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TECHNICAL REPORT 3252
NOVEMBER 2021

Pulsed Toxicity Exposure Methodology Summary of Results: Interlaboratory Calibration Exercise

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ADMINISTRATIVE INFORMATION

The work described in this report was performed by the Energy and Environmental Sustainability branch of the Advanced Systems and Applied Sciences division, Naval Information Warfare Center Pacific (NIWC Pacific), San Diego, CA. The Environmental Security Technology Certification Program (ESTCP), Project ER-201727, provided funding for this Basic Applied Research project. Further assistance was provided by Wood Environment and Infrastructure Solutions, Inc.; University of California, Davis, Granite Canyon Laboratory; Loyola University, Chicago; Enthalpy Analytical, Inc.; Nautilus Environmental Company, Inc.; and Tetra Tech, Inc.

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EXECUTIVE SUMMARY

Episodic discharges (e.g. stormwater, dry-dock discharges, and pesticide applications, etc.) require environmentally-relevant, scientifically-defensible, and conservative toxicity test designs to assess potential for receiving water impacts. Currently, permittees in highly industrialized areas are regularly required to conduct 96 hour (or longer) toxicity tests on discharges associated with events that are often less than 24 hours in duration. Existing EPA whole effluent toxicity (WET) test methods developed to assess continuous point source discharges are now being applied to episodic discharges as well. However, these methods do not adequately reflect episodic discharge conditions at either the point of compliance (i.e. storm drain) or as it mixes with the receiving environment (e.g. a riverine or marine system), which can result in an overestimation of toxicity at a given site.

In order to capture representative toxicity at a site, an alternative toxicity test approach is described, incorporating pulsed exposures to end-of-pipe samples. Following pulsed exposures, organisms are transferred to uncontaminated seawater (or receiving water) for the remainder of standard test period. This method was developed under The Environmental Security Technology Certification Program (ESTCP), Project ER-201727.

This report presents the results of an Interlaboratory Calibration Study conducted in order to assess the ability of the modified methodology to provide consistent and defensible data for the assessment of episodic discharges. The study characterized: 1) Completion Rate; 2) False Positive Rate; 3) Precision; and 4) Repeatability. The following three WET methods that were modified for pulsed exposures and included in this study were:

- Cladoceran, *Ceriodaphnia dubia*, acute test (*Ceriodaphnia* acute)
- Mysid, *Americamysis bahia*, acute test (Mysid acute)
- Purple Sea Urchin, *Strongylocentrotus purpuratus*, larval development/short-term chronic (Purple sea urchin chronic)

In total, 12 laboratories were involved in the study, with 7, 8 and 7 laboratories participating in the daphnia, mysid and the purple sea urchin pulsed exposure test methods, respectively. Each participating laboratory was required to prequalify for each test method by documenting their capabilities, experience and proficiency, and quality assurance processes in order to meet the needs of the study.

For each test method, 2 referee laboratories were involved in the Calibration Study. The referee laboratories were responsible for preparing bulk test samples, conducting preliminary testing of the samples and distribution of the “blind” samples to the participating laboratories. The following five samples were prepared for each test method: 1) a blank control water; 2) a non-toxic stormwater sample; 3) a toxic stormwater sample; and 3) a toxic metal-spiked laboratory control sample, and 5) a blind duplicate of the metal-spiked laboratory control.

Participant laboratories were instructed to perform 26 hr pulsed exposure toxicity testing on each of the 5 blind samples using instructions provided in a pulsed exposure toxicity testing standard operating procedure. Each sample was tested using a 5 concentration dilution series (i.e. 6.25, 12.5, 25, 50 & 100% concentration of whole sample). Each blind sample was also tested under standard static exposure conditions with 6.25 & 100% concentrations. Additionally, concurrent reference toxicant tests were conducted by each laboratory for each species study they participated in. Interlaboratory Calibration testing was conducted from July to August 2020.

Once all testing was completed, participant laboratories submitted all data using an electronic data deliverable provided by the project team. The project team reviewed all data to ensure that all required information was provided. Results were then compiled, and method performance characteristics were calculated for each pulsed exposure test method.

An overall summary of results from the Interlaboratory Calibration Study is provided in Table ES-1. All laboratories that participated in the study successfully completed both pulsed and standard test methods and met standard EPA laboratory test acceptability criteria including control performance. Two contracted laboratories that planned to initiate the daphnia tests were unable to do so properly due to a lack of suitable in-house neonates to initiate all tests at once. In addition, one participating laboratory provided questionable supporting water quality results and had insufficient documentation to verify that required test methods were followed and thus their data was excluded from the study.

In addition to successful completion of the tests, two additional key metrics for this evaluation included the frequency of false positives (declaring a clean sample toxic), and interlaboratory precision measured as the percent coefficient of variation (% CV) among labs. False positive rates for the negative control (Sample 1) were 0% for all tests conducted. Overall false positive rate, including the non-toxic stormwater sample (Sample 2), was 2.3%. Precision for the test methods was determined by comparing % CVs based on both laboratory control performance and dose response relationships measured using calculated median lethal or median effect (LC_{50} or EC_{50}) concentration values. Confidence intervals surrounding the point estimates were also evaluated when a dose response was observed. Interlaboratory CVs of LC_{50}s ranged from 25.6 to 70.1% for acute test methods using *Ceriodaphnia* and mysid shrimp. Interlaboratory CVs of EC_{50}s for the short-term chronic tests using the purple sea urchin ranged from 50.4 to 59.2%. Furthermore, the pooled mean LC_{50}/EC_{50} values for the pulsed exposure tests for the two duplicate samples were not statistically different from each other for all three test species indicating good repeatability for intra-laboratory performance.

Table ES- 1.Summarized test results from ESTCP ER-201727 Pulsed Exposure Interlaboratory Calibration Study.

Test Method	Exposure Duration	Successful Test Completion Rate (%)	Negative Control False Positive Rate (%)	Interlaboratory Precision (%CV)		Intra-Laboratory Relative Percent Difference (%)
				6% Sample	LC_{50}/EC_{50}	
Ceriodaphnia acute	26 hr	100	0	35.9	67.2	9.8
	Standard/ 96hr	100	0	41.1	-	-
Mysid acute	26 hr	100	0	6.6	28.7	19.0
	Standard/ 96hr	100	0	5.7	-	-
Purple sea urchin chronic	26 hr	100	0	7.5	55.4	17.3
	Standard/ 96hr	100	0	26.1	-	-

ACRONYMS

ASTM	American Society for Testing and Materials
CETIS	Comprehensive Environmental Toxicity Information System
CuSO4	Copper Sulfate
CV	Coefficient of Variation
DO	Dissolved Oxygen
DOC	Dissolve Organic Carbon
DMW	Diluted Mineral Water
DoD	Department of Defense
EC50	Median-Effect Concentration
ELAP	Environmental Laboratory Accreditation Program
EPA	Environmental Protection Agency
ESTCP	Environmental Security Technology Certification Program
HDPE	High Density Polyethylene
ICS	Interlaboratory Calibration Study
LC50	Median Lethal-Effect Concentration
LOEC	Lowest Observed Effect Concentration
MSD	Minimum Significant Difference
NIWC Pacific	Naval Information Warfare Center Pacific
NOEC	No Observed Effect Concentration
NPDES	National Pollutant Discharge Elimination System
PMSD	Percent Minimum Significant Difference
RPD	Relative Percent Difference
SERDP	Strategic Environmental Research and Development Program
SOP	Standard Operating Procedure
QA	Quality Assurance
QC	Quality Control
TAC	Test Acceptability Criteria
TST	Test of Significant Toxicity
USEPA	United States Environmental Protection Agency
WET	Whole Effluent Toxicity

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CONTENTS

FIGURES

TABLES

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1. INTRODUCTION

This report presents the results of a Pulsed Exposure Toxicity Interlaboratory Calibration Study (ICS) conducted in order to assess the ability of the modified pulsed exposure toxicity methodology to provide consistent and defensible data for the assessment of episodic discharges. The study characterized 1) Completion Rate; 2) False Positive Rate; and 3) Precision. The following three USEPA whole effluent toxicity (WET) methods that were modified for pulsed exposures and included in this study were:

- **Cladoceran, *Ceriodaphnia dubia,* acute test (*Ceriodaphnia* acute)**
    - USEPA. 2002a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, U.S. Environmental Protection Agency, Office of Water, Washington DC. EPA/821/R-02/012.
- **Mysid, *Americamysis bahia,* acute test (mysid acute)**
    - USEPA. 2002a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, U.S. Environmental Protection Agency, Office of Water, Washington DC. EPA/821/R-02/012.
- **Purple Sea Urchin, *Strongylocentrotus purpuratus,* larval development/short-term chronic (purple urchin chronic)**
    - USEPA. 1995. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms. U.S. Environmental Protection Agency, Office of Research and Development, Cincinnati, Ohio. EPA/600/R-95/136.

This effort is conducted in support of Environmental Security Technology Certification Program (ESTCP) project ER-201727, entitled “Derivation and Demonstration of an Environmentally Relevant Approach for Stormwater Toxicity Testing Compliance Monitoring”.

1.1 BACKGROUND

Industrial and municipal discharges are required to comply with increasingly stringent water quality requirements for stormwater runoff and other episodic (non-continuous) discharges. These requirements generally include end-of-pipe monitoring, enforced by National Pollutant Discharge Elimination System (NPDES) permits, prior to mixing in the receiving water. Regulatory concern with stormwater discharges is associated with the Clean Water Act’s goal to prevent discharge of toxicants in toxic amounts (USEPA 1972). As a result, the existing EPA whole effluent toxicity (WET) test methods developed to assess continuous point source discharges (USEPA 1995, 2002a, 2002b, 2002c) are now also being applied to various episodic discharges, such as stormwater. These industrial permittees, in fact, are frequently unable to comply with current stormwater NPDES requirements using current test methodologies, even with the implementation of Best Management Practices (BMPs; Katz et al. 2006). 30% of the samples tested from the study by Katz et al (2006) were acutely toxic at the end of pipe using a t-test. These samples were collected from multiple Naval Stations in San Diego during 11 storm events from 2002 to 2005. The current exceedance rate for acute toxicity at end of pipe Naval Base locations using the newer TST approach with allowance for a 50% effect in undiluted sample is approximately 20% depending on the specific location and year. Consistent acute toxicity exceedances at a few specific locations have recently resulted in the creation and implementation of Toxicity Reduction Evaluation Plans at Naval Base Coronado and Naval Base Point Loma in 2017. Every sample tested for chronic toxicity at the end of pipe during

the past two years has resulted in a toxic response, while samples collected from adjacent receiving waters have been nontoxic.

Furthermore, passing or failing toxicity and chemical concentration limits in any given end-of-pipe stormwater discharge will vary depending on a multitude of factors such as the size and intensity of the storm, antecedent dry period, timing when the sample was taken, and type of sample (grab or composite) as shown effectively in pollutograph studies (e.g. Kayhanian et al. 2008). Timing and flows can also impact toxicity in typical wastewater discharges as well, but generally the chemical and physical properties of these flows will be much more uniform over time. These variables increase the complexity with regard to interpreting the results for a given end-of-pipe stormwater sample, necessitating a more realistic and standardized sample collection and toxicity exposure regime. The key element of exposure duration and integration with receiving waters is not adequately addressed using current methodologies to assess episodic discharges.

Toxicity tests are desirable because: (1) they take into account contaminant bioavailability in a sample, which can vary from the bioavailability in uncontaminated filtered water used to develop water quality criteria (Stephan et al. 1985); and (2) they incorporate the potential for adverse effects associated with exposure to complex mixtures (i.e. multiple contaminants), many of which may not be monitored. However, regulators often require monitoring of stormwater from end-of-pipe samples during the first flush (first few hours of rainfall) and evaluation of these samples with laboratory toxicity tests using a continuous (i.e. static or static-renewal) exposure of up to seven days, depending on the species and test endpoint. This methodology does not adequately replicate the dynamic nature of the stormwater exposure at either the point of compliance or as it mixes with the receiving environment (Katz et al. 2006; Stransky et al., 2015, Rosen et al. 2019), which may have substantially different physical and chemical properties than the runoff itself. The limitations of using current WET methods for stormwater testing at end-of-pipe for compliance has been recognized by regulators, as reflected in a new NPDES Permit for Naval Base San Diego (NBSD; Permit R9-2013-0064). This permit includes a provision where a special chronic toxicity study may be conducted to propose modifications to the Water Quality Control Plan for the San Diego Region (Basin Plan) to incorporate mixing zone dilution credits, recognizing that end-of-pipe monitoring is not representative of actual conditions in the bay. Use of a more applicable and representative toxicity test method would provide a sound basis for supporting such a change.

A more realistic assessment of the toxicological impacts of stormwater runoff, or other episodic discharges (e.g. intermittent discharges of stormwater and relief water from dry dock outfalls at Naval shipyards, chlorinator/dechlorinator cooling water from pier side ship, etc.) on beneficial uses in the receiving waters is critical. This would support decisions related to the need and prioritization of appropriate Best Management Practices (BMPs), and meaningful compliance with Clean Water Act goals. Results from pulsed toxicity exposures have been well documented in the peer-reviewed literature as a means of improving the characterization of exposure and potential for toxicity associated with episodic contaminant exposures (Dupuis and Kreutzberger 2003, Butcher et al. 2006, Diamond et al. 2006, Hoang et al. 2007a, 2007b, 2007c, Angel et al. 2010, Stransky et al., 2015) however, the development and application of standardized protocols that are accepted by the regulatory community are currently lacking.

2. STUDY DESIGN AND OBJECTIVES

2.1 OBJECTIVES

The objective of this study was to provide data required to support and validate a proposed standardized pulsed exposure toxicity test design to assess impacts to receiving waters related to episodic discharges such as stormwater runoff. This objective was met through the conduct of a Pulsed Exposure Toxicity Interlaboratory Calibration Study (ICS) using the pulsed exposure methods for standard USEPA marine and freshwater test species.

The specific goals in conducting the pulsed toxicity exposure Interlaboratory Calibration Study were:

1. Evaluate the ability of laboratories to successfully execute the pulsed exposure toxicity methods for each species (completion rate);
2. Evaluate the rate at which laboratories detected toxicity when evaluating non-toxic samples (false positive rate);
3. Characterize the interlaboratory variability of the 3 modified pulsed exposure test methods for each species through the comparison of coefficients of variation (CVs) for median lethal effect and median sublethal effect (LC_{50} and EC_{50}) concentration values and ranges for no observed effect concentration (NOEC) values and 95% confidence intervals.
4. Assess repeatability by comparing results obtained for both pulsed and static exposures in blind duplicate samples.

The ESTCP project team modeled the Pulsed Exposure Toxicity ICS after the USEPA's Interlaboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Vol. 1 (USEPA 2001).

2.2 GENERAL STUDY DESIGN

The Pulsed Exposure Toxicity ICS was conducted with the following tasks to achieve the objectives of the study and are described more fully in the sections below:

- Laboratory Procurement (Section 1);
- Sample Preparation (Section 4);
- Preliminary Testing (Section 5);
- Sample Distribution (Section 1);
- Interlaboratory Calibration Study Testing (Section 7);
- Data Review and Analysis (Section 1).

2.2.1 Study Management

The ESTCP project team from the Naval Information Warfare Center (NIWC) Pacific and Wood Environment & Infrastructure Solutions, Inc. (Wood) provided overall management and technical oversight of the Pulsed Exposure Toxicity ICS. Coordination of study activities, data review and report preparation were also performed by the ESTCP project team. Laboratory procurement was performed by Edmond Scientific Company.

The bioassay laboratories at NIWC and Wood also served as the referee laboratories for both the preliminary testing and for the Pulsed Exposure ICS testing.

2.2.2 Methods Evaluated

The ESTCP Pulsed Exposure Toxicity ICS evaluated three test methods. These included an acute freshwater, an acute marine and a short-term chronic marine method. The following three USEPA WET methods that were modified for pulsed exposures and included in this study were:

- Cladoceran, *Ceriodaphnia dubia*, acute test (*Ceriodaphnia* acute) – 96-hour Survival
    - USEPA. 2002a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, U.S. Environmental Protection Agency, Office of Water, Washington DC. EPA/821/R-02/012.
- Mysid, *Americamysis bahia*, acute test (mysid acute) – 96-hour Survival
    - USEPA. 2002a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, U.S. Environmental Protection Agency, Office of Water, Washington DC. EPA/821/R-02/012.
- Purple Sea Urchin, *Strongylocentrotus purpuratus*, larval development/short-term chronic (purple sea urchin chronic) – 96-hour Embryo Development
    - USEPA. 1995. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms. U.S. Environmental Protection Agency, Office of Research and Development, Cincinnati, Ohio. EPA/600/R-95/136.

