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March 2020

Standard Operating Procedures - Pulsed Exposure Methodology

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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 Navy Environmental Sustainability Development to Integration (NESDI) Program, Project #547, and the Environmental Security Technology Certification Program (ESTCP), Project ER-201727, provided funding for this Basic Applied Research project.

Released by
Robert George, Branch Head
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Under authority of
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RJP

EXECUTIVE SUMMARY

This Standard Operating Procedure was generated in support of project #547 “Demonstration of Improved Toxicity Methodology to Link Stormwater Discharges to Receiving Water Impacts at Navy Sites” as supported by the Navy Environmental Sustainability Development to Integration (NESDI) Program and project #ER-201727 “Derivation and Demonstration of an Environmentally Relevant Approach for Stormwater Toxicity Testing Compliance Monitoring” as supported by the Environmental Security Technology Certification Program (ESTCP).

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1. PROCEDURES FOR CONDUCTING TOXICITY TESTS

This document describes standard operating procedures for conducting pulsed exposure toxicity testing for evaluation of episodic and/or ephemeral discharges such as those associated with stormwater runoff. These methods are slight modifications to existing whole effluent toxicity (WET) testing protocols designed for continuous flow discharges. Test methods included herein are the chronic toxicity test using the purple sea urchin (*Strongylocentrotus purpuratus*) and acute toxicity tests using mysid shrimp (*Americamysis bahia*), water fleas (*Ceriodaphnia dubia*) and the freshwater amphipod (*Hyalella azteca*).

1.1 CHRONIC EMBRYO-LARVAL DEVELOPMENT TOXICITY TEST WITH PURPLE SEA URCHINS (*STRONGYLOCENTROTUS PURPURATUS*)

1.1.1 Objective

This method estimates the chronic toxicity of pulsed exposures of effluent and receiving waters to embryos of the purple sea urchin (*Strongylocentrotus purpuratus*). The test endpoint is normal embryo development. This method was modified from "US EPA's Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. First edition" (EPA/600/R-95/136, August 1995) and ASTM E1563-98 (2012) "Standard Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryos".

1.1.2 Materials And Supplies

- Salinity, DO and pH meter – for determining salinity, dissolved oxygen and pH for routine physical and chemical measurements
- Thermometers – laboratory grade for measuring water temperatures
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic lab ware, 50-1,000 mL for making test solutions.
- Volumetric flasks – Class A, borosilicate glass or non-toxic plastic lab ware, 100-1,000 mL for making test solutions
- Beakers, 50 - 100 mL borosilicate glass for collecting sperm from sea urchins
- Beakers, 250 mL for rinsing and settling sea urchin eggs and to hold final egg stock solutions. Larger glass beakers of Erlenmeyer flasks may also be used for the final stock solution depending on the volume of stock required
- Plastic or glass holding tanks for sea urchins – 4 to 20 L recommended supplied with aeration
- Standard test chambers – 5 dram (26 mL) or 12 dram (45 ml) glass scintillation or shell vials and caps to retain and preserve contents from the pulsed exposure replicates at the end of the full exposure period
- Pulsed Exposure test chambers – 400 mL high density polyethylene (HDPE) tri-pour beakers, 16 to 20 oz disposable clear plastic cups, or other similar material as needed per specific constituents of interest – pre-soaked in dilution water
- 25 µm Nitex screen polycarbonate tubes (inner test chamber to facilitate transfer of organisms to clean water after the pulsed exposure)
- Nitex mesh screens (37 and 80 µm) for rinsing gametes (optional)
- Nitex mesh screen (≤25 µm) for separating sperm from the eggs after fertilization
- Dilution water – 0.45 µm Filtered Seawater (FSW), artificial seawater (ASW) created using Crystal Sea® (Forty Fathoms) sea salts mixed with de-ionized water, or hypersaline brine

made from 0.45 µm filtered natural seawater and diluted with deionized water; all stored at 15°C 0.5 M KCl stock solution for spawning organisms
- Pipettes, automatic – adjustable or fixed, to cover a range of 0.1 to 5 mL and pipette tips
- Wash bottles – for reagent water, dilution water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes
- Inverted or compound microscope and slides – for inspecting gametes and counting embryos and larvae.
- Counter, two unit, 0-999 – for recording counts of embryos and larvae
- Hemocytometer – for counting sperm
- Sedgewick Rafter counting chamber – for counting eggs
- Siphon hose – for removing water from settled eggs
- Data sheets
- Parafilm
- Environmental chamber or incubator (15±1°C, ambient lighting 16 hours light/ 8 hours dark)
- Acrylic sheets – for covering test chambers
- Reference toxicant solution (lab-specific; copper chloride or copper sulfate is used at NIWC and Wood).

1.1.3 Methods

A. Obtaining and Holding Organisms

Obtain ripe sea urchins from local urchin supplier at least one day prior to test setup and hold in tanks of sufficient size to maintain good water quality (6 – 20 L minimum depending on the length of time in holding). Seawater for holding may consist of raw filtered seawater from a clean source (flow-through or static), or created using artificial salts (Crystal Sea® (Forty Fathoms) sea salts are recommended). Salinity of the seawater should be 32 ± 2 ppt and organisms should be held at a temperature of 12 – 15°C) until they are needed for testing. Continuous aeration should be provided using a glass air stone. An overhead or canister filter is also recommended to help maintain good water quality for tanks held static. Kelp should be added to tanks as a food supply when held for more than a few days. Holding and conditioning tanks should be drained and sprayed with fresh seawater at least once weekly to prevent accumulation of organic matter and bacteria. Dead animals should be removed daily.

