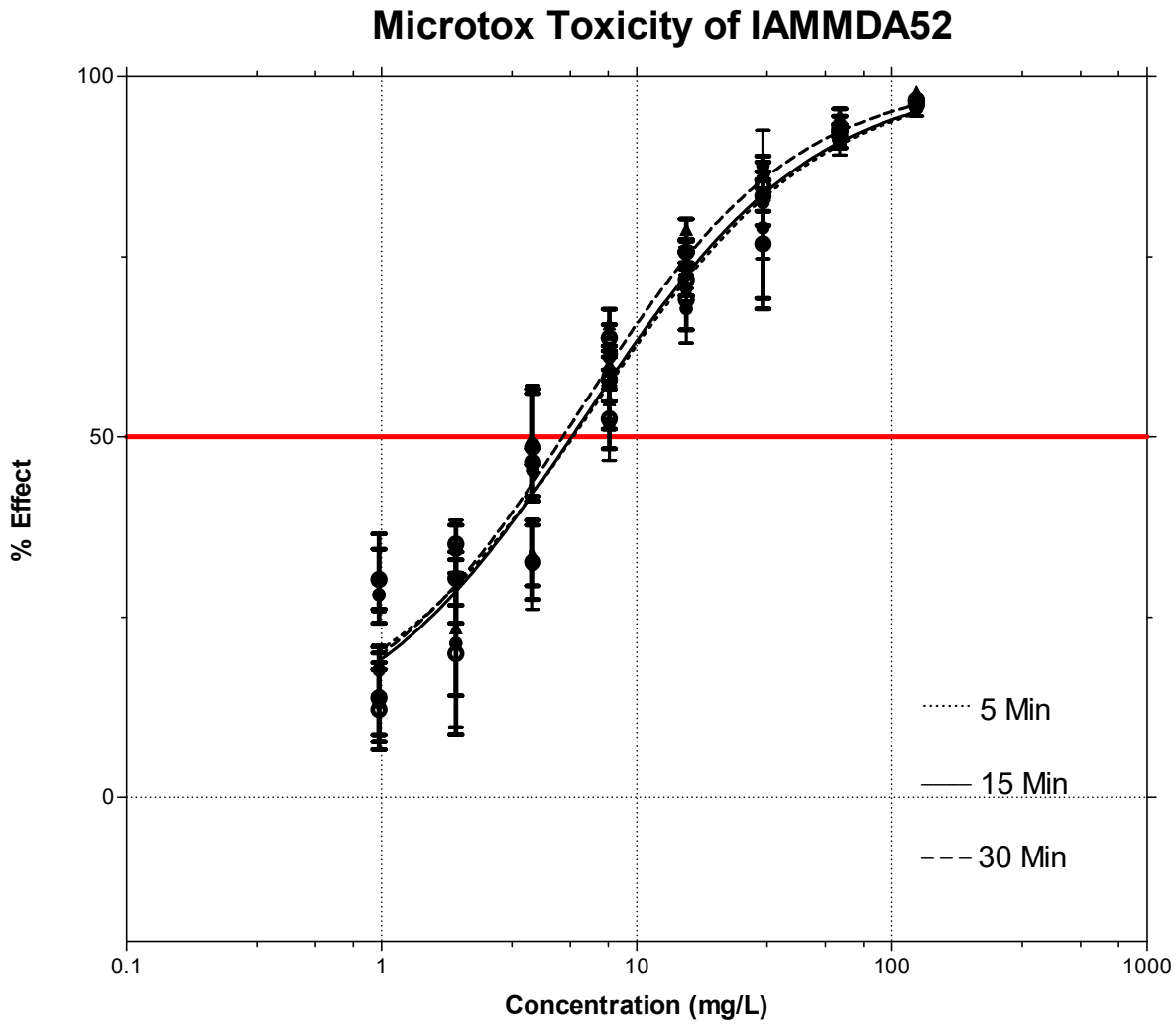
APPENDIX K

IAMMDA52 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*
0.098			
0.195			
0.391			
0.781			
1.56			
3.123			
6.25			
12.5			

Note:
*Working concentrations were not available at the time of the report due to no available methods for concentration analysis by LAB.

IAMMDA52 EC_{50} (mg/L; 95% CI)		
5 minute	15 minute	30 minute
6.77 [4.52-10.12]	6.29 [4.08-9.73]	5.97 [3.97-8.97]


Microtox Toxicity of IAMMDA52
100
50
0
% Effect
0.1
1
10
100
1000
Concentration (mg/L)
5 Min
15 Min
30 Min