Pulsed exposure modifications and procedures were outlined in Standard Operating Procedures: Pulsed Exposure Methodology (Colvin et al. 2020; Appendix A). In brief, for pulsed exposures, test organisms were initially exposed to the each of the five blind samples for 26 h. After the exposure period, the organisms were transferred to uncontaminated laboratory control water for the remainder of the standard test duration.

Standard static and static-renewal exposures were evaluated concurrently by each laboratory for comparison. Concurrent reference toxicant tests were also conducted for each species by each participating laboratory as a standard quality assurance/quality control (QA/QC) measure to evaluate sensitivity of the test species to a single toxicant compared to historical results.

2.2.3 Samples

For each test method, four samples were prepared in bulk by the ESTCP project team.

1. A blank sample – a negative control consisted of laboratory water obtained from the Wood referee laboratory appropriate for a given test method;
2. A non-toxic stormwater sample – collected from the San Diego region (salted with Crystal Sea Marine Mix © as appropriate for the marine species test methods);
3. A toxic stormwater sample – this sample was spiked with copper sulfate to ensure a consistent measurable toxic effect (salted with Crystal Sea Marine Mix © as appropriate for the marine species test methods);
4. A copper-spiked sample – a positive control prepared using referee laboratory water spiked with copper sulfate at a concentration that resulted in a consistent measurable toxic dose response for the particular species;

5. A fifth duplicate sample was sent to each laboratory for a total of 5 samples tested. This duplicate was the positive control for every laboratory (Sample 4).

Samples were distributed to participant laboratories as whole undiluted samples. Laboratory control water for the marine tests consisted of natural seawater at a salinity of approximately 33 ppt collected from Scripps Pier, La Jolla, CA. Control water for the freshwater tests was prepared by the Wood Bioassay Laboratory by diluting Perrier© water with deionized (DI) water.

2.2.4 Schedule

The Pulsed Exposure ICS was performed from July through August 2020. The testing schedule is shown below in Table 2-1. All four samples and the duplicate sample were tested simultaneously for each method. Samples were shipped with ice priority overnight to each respective laboratory. Acute testing with the mysid shrimp took place on two separate dates to accommodate supply limitations. The vendor of the organisms, Aquatic Biosystems, Inc. (Fort Collins, CO), was limited in their ability to supply enough mysid shrimp for the entire study during a single testing week. To accommodate, the participant laboratories were broken up into two testing groups with a referee laboratory testing concurrently to both sets of tests.

Table 2-1. Pulsed Exposure Interlaboratory Calibration Study Schedule.

ICS Task	Sample Shipment Date	Planned Testing Dates (start date)	Actual Testing Dates (start date)
Mysid acute testing – Group 1	8 Jul 2020	9 Jul 2020	9 Jul 2020
Mysid acute testing – Group 2	15 Jul 2020	16 Jul 2020	16-17 Jul 2020[A]
Purple sea urchin chronic testing	22 Jul 2020	23 Jul 2020	23 Jul 2020
Ceriodaphnia acute testing	10 Aug 2020	11 Aug 2020	11-12 Aug 2020[B]

[A] For a single participant laboratory, samples were received as planned; however, shipment of organisms was delayed by one day.

[B] For a single participant laboratory, the shipment of samples was delayed by one day, resulting in the test being initiated one day after the planned date.

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3. LABORATORY PROCUREMENT

3.1 IDENTIFICATION AND SOLICITATION OF POTENTIAL PARTICIPANT LABORATORIES

National or state-certified toxicology laboratories for the species tested were sought out to participate in the intercalibration study. The parent list of laboratories and primary contacts were derived from those that have participated in the North America Society of Environmental Toxicology and Chemistry (SETAC) Aquatic Toxicity Testing Interest Group (ATTIG) during an initial meeting at the North America National SETAC conference in Toronto, Canada in 2019 and thereafter. A total of 14 laboratories in the United States and one laboratory in Canada were contacted via phone calls and email. Of those contacted a total of 10 of laboratories in the United States and the one laboratory in Canada expressed interest in participation.

3.2 PREQUALIFICATION REQUIREMENTS AND SELECTION OF PARTICIPANT LABORATORIES

After confirming those laboratories that were interested in the study a request for qualifications (RFQ) and costs package was sent out to each laboratory. A third-party contractor, Edmond Scientific Company, managed the RFQ coordination and cost negotiations with each interested laboratory. The following information was requested of each laboratory to be submitted to be evaluated and qualified to participate:

1. A brief description of each laboratory's approach to ensuring high quality data that will meet established Test Acceptability Criteria (TAC) for standard EPA WET test methods.
2. The source and physical characteristics of culture water (pH, hardness, conductivity) and source of food for daphnid acute survival tests? Are daphnia cultured in house or purchased from a supplier?
3. Acknowledgement of the ability to use Bioassay grade Crystal Sea Marine Mix to create artificial seawater for sea urchin and mysid toxicity testing,
4. Copies of laboratory control charts for each species of interest. If there were any inconsistencies or deviations (e.g. excessive exceedances, not performed frequently, etc.), a narrative of how these inconsistencies have been resolved was requested.
5. Reports of the most recent Discharge Monitoring Report-Quality Assurance (DMRQA) study for acute mysid and/or daphnid tests. If not performed, please explain why. DMRQA studies are not conducted for purple sea urchin embryo development.
6. Confirm the ability to receive next day deliveries (Y/N)?
7. Copies of State Environmental Laboratory Accreditation Program (ELAP) and/or National Environmental Laboratory Accreditation Program (NELAP) certificates with fields of testing including the methods/species proposed for testing.
8. Any questions or concerns with the Pulsed Exposure SOP.

3.3 SELECTION OF PARTICIPANT LABORATORIES

Upon receiving laboratory qualification packages, a team of scientists from NIWC, Wood, and Edmond Scientific independently reviewed each submittal and scored each laboratory using a rubric as shown in Table 3-1 and Table 3-2. Each reviewer provided written comments along with scores

for each category. The scores and written comments were collated and tallied followed by a team call to review and discuss the proposals and scores prior to finalizing laboratories to be included in the study. A goal for the program was to include a minimum of 5 and up to 7 qualified laboratories for each test procedure. All selected laboratories were paid to participate in the study which limited the total number of participating laboratories possible for this program.

A list of selected participant laboratories and species tested by each is shown in Table 3-3.

Table 3-1. Pulsed Exposure Participating Laboratory Scoring Criteria.

Technical Capability (50 points)			
Experience/Capability (20 points)	Submittal Completeness (20 points)	Comprehension/Workplan reflects understanding of SOP (10 points)	
Staff Qualifications (40 points)			
Qualifications of Proposed Lead? (20 points)	Qualifications of Proposed Supporting Staff? (10 points)	Overall Years of Experience? (5 points)	Staff Redundancy (COVID-19 considerations)? (5 points)
Past Performance (10 points)			
Within 5 years? Y/N	Relevant Experience? (5 points)	Number of Citations/ Presentations Related to Pulsed Exposures (5 points)	

Table 3-2. Scoring Rubric for Each Sub-Category.

20 points	**10 points**	**5 points**	
15-20	8-10	5	Far Exceeds Requirements - Excellent
10-15	6-8	4	Exceed Requirements - Very Good
5-10	4-6	3	Meets Requirements - Acceptable
0-5	0-4	1-2	Does Not Meet Requirements - Unacceptable

Table 3-3. Selected Laboratories for the Pulsed Exposure Interlaboratory Study and Species Tested.

Laboratory	Location	Test Species		
		Ceriodaphnia	**Mysid**	**Purple Sea Urchin**
Aquatic Bioassay Consulting (ABC)	Ventura, CA	X	X	X
EA Engineering	Hunt Valley, MD	X	--	X
EcoAnalysts	Port Gamble, WA	X	X	X
Enthalpy	San Diego, CA	X	X	X
GEI Consultants	Denver, CO	X	--	--
Great Lakes Environmental Co (GLEC)	Columbus, OH	X	--	--
McCampbell Analytical Inc.	Pittsburg, CA	--	X	X
Nautilus Environmental Company	Burnaby, BC	X	X	X
Pacific EcoRisk	Martinez, CA	X	X	X
Tetra Tech	Owings Mills, MD	X	--	--
Naval Information Warfare Center (NIWC)[A]	San Diego, CA	--	X	X
Wood Environment & Infrastructure Solutions Inc. (Wood)[A]	San Diego, CA	X	X	X

[A] – referee laboratory

3.4 REFEREE LABORATORIES

Two referee laboratories supported the Pulsed Exposure Intercalibration Study; project partners Naval Information Warfare Center (NIWC) Pacific and Wood Environment & Infrastructure (Wood). Both Wood and NIWC maintain State of California Environmental Laboratory Accredited facilities located in San Diego CA. Referee laboratories were responsible for collecting and preparing bulk test samples, conducting preliminary testing to verify appropriate and stable toxicological responses in each sample prior to distribution, and distributing blind test sample aliquots to participant laboratories. The referee laboratories also performed testing of the blind samples concurrent with all participant laboratories for comparison purposes for those species respectively certified for: all three test species at Wood and purple sea urchin and mysid tests at NIWC.

Referee laboratories prepared the following five test sample types for each test method as described further in Section 4: blank sample (negative control); nontoxic stormwater, toxic stormwater, reference toxicant sample (positive control), and a duplicate sample (reference toxicant

sample). Referee laboratories distributed a combination of these test sample types with blind sample IDs to participant laboratories for testing as shown in Table 3-3.

Following testing, participant laboratories submitted all test data for analyzed samples to NIWC and Wood for independent review and subsequent inter and intra-laboratory data comparison calculations. A fillable electronic spreadsheet was provided to all participating laboratories to ensure consistent and complete reporting of all requested information. All test data was reviewed to verify that all pertinent information was provided, tests were conducted in accordance with the EPA WET method manuals and the modified Pulsed Exposure SOP, and test results were accurately calculated. Following test review, results were compiled and method performance characteristics (interlaboratory variability, successful test completion rate, and false positive rate) were calculated for each test method as described further in Section 1.

3.5 PARTICIPANT LABORATORY TRAINING AND MEETINGS

After selection of participant laboratories several meetings were held specific to each test species to summarize the pulsed exposure method in person and to cover any questions, concerns, or clarifications needed in the SOPs that were provided along with the RFQ. A training video that was developed by NIWC and Wood was also played showing various acceptable methods that may be used to transfer test organism to clean laboratory water after the initial pulsed exposure. Following meetings and follow up communications to clarify any outstanding items, a revised set of final SOPs were provided and redistributed to all participating laboratories for reference. Finally, testing dates were selected that would best accommodate the multiple laboratory schedules. Given the amount of work required to conduct the full set standard and pulsed exposure tests for each species, the testing periods were spread out over time as shown in Table 2-1 to avoid overlap in efforts among the three test species.

Each participating laboratory was assigned a random number to keep the identity of the laboratory associated with each dataset blind throughout the study including all data analysis and reporting efforts.

4. SAMPLE PREPARATION

For each test method, four samples were prepared in bulk in precleaned and rinsed 151 L (40 gallon) HDPE carboys by the project team at the Wood Bioassay Laboratory. Carboys were cleaned with hot tap water and Alconox® detergent and rinsed thoroughly with tap water and DI water prior to use. As previously mentioned, the sample types are listed below:

1. A blank sample – a negative control consisted of laboratory water obtained from the referee laboratory appropriate for a given test method;
2. A non-toxic stormwater sample – collected from the San Diego region (salted with Crystal Sea Marine Mix © as appropriate for the marine species a given test methods);
3. A toxic stormwater sample – this sample was spiked with copper sulfate to ensure a consistent measurable toxic effect (salted with Crystal Sea Marine Mix© as appropriate for the marine species test methods);
4. A copper-spiked sample – a positive control prepared using referee laboratory water spiked with copper sulfate at a concentration that resulted in a consistent measurable dose response for the particular species;
5. A fifth duplicate sample was sent to each laboratory for a total of 5 samples tested. This duplicate was the positive control for every laboratory (Sample 4).

Sample preparation for each method evaluated in the ICS is described in this section. The stormwater sample was collected on 6 April 2020 from a public roadway located in central San Diego (at approximately 4360 north Morena Blvd. Approximately 200L of stormwater runoff was collected by the project team. The concentration of dissolved organic carbon (DOC) was 5.5 mg/L. Concentrations of total and dissolved Cu were 7.4 and 5.8 µg/L, respectively (Weck Analytical Laboratory, USEPA Method 200.8).

All samples types were subsampled at the time of sample distribution for water quality characterization (i.e. salinity, conductivity, dissolved oxygen [DO], temperature, pH). Samples were sent to Weck Analytical Laboratory for measurements of dissolved copper (USEPA method 200.8).

4.1 FRESHWATER METHODS – *CERIODAPHNIA* ACUTE

The laboratory control water that was used in the freshwater studies consisted of diluted mineral water (DMW) prepared by the referee laboratory, Wood Bioassay Laboratory. The DMW sample was prepared by diluting Perrier ® mineral water with deionized water (DI) per EPA guidance (USEPA 2002). The sample was thoroughly mixed and aerated prior to use for preliminary testing and distribution to the participant laboratories. The DMW was used as the negative control sample (Sample #1) and as the base for preparing the positive control sample (Sample #4).

Approximately 25 L of positive control sample (Sample #4) was prepared by the addition of 2.5 mL of 1000 mg/L copper sulfate ($CuSO_4$) stock solution to create a high nominal concentration of 100 µg/L copper (Cu). The stormwater sample was determined to be non-toxic as part of the preliminary testing (Section 5) and thus was tested as-is for Sample #2. To prepare Sample #3, the toxic stormwater sample, 14.4 mL of the 1000 µg/L $CuSO_4$ stock solution was added to 36L of the stormwater sample to create a nominal high concentration of 400 µg/L Cu. Preliminary pre-intercalibration study testing determined that the 400 µg/L sample was more toxic than desired and thus a 25% dilution of the sample was made through the addition of DMW. This resulted in an approximate concentration of 300 µg/L Cu. This too was determined through preliminary testing still

be more toxic than desired and thus a second 25% dilution was made with DMW for a final nominal concentration of approximately 200 µg/L Cu. Final definitive testing resulted in a dose response that was acceptable, and Sample #3 was deemed ready for distribution to participant laboratories for the ICS.

The spiked and unspiked stormwater samples were stored in the dark at 4°C and spiked and unspiked laboratory waters were stored in the dark at 15°C prior to distribution to cubitainers for sample shipment to participant laboratories. Each laboratory received between 2.5 and 7 L of each type of sample depending on the species being tested. Water quality characteristics of the freshwater samples as prepared are shown in Table 4-1.

Table 4-1. Water Quality Characterization of Freshwater Samples.

Sample #/ Type	DO (mg/L)	pH (pH units)	Conductivity (µS/cm)	Alkalinity ($CaCO_3$/L)	Hardness ($CaCO_3$/L)	Targeted Nominal Cu (µg/L)	Dissolved Cu (µg/L)
1 Negative Control	9.6	7.71	209	76	91	0.0	1.6
2 Non-toxic Stormwater	9.8	6.58	54	62	26	0.0	7.5
3 Toxic Stormwater	10.6	7.61	400	79	150	200	170
4 Positive Control	9.6	8.15	209	68	88	100	95
5 Duplicate Sample	9.6	8.11	191	65	87	100	88

4.2 MARINE METHODS – MYSID (*AMERICAMYSIS*) ACUTE & PURPLE SEA URCHIN (*STRONGYLOCENTROTUS*) CHRONIC

The laboratory control water, the negative control/Sample #1, used for both marine methods consisted of seawater collected from Scripps Institution of Oceanography (SIO), referred hereafter as filtered seawater (FSW). Raw seawater at SIO is collected from an intake near the end of the 1084-foot Ellen Browning Scripps Memorial Pier in La Jolla, CA and is pulled through a series of large sand filters prior to distribution points. This water was collected from a tap at SIO and transported to Wood in a 210-gallon high-density polypropylene (HDPE) tank and then pumped into a 250-gallon HDPE holding tank at the Wood laboratory located in San Diego, CA. The seawater tank at Wood is plumbed to a recirculating system with an Aquatop® brand canister filter with UV light for sterilization, and carbon, zeolite and multiple foam and fiber filters capable of removing particulates approximately 20 microns in size. Continuous aeration is also supplied to the FSW holding tank.