B. Species Identification Check

Field-collected urchins must be identified to species using the following dichotomous key to confirm they are *Strongylocentrotus purpuratus*. If an urchin cannot be confirmed to be *S. purpuratus*, they cannot be used in this procedure.

West Coast (Eastern Pacific, California) Urchin Identification Key:

1a. Urchin is green (without purple on spines) → *Strongylocentrotus droebachiensis*

1b. Urchin is purple to red → go to 2

2a. Spines as long as the test is wide, red in color → *Strongylocentrotus franciscanus*

2b. Spines shorter than the test is wide, purple in color → *Strongylocentrotus purpuratus*

C. Spawning and Fertilization

1. Pour 20-30 mL seawater into 3 to 5 100 – 200 mL beakers for females and 25 mL in 100 mL beakers for males and place in 15°C incubator. These beakers will be used to collect the gametes (Step C.7).
2. Select a sufficient number of sea urchins (based on previous spawning success) so that three animals of each sex are likely to provide gametes of acceptable quality and quantity.
3. Care should be exercised when removing urchins from holding tanks, preventing unnecessary damage to tube feet. Carefully place urchin in a container lined with moist paper towels.
4. Prior to injection with KCl, rinse each urchin thoroughly with filtered, 15°C seawater. Place sea urchins with the oral cavity on top on trays with several layers of moistened (with filtered seawater) paper towels.
5. Inject 0.5 mL of 0.5M KCl into oral cavity of each urchin, cleaning needle with hot water between injections if sex of organism is not known to prevent cross contamination. Record injection time on data sheet. 0.5M KCl is prepared by dissolving 37.28 g KCl in 1 L filtered natural seawater or artificial seawater.
6. Swirl each organism for a few seconds then place back on moist paper towels.
7. When gametes begin to shed, note time, and separate sexes. In the 15°C environmental room or water bath at the same temperature place a single male onto each of the three 50-100 mL beakers and each female onto the three 100 – 250 mL beakers both oral side up. Carefully rinse eggs and sperm off each organism into individual beakers with a wash-bottle containing clean dilution water. It is optimal to obtain gametes from at least 3 spawning individuals of each sex if possible. The water for rinsing the eggs and sperm should also be at the appropriate test temperature (15±1°C).
8. Sperm should be passed through a 37-µm screen to remove debris. Sperm will pass through the screen while debris will be retained.
9. After confirming good motility of each sperm sample under the microscope (400x), combine equal quantities from up to four males, and store in refrigerator or on ice until use within 4 h. Sperm that do not exhibit good motility should not be used for testing and not combined with sperm from other males.
10. Eggs should be passed through an 80-µm screen to remove debris. The eggs will pass through the screen and debris will be retained.
11. Observe egg quality under the microscope for each spawning female. Healthy appearing eggs should be uniform round in shape with full and uniform internal texture. Select only those females with eggs that have a healthy appearance
12. Confirm fertilization success for each female by placing a drop of eggs onto a well slide with a small amount of sperm. Check for fertilization membrane after 5-10 min. Greater than 95% fertilization is recommended for a successful test. Discard any egg stocks with < 95% fertilization.
13. To determine the egg density the egg stock will need to be diluted. Always cut pipette tip so that the bore is at least 2 mm wide. The concentration of the egg stock can be determined by counting three 10 – 20-µL samples of the stock at 100X magnification

or using a Sedgewick Rafter counting chamber at a similar magnification. Prepare egg stock in dilution water to achieve a final target concentration of 2,000 eggs/mL for the Pulsed Exposure replicates with 250 ml solution volumes. A separate 400 egg/mL stock may be prepared for any current standard Static Exposures, as well as the internal laboratory reference toxicant test. Verify final prepared stock solution(s) by counting eggs once again (a minimum of 3 independent replicate counts is recommended).

D. Sperm Dilution

Sperm should be added at a density of approximately 10^5 to 10^7 sperm/mL in the final mixture. Sperm density can be confirmed with a hemocytometer or spectrophotometer if an internal curve has been documented and verified. With experience, precise sperm counts are not necessary (sperm should make diluted egg stock very slightly cloudy).

E. Fertilization and Embryo Stock Preparation

1. Add a calculated volume of sperm stock to each egg stock to create an approximate 500:1 sperm to egg ratio and mix gently. Wait 10 minutes and check for fertilization. If fertilization exceeds 90% in one or more egg stocks, combine the eggs from each into a single test stock solution. If fertilization is not at least 90% in any of the egg stocks, add a second volume of sperm stock, wait 10 minutes and re-check. If fertilization is still not 90% in any of the egg stocks, the test must be restarted with different gametes. Once again, with experience, the amount of sperm to add can be estimated eliminating the need for precise counts.
2. After fertilization and preparation of a combined egg stock, excess sperm, bacteria and protozoans should be removed by pouring embryos onto a 25-µm or smaller screen, washing delicately with dilution water, then backwashing into a suitable container with dilution water. Another acceptable option is to let the embryos settle for approximately 10 min. and then carefully siphon and replace approx. 90% of the overlying water with clean dilution water of the appropriate test temperature and repeat 2 more times. Confirm and re-adjust embryo density to approximately 2,000 embryos/mL for the pulsed exposure test replicates following the procedure above in Section C.13.
3. Maintain the resulting embryo suspension at 15°C in an incubator and keep the embryos suspended by swirling the container frequently. The test should be initiated within four hours of fertilization[1].