For the mysid acute testing, the positive control, Sample #4, was prepared in two separate batches as the ICS testing was conducted on two separate dates. For both batches, the targeted high concentration of Cu was 800 µg/L. The first batch was prepared by the addition of 51.2 mL of 1000

µg/L Cu stock solution to 64 L of FSW. The second batch was prepared by the addition of 68 mL of the 1000 µg/L Cu stock solution to 85 L of FSW. For the purple sea urchin chronic testing, Sample #4 was prepared through the addition of 14 mL of the 1000 µg/L Cu stock solution to 70L of FSW.

For both the acute and chronic test methods for Samples #2 and #3, the salinity of the stormwater sample was increased by the addition of Crystal Sea ® artificial sea salts. Target salinity for the mysid and the purple sea urchin tests were 30 and 32 ppt, respectively. Sample #2 was then deemed ready for distribution for the ICS. For the mysid tests for Sample #3, 128 mL of the 1000 µg/L Cu stock solution was added to 80 L of the salted stormwater sample for a targeted nominal concentration of 1600 µg/L Cu. For the purple sea urchin test for Sample #3, 37.6 mL of the 1000 µg/L Cu stock solution was added to 47 L of the salted stormwater sample for a targeted nominal concentration of 800 µg/L Cu. Preliminary testing determined that this sample resulted in a greater than desired toxic response and therefore a 50% dilution using FSW was performed on the sample to achieve an approximate nominal concentration of 400 µg/L Cu.

All stormwater samples were stored in the dark at 4°C and spiked and unspiked laboratory control waters at 15°C until ready for distribution to cubitainers for sample shipment to participant laboratories. Each laboratory received approximately 7 L of each sample type for the mysid test and 4 L of each sample for the purple sea urchin tests. Water quality characteristics of the marine samples for the mysid and purple sea urchin tests are shown in Table 4-2 and Table 4-3, respectively.

Table 4-2. Water Quality Characterization of Marine Samples – Mysid (*Americamysis*) Acute Test.

Sample #/ Type	DO (mg/L)	pH (pH units)	Salinity (ppt)	Targeted Nominal Cu (µg/L)	Dissolved Cu (µg/L)
1 Negative Control	8.5	7.92	32.1	0.0	5.2
2 Non-toxic Stormwater	9.3	7.79	29.8	0.0	ND
3 Toxic Stormwater	9.8	7.69	29.4	1600	1000
4 Positive Control	9.0	7.90	32.0	800	580
5 Duplicate Sample	9.0	7.86	32.3	800	580

ND – Non-detect at method reporting limit of 5.0 µg/L.

Table 4-3. Water Quality Characterization of Marine Samples – Purple Sea Urchin Chronic Test.

Sample #/ Type	DO (mg/L)	pH (pH units)	Salinity (ppt)	Targeted Nominal Cu (µg/L)	Dissolved Cu (µg/L)
1 Negative Control	8.6	7.98	32.5	0	10
2 Non-toxic Stormwater	8.9	7.98	29.6	0	ND
3 Toxic Stormwater	8.5	7.90	31.0	400	390
4 Positive Control	9.2	7.96	32.5	200	210
5 Duplicate Sample	8.8	7.96	32.1	200	230

ND – Non-detect at method reporting limit of 10.0 µg/L.

5. PRELIMINARY TESTING

Preliminary testing of all samples used in the Pulsed ICS were conducted by the referee laboratory, Wood Bioassay Laboratory. Testing was conducted to confirm control and nontoxic stormwater samples were stable and could achieve control acceptability criteria, and that copper-spiked laboratory water and stormwater resulted in a sufficient repeatable toxic dose response for each sample for use in the Pulsed ICS. Previous testing (Rosen et al. 2019; Colvin et al. 2021) has shown a decrease in the sensitivity for pulsed exposures relative to standard, static exposures. Thus, preliminary testing included both standard continuous exposures and the modified and pulsed exposure method for each sample and method combination.

Laboratory control water (negative control), appropriate for each species, was tested to ensure a lack of toxicity and the stormwater sample that was collected for this study was tested to determine relative toxicity, if any, for each species using the pulsed exposure methodology. The stormwater sample exhibited no toxicity for any of the species evaluated for either the pulsed or standard methods. Both the laboratory control water and the stormwater sample were then spiked with copper sulfate ($CuSO_4$) specific for each species to create the positive control sample and the toxic stormwater sample. Spiked concentrations for both samples were based on analytical chemistry properties and historical results from previous spiking studies (Rosen et al. 2019; Colvin et al. 2021).

Definitive testing of the spiked samples was then conducted for each species to ensure a desired dose response for use in the ICS. For the freshwater testing, Sample #3: the toxic stormwater sample, definitive testing determined that the first preparation of the sample was more toxic than desired and thus a 25% dilution of the sample was made through the addition of DMW. This too was determined to be result in more toxic dose response than desired and thus a second 25% dilution was made with DMW for a final nominal concentration of approximately 200 µg/L Cu. Final definitive testing resulted in a dose response that was acceptable, and the sample was deem ready for distribution to participant laboratories for the ICS.

No further sample manipulations were required for the samples prepared for the mysid acute testing as preliminary testing showed that acceptable dose responses were observed for each sample tested. For the samples prepared for the purple sea urchin short-term chronic tests, Sample #3: the toxic stormwater sample, a 50% dilution of the sample was conducted following the first round of preliminary testing showed that the sample was more toxic than desired. The targeted nominal concentration was then 400 µg/L Cu. The second round of definitive testing with the purple urchins resulted in an appropriate dose response and the sample was deemed ready for distribution to participant laboratories.

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6. PACKAGING AND DISTRIBUTION OF TEST SAMPLES

Laboratories participating in the ICS each received five blind samples, one of which was a duplicate of the spiked laboratory water sample (#4). For each method tested, the sample ID number was randomized so that participant laboratories would not be aware of potential response if they were involved with multiple methods.

6.1 PACKAGING AND SHIPMENT OF SAMPLES

Bulk test samples were distributed to appropriately sized LDPE cubitainers that were rinsed with sample water three times prior to the final fill. Laboratories participating in the freshwater *Ceriodaphnia* acute testing received approximately 2.5 L of each sample, with all 5 samples packaged and shipped in a single 48-quart (45 L) cooler. Each laboratory received approximately 7 L sample for mysid acute tests and 4L for purple sea urchin chronic tests. Samples for each species were shipped to each laboratory in two large 48 quart coolers. Cubitainers were labeled both on the body of the container as well as on the lid (Figure 6-1). All cubitainers were capped with zero head space and placed in coolers lined with heavy duty plastic bags. Wet ice was added thoroughly to surround all samples and plastic bags were then secured by a knot or zip tie. Plastic bags were used to minimize potential leakage of wet ice and samples during shipment. Chain-of-custody (COC) forms were placed in large Ziploc bags and laid on top of the samples within the coolers. Coolers were then secured with shipping tape and pre-printed shipping labels secured to the top of each cooler. Coolers were labeled by the random number that was assigned to each Laboratory. All coolers were shipped priority overnight via FedEx to laboratories located outside the San Diego region. Laboratories co-located in the vicinity of San Diego; CA received coolers via hand-delivery by NIWC personnel. Referee laboratories, which were located in San Diego, CA, kept samples in coolers overnight and "received" the samples on the following day to mimic overnight shipment.

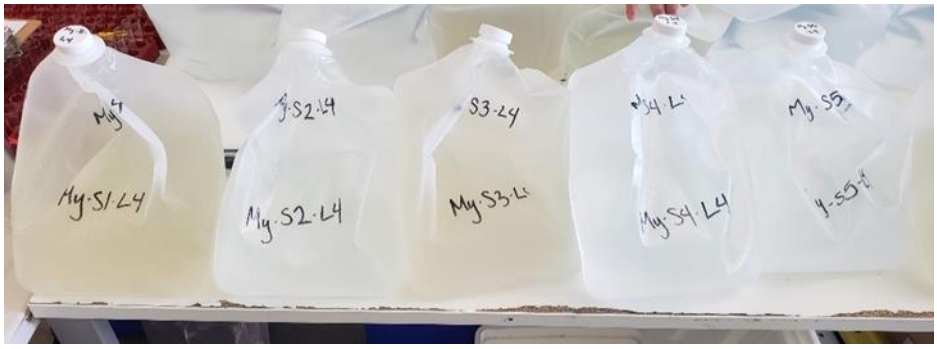

Figure 6-1. Sample preparation for one of the mysid shrimp testing dates.

6.2 PROCUREMENT AND DISTRIBUTION OF MYSID SHRIMP

Americamysis bahia were provided to each participant laboratory by the project team by Aquatic Biosystems, Inc. (ABS, Fort Collins, CO). In order to meet the quantities necessary for all laboratories to test the samples with a similar batch of organisms, testing was broken into two testing weeks with mysid shrimp shipped priority overnight from ABS to each participant laboratory on the Wednesday of each testing week. Four laboratories, including a referee laboratory, participated in the first round of testing with mysids shipped on 8 July 2020. Five laboratories, including a referee laboratory, participated in the second round of testing with mysids shipped on 15 July 2020. For each round of testing, mysids were in the age range of 2 to 3 days old upon shipment. Mysids were used for test initiations on the day they were received, when they were 3 to 4 days old.

6.3 COLLECTION AND DISTRIBUTION OF PURPLE SEA URCHINS

Purple sea urchins, *Strongylocentrotus purpuratus*, were provided along with the samples to each of the laboratories participating in the short-term chronic testing. Purple sea urchins were field collected from kelp beds located offshore of Point Loma, CA on 23 July 2020 and held in flow-through seawater tanks at the NIWC Bioassay Laboratory. On the Wednesday of the testing week, purple sea urchins were brought to the Wood Bioassay Laboratory in coolers with blue ice and seawater dampened clean rags. Approximately 18 randomly selected urchins were packaged in plastic shoe box containers with blue ice and seawater dampened clean rags to minimize jostling in transit (Figure 6-2). These boxes with the test organisms were placed in one of the two coolers exterior to the plastic bags filled with the samples and wet ice that were sent to each participant laboratory.

Figure 6-2. Packaging of purple sea urchins for the ICS effort.

6.4 SAMPLE TRACKING

Tracking numbers for each testing effort was provided by email to each participant laboratory. Upon receipt, each laboratory was responsible for documenting the condition of the samples (i.e. temperature, dissolved oxygen, pH, conductivity or salinity, hardness, alkalinity).

6.5 PROBLEMS ENCOUNTERED IN SAMPLE/ORGANISM DISTRIBUTION

Shipment of all samples occurred during a time of extreme uncertainty due to COVID-19 pandemic circumstances that limited shipping carriers relative to prior-pandemic conditions. Fortunately, only one set of samples for the *Ceriodaphnia* testing and one shipment of mysids were delayed in shipment. Temperatures of the delayed samples for the *Ceriodaphnia* test were slightly elevated upon receipt by the testing laboratory (7.8°C versus a target of 4 ± 2 °C), however this deviation was considered minor and testing proceeded, with results that were deemed acceptable for reporting purposes.

For a single laboratory, mysid shrimp were delayed in transit and were delivered a day late. The mysids received were deemed healthy and appropriate for testing as they were still within an appropriate age range.

7. INTERLABORATORY TESTING

Prior to testing, a kick-off meeting was held on 17 June 2020 with all participant laboratories. This meeting provided introductions, a background review of the ESTCP Pulsed Exposure project and objectives of the Interlaboratory Calibration Study (ICS). Method specific meetings were also held in June and July 2020 to discuss particulars regarding a given method and to allow time for laboratories to review the Pulsed Exposure Standard Operating Procedure (SOP) and ask questions for clarification as also described in Section 3.5.

Following testing, laboratories were expected to input their results into an Excel-based Electronic Data Deliverable (EDD) provided by the project team. Further details of the EDD and reporting are detailed in Section 1.

7.1 GENERAL TESTING REQUIREMENTS

For each of the five samples, including the duplicate sample, laboratories were expected to conduct toxicity testing using general guidance and species-specific standard methods (Section 2.2.2). Each sample was tested using a 5 concentration dilution series (6.25, 12.5, 25, 50 & 100% concentration of whole sample). Each sample was also tested under standard static exposure conditions with 6.25 & 100% concentrations. Samples were to be tested alongside a laboratory control that was prepared in accordance with EPA WET protocols following specific methods employed by each individual participant laboratory. Acceptable physical and chemical water quality parameters were consistent with EPA WET guidance and were also provided in the SOP and slides that were shared with participating laboratories during the pre-testing meetings.

Test specifications and acceptability criteria for each method are shown in Table 7-1 for *Ceriodaphnia*, Table 7-2 for mysid shrimp, and Table 7-3for purple sea urchin embryo development. In addition to specifications detailed in the tables, laboratories were required to conduct daily observations of organism response for the acute *Ceriodaphnia* and mysid tests. Any deviations to the test methods were documented on the EDD and within the QAQC report provided by each laboratory.

In addition, concurrent reference toxicant tests were to be performed by each laboratory according to internal procedures employed by each individual participant laboratory.

7.2 METHOD-SPECIFIC REQUIREMENTS

The Pulsed Exposure testing requires slight adjustments to standard methods and are outlined in the Pulsed Exposure SOP that was provided as part of this ICS. A video was also made available to laboratories to present procedures for conducting the transfer of organisms at the appropriate time.

In particular, the use of screen tubes to facilitate the transfer of organisms (i.e. sea urchin embryos or mysid shrimp) is a new aspect to the methods that were tested in this effort. In brief, test solutions are poured into test replicate chambers. Clear Acrylic tubes with a 25-µm Nitex screen glued to one end as shown in Figure 7-1 are placed within each replicate test beaker and organisms then are introduced into the screen tubes. Following the pulsed exposure duration (26 hour pulsed exposure), screen tubes, with the organisms inside, are removed from replicate chambers containing the test solutions. Each screen tube is gently rinsed with clean artificial seawater (ASW) and placed into clean, pre-rinsed tri-pour beakers containing clean ASW for the remainder of the test. The transfer can occur by placing the screen tubes in a separate set of labeled beakers with ASW prepared ahead of time, or by placing them back in the same container after a quick rinse with ASW. A team of two is suggested for the second method approach. As a quality control measure to ensure that the pulsed

exposure transfer methods do not negatively impact the organisms, the same transfer procedure was also be conducted for the concurrent laboratory control treatment.

For the pulsed exposures using mysid shrimp, an alternative method was permitted whereby the entire contents of each replicate (solution and shrimp) were poured through a 100-µm or smaller mesh screen. Mysids retained on the screen were then rinsed with ASW and then promptly rinsed into recipient beakers with clean ASW to continue the exposure for the remainder of the test period.

Termination of the pulsed exposure tests using sea urchin embryos tests are performed by removing the screen tubes with embryos inside, and gently rinsing the embryos into pre-labeled glass scintillation vials. Vials are then preserved with the addition of 1.0 mL of 10% buffered formalin for each 10 ml of solution retained and evaluated for normal versus abnormal development per the EPA WET standard protocol.

Termination of the pulsed exposure test using mysid shrimp were performed by carefully pouring the contents of each replicate chamber through a Nitex sieve (100-µm or smaller), followed by a through rinse with ASW and then into a secondary container (e.g. a Pyrex glass baking dish) where surviving mysids were enumerated.

For pulsed exposures using *Ceriodaphnia*, tests were conducted in 30 to 40 mL plastic soufflé cups or 5-dram (26-ml) glass scintillation vials without the use of a screen tube. At the end of the 26-hour pulsed exposure daphnia were individually transferred using a wide bore pipette into a new solution with control water for the remaining duration of the test period. This transfer method is the same as that conducted for the standard EPA WET protocol requiring a 48-hr water renewal for 96-hr acute exposures (EPA 2002a).

Figure 7-1. Nitex mesh screen tube and tri-corner beaker used for housing and transferring organisms during pulsed exposure toxicity testing.

Table 7-1. Water Flea (*Ceriodaphnia dubia*) Acute Survival Toxicity Test Specifications.

Subject	Detail
Test organism	*Ceriodaphnia dubia* (water flea)
Test endpoints	Survival
Test solution renewal	Pulsed exposure: at 26-hr (transfer to clean culture water); Static exposure: at 48-hr
Feeding	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test. Feeding to also occur once at 48-hr during the test.
Test chamber size/type	30 – 40 mL plastic soufflé cups or 5 dram (26-ml) glass scintillation vials
Test solution volume	20 mL
Test temperature	25 ± 1°C
Aeration	None
Light quality	Ambient laboratory illumination
Light intensity	10 – 20 µE/m^2/s (Ambient laboratory levels)
Photoperiod	16 hours light/ 8 hours dark
No. of organisms per chamber	5
Age of test organism	<24 hour
No. of replicates	5
Dilution water	Moderately hard dilute mineral water (DMW)
Test concentrations	Pulsed exposures: 0 (control), 6.25, 12.5, 25, 50, & 100%; Static exposures: 0 (control), 6.25 and 100%
Test duration	Pulsed exposures: 26 hours to test solution followed by transfer to clean culture water, 96-hour total test duration; Static exposures: 96-hour
Test acceptability criteria	≥ 90% survival in controls
Test protocol	Pulsed exposures: Colvin et al. 2020; Static exposures: EPA 821/R-02/012, 2002

Table 7-2. Mysid Shrimp (*Americamysis bahia*) Acute Survival Toxicity Test Specifications.

Subject	Detail
Test organism	*Americamysis bahia* (Mysid shrimp)
Test endpoints	Survival
Test solution renewal	Pulsed exposure: at 26-hr (transfer to clean synthetic seawater); Static exposure: at 48-hr
Feeding	Feed 75-100 newly hatched *Artemia* nauplii per shrimp twice daily, morning and evening
Test chamber size/type	400 mL polyethylene (HDPE) tri-pour containers with polycarbonate screen tubes with 25 µm mesh or other suitable material to capture and rinse organisms during transfers
Test solution volume	200 mL
Test temperature	20 ± 1°C
Test salinity	32 ± 2 ppt
Aeration	None, unless DO concentrations fall below 4.0 mg/L, then aerate all chambers
Light quality	Ambient laboratory illumination
Light intensity	10 – 20 µE/m^2/s (Ambient laboratory levels)
Photoperiod	16 hours light/ 8 hours dark
No. of organisms per chamber	5
Age of test organism	1 – 5 days; 24-h range in age
No. of replicates	6
Dilution water	Artificial seawater made with Crystal Sea® (Forty Fathoms) Bioassay Grade sea salts mixed with de-ionized water (32 ± 2 ppt)
Test concentrations	Pulsed exposures: 0 (control), 6.25, 12.5, 25, 50, & 100%; Static exposures: 0 (control), 6.25 and 100%
Test duration	Pulsed exposures: 26 hours to test solution followed by transfer to clean synthetic seawater, 96-hour total test duration; Static exposures: 96-hour
Test acceptability criteria	≥ 90% survival in controls
Test protocol	Pulsed exposures: Colvin et al. 2020; Static exposures: EPA 821/R-02/012, 2002

Table 7-3. Purple Sea Urchin (*Strongylocentrotus purpuratus*) Embryo-Larval Development Toxicity Test Specifications.

Subject	Detail
Test organism	*Strongylocentrotus purpuratus*
Test endpoints	Embryo Development Rate (Proportion Normal)
Test solution renewal	Pulsed exposure: at 26-hr (transfer to clean synthetic seawater); Static exposure: none
Feeding	None
Test chamber size/type	400 mL polyethylene (HDPE) tri-pour containers with polycarbonate screen tubes with 25 µm mesh
Test solution volume	250 mL
Test temperature	15 ± 1°C
Test salinity	32 ± 2 ppt
Light quality	Ambient laboratory illumination
Light intensity	10 – 20 µE/m^2/s (Ambient laboratory levels)
Photoperiod	16 hours light/ 8 hours dark
No. of organisms per chamber	Pulsed exposure: 2000 eggs/ chamber Static exposure: 250 eggs/vial Appropriate sperm density to provide > 90% fertilization success (determined in a pre-test trial)
No. of replicates	5
Dilution water	Artificial seawater made with Crystal Sea® (Forty Fathoms) Bioassay Grade sea salts mixed with de-ionized water (32 ± 2 ppt)
Test concentrations	Pulsed exposures: 0 (control), 6.25, 12.5, 25, 50, & 100%; Static exposures: 0 (control), 6.25 and 100%
Test duration	Pulsed exposures: 26 hours to test solution followed by transfer to clean synthetic seawater, 96-hour total test duration; Static exposures: 96-hour
Test acceptability criteria	≥ 80% normal development in surviving controls; < 25% Minimum Significant Difference (MSD)
Test protocol	Pulsed exposure: Colvin et al. 2020; Static exposure: Modified from EPA 600/R-95/136 (USEPA 1995)

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8. DATA REPORTING AND EVALUATION

8.1 REPORT SUBMISSION

Within three weeks of test termination, laboratories were requested to submit their testing results to the ESTCP project team using an Excel-based Electronic Data Deliverable (EDD) that was provided by the project team. Fields of entry included water quality information of the samples upon receipt and daily water quality measurements during the course of the toxicity tests (i.e. DO, pH, salinity or conductivity and pH). Information on the testing dates/times and individual replicate performance was documented in the EDD as well. To assist laboratories that may have questions about methods or how to input data into the provided EDD, the project team provided open support hours via MS Teams on a weekly basis. A brief report was also requested of each laboratory following testing to document the test acceptability of their methods and whether their reference toxicant test met internal quality control requirements. EDD submissions were received from all participant laboratories where tests were completed. Table 8-1 lists the number of EDD reports received from laboratories and the number that were ultimately used for analysis of method performance.

For the *Ceriodaphnia* acute method, two laboratories were unable to complete testing due to complications with the in-house organism cultures that resulted in the tests failing to meet test acceptability criteria (TAC) for the controls. Data for the *Ceriodaphnia* acute method was excluded for a third laboratory due to initiation issues. The reported dose responses (e.g. lack of dose responses) from this laboratory were considered extreme outliers and warranted discussions with the laboratory to investigate any issues that may have been encountered. It was determined that a new employee at the laboratory added an excessive amount of culture water along with the organisms that diluted the samples and resulted in the lack of dose responses that were reported. As a result of these deviations, interlaboratory calibration data for the *Ceriodaphnia* acute method included results from 6 laboratories and the referee laboratory.

For the mysid acute method, data from 9 laboratories was received. Data from a single laboratory was excluded due to a lack of dose response observed in the reported results for the spiked “toxic” samples. Discussions with the laboratory failed to provide a resolution and a lack of confidence in the data reported led the project team to exclude this dataset. As a result, interlaboratory calibration data for the acute mysid acute method included results from 6 laboratories and 2 referee laboratories.

For the short-term chronic sea urchin embryo development method, data from 8 laboratories was received. Data from a single laboratory was excluded due to the concurrent reference toxicant test failing to meet the internal TAC for that laboratory. This rendered accompanying tests invalid and thus the data were excluded. As a result, interlaboratory calibration data for the chronic purple sea urchin test method included results from 5 laboratories and 2 referee laboratories.

Table 8-1. Interlaboratory Calibration Reporting Summary.

Test Method	Number of Reports Received	Number of Reports that Met TAC/QAQC Checks
Ceriodaphnia acute	10	7
Mysid acute	9	8
Purple sea urchin chronic	8	7

8.2 DATA REVIEW

Electronic Data Deliverables (EDD) reports from each laboratory was reviewed by the project team. For each section of the EDD, data was reviewed to ensure that all required information was included. If any deviations were observed, the project team contacted the laboratory and requested additional information and/or clarification.

8.2.1 Data Accuracy and Quality Check

Each section of the EDD was reviewed to determine if data were accurate and provided in a manner as requested. The sections of the EDD were as follows: 1) *ToxBatch*; 2) *Sample Receipt*; 3) *Daily Water Quality*; and 4) *Toxicity Results*. Each section allowed for additional comments for the laboratories to report deviations from the protocols or other observations. For the *ToxBatch* section, data was reviewed to ensure that both standard and pulsed exposure methods were conducted and that test durations were appropriate. Laboratories were instructed to report whether their laboratory controls for each method met TAC. The *Sample Receipt* section was reviewed to ensure that the five samples were received appropriately and that water quality measurements upon receipt were deemed acceptable. To ensure that there were no confounding issues during testing, the *Daily Water Quality* section was reviewed for acceptable water quality parameters (i.e. DO, temperature, pH). In several instances, there were deviations where dissolved oxygen measurements exceeded the recommended 9.0 mg/L. The referee laboratories also reported this deviation and since control and sample responses were as expected, the tests were deemed acceptable. The final section that was reviewed by the project team was the *Toxicity Results*. The structure of the submitted data was examined to see if the appropriate number of data points for each test was present (i.e. number of reported tests, number of replicates, number of organisms per replicate). In one instance, a laboratory initiated 6 replicates for each sample for the *Ceriodaphnia* acute test, rather than the 5 replicates that were instructed. This deviation was not considered grounds for removal of the data set. Occasionally for both the *Ceriodaphnia* and mysid acute tests, individual replicates were initiated with 6 organisms rather than the instructed 5 organisms per replicate. Again, these deviations were not considered grounds for removal of the data.

Each participant laboratory was required to conduct a concurrent reference toxicant test pursuant to each laboratory's internal protocols. Laboratories were instructed to provide a narrative summary report of whether the results fell within two standard deviations of the laboratory's control chart limit.

8.2.2 Effect Concentration Calculation

Once data were deemed appropriate and acceptable, the project team conducted statistical analyses using CETIS Analytical Software (Tidepool Scientific, LLC., v1.9.7.9). Data were copied from the EDD into the CETIS software to reduce transcription errors. Statistical methods for analysis were selected according to the WET method manuals using the USEPA Automated Decision Tree options in the CETIS software. The endpoints and effect concentrations evaluated for each test method are displayed in Table 8-2.

The Test for Significant Toxicity (TST; USEPA 2010) was the statistical method used for comparisons for both standard exposure testing where point estimates were not calculatable (due to only 6.25 & 100% concentrations being tested), and for the 100% concentration of the pulsed exposures.

Table 8-2. Endpoints and Effect Concentrations Evaluated for each Test Method in the ICS.

Test Method	Exposure Duration	Point Estimate	TST (100%)	NOEC & LOEC	CV	% Difference from Control (100%)
Ceriodaphnia acute	Pulsed	✓ (LC_{50})	✓	✓	✓ (LC_{50}, 6.25% & 100%)	✓
	Standard	-	✓	-	✓ (6.25% & 100%)	✓
Mysid acute	Pulsed	✓ (LC_{50})	✓	✓	✓ (LC_{50}, 6.25% & 100%)	✓
	Standard	-	✓	-	✓ (6.25% & 100%)	✓
Purple sea urchin chronic	Pulsed	✓ (EC_{50})	✓	✓	✓ (EC_{50}, 6.25% & 100%)	✓
	Standard	-	✓	-	✓ (6.25% & 100%)	✓

CV – Coefficient of Variation; NOEC – No Observed Effect Concentration; LOEC – Lowest Observed Effect Concentration; TST – Test for Significant Toxicity; LC_{50} – Median Lethal Concentration; EC_{50} – Median Effective Concentration

8.3 DATA ANALYSIS

Valid data from all tests (Table 8-1) were used in the determination of the objectives of this effort as outlined in Section 2.1. Shown below again are the specific objectives of this effort:

1. Evaluate the ability of laboratories to successfully execute the pulsed exposure toxicity methods for each species (completion rate);
2. Evaluate the rate at which laboratories detected toxicity when evaluating non-toxic samples (false positive rate);
3. Characterize the interlaboratory variability of the 3 modified pulsed exposure test methods for each species through the comparison of coefficients of variation (CVs) for the LC_{50} and EC_{50} values and ranges for NOEC values and 95% confidence intervals.

Referee laboratory results were included in determinations of successful test completion rates, false positive rates, and precision for methods. The decision to include referee results was made because testing was conducted concurrently to the participant laboratories and the identity of the samples were blinded to the referee laboratories until completion of the testing.

For samples that elicited a dose response, point estimates (i.e. LC_{50} and EC_{50}) were calculated and the NOEC and LOEC determined. The coefficient of variation (CV) was also determined for each test method, sample and endpoint evaluated as well as for the 6.25 and 100% concentrations for both the pulsed exposure and standard method.

8.3.1 Successful Test Completion Rate

The test completion rate was calculated for each test method evaluated as the percentage of successful initiated tests by species that met TAC as specified in the Pulsed Exposure SOP. Participant laboratories that failed to meet TAC for reasons unrelated to the pulsed exposure method were not included in completion rate calculations or further statistical analyses.

8.3.2 False Positive Rate

A false positive rate was calculated for each test method based on results using the TST hypothesis test and point estimate (i.e. LC_{50}/EC_{50}) values. A test was considered to show toxicity if any of these endpoints indicated a deleterious effect when in fact there should be none (i.e. a TST fail, or an EC_{50} or LC_{50} value <100%).

8.3.3 Precision

Precision determination for the Pulsed Exposure test methods followed similar analytical methods used by the USEPA's Interlaboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Vol. 1 (USEPA 2001). First, for each method performed, the *h* statistic was calculated for each laboratory and sample type (ASTM 1997) to determine if any laboratory data is an outlier (0.5% significance level). If a test result was identified as an outlier, the data file received from the laboratory was reviewed to determine if there was a reasonable cause for the result. For *Ceriodaphnia* and mysids, two tests were identified as having potential outliers however this data was retained for subsequent analysis purposes as no reasonable cause for the anomalous data was discovered. No outliers were detected using the purple sea urchin embryo development method. As a result, all data submitted that met standard EPA TAC was used for further analysis.

Precision was then estimated through analysis of the CV of the point estimates (i.e.LC_{50} or EC_{50}) for samples that elicited a dose response (i.e. Samples # 3, 4 & 5). Precision for samples that did not elicit toxicity for a given method was not determined. For example, precision calculations were not determined for the negative control/blank sample, Sample #1, not for the non-toxic stormwater sample, Sample #2.

Following similar methods as USEPA 2001, for samples that elicited a toxic response, when a calculated result was determined to be greater than the test concentration range (i.e. >100% concentration) or less than the test concentration range (i.e. <6.25%), the values were set to the closest concentration tested. For example, if the LC_{50} value for a given sample was estimated through the CETIS analysis to be >100% concentration, for further analytical purposes, the LC_{50} value used was 100%. Likewise, if the LC_{50} value for a given sample was estimated to be <6.25%, the value used for further analyses was 6.25%. This application was only performed once for Lab 1, Sample 5, where the calculated LC_{50} was >100% concentration.

For the NOEC and LOEC values for each method and sample tested, precision was described by the range of NOEC and LOEC values. The percent minimum significant differences (PMSDs) were also evaluated for the purple sea urchin embryo development data (short-term chronic) to determine within-test variability and test sensitivity. These values were all derived from the statistical results generated by the CETIS analytical software used. PMSDs for the pulsed exposures ranged from 0.79 to 18.6%.

8.3.4 Repeatability

An evaluation of same day repeatability was performed by comparing results obtained in the duplicate positive control copper-spiked laboratory control waters, Samples 4 and 5. To assess within laboratory variability, the toxic response endpoints (TST results and LC_{50}s/EC_{50}s) were compared for consistency between the two samples. To compare variability among the laboratories, point estimate LC_{50}/EC_{50} values were statistically compared for the pulsed exposures using a paired two-sample t-test with calculated values for each laboratory representing the replicate values (n=7 for *Ceriodaphnia* and purple sea urchin tests, and n=8 for mysid shrimp).

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9. RESULTS

9.1 *CERIODAPHNIA* ACUTE TEST METHOD RESULTS

A total of 9 participant laboratories and a referee laboratory conducted the *Ceriodaphnia* acute pulsed exposure test method. As previously described in Section 8.1, data from three participant laboratories were either not reported or excluded due to reasons not related to the pulsed exposure methodology. Therefore, a total of 7 datasets were available for analysis. The ICS effort tested a total of 14 blank samples, 28 spiked laboratory control samples and 28 stormwater samples (including spiked and unspiked stormwater), using both pulsed exposure and standard methodologies. For each sample tested, a 96-hr LC_{50} was generated and other descriptive statistics are shown in Table 9-1 through Table 9-6.

9.1.1 Successful Test Completion Rate

Data from the 7 laboratories resulted in a total of 70 acute tests, all of which were completed successfully. All controls for all samples tested across all participant laboratories met test acceptability criteria of at least 90% survival. The resulting test completion rate was therefore 100% for the *Ceriodaphnia* acute protocol.