F. Conducting the Test

Test Preparation: Day -1 or Prior

1. The test should begin within 36 hours of sample collection (USEPA 1995).
2. Prepare randomization table using Excel, CETIS, or other similar software and keep in safe place for referral at test termination.
3. Label test chambers according to the randomization table.
4. Recommend soaking all pre-cleaned screen tubes in laboratory dilution water overnight given the high surface area of the screens and added potential for contamination from the air during storage.

Sample Preparation and Test Initiation: Day 0

1. Prepare reference toxicant dilutions and sample dilutions as required. Reference toxicant exposures are made according to species sensitivity. Approximately 1,500 mL of each concentration is prepared in order to accommodate for test exposures and to allow for excess as needed for water quality measurements in a surrogate beaker if desired (assuming five replicates per sample).
2. Add 250 mL of each test concentration to each test replicate chamber (400 mL tri-pour beakers or 16 to 20 oz disposable clear plastic cups recommended). In general, five replicates per treatment are used. Gently place a clean 25-µm Nitex screen tube into each test replicate. Ensure that no air bubbles are present under the screen. Place all test replicates in the 15°C environmental chamber on a shelf under ambient light in numerical order resulting in a randomized pattern. Cover with acrylic or plexiglas.
3. Following sample preparation and equilibration, water quality measurements of dissolved oxygen, pH, temperature, and salinity) are recorded in a single replicate container for each treatment (or surrogate chamber if used) at the initiation of the test.
4. Once test chambers/solutions have reached 15°C, add embryos using a 1-ml pipette with the tip removed to increase the bore size. Pulsed Exposures with Screen Tubes: Inoculate each screen tube with 1 mL of the 2,000 embryo/ml; Static Exposure and Reference Toxicant Test: Inoculate each replicate with 0.5 mL of a 400 embryo/mL stock. Add embryos by dispensing the stock solution just above the water's surface, being sure to avoid dispensing embryos along the sides of the screen tube. Be sure that embryo stock is always homogenized. This is accomplished by frequently mixing the contents of the flask with a combination of gentle swirling and back and forth motions or using a perforated plunger. Record the date and time that the test is initiated.

Day 1: Pulsed Exposure Organism Transfer

1. Following the pulsed exposure duration (i.e. 6, 12 or 26-hour pulse), screen tubes are removed from replicate chambers containing the test solutions. Each screen tube is gently rinsed with lab dilution water or receiving water (RW) and placed into clean, pre-rinsed, pre-labeled test chambers containing lab dilution water or RW as appropriate for the remainder of the test period. The transfer can occur by placing the screen tubes in a separate set of labeled beakers with dilution water or RW prepared ahead of time, or by placing them back in the same container after a quick rinse with dilution water or RW. A team of two is suggested for the second method approach. For both methods the dilution water or RW must be acclimated to the same test temperature (15±1°C) prior to the transfer.
2. As a quality control measure to ensure that the pulsed exposure transfer methods do not negatively impact the embryos, the same transfer procedure will also be conducted on a set of lab control test replicates.
3. Measure and record physical parameters (temp., salinity, DO, pH) in the dilution water or RW prior to transfer of the embryos to the new solution (Tinitial measurements at each renewal time).
4. Measure and record physical parameters (temp., salinity, DO, pH) in each surrogate water quality container from existing solutions (Tfinal measurements at each renewal time).

Test Maintenance: Daily

Measure and record physical parameters (temp., salinity, DO, pH) in a single replicate test chamber or a surrogate chamber from each lab control and test concentration.

G. Test Termination

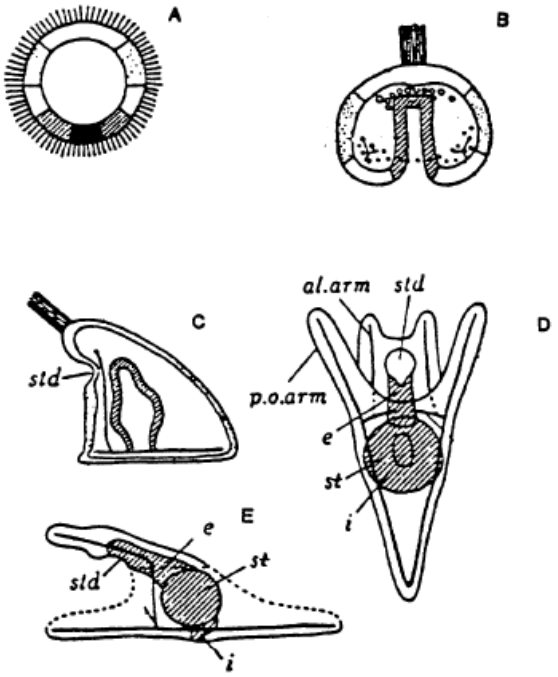
1. The embryos should be incubated for 72 hours in the test chambers at 15°C and at ambient laboratory light levels (16-hour light and 8-hour dark). If controls have not achieved the pluteus stage, the exposure can be extended up to 96 hours. Test acceptability is ≥ 80% normal development in the surviving lab controls.
2. Terminate the pulsed exposure tests by removing the screen tubes and gently rinsing contents into pre-labeled 7 or 12-dram glass scintillation vials. Preserve with the addition of 1.0 mL of 10% buffered formalin for each 10 ml of solution retained.
3. Record the date and time of test termination for each individual test.