9.1.2 False Positive Rate

The 7 datasets from laboratories produced 14 valid tests for the negative control/blank sample (Sample #1). None of the laboratories identified Sample #1 as exhibiting toxicity using either standard static or pulsed exposures based on a comparison between survival in this sample and survival in concurrent laboratory controls using the TST hypothesis test methods. An LC_{50} point estimate value was calculated only for the pulsed exposure method as these tests were performed using a full 5 concentration dilution series. LC_{50} values calculated for Sample #1 for all pulsed exposure tests were >100%, also indicating no toxicity and no false positives (Table 9-1). Consequently, the resulting false positive rate for both the standard static and pulsed exposure methods in the negative control/blank sample was 0% for acute survival of *Ceriodaphnia*.

Sample #2, the non-toxic stormwater sample, was also evaluated for false positive results from the 14 valid tests that were produced. One of the 7 laboratories (Laboratory 10) identified this sample to be toxic using the pulsed exposure approach based on the TST (Fail) with mean survival of 68% (Table 9-2). However, this sample was also identified by the same laboratory as being non-toxic using the standard static exposure method. Opposite of that observed for Laboratory 10, a different laboratory (Laboratory 7) identified Sample #2 as toxic using the standard static exposure (0% survival), but non-toxic using the pulsed exposure method on the same sample (100% survival). The reason for these discrepancies remains unknown despite discussions with each laboratory and a thorough review of all supporting information.

No other laboratories identified Sample #2 as exhibiting toxicity using either standard static or pulsed exposure methods. As a result, 1 of the 7 tests using both the standard static and pulsed exposure methods resulted in a false positive, or a rate of 14.3%.

Combining results for both non-toxic laboratory control and storm water samples resulted in 1 test out of 14 (7.1%) resulting in a positive test result for both the static and pulsed exposure methods.

9.1.3 Precision

Point estimate CV values were calculated for the *Ceriodaphnia* pulsed exposure acute test for the three samples that elicited a dose response with the organisms; Samples 3, 4 and 5 as summarized in Table 9-6. Box plots of the LC_{50} values that were generated from the 7 participant laboratories are shown in Figure 9-1. Two laboratory test results (one for Sample #3 and one for Sample #4) were considered to be statistical outliers although these data (identified as separate dots above the bars in Figure 9-1) were retained for all analytical purposes as described previously in Section 8.3.3.

CVs for the LC_{50} values were 70.1, 67.0 and 64.4% for Samples 3,4 and 5, respectively. The average CV for all samples combined was 67.2%. Due to the relatively low LC_{50} values for all three samples (< 30%), a small change in response can lead to elevated CV calculations; as such, this factor should be accounted for when interpreting this data.

To further understand the variability associated with the pulsed exposure method relative to that observed using the static standard method, CVs obtained for the 6.25% concentration among all laboratories for all five samples tested were compared between the two methods as shown in Table 9-6. CVs for the 6.25% concentrations ranged from 4.6 to 71.8% and 8.6 to 75.1% for the pulsed and standard exposures, respectively. Averaging the CVs based on total variance for all sample types for the 6.25% concentration, a total CV of 28.7 and 39.0% was obtained for the *Ceriodaphnia* acute pulsed and standard exposures, respectively.

For both pulsed and standard exposures combined, CVs for the 6.25% concentration were lower for the non-toxic samples (mean of 7.9%) than for the toxic samples (mean 51.1%). Additionally, CVs were lower in spiked laboratory water Samples #4 and 5 (mean of 40.0%) than that observed for the toxic stormwater samples (73.5%).

9.1.4 Repeatability

Toxic response endpoints (TST results and point estimate LC_{50}s values) were compared for consistency between the two duplicate positive control copper-spiked laboratory control waters. Results within each laboratory for the pulsed exposures were identical using the TST approach (all considered toxic). Calculated point estimate values were also very comparable between the two samples within each laboratory as shown in Table 9-4 and Table 9-5. Variability among the laboratories point estimate LC_{50} values were statistically compared for the pulsed exposures using a paired two-sample t-test. Point estimate pulsed exposure LC_{50} results for duplicate Samples #4 and 5 for each laboratory are shown in Figure 9-2. Mean LC_{50} values among all laboratories pooled were very comparable (11.4 and 11.7% sample for Sample #4 and 5, respectively), and not statistically different (paired t-test, $p = 0.59$, $n=7$).

As a final comparability measure for both pulsed and standard static exposures, the combined mean % survival results among all laboratories for each treatment dilution are shown in 3. These results likewise show good comparability in results between the two duplicate samples for all dilutions tested (pulsed and static).

Table 9-1. Results for *Ceriodaphnia* Acute Tests for the Negative Control (Sample #1).

Lab ID	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25 %	Mean Survival @ 100% (%)	TST Result	CV @ 6.25 %
1	>100	100	>100	88.0	Non-toxic	22.0	100	Non-toxic	0
2	>100	100	>100	100	Non-toxic	0	96.0	Non-toxic	24.9
6	>100	100	>100	100	Non-toxic	0	93.3	Non-toxic	0
7	>100	100	>100	100	Non-toxic	11.9	92.0	Non-toxic	9.3
8	>100	100	>100	100	Non-toxic	0	96.0	Non-toxic	0
10	>100	100	>100	88.0	Non-toxic	9.3	92.0	Non-toxic	9.3
12*	>100	100	>100	96.0	Non-toxic	23.3	88.0	Non-toxic	19.9
Min	>100	100	>100	88.0	--	0	88.0	--	0
Max	>100	100	>100	100	--	23.3	100	--	24.9
Median	>100	100	>100	100	--	9.3	93.3	--	9.3
Mean	>100	100	>100	96.0	--	9.5	93.9	--	9.1
False positives	0	--	--	--	--	--	0	--	--
False positive rate	0%	--	--	--	--	--	0%	--	--

"--": value not calculatable. *Referee laboratory

Table 9-2. Results for *Ceriodaphnia* Acute Tests for the Non-toxic Stormwater Sample (Sample #2).

Lab ID	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	**NOEC**	**LOEC**	**Mean Survival @ 100% (%)**	**TST Result**	**CV @ 6.25 %**	**Mean Survival @ 100% (%)**	**TST Result**	**CV @ 6.25 %**
1	>100	100	>100	100	Non-toxic	20.3	100	Non-toxic	9.3
2	>100	100	>100	100	Non-toxic	0	96.0	Non-toxic	11.9
6	>100	100	>100	100	Non-toxic	0	96.7	Non-toxic	8.5
7	>100	100	>100	100	Non-toxic	0	**0.0**	**Toxic**	19.9
8	>100	100	>100	100	Non-toxic	0	100	Non-toxic	0
10	>100	100	>100	**68[A]**	**Toxic**	11.9	84.0	Non-toxic	10.7
12*	>100	100	>100	100	Non-toxic	0	96.0	Non-toxic	0
Min	>100	100	>100	68.0	--	0	0.0	--	0
Max	>100	100	>100	100.0	--	20.3	100	--	19.9
Median	>100	100	>100	100.0	--	0	96.0	--	9.3
Mean	>100	100	>100	95.4	--	4.6	81.8	--	8.6
False positives	0	--	--	1	--	--	1	--	--
False positive rate	0%	--	--	14.3%	--	--	14.3%	--	--

Values in **bold** indicate a statistically significant decrease compared to the control. "--": value not calculatable. *Referee laboratory

Table 9-3. Results for *Ceriodaphnia* Acute Tests for the Toxic Stormwater Sample (Sample #3).

Lab ID	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	**NOEC**	**LOEC**	**Mean Survival @ 100% (%)**	**TST Result**	**CV @ 6.25%**	**Mean Survival @ 100% (%)**	**TST Result**	**CV @ 6.25 %**
1	4.5	<6.25	6.25	**0.0**	**Toxic**	63.9	**0.0**	**Toxic**	60.9
2	3.4	<6.25	6.25	**0.0**	**Toxic**	137	**0.0**	**Toxic**	--
6	18.4	12.5	25	**0.0**	**Toxic**	11.1	**0.0**	**Toxic**	11.1
7	6.0	<6.25	6.25	**0.0**	**Toxic**	74.7	**0.0**	**Toxic**	70.7
8	8.5	6.25	12.5	**0.0**	**Toxic**	25.0	**0.0**	**Toxic**	34.4
10	3.6	<6.25	6.25	**0.0**	**Toxic**	149	**0.0**	**Toxic**	224
12*	7.8	6.25	12.5	**0.0**	**Toxic**	42.1	**0.0**	**Toxic**	50.0
Min	3.4	<6.25	6.25	0.0	--	11.1	0.0	--	11.1
Max	18.4	12.5	25	0.0	--	149	0.0	--	224
Median	6.0	--	--	0.0	--	63.9	0.0	--	55.5
Mean	7.5	--	--	0.0	--	71.8	0.0	--	75.1

Values in **bold** indicate a statistically significant decrease compared to the control. "**--**": value not calculatable. *Referee laboratory

Table 9-4. Results for *Ceriodaphnia* Acute Tests for the Spiked Laboratory Water (Sample #4).

Lab ID	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	**NOEC**	**LOEC**	**Mean Survival @ 100% (%)**	**TST Result**	**CV @ 6.25%**	**Mean Survival @ 100% (%)**	**TST Result**	**CV @ 6.25 %**
1	8.2	6.25	12.5	**0.0**	**Toxic**	22.0	**0.0**	**Toxic**	12.5
2	8.8	6.25	12.5	**0.0**	**Toxic**	0	**0.0**	**Toxic**	224
6	27.5	25.0	50.0	**0.0**	**Toxic**	0	**0.0**	**Toxic**	8.5
7	10.3	6.25	12.5	**0.0**	**Toxic**	11.9	**0.0**	**Toxic**	22.0
8	13.0	<6.25	6.25	**0.0**	**Toxic**	11.8	**0.0**	**Toxic**	20.3
10	4.4	<6.25	6.25	**0.0**	**Toxic**	139	**0.0**	**Toxic**	52.7
12*	7.2	<6.25	6.25	**0.0**	**Toxic**	40.8	**0.0**	**Toxic**	30.6
Min	4.4	<6.25	6.25	0.0	--	0	0.0	--	8.5
Max	27.5	25	50	0.0	--	139	0.0	--	224
Median	8.8	--	--	0.0	--	11.9	0.0	--	22.0
Mean	11.4	--	--	0.0	--	32.2	0.0	--	52.9

Values in **bold** indicate a statistically significant decrease compared to the control. "**--**": value not calculatable. *Referee laboratory

Table 9-5. Results for *Ceriodaphnia* Acute Tests for the Duplicate Spiked Laboratory Water (Sample #5).

Lab ID	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	**NOEC**	**LOEC**	**Mean Survival @ 100% (%)**	**TST Result**	**CV @ 6.25%**	**Mean Survival @ 100% (%)**	**TST Result**	**CV @ 6.25 %**
1	7.9	<6.25	6.25	**0.0**	**Toxic**	24.9	**0.0**	**Toxic**	34.3
2	9.3	6.25	12.5	**0.0**	**Toxic**	0	**0.0**	**Toxic**	149
6	26.6	25	50	**0.0**	**Toxic**	8.5	**0.0**	**Toxic**	8.5
7	8.7	6.25	12.5	**0.0**	**Toxic**	10.7	**0.0**	**Toxic**	9.3
8	16.9	12.5	25	**0.0**	**Toxic**	12.5	**0.0**	**Toxic**	19.9
10	4.4	<6.25	6.25	**0.0**	**Toxic**	81.4	**0.0**	**Toxic**	100
12*	8.4	<6.25	6.25	**0.0**	**Toxic**	39.5	**0.0**	**Toxic**	25.0
Min	4.4	<6.25	6.25	0.0	--	0	0.0	--	8.5
Max	26.6	25	50	0.0	--	81.4	0.0	--	149
Median	8.7	--	--	0.0	--	12.5	0.0	--	25.0
Mean	11.7	--	--	0.0	--	25.4	0.0	--	49.4

Values in **bold** indicate a statistically significant decrease compared to the control. "**--**": value not calculatable. *Referee laboratory

Table 9-6. Coefficient of Variation (CV) Values from the *Ceriodaphnia* Acute Pulsed Exposure Method.

Sample #/ Type	CV of LC_{50} (%)	CV of 6.25% Concentration (%)	
		Pulsed	Standard
1 Negative Control	--	9.5	9.1
2 Non-toxic Stormwater	--	4.6	8.6
3 Toxic Stormwater	70.1	71.8	75.1
4 Positive Control	67.0	32.2	52.9
5 Positive Control Duplicate Sample	64.4	25.4	49.4
Mean	67.2	28.7	39.0

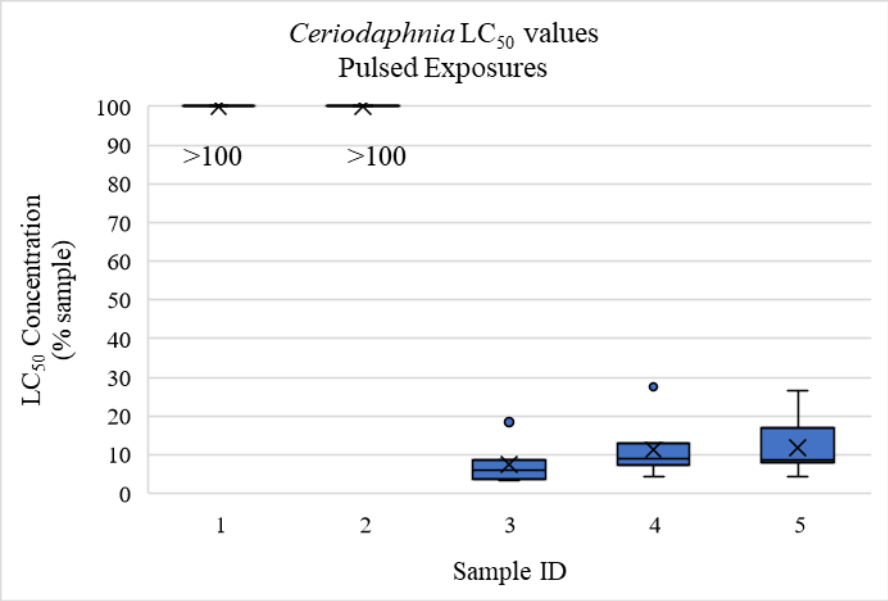

Figure 9-1. Mean point estimate values for the *Ceriodaphnia* pulsed exposures. "X" indicates the mean LC_{50} value; dots indicate outliers as determined through *h* statistics.

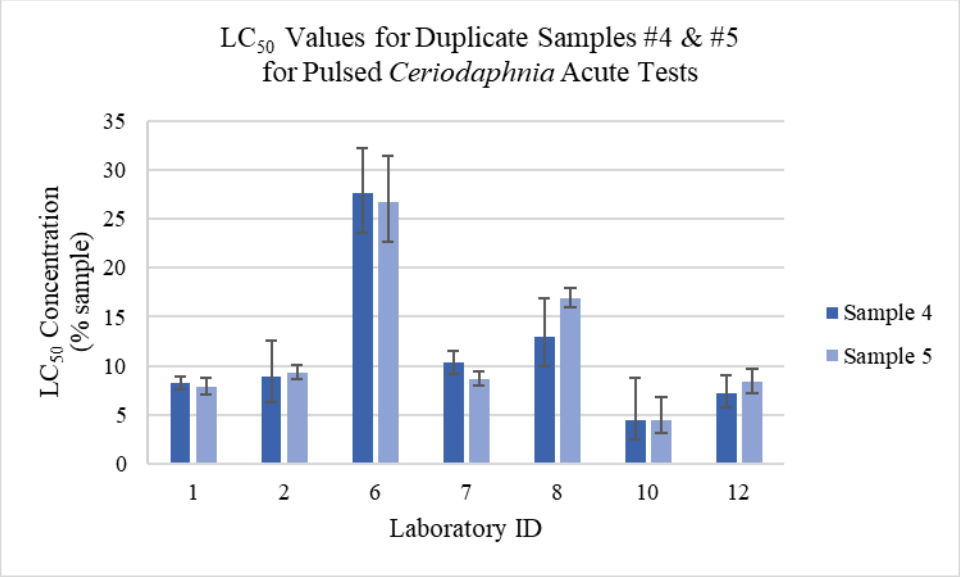

Figure 9-2. Point estimate pulsed exposure LC_{50} results for duplicate Samples #4 and 5 for the *Ceriodaphnia* pulsed exposures. Error bars are the 95% upper and lower confidence intervals.

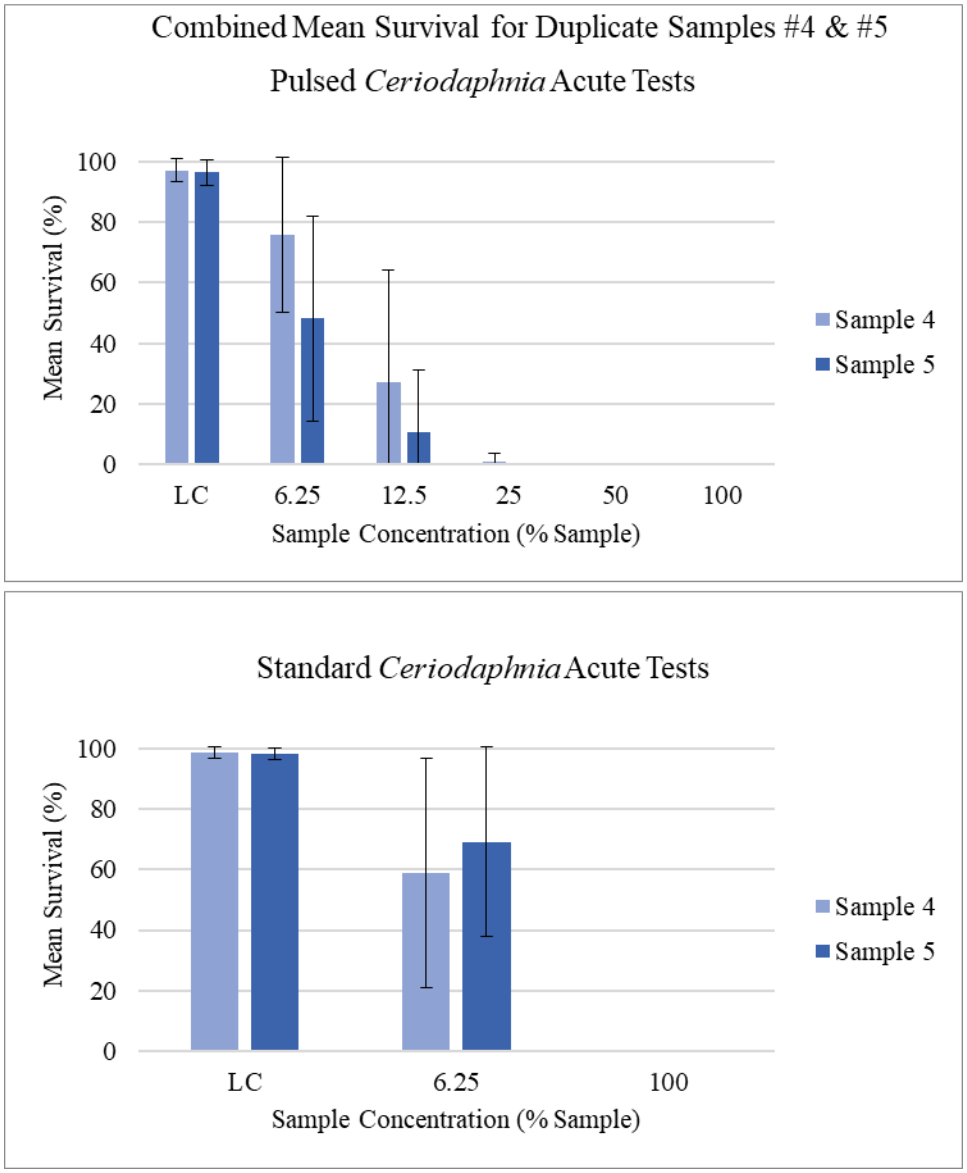

Figure 9-3. The combined mean % survival results among all laboratories for each treatment dilution for duplicate Samples #4 and 5 for the *Ceriodaphnia* pulsed (TOP) and standard (BOTTOM) exposures. Error bars indicate the standard deviations.

9.2 MYSID ACUTE TEST METHOD RESULTS

A total of 7 participant laboratories and 2 referee laboratories conducted the mysid acute pulsed and standard static exposure test methods side-by-side. As mentioned in Section 8.1, results from a single laboratory was excluded due to reasons not related to the pulsed exposure methodology. Therefore, a total of 8 datasets were used for this analysis. A total of 16 blank samples, 30 spiked laboratory control samples and 30 stormwater samples, using both pulsed exposure and standard methodologies were tested using the acute mysid shrimp protocol. For each sample tested, a 96-hr LC_{50} was generated and other descriptive statistics are shown in Table 9-7 through Table 9-12.

9.2.1 Successful Test Completion Rate

Data from the 8 laboratories resulted in a total of 80 acute tests, all of which were completed successfully. All controls for all samples tested across all participant laboratories met test acceptability criteria of at least 90% survival. The resulting test completion rate was thus 100% for the mysid acute test protocol.

9.2.2 False Positive Rate

The 8 datasets from laboratories produced 16 valid tests for the negative control/blank sample (Sample #1). None of the laboratories identified Sample #1 as exhibiting toxicity using either standard static or pulsed exposures based on a comparison between survival in this sample and survival in concurrent laboratory controls using the TST hypothesis test methods. An LC_{50} point estimate value was calculated only for the pulsed exposure method as these tests were performed using a full 5 concentration dilution series. LC_{50} values calculated for Sample #1 for all pulsed exposure tests were >100%, also indicating no toxicity and no false positives (Table 9-7). Consequently, the resulting false positive rate for both the standard static and pulsed exposure methods in the negative control/blank sample was 0% for acute survival of mysid shrimp.

Similarly, Sample #2, the non-toxic stormwater sample, was also evaluated for false positive results from the 16 valid tests that were produced. None of the laboratories identified Sample #2 as exhibiting toxicity using either standard static or pulsed exposures based on a comparison between survival in this sample and survival in concurrent laboratory controls using the TST hypothesis test methods. LC_{50} values for Sample #2 for all pulsed exposure tests were >100%, also indicating no toxicity and no false positives (Table 9-8). Consequently, the resulting false positive rate for both the standard static and pulsed exposure methods in the non-toxic stormwater sample was also 0% for acute survival of mysid shrimp.

9.2.3 Precision

CVs for the LC_{50} values were 25.6, 23.3 and 37.2% for Samples 3,4 and 5, respectively. The average CV for all samples combined was 28.7%. Box plots of the LC_{50} values that were generated from the 7 participant laboratories are shown in Figure 9-4. Two laboratory test results (one for Sample #3 and one for Sample #5) were considered to be statistical outliers although these data (identified as separate dots above the bars in Figure 9-4) were retained for all analytical purposes as described previously in Section 8.3.3.

To further understand the variability associated with the pulsed exposure method relative to that observed using the static standard method, CVs obtained for the 6.25% concentrations among all laboratories for all five samples tested were compared between the two methods as shown in Table 9-12. CVs for the 6.25% concentrations ranged from 2.8 to 11.4% and 1.1 to 14.9% for the pulsed and standard exposures, respectively.

Averaging the CVs based on total variance for all sample types for the 6.25% concentrations, a total CV of 6.6 and 5.7% was obtained for the mysid acute pulsed and standard exposures, respectively.

For both pulsed and standard exposures combined, CVs were lower for the non-toxic samples (mean of 3.3%) than for the toxic samples (mean 8.1%). The spiked laboratory water, Samples #4 and 5, had a mean CV of 6.8% and the toxic stormwater samples had a mean CV of 10.6%.

9.2.4 Repeatability

Toxic response endpoints (TST results and point estimate LC_{50}s values) were compared for consistency between the two duplicate positive control copper-spiked laboratory control waters. Results within each laboratory for the pulsed exposures were identical using the TST approach (all considered toxic). Calculated point estimate values were also very comparable between the two samples within each laboratory as shown in Table 9-10 and Table 9-11. Variability among the laboratories point estimate LC_{50} values were statistically compared for the pulsed exposures using a paired two-sample t-test. Point estimate pulsed exposure LC_{50} results for duplicate Samples #4 and 5 are shown in Figure 9-5. Mean LC_{50} values among all laboratories pooled were very comparable (46.7 and 47.9% sample for Sample s#4 and 5, respectively), and not statistically different (paired t-test, $p = 0.21$, $n=8$).

As a final comparability measure for both pulsed and standard static exposures, the combined raw % survival results among all laboratories for each treatment dilution are shown in Figure 9-6. These results likewise show good comparability in results between the two duplicate samples for all dilutions tested (pulsed and static).

Table 9-7. Results for Mysid Acute Tests for the Negative Control (Sample #1).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25 %
1	>100	100	>100	100	Non-toxic	0	96.7	Non-toxic	0
2	>100	100	>100	100	Non-toxic	0	96.7	Non-toxic	0
3	>100	100	>100	96.7	Non-toxic	11.1	96.7	Non-toxic	0
6	>100	100	>100	96.7	Non-toxic	8.5	100	Non-toxic	0
7	>100	100	>100	96.7	Non-toxic	8.45	96.7	Non-toxic	0
9	>100	100	>100	100	Non-toxic	8.5	100	Non-toxic	0
11*	>100	100	>100	100	Non-toxic	0	100	Non-toxic	8.5
12*	>100	100	>100	93.3	Non-toxic	8.5	100	Non-toxic	0
Min	>100	100	>100	93.3	--	0.0	96.7	--	0.0
Max	>100	100	>100	100	--	11.1	100	--	8.5
Median	>100	100	>100	98.4	--	8.5	98.4	--	0.0
Mean	>100	100	>100	97.9	--	5.6	98.4	--	1.1
False positives	0	--	--	--	--	--	0	--	--
False positive rate	0%	--	--	--	--	--	0%	--	--

"--": value not calculatable. *Referee laboratory

Table 9-8. Results for Mysid Acute Tests for the Non-toxic Stormwater Sample (Sample #2).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25 %
1	>100	100	>100	100	Non-toxic	0	100	Non-toxic	8.5
2	>100	100	>100	93.3	Non-toxic	11.1	100	Non-toxic	0
3	>100	100	>100	100	Non-toxic	0	96.7	Non-toxic	0
6	>100	100	>100	100	Non-toxic	0	96.7	Non-toxic	0
7	>100	100	>100	96.7	Non-toxic	0	100	Non-toxic	11.1
9	>100	100	>100	100	Non-toxic	0	100	Non-toxic	0
11*	>100	100	>100	96.7	Non-toxic	0	100	Non-toxic	0
12*	>100	100	>100	93.3	Non-toxic	11.1	96.7	Non-toxic	11.1
Min	>100	100	>100	93.3	--	0.0	96.7	--	0.0
Max	>100	100	>100	100	--	11.1	100	--	11.1
Median	>100	100	>100	98.4	--	0.0	100.0	--	0.0
Mean	>100	100	>100	97.5	--	2.8	98.8	--	3.8
False positives	0	--	--	0	--	--	0	--	--
False positive rate	0%	--	--	0%	--	--	0%	--	--

"--": value not calculatable. *Referee laboratory

Table 9-9. Results for Mysid Acute Tests for the Toxic Stormwater Sample (Sample #3).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25 %
1	60.6	12.5	25	**40**	**Toxic**	**0**	**0.0**	**Toxic**	8.5
2	35.4	12.5	25	**6.7**	**Toxic**	8.5	**0.0**	**Toxic**	8.5
3	33.5	12.5	25	**16.7**	**Toxic**	11.1	**0.0**	**Toxic**	18.1
6	30.7	12.5	25	**10**	**Toxic**	0	**0.0**	**Toxic**	11.1
7	37.7	25	50	**13.3**	**Toxic**	9.8	**0.0**	**Toxic**	42
9	38.6	12.5	25	**6.7**	**Toxic**	8.5	**3.3**	**Toxic**	0
11*	32.2	12.5	25	**10**	**Toxic**	12.2	**0.0**	**Toxic**	18.8
12*	33.0	12.5	25	**0.0**	**Toxic**	0	**0.0**	**Toxic**	12.2
Min	30.7	12.5	25	0.0	--	0	0.0	--	0
Max	60.6	25	50	40.0	--	12.2	3.3	--	42.0
Median	34.4	--	--	10.0	--	8.5	0.0	--	11.7
Mean	37.7	--	--	12.9	--	6.3	0.4	--	14.9

Values in **bold** indicate a statistically significant decrease compared to the control. "--": value not calculatable. *Referee laboratory

Table 9-10. Results for Mysid Acute Tests for the Spiked Laboratory Water (Sample #4).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25 %
1	65.6	50	100	**13.3**	**Toxic**	8.5	**0.0**	**Toxic**	0
2	42.4	25	50	**13.3**	**Toxic**	8.5	**3.3**	**Toxic**	0
3	34.7	12.5	25	**13.3**	**Toxic**	8.5	**0.0**	**Toxic**	0
6	37.4	12.5	25	**10**	**Toxic**	11.1	**0.0**	**Toxic**	0
7	37.7	25	50	**3.3**	**Toxic**	18.6	**0.0**	**Toxic**	11.1
9	50.9	25	50	**20**	**Toxic**	17.5	**0.0**	**Toxic**	11.1
11*	47.3	25	50	**16.7**	**Toxic**	0	**0.0**	**Toxic**	8.5
12*	57.8	25	50	**20**	**Toxic**	18.6	**6.7**	**Toxic**	0
Min	34.7	12.5	25	3.3	--	0.0	0.0	--	0.0
Max	65.6	50	100	20.0	--	18.6	6.7	--	11.1
Median	44.8	--	--	13.3	--	9.8	0.0	--	0.0
Mean	46.7	--	--	13.7	--	11.4	1.3	--	3.8

Values in **bold** indicate a statistically significant decrease compared to the control. "--": value not calculatable. *Referee laboratory

Table 9-11. Results for Mysid Acute Tests for the Duplicate Spiked Laboratory Water (Sample #5).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	LC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%
1	>100	50	100	**53.3**	**Toxic**	0	**0.0**	**Toxic**	8.5
2	58.5	25	50	**10**	**Toxic**	8.5	**0.0**	**Toxic**	0
3	39.2	12.5	25	**16.7**	**Toxic**	11.9	**0.0**	**Toxic**	11.9
6	59.5	25	50	**26.7**	**Toxic**	11.1	**3.3**	**Toxic**	18.6
7	36.9	25	50	**13.3**	**Toxic**	8.45	**0.0**	**Toxic**	0
9	51.8	25	50	**6.7**	**Toxic**	8.5	**0.0**	**Toxic**	0
11*	46.8	25	50	**3.3**	**Toxic**	8.5	**0.0**	**Toxic**	0
12*	42.5	25	50	**0.0**	**Toxic**	0	**0.0**	**Toxic**	0
Min	36.9	12.5	25	0.0	--	0.0	0.0	--	0.0
Max	59.5	50	100	53.3	--	11.9	3.3	--	18.6
Median	46.8	--	--	11.7	--	8.5	0.0	--	0.0
Mean	47.9	--	--	16.3	--	7.1	0.4	--	4.9

Values in **bold** indicate a statistically significant decrease compared to the control. "--": value not calculatable. *Referee laboratory

Table 9-12. Coefficient of Variation (CV) Values from the Mysid Acute Pulsed Exposure Method.

Sample #/ Type	CV of LC_{50} (%)	CV of 6.25% Concentration (%)	
		Pulsed	Standard
1 Negative Control	--	5.6	1.1
2 Non-toxic Stormwater	--	2.8	3.8
3 Toxic Stormwater	25.6	6.3	14.9
4 Positive Control	23.3	11.4	3.8
5 Duplicate Positive Control	37.2	7.1	4.9
Mean	28.7	6.6	5.7

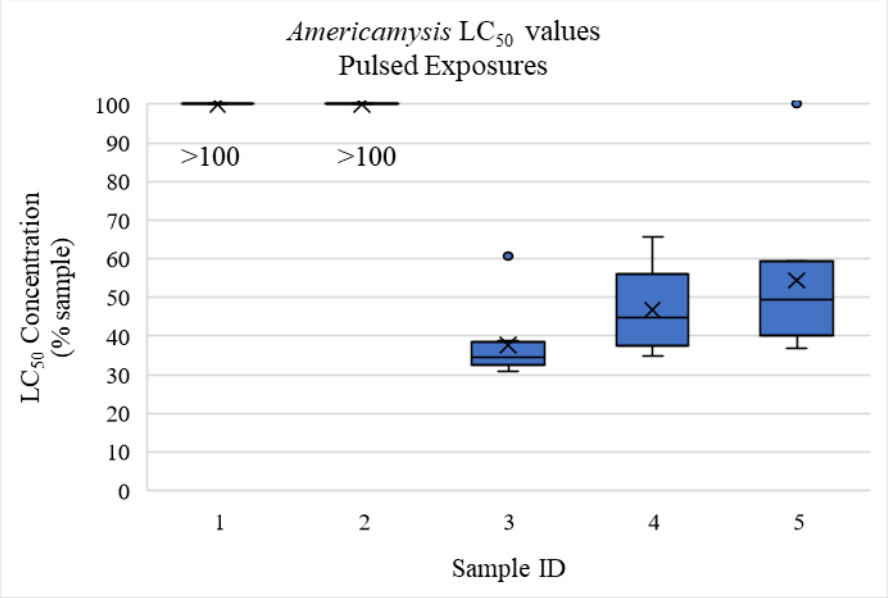

Figure 9-4. Mean point estimate values for the *Americamysis* pulsed exposures. “X” indicates the mean LC_{50} value; dots indicate outliers as determined through *h* statistics.