H. Assessment of Embryo Development and Data Reporting

Within 14-days, evaluate normal development of the first 100 larvae in each replicate test chamber. Normal embryos have a pyramid shape with a pair of skeletal rods that extend at least half the length of the long axis of the larvae (see Figures 1 and 2 below for examples of both normal and abnormal embryos at 72 to 96-hours post fertilization).

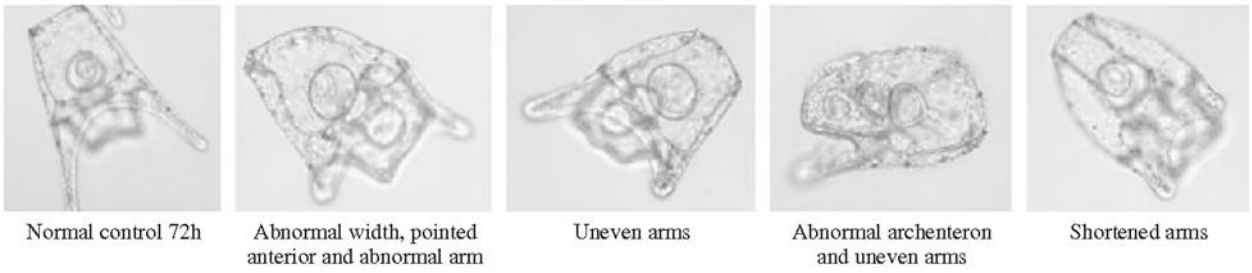
I. Analyzing Data

Using CETIS or other appropriate statistical software package, enter data retrieved from enumeration of larvae to determine the EC_{50}, LOEC, NOEC, or other desired statistical endpoint depending on the goal of the study.



Detailed in figure above: A. blastula; B. gastrula, C. prism; D. normal pluteus (frontal view); E. normal pluteus (lateral view). al arm: anterior lateral arm; e: esophagus; i: intestine; st: stomach; std: stomodaeum (EPA 2002).

Figure 1. Stages of sea urchin embryo development (normal).



Note missing or deformed arms, underdeveloped or malformed body shape, and incomplete or missing gut (Reichard-Brown et al. 2009).

Figure 2. Examples of normal and abnormal sea urchin embryos at 72 to 96-hours post fertilization.

1.2 ACUTE SURVIVAL TOXICITY TEST WITH JUVENILE MYSID SHRIMP (*AMERICAMYSIS BAHIA*)

1.2.1 Objective

This method estimates the acute (96 hour) toxicity of pulsed exposures of effluent and receiving waters to the mysid (*Americamysis bahia*) using three to five-day old juveniles. The test endpoint is percent survival. This method was modified from the US EPA's "Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition" (EPA/821/R/02/012, October 2002). Note: While the methods below describe only 20°C, this test may be performed at either 20 or 25°C.

1.2.2 Materials and Supplies

- Plastic or glass holding tanks – 3 to 6 L
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic lab ware, 50-1,000 mL for making test solutions
- Salinity, DO and pH meter – for determining salinity, dissolved oxygen and pH for routine physical and chemical measurements
- Thermometers – laboratory grade for measuring water temperatures
- Test chambers – 400 mL high density polyethylene (HDPE), tri-pour beakers, 16 – 20 oz disposable plastic drinking cups, or other material as needed per specific constituents of interest – pre-soaked in dilution water
- 25 µm Nitex screen polycarbonate tubes (inner test chamber to facilitate transfer of organisms to clean water after the pulsed exposure). Alternatively, a single 25 µm Nitex screen (with or without the tube) can be used to transfer mysids if inner polycarbonate tubes are not used.
- Dilution water – 0.45 µm Filtered Seawater (FSW), artificial seawater (ASW) created using Crystal Sea® (Forty Fathoms) sea salts mixed with de-ionized water, or hypersaline brine made from filtered natural seawater and diluted with deionized water; all stored at 20°C
- Pipettes, automatic – adjustable or fixed, to cover a range of 0.1 to 5 mL and pipette tips
- Disposable plastic transfer pipettes with tips cut off (for capturing shrimp)
- Beakers – Class A, borosilicate glass or non-toxic plastic lab ware, 1 to 2 L for making test solutions
- Wash bottles – for reagent water, dilution water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes
- Data sheets
- Brine Shrimp, *Artemia salina* (< 24-hr old post-hatch culture) to feed mysids
- Separatory funnels, 2 L – two for culturing *Artemia*
- Light box – for examining/counting organisms
- Environmental chamber or incubator (20 ±1°C, ambient laboratory lighting 16-hours light/ 8-hours dark)
- Acrylic or Plexiglas sheets – for covering test chambers
- Reference toxicant solution (lab-specific; copper chloride or copper sulfate is used at NIWC and Wood).