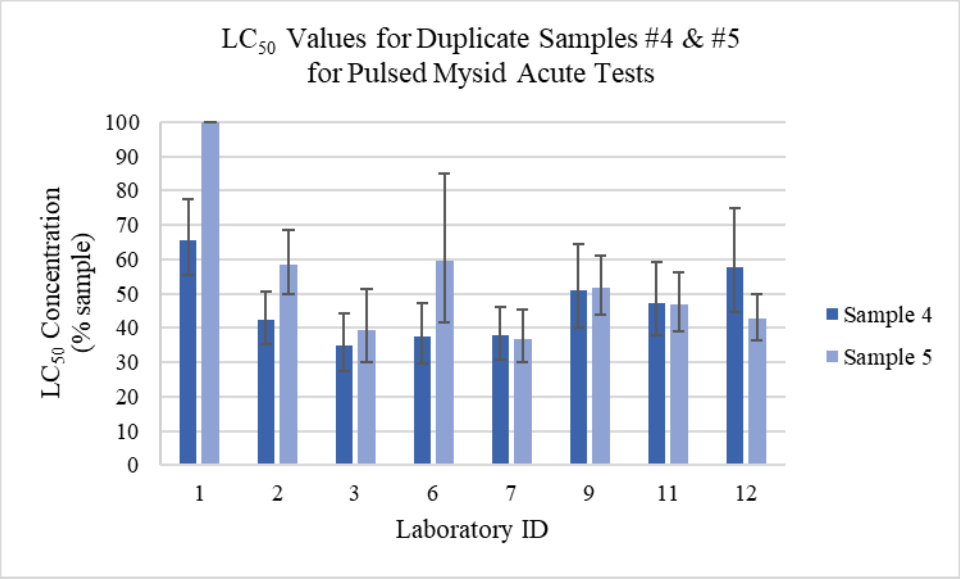

Figure 9-5. Point estimate pulsed exposure LC_{50} results for duplicate Samples #4 and 5 for the Mysid pulsed exposures. Error bars are the 95% upper and lower confidence intervals.

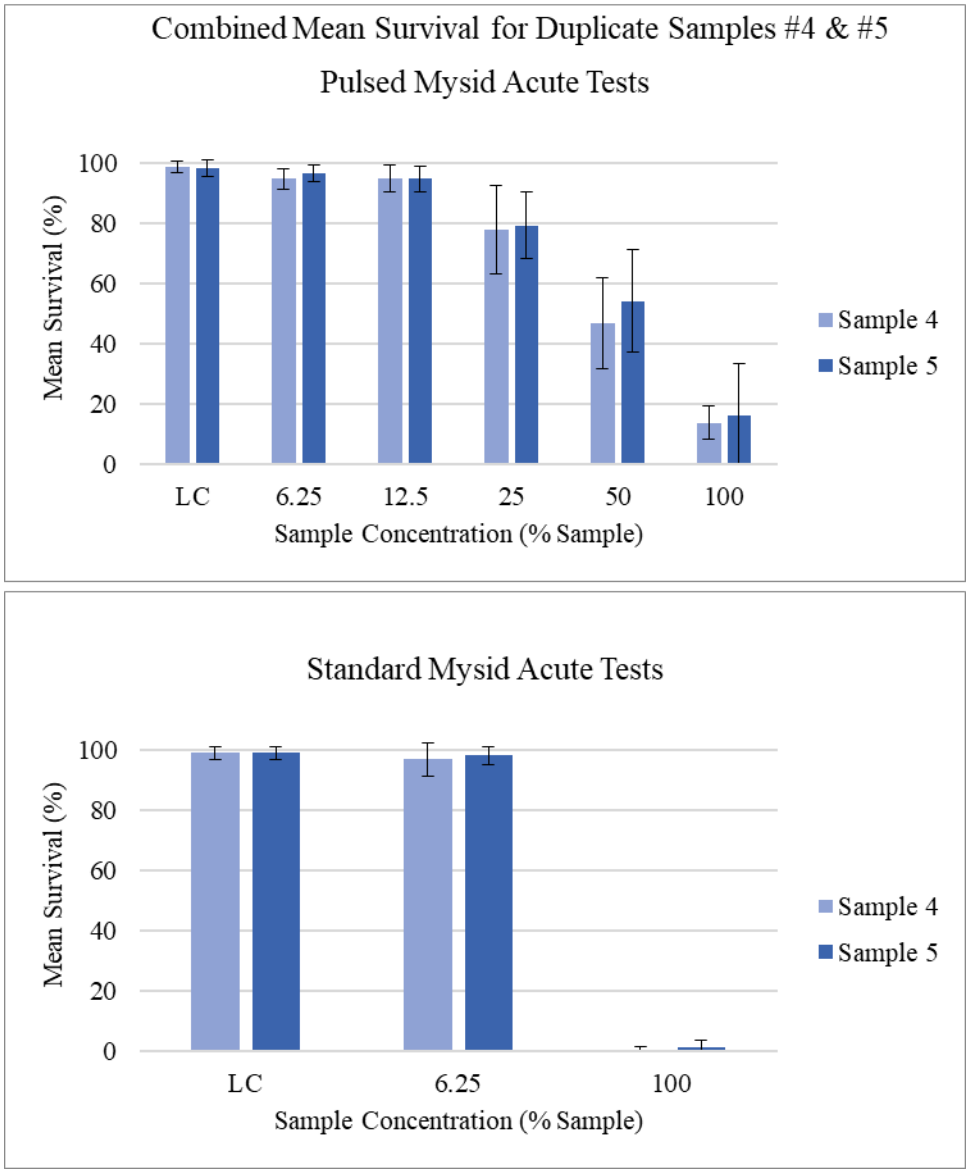

Figure 9-6. The combined mean % survival results among all laboratories for each treatment dilution for duplicate Samples #4 and 5 for the Mysid pulsed (TOP) and standard (BOTTOM) exposures. Error bars represent the standard deviations.

9.3 PURPLE SEA URCHIN CHRONIC TEST METHOD RESULTS

A total of 6 participant laboratories and 2 referee laboratories conducted the short-term chronic pulsed exposure test method using the purple sea urchin (embryo development). As previously mentioned in Section 8.1, data from a single participant laboratory was excluded due to reasons not related to the pulsed exposure methodology. Therefore, a total of 7 datasets were available for analysis for this test species. A total of 14 blank samples, 28 spiked laboratory control samples and 28 stormwater samples, using both pulsed exposure and standard methodologies were tested using the purple sea urchin test method. For each sample tested, a 96-hr EC_{50} was generated and other descriptive statistics are shown in Table 9-13 through Table 9-17.

9.3.1 Successful Test Completion Rate

Data from the 7 laboratories resulted in a total of 70 acute tests, all of which were completed successfully. All controls for all samples tested across all participant laboratories met test acceptability criteria of at least 80% normal embryo-larval development. The resulting test completion rate was thus 100% for the sea urchin embryo development test procedure.

9.3.2 False Positive Rate

The 7 datasets from laboratories produced 14 valid tests for the negative control/blank sample (Sample #1). None of the laboratories identified Sample #1 as exhibiting toxicity using either standard static or pulsed exposures based on a comparison between survival in this sample and survival in concurrent laboratory controls using the TST test methods. An LC_{50} point estimate value was calculated only for the pulsed exposure method as these tests were performed using a full 5 concentration dilution series. LC_{50} values calculated for Sample #1 for all pulsed exposure tests were >100%, also indicating no toxicity and no false positives (Table 9-13). Consequently, the resulting false positive rate for both the standard static and pulsed exposure methods in the negative control/blank sample was 0% for the chronic purple sea urchin embryo development test.

Similarly, Sample #2, the non-toxic stormwater sample, was also evaluated for false positive results from the 14 valid tests that were produced. None of the laboratories identified Sample #2 as exhibiting toxicity using either standard static or pulsed exposures based on a comparison between survival in this sample and survival in concurrent laboratory controls using the TST test methods. LC_{50} values for Sample #2 for all pulsed exposure tests were >100%, also indicating no toxicity and no false positives (Table 9-14). Consequently, the resulting false positive rate for both the standard static and pulsed exposure methods in the non-toxic stormwater sample was also 0% for acute survival of mysid shrimp.

9.3.3 Precision

Point estimate CV values were calculated for the pulsed exposure tests using purple sea urchins for the three samples that elicited a dose response with the organisms; Samples 3, 4 and 5 as summarized in Table 9-18. Box plots of the LC50 values that were generated from the 7 participant laboratories are shown in Figure 9-7.

CVs values were 56.5, 59.2 and 50.4% for Samples 3,4 and 5, respectively. The average CV for all samples combined was 55.4%.

To further understand the variability associated with the pulsed exposure method relative to that observed using the static standard method, CVs obtained for the 6.25% concentrations among all laboratories for all five samples tested were compared between the two methods as shown in Table 9-18. CVs for the 6.25% concentrations ranged from 3.2 to 11.7% and 2.0 to 56.1% for the pulsed and standard exposures, respectively.

Averaging the CVs based on total variance for all sample types for the 6.25% concentrations, a total CV of 7.5 and 26.1% was obtained for the purple sea urchin short-term chronic pulsed and standard exposures, respectively.

For both pulsed and standard exposures combined, CVs were lower for the non-toxic samples (mean of 3.8%) than for the toxic samples (mean 25.5%). The spiked laboratory water, Samples #4 and 5, had a mean CV of 23.5% and the toxic stormwater samples had a mean CV of 29.4%.

9.3.4 Repeatability

Toxic response endpoints (TST results and point estimate EC_{50}s values) were compared for consistency between the two duplicate positive control copper-spiked laboratory control waters. Results within each laboratory for the pulsed exposures were identical using the TST approach (all considered toxic. Calculated point estimate values were also very comparable between the two samples within each laboratory as shown in Table 9-16 and Table 9-17. Variability among the laboratories point estimate EC_{50} values were statistically compared for the pulsed exposures using a paired two-sample t-test. Point estimate pulsed exposure EC_{50} results for duplicate Samples #4 and 5 are shown in Figure 9-8. Mean LC_{50} values among all laboratories pooled were very comparable (18.3 and 17.4% sample for Sample #4 and 5, respectively), and not statistically different (paired t-test, $p = 0.36$, $n=7$).

As a final comparability measure for both pulsed and standard static exposures, the combined raw % survival results among all laboratories for each treatment dilution are shown in Figure 9-9. These results likewise show good comparability in results between the two duplicate samples for all dilutions tested (pulsed and static).

Table 9-13. Results for Purple Sea Urchin Chronic Tests for the Negative Control (Sample #1).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	EC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%
1	>100	100	>100	97.4	Non-toxic	0.9	96.4	Non-toxic	0.9
2	>100	100	>100	84.8	Non-toxic	4.7	89.2	Non-toxic	3.1
3	>100	100	>100	95.0	Non-toxic	4.6	91.0	Non-toxic	3.8
7	>100	100	>100	98.6	Non-toxic	2.0	80.4	Non-toxic	2.9
9	>100	100	>100	94.7	Non-toxic	3.8	88.2	Non-toxic	1.3
11*	>100	100	>100	100	Non-toxic	0.9	99.4	Non-toxic	0.5
12*	>100	100	>100	97.4	Non-toxic	5.6	95.4	Non-toxic	1.5
Min	>100	100	>100	84.8	--	0.9	80.4	--	0.5
Max	>100	100	>100	100	--	5.6	99.4	--	3.8
Median	>100	100	>100	97.4	--	3.8	91.0	--	1.5
Mean	>100	100	>100	95.4	--	3.2	91.4	--	2.0
False positives	0	--	--	--	--	--	0	--	--
False positive rate	0%	--	--	--	--	--	0%	--	--

"--": value not calculatable. *Referee laboratory

Table 9-14. Results for Purple Sea Urchin Chronic Tests for the Non-toxic Stormwater Sample (Sample #2).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	EC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%
1	>100	100	>100	92.0	Non-toxic	0.9	98.2	Non-toxic	1.3
2	>100	100	>100	81.0	Non-toxic	4.9	89.0	Non-toxic	4.1
3	>100	100	>100	91.0	Non-toxic	1.2	91.8	Non-toxic	2.2
7	>100	100	>100	94.4	Non-toxic	31.2	95.4	Non-toxic	3.1
9	>100	100	>100	92.3	Non-toxic	1.9	98.7	Non-toxic	10.5
11*	>100	100	>100	99.2	Non-toxic	1.7	99.6	Non-toxic	0.5
12*	>100	100	>100	89.8	Non-toxic	5.1	96.8	Non-toxic	0.9
Min	>100	100	>100	81.0	--	0.9	89.0	--	0.5
Max	>100	100	>100	99.2	--	31.2	99.6	--	10.5
Median	>100	100	>100	92.0	--	1.9	96.8	--	2.2
Mean	>100	100	>100	91.4	--	6.7	95.6	--	3.2
False positives	0	--	--	--	--	--	0	--	--
False positive rate	0%	--	--	--	--	--	0%	--	--

"--": value not calculatable. *Referee laboratory

Table 9-15. Results for Purple Sea Urchin Chronic Tests for the Toxic Stormwater Sample (Sample #3).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	EC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%
1	14.8	6.25	12.5	0.0	**Toxic**	1.6	0.0	**Toxic**	--
2	4.1	<6.25	6.25	0.0	**Toxic**	13.8	0.0	**Toxic**	--
3	16.2	6.25	12.5	30.6	**Toxic**	3.2	0.0	**Toxic**	15.4
7	3.9	<6.25	6.25	0.0	**Toxic**	22.1	0.0	**Toxic**	--
9	8.9	<6.25	6.25	0.0	**Toxic**	32.3	0.0	**Toxic**	--
11*	19.9	12.5	25	0.0	**Toxic**	0.5	0.0	**Toxic**	17.9
12*	8.8	<6.25	6.25	0.0	**Toxic**	8.1	0.0	**Toxic**	108
Min	3.9	<6.25	6.25	0.0	--	0.5	0.0	--	15.4
Max	19.9	12.5	25	30.6	--	32.3	0.0	--	108
Median	8.9	--	--	0.0	--	8.1	0.0	--	17.9
Mean	10.9	--	--	4.4	--	11.7	0.0	--	47.2

Values in **bold** indicate a statistically significant decrease compared to the control. "--": value not calculatable. *Referee laboratory

Table 9-16. Results for Purple Sea Urchin Chronic Tests for the Spiked Laboratory Water (Sample #4).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	EC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%
1	26.7	12.5	25	0.0	**Toxic**	1.6	0.0	**Toxic**	31.1
2	4.9	<6.25	6.25	0.0	**Toxic**	18.3	0.0	**Toxic**	67.4
3	26.0	12.5	25	6.0	**Toxic**	3.1	0.0	**Toxic**	2.4
7	7.8	<6.25	6.25	0.0	**Toxic**	8.4	0.0	**Toxic**	--
9	12.9	<6.25	6.25	0.0	**Toxic**	8.6	0.0	**Toxic**	179
11*	34.1	12.5	25	0.0	**Toxic**	0.5	0.0	**Toxic**	0.6
12*	15.8	6.25	12.5	0.0	**Toxic**	8.7	0.0	**Toxic**	56.0
Min	4.9	6.25	6.25	0.0	--	0.5	0.0	--	0.6
Max	34.1	12.5	25	6.0	--	18.3	0.0	--	179
Median	15.8	--	--	0.0	--	8.4	0.0	--	43.6
Mean	18.3	--	--	0.9	--	7.0	0.0	--	56.1

Values in **bold** indicate a statistically significant decrease compared to the control. "--": value not calculatable. *Referee laboratory

Table 9-17. Results for Purple Sea Urchin Chronic Tests Method for the Duplicate Spiked Laboratory Water (Sample #5).

Lab ID Sample #	Pulsed Exposure Methodology						Standard Methodology		
	EC_{50} (% sample)	NOEC	LOEC	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%	Mean Survival @ 100% (%)	TST Result	CV @ 6.25%
1	30.7	12.5	25	0.0	**Toxic**	0.9	0.0	**Toxic**	36.0
2	5.2	<6.25	6.25	0.0	**Toxic**	28.1	0.0	**Toxic**	20.8
3	22.2	<6.25	6.25	0.0	**Toxic**	4.1	0.0	**Toxic**	3.4
7	8.0	<6.25	6.25	0.0	**Toxic**	23.6	0.0	**Toxic**	4.4
9	17.8	<6.25	6.25	0.0	**Toxic**	3.4	0.0	**Toxic**	61.0
11*	22.2	12.5	25	0.0	**Toxic**	0.5	0.0	**Toxic**	0.5
12*	16.0	6.25	12.5	0.0	**Toxic**	3.5	0.0	**Toxic**	26.6
Min	5.2	<6.25	6.25	0.0	--	0.5	0.0	--	0.5
Max	30.7	12.5	25	0.0	--	28.1	0.0	--	61.0
Median	17.8	--	--	0.0	--	3.5	0.0	--	20.8
Mean	17.4	--	--	0.0	--	9.1	0.0	--	21.8

Values in **bold** indicate a statistically significant decrease compared to the control. "--": value not calculatable. *Referee laboratory

Table 9-18. Coefficient of Variation (CV) Values from the Purple Sea Urchin Short-term Chronic Pulsed Exposure Method.

Sample #/ Type	CV of EC_{50} (%)	CV of 6.25% Concentration (%)	
		Pulsed	Standard
1 Negative Control	-	3.2	2.0
2 Non-toxic Stormwater	-	6.7	3.2
3 Toxic Stormwater	56.5	11.7	47.2
4 Positive Control	59.2	7.0	56.1
5 Duplicate Positive Control	50.4	9.1	21.8
Mean	55.4	7.5	26.1

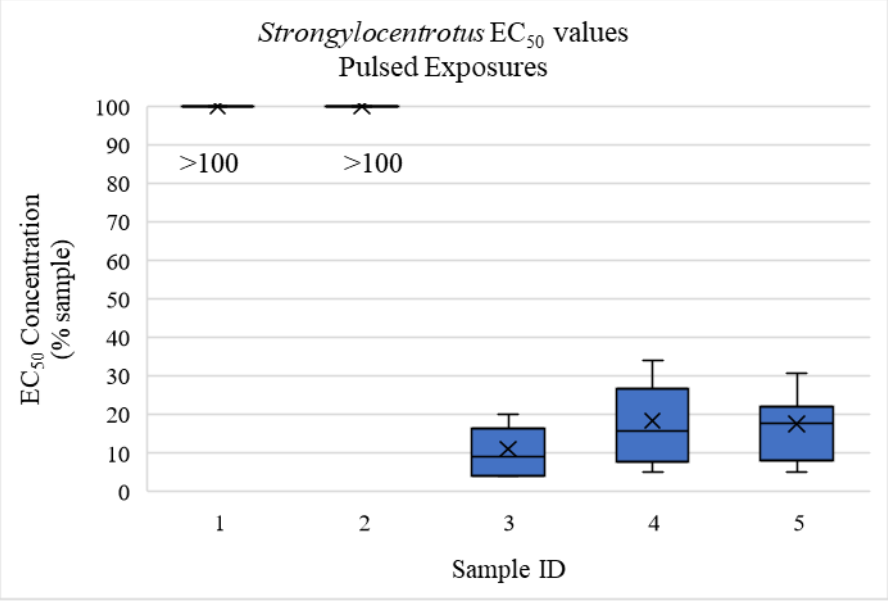

Figure 9-7. Mean point estimate values for the *Strongylocentrotus* pulsed exposures. “X” indicates the mean EC_{50} value.

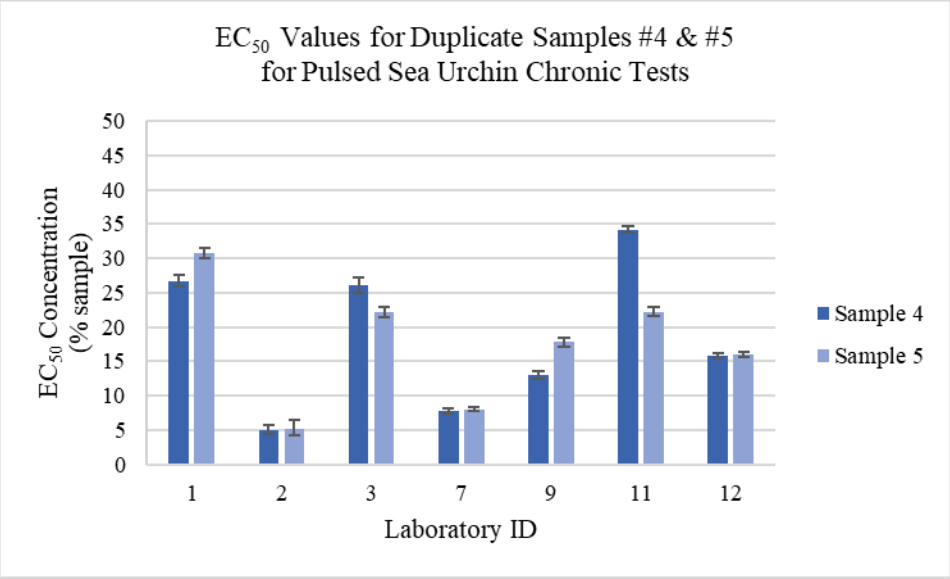

Figure 9-8. Point estimate pulsed exposure EC_{50} results for duplicate Samples #4 and 5 for the Sea Urchin pulsed exposures. Error bars are the 95% upper and lower confidence intervals.

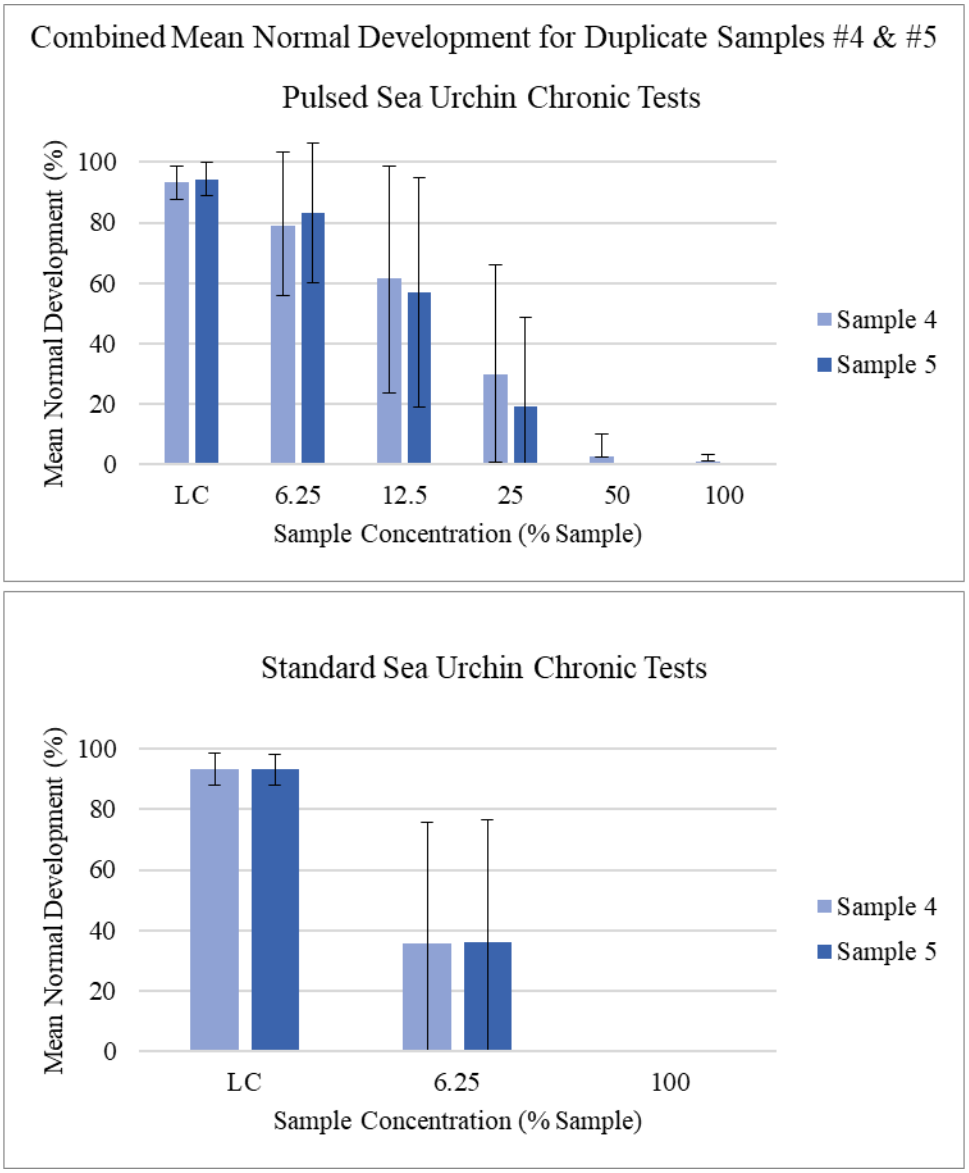

Figure 9-9. The combined mean % survival results among all laboratories for each treatment dilution for duplicate Samples #4 and 5 for the Sea Urchin pulsed (TOP) and standard (BOTTOM) exposures. Error bars represent the standard deviations.

9.4 RESULTS SUMMARY

This section summarizes the results of the Pulsed Exposure Toxicity Interlaboratory Calibration Study.

9.4.1 Successful Test Completion Rate

The test completion rates calculated for each test method were evaluated as the percentage of tests successfully initiated and terminated meeting all TAC (Section 8.3.1). Completion rates were 100% for all methods for both the pulsed exposure and the standard methods as summarized in Table 9-19 reflecting a very successful outcome for the study.

Table 9-19. Successful Test Completion Rates for Test Methods Evaluated in the Pulsed Exposure Toxicity Interlaboratory Calibration Study.

Test Method	Exposure Duration	N	No. of Invalid Tests	Successful Test Completion Rate (%)
Ceriodaphnia acute	26 hr	35	0	100
	Standard/ 96hr	35	0	100
Americamysis acute	26 hr	40	0	100
	Standard/ 96hr	40	0	100
Strongylocentrotus chronic	26 hr	35	0	100
	Standard/ 96hr	35	0	100

9.4.2 False Positive Rate

The false positive rate was calculated as the number of valid test results that indicated a deleterious effect when in fact there should be none (i.e. a statistical effect in an undiluted blank/control sample). Table 9-20 summarizes the false positive rates observed for all test methods for both pulsed exposure and standard exposures. For both static and pulsed exposures, the false positive rate for the *Ceriodaphnia* acute method was 7.12%. The false positive results for the *Ceriodaphnia* were observed only for the non-toxic stormwater sample. There were no false positive results for the laboratory blank/control water for either standard static or pulsed exposures.

False positive rates were 0% for the both the mysid acute and purple sea urchin chronic test methods for both sample types and for both exposure scenarios.

Table 9-20. False Positive Rates for Test Methods Evaluated in the Pulsed Exposure Toxicity Interlaboratory Calibration Study.

Test Method	Exposure Duration	N	False Positive Rate (%)		
			Sample 1 Negative Control	Sample 2 Non-toxic Stormwater	TOTAL
Ceriodaphnia acute	26 hr	14	0.00	14.3	7.12
	Standard/ 96hr	14	0.00	14.3	7.12
Mysid acute	26 hr	16	0.00	0.00	0.00
	Standard/ 96hr	16	0.00	0.00	0.00
Purple sea urchin chronic	26 hr	14	0.00	0.00	0.00
	Standard/ 96hr	14	0.00	0.00	0.00

9.4.3 Precision

The precision of test methods evaluated in the Pulsed Exposure Interlaboratory Calibration Study was estimated by calculating a CV for point estimates (i.e., LC_{50}s and EC_{50}s) for samples that elicited a toxic response. Additionally, to compare the pulsed exposure method to standard continuous exposure methods, the CVs of the 6.25 and 100% concentrations were calculated for each sample. CVs calculated for each test method and for each exposure scenario are summarized in Table 9-21.

Variability observed in the Pulsed Exposure Interlaboratory Calibration Study averaged 67.2 and 22.6% for LC_{50} values for the *Ceriodaphnia* and mysid acute tests for all sample types. Variability averaged 55.5% for EC_{50} values for the short-term chronic tests for all sample types.

For comparison, USEPA (2001) reported multi-laboratory precision CVs for acute WET test methods that ranged from 20.0 to 38.5% and averaged 28.4%. Previous CVs reported from USEPA (1988) were 22-167% (with a weighted mean of 50%) for acute WET methods testing reference toxicants.

Table 9-21. Mean Precision Estimates (CVs) for Test Methods Evaluated in the Pulsed Exposure Toxicity Interlaboratory Calibration Study.

Test Method	Exposure Duration	CV* (%)	
		LC_{50} or EC_{50}	6.25% Concentration
Ceriodaphnia acute	26 hr	67.2	28.7
	Standard/ 96hr	--	39.0
Mysid acute	26 hr	28.7	6.6
	Standard/ 96hr	--	5.7
Purple sea urchin chronic	26 hr	55.4	7.5
	Standard/ 96hr	--	26.1

*CVs presented are based on total variance and averaged across sample types.

9.4.4 Repeatability

Test repeatability for both pulsed and static exposures was evaluated by comparing toxic response endpoints (TST results and point estimate LC_{50}s/EC_{50}s) between the two duplicate positive control copper-spiked laboratory control waters (Samples 4 and 5). Results for the duplicate samples were found to be all very comparable both within each laboratory and between laboratories. All laboratories identified these two samples as toxic using the TST approach. Furthermore, between laboratories the pooled mean LC_{50}/EC_{50} values for the pulsed exposure tests for the two duplicate samples were all not statistically different from each other for all three test species. Intra-laboratory repeatability was calculated by looking at the relative percent differences (RPD) between the duplicate samples for each laboratory and method tested (Table 9-22). Mean RPDs for the methods tested were all below 20%.

Table 9-22. Intra-Laboratory Relative Percent Difference (%) for Duplicates Samples.

Laboratory ID	*Ceriodaphnia* Acute	Mysid Acute	Purple Sea Urchin Chronic
1	4.3	34.4	13.0
2	5.4	27.6	4.8
3	-	11.6	17.2
6	3.4	37.1	-
7	18.4	2.2	3.0
8	23.0	-	-
9	-	1.7	27.3
10	0	-	-
11	-	1.2	54.0
12	13.8	36.0	1.5
AVERAGE	**9.8**	**19.0**	**17.3**

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14. ABSTRACT

Episodic discharges (e.g. stormwater, dry-dock discharges, and pesticide applications, etc.) require environmentally-relevant, scientifically-defensible, and conservative toxicity test designs to assess potential for receiving water impacts. Currently, permittees in highly industrialized areas are regularly required to conduct 96-hour (or longer) toxicity tests on discharges associated with events that are often less than 24 hours in duration. Existing EPA whole effluent toxicity (WET) test methods developed to assess continuous point source discharges are now being applied to episodic discharges as well. However, these methods do not adequately reflect episodic discharge conditions at either the point of compliance (i.e. storm drain) or as it mixes with the receiving environment (e.g. a riverine or marine system), which can result in an overestimation of toxicity at a given site. In order to capture representative toxicity at a site, an alternative toxicity test approach is described, incorporating pulsed exposures to end-of-pipe samples. Following pulsed exposures, organisms are transferred to uncontaminated seawater (or receiving water) for the remainder of standard test period. This presentation presents the results of an Interlaboratory Calibration Study conducted in order to assess the ability of the modified methodology to provide consistent and defensible data for the assessment of episodic discharges. The study characterized 1) Completion Rate; 2) False Positive Rate; and 3) Precision on three WET methods that were modified for pulsed exposures and included acute tests with *Ceriodaphnia dubia* and *Americamysis bahia* and the larval development/short-term chronic test using *Strongylocentrotus purpuratus*.

15. SUBJECT TERMS

Whole effluent toxicity; episodic discharges; acute toxicity; stormwater toxicity; interlaboratory calibration

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