1.2.3 Methods

A. Obtaining, Holding and Feeding Organisms

1. Mysids should be 1-3 days old at time of shipping, so that they will be between 3–5 days old at the start of the test.
2. Prepare *Artemia* culture on day of mysid order so that there are freshly hatched nauplii available when the mysids arrive. Hatching takes 24–36 hour at 25°C.
3. Upon receipt of mysids, open plastic bag and measure and record arrival temperature, pH, DO and salinity.
4. Provide gentle aeration by placing an air stone in the bag.
5. Transfer mysids to a large plastic holding tank (3–6 L) in a 20°C temperature-controlled room, incubator, or water bath. The easiest way to transfer is to place open plastic bag in holding tank and cut open bottom of bag with a razor blade. Gently pull up on bag, releasing mysids into the container. A squirt bottle filled with filtered seawater can be used to help get mysids off plastic and into the tank. Remove dead by siphoning out of tank with Tygon tubing.
6. Perform approximately 50% water change with laboratory dilution seawater adjusted to 20°C. Be sure seawater is within 2 ‰ and 2°C of the arriving conditions. If the salinity is below the desired level (usually 30 ‰), adjust by no more than 2 ‰ per day.
7. Collect newly hatched *Artemia* nauplii. Pipette nauplii so that each mysid receives approximately100 nauplii per day.
8. Each day prior to initiation of the test, monitor the condition of the mysids in holding tanks, remove any dead, record physical parameters (temp, pH, salinity, DO), perform a 50% water change with 20°C dilution water of the appropriate salinity, and feed. The final salinity in the holding bowl before test initiation should be 32±2ppt.

B. Conducting the Test

Test Preparation: Day -1 or Prior

1. Test should begin within 36 hours of sample collection (USEPA 2002).
2. Prepare randomization table using Excel, CETIS, or similar software and keep in safe place for referral at test termination.
3. Label test chambers according to the randomization table.
4. Recommend soaking all pre-cleaned screen tubes in laboratory dilution water overnight given the high surface area of the screens and added potential for contamination from the air during storage.

Sample Preparation and Test Initiation: Day 0

1. Prepare reference toxicant dilutions and sample dilutions as required. Approximately 2,000 mL of each concentration is prepared in order to accommodate for test exposures and to allow for excess for water quality measurements in a surrogate beaker if desired.
2. For all samples, add 250 mL of each concentration to each test chambers. In general, five to six replicates per treatment are recommended. To prevent potential confusion, isolate different pulsed exposure durations into groups.

3. Gently place a 25-µm Nitex screen tube into each test replicate. Ensure that no air bubbles are present under the screen. An optional procedure may be conducted without screen tubes as well; in this case mysids are counted and added directly to a replicate test chamber without the screen tube.
4. Place all test replicates in the environmental chamber on a shelf under ambient light in a 20°C temperature-controlled facility. Place the test chambers in numerical order resulting in a randomized pattern.
5. Cover and allow solutions to equilibrate to the test temperature as needed until within the protocol range of 20 ±1°.
6. Following equilibration, water quality measurements (dissolved oxygen, pH, temperature, and salinity) should be recorded from the "A" replicate for each treatment, or in a surrogate replicate, at the initiation of the test and daily thereafter until termination.
7. Randomly distribute 5 mysids to each test chamber inside the Nitex screen tube, using a 1 or 5 mL plastic disposable pipette with the lowest 0.5 cm cut off to increase the bore size to prevent injury. It is generally easiest to track and count mysids with the holding tank and beakers on a light table. Record the date and time that the test is initiated.
8. Feed all replicates with *Artemia*.
9. Cover test chambers with acrylic or Plexiglas cover sheet to reduce evaporation potential and potential for cross-contamination.

Test Maintenance: Daily

1. Measure and record physical parameters (temp., salinity, DO, pH) (refer to randomization table to determine "A" replicate for each treatment/concentration). If DO is below 4.0 mg/L in any concentration for a test, aerate all beakers for that test. Provide a gentle bubble rate (no more than 100 bubbles/minute).
2. Note and remove any mortalities and record surviving organisms on datasheet.
3. Feed *Artemia* nauplii twice daily.

Day 1: Pulsed Exposure Organism Transfer

1. Following the pulsed exposure duration (i.e. 6, 12, 26-hour pulse), screen tubes are removed from each test chambers containing test solutions and mysid shrimp are transferred to clean dilution water or RW to assess latent mortality throughout the remaining duration of the test. After removing the screen tube from each test chamber, rinse gently with dilution water or RW before placing into a clean, pre-rinsed, pre-labeled replicate test chamber containing dilution water or RW as appropriate for the remainder of the test period
2. Another option if screen tubes are not available is to test the mysids in replicate test chambers without the inner screens and after the pulsed exposure by gently pouring the contents of each replicate test chamber through a 25 µm Nitex mesh screen, rinsing with dilution water or RW, and then rinsing the mysids into a clean pre-labeled replicate test chamber with lab dilution water or RW as appropriate.
3. The mysids (with or without screen tubes) can be rinsed into a separate set of labeled beakers with dilution water or RW prepared ahead of time, or by placing them back in

the same container after a quick rinse with dilution water. A team of two is suggested for the second method approach. For both methods the dilution water or RW must be acclimated to the same test temperature (20±1°C) prior to the transfer.

4. As a quality control measure to ensure that the pulsed exposure transfer methods do not negatively impact the mysids, the same transfer procedure must also be conducted on an additional artificial seawater lab control (Method Lab Control).
5. Measure and record physical water quality parameters (temperature, conductivity, DO, and pH) in the dilution water or RW prior to transfer of the mysids to the new solution ($T_{initial}$ measurements at each renewal time).
6. Measure and record physical water quality parameters (temperature, conductivity, DO, pH) in the "A" replicate or surrogate test chambers with the pulsed exposure solution after transferring mysids (T_{final} measurements at each renewal time); refer to randomization table to determine "A" replicate for each treatment/concentration).

C. Test Termination

The mysid acute test is terminated 96±2 hours following the initiation of the test.

1. Measure and record physical parameters (temp., salinity, DO, pH) (refer to randomization table to determine "A" replicate for each treatment/concentration).
2. Make final mortality observations and record.
3. Terminate tests by pouring contents of beakers through a sieve into a secondary container. Surviving mysids should be sacrificed by freezing or other humane methods.

D. Analyzing Data

Using CETIS or other appropriate statistical software package, enter data retrieved from the Excel files to determine the LC_{50}, LOEC, NOEC, or other desired statistical endpoint depending on the goal of the study.

1.3 ACUTE SURVIVAL TOXICITY TEST WITH NEONATE WATER FLEAS (*CERIODAPHNIA DUBIA*)

1.3.1 Objective

This method estimates the acute (96 hour) toxicity of pulsed exposures of effluent and receiving waters to water fleas (Ceriodaphnia dubia) using less than 24-hour old neonates. The test endpoint is percent survival. This method was modified from the US EPA's "Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition" (EPA/821/R/02/012, October 2002).

1.3.2 Materials and Supplies

- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic lab ware, 50–1000 mL for making test solutions
- Conductivity, hardness, DO and pH meter – for determining conductivity, dissolved oxygen and pH for routine physical and chemical measurements
- Thermometers – laboratory grade for measuring water temperatures
- Standard and pulsed exposure test chambers – 30 to 40 mL soufflé cups or 5 dram (26-ml) glass scintillation vials
- Dilution water – moderately hard water prepared using 4 parts Nanopure water and 1 parts Perrier ® maintained at 25°C (often also referred to as 8:2 dilution water).
- Yeast, trout chow and cerophyll (YTC) mixture and *Selenastrum* algae for feeding
- Pipettes, automatic – adjustable or fixed , to cover a range of 0.1 to 5 mL and pipette tips (for feeding and internal reference toxicant tests).
- Disposable plastic transfer pipettes with tips cut off (for capturing/counting organisms)
- Beakers – Class A, borosilicate glass or non-toxic plastic lab ware, 0.5 to 2 L for making test solutions and for holding neonate *Ceriodaphnia*
- Wash bottles – for reagent water, dilution water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes
- Data sheets
- Environmental chamber or incubator (25 ± 1°C, ambient laboratory lighting 16-hours light/ 8- hours dark)
- *Ceriodaphnia* culture – maintained in-house or received day prior to test
- Acrylic or Plexiglas sheets – for covering test chambers
- Shallow glass dish for counting out *Ceriodaphnia*

1.3.3 Methods

A. Preparation of Dilution Water

Create moderately hard water for test dilutions, cultures, and control. To create moderately hard water Nanopure water is mixed with Perrier® water in a 4:1 ratio. Alternatively, synthetic freshwater can be prepared through the addition of reagent grade chemicals as indicated methods described in USEPA 2002. Using these methods hardness (measured as $CaCO_3$) should be between the target of 80-100 mg/L, pH between 7.9 and 8.3, and alkalinity between 57 and 64 mg/L.

B. Obtaining, Holding and Feeding Organisms

1. *Ceriodaphnia* can be cultured in-house or a culture can be purchased from a reputable supplier (i.e. Aquatic Biosystems, Fort Collins, CO or Aquatic Research Organisms, Hampton, NH). Culture boards are prepared and maintained/renewed daily. Neonates for testing are isolated from culture boards on Day 0 of the test.
2. Neonates (< 24 hr old) are collected and sorted into a small glass dish, fed a 1:1.5 mixture of YTC and *Selenastrum,* and set aside for 2 hours at 25°C prior to test initiation.

C. Conducting the Test

Test Preparation: Day -1 or Prior

1. Test should begin within 36 hours of sample collection for field-collected samples (USEPA 1995) and on the day of dilution prep for the reference toxicant tests.
2. Prepare randomization table using Excel, CETIS, or other similar program and keep in safe place for referral at test termination.
3. Label test chambers according to randomization table.
4. Prepare dilution water 1 to 2 days prior to testing and aerate continuously with a glass air stone to allow complete mixing/ acclimation. Re-check hardness, pH, and conductivity to confirm it is within the protocol range on the day of test initiation

Sample Preparation and Test Initiation: Day 0

1. Prepare reference toxicant dilutions and sample dilutions as required. Approximately 250 mL of each concentration is prepared in order to accommodate for test exposures and to allow for excess as a surrogate for water quality readings and for analytical chemistry as needed.
2. Two controls should be prepared and tested concurrently. One of culture water and a second of culture water with the conductivity adjusted to reflect the highest conductivity tested among the samples; termed “conductivity control”. Conductivity is increased in the conductivity control through the addition of 0.45 µm filtered seawater one drop at a time until appropriate conductivity is reached. Recommended conductivity range for testing with *Ceriodaphnia* is 100 – 1,900 µS/cm; substitute with *H. azteca* if conductivity is >2,500 µS/cm (recommended from California Surface Water Ambient Monitoring Program (SWAMP) July 2018).
3. For all samples, add 20 mL of each concentration to pre-labeled and randomized test chambers. In general, five replicates per treatment are used (the minimum protocol requirement is 4).
4. Place all test replicates in the environmental chamber on a shelf under ambient light in a 25°C temperature-controlled facility. Place the test chambers in numerical order resulting in a randomized pattern. Cover and place test chambers and allow them to equilibrate for at least 30 minutes. To prevent potential confusion, isolate different pulsed exposure durations into groups.
5. Following equilibration, water quality measurements (e.g. dissolved oxygen, pH, temperature, conductivity, alkalinity and hardness) should be recorded from a surrogate container for each treatment at the initiation of the test and daily thereafter until

termination. Approximately 150 mL of solution should be remaining from each treatment/concentration to allow for sufficient volume in the surrogate cup for water quality measurements.

6. Randomly distribute 5 *Ceriodaphnia* neonates to each test chamber, using a 1 or 5 mL plastic disposable pipette with the lowest 0.5 cm cut off to increase the bore size and prevent injury. It is important to minimize the volume of culture water added with each neonate to avoid dilution of the test sample. One technique is to first count out 5 neonates into a separate soufflé cup, remove excess water with a pipette, and carefully use the pipette to rinse the neonates into the test solution using the test solution itself. Rinse the pipette thoroughly with DI prior between each transfer. It is generally easiest to track and count neonates with the holding container and test chambers on a light table or with the use of a dissecting microscope. Record the date and time that the test is initiated.
3. Cover test chambers with acrylic or Plexiglas cover sheet to reduce evaporation potential and potential for cross-contamination.

Test Maintenance: Daily

1. Measure and record physical parameters (temperature, conductivity, DO, pH) in each surrogate water quality container.
2. Note and remove any mortalities and record surviving organisms on datasheet. Immobile *Ceriodaphnia* that do not respond to a stimulus of a gentle stream of water are considered dead.
3. Add 100 µL *Selenastrum* and 100 µL YTC as a food source at approximately 48-hr post-test initiation.

Day 1: Pulsed Exposure Organism Transfer

1. Following the pulsed exposure duration (i.e. 6, 12 or 26-hour pulsed exposure), Ceriodaphnia neonates are removed from their respective test chambers with a disposable transfer pipette (with the tip cut off) into new, pre-labeled test chambers containing laboratory dilution water for the remainder of the test period to assess latent mortality throughout the remaining duration of the test.
2. As a quality control measure to ensure that the pulsed exposure transfer methods do not negatively impact the Ceriodaphnia, the same transfer procedure must also be conducted on an additional lab control (Method Lab Control).
3. Prepare new exposure chambers containing lab dilution water. Measure and record physical parameters (temperature, conductivity, DO, pH); this is termed the Tinitial measurements at each renewal time).
4. Measure and record physical parameters (temperature, conductivity, DO, pH); in surrogate water quality container from existing solutions. This is termed the Tfinal measurements at each renewal time).
5. After two hours, use a dissecting microscope to transfer test organisms into matching new, pre-filled test chambers.
6. Cover with acrylic or Plexiglas sheet and return to 25°C temperature-controlled facility.

D. Test Termination

The *Ceriodaphnia* acute test is terminated 96 ±2 hours following the initiation of the test.

1. Make final mortality observations from each replicate and record.
2. Measure and record physical parameters (temperature, conductivity, DO, pH) by taking a sample from each replicate and combining into pre-label water quality containers.

E. Analyzing Data

Using CETIS or other appropriate statistical software package, enter data retrieved from the Excel files to determine the LC_{50}, LOEC, NOEC, or other desired statistical endpoint depending on the goal of the study .

1.4 ACUTE SURVIVAL TOXICITY TEST WITH THE FRESHWATER AMPHIPOD (*HYALELLA AZTECA*)

1.4.1 Objective

This method estimates the acute (96 hour) toxicity of pulsed exposures of effluent and receiving waters to the freshwater amphipod (Hyalella azteca) using 7 to 14 day old juveniles. The test endpoint is percent survival. This method was modified from the US EPA's "Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition" (EPA/821/R/02/012, October 2002).

1.4.2 Materials and Supplies

- Plastic holding tanks – 3 to 6 L
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic lab ware, 50–1,000 mL for making test solutions
- Conductivity, DO and pH meter – for determining conductivity, dissolved oxygen and pH for routine physical and chemical measurements
- Thermometers – laboratory grade for measuring water temperatures
- Standard and pulsed exposure test chambers – 400 mL high density polyethylene (HDPE) tri-pour beakers, 16 - 20 oz or similar clear disposable drinking cups, or other material as needed per specific constituents of interest – pre-soaked in dilution water
- 25 µm Nitex screen polycarbonate tubes (inner test chamber to facilitate transfer of organisms to clean water after the pulsed exposure). Alternatively, a single 25 µm Nitex screen (with or without the tube) can be used to transfer mysids if inner polycarbonate tubes are not used.
- Dilution water – Moderately hard water prepared by mixing 4 parts Nanopure water with 1 part Perrier® (also referred to as 8:2); maintained at23°C
- Ground TetraMin® fish flake food or mixture of yeast, Cerophyll and trout chow (YTC) for feeding
- Pipettes, automatic – adjustable or fixed, to cover a range of 0.1 to 1.0 mL and pipette tips
- Disposable plastic transfer pipettes with tips cut off (for handling organisms)
- Beakers- Class A, borosilicate glass or non-toxic plastic lab ware, 1 to 2L for making test solutions
- Wash bottles – for reagent water, dilution water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes
- Data sheets
- Light box – for examining organisms
- Environmental chamber or incubator (23±1°C, ambient laboratory lighting 16-hours light/ 8-hours dark)
- Acrylic or Plexiglas sheets – for covering test chambers
- Reference toxicant solution (copper chloride is used at the Wood laboratory)

1.4.3 Methods

A. Preparation of Dilution Water

1. A few different options are available for control and dilution water. The type of water (hardness and pH in particular) should be catered to best match the test samples of interest if possible. A common method includes the creation of moderately hard water by mixing Nanopure water with Perrier® water in a 4:1 ratio. Hardness of this dilution water should be between 80 and 100 mg/L as $CaCO_3$. pH between 7.9 and 8.3, and alkalinity between 57 – 64 mg/L.
2. Alternatively, synthetic freshwater can be prepared through the addition of reagent grade chemicals as indicated methods described in USEPA 2002 or filtration of tap water to achieve a desired hardness.
3. Make dilution water at least 1 to 2 days prior to testing and aerate continuously with a glass air stone to allow complete mixing/ acclimation. Re-check hardness, pH, and conductivity to confirm it is within the desired range on the day of test initiation.

B. Obtaining, Holding and Feeding Organisms

1. Amphipods should be 5–12 days old at time of shipping, so that they will be 7–14 days old at start of test. Age difference among organisms used should not be greater than 2 days.
2. Upon receipt of amphipods, open plastic bag and measure arrival temperature, pH, DO and conductivity.
3. Provide gentle aeration by placing an air stone in the bag.
4. Transfer amphipods to a large plastic holding tank (3– L) in a 23°C temperature-controlled room, incubator, or water bath. The easiest way to transfer is to place the open plastic bag in a holding tank and cut open bottom of bag with a razor blade. Gently pull up on bag, releasing amphipods into the container. A squirt bottle filled with 4:1 culture water can be used to help get amphipods off plastic and into the tank. Remove dead by siphoning out of tank with Tygon tubing.
5. Perform approximately 50% water change with 4:1 culture water adjusted to 23°C.
6. Each day prior to initiation of the test, monitor the condition of the amphipods in holding tanks, remove any dead, record physical parameters (temp, pH, conductivity, DO), perform a 50% water change with 23°C culture water of the appropriate salinity, and feed.

C. Conducting the Test

Test Preparation: Day -1 or Prior

1. Test should begin within 36 hours of sample collection for field-collected samples (USEPA 2002).
2. Prepare randomization table using Excel, CETIS, or similar program and keep in safe place for referral at test termination.
3. Label test chambers according to randomization table.

Sample Preparation and Test Initiation: Day 0

1. Prepare reference toxicant dilutions and sample dilutions as required. Approximately 2,000 mL of each concentration is generally prepared in order to accommodate for test exposures and to allow for excess as needed for measurement of water quality parameters if the use of a surrogate chamber is desired.
2. For all samples, add 250 mL of each concentration to labeled pre-randomized test chambers. Typically, six replicates per treatment are used (minimum protocol requirement is 4). To prevent potential confusion, isolate different pulsed exposure durations into groups.
3. Gently place a 25 µm Nitex screen tube into each test replicate. Ensure that no air bubbles are present under the screen. Cover and place test chambers with solutions in a 23°C temperature-controlled facility to equilibrate for at least 30 minutes. An optional procedure may be conducted without screen tubes as well; in this case amphipods are counted and added directly to a replicate test chamber without the screen tube.
4. Following equilibration, water quality measurements (e.g. dissolved oxygen, pH, temperature, conductivity, alkalinity and hardness) should be recorded from the "A" replicate for each treatment at the initiation of the test and daily thereafter until termination.
5. Place all test replicates in the environmental chamber on a shelf under ambient light in a 23°C temperature-controlled facility or water bath. Place the test chambers in numerical order resulting in a randomized pattern.
6. Randomly distribute 5 amphipods to each test chamber inside the Nitex screen tube, using a 5 mL plastic pipette with the lowest 0.5 cm cut off to prevent injury. It is generally easiest to track and count amphipods with the holding tank and beakers on a light table. Record the date and time that the test is initiated.
7. Cover test chambers with acrylic or Plexiglas cover sheet to reduce evaporation potential and cross-contamination potential.

Test Maintenance: Daily

1. Measure and record physical parameters (temperature, conductivity, DO, pH) (refer to randomization table to determine "A" replicate for each treatment/concentration). If DO is below 4.0 mg/L in any concentration for a test, aerate all beakers for that test. Provide a gentle bubble rate (no more than 100 bubbles/minute).
2. Note and remove any mortalities and record surviving organisms on datasheet.
3. Feed 0.5 mL YCT or TetraMin® fish food flake solution at 48 hour during test. Add 4.0 g of grounded TetrMin® fish food to 100 mL dilution water and stir vigorously while subsampling with a 1-ml disposable pipette for feeding.

Day 1: Pulsed Exposure Organism Transfer

1. Following the pulsed exposure duration (i.e. 6, 12, or 26-hr hour pulsed exposure), screen tubes with the amphipods are removed from each test chamber containing test solutions and are transferred to clean dilution water or RW to assess latent mortality throughout the remaining duration of the test. After removing the screen tube from each test chamber each screen tube is gently rinsed with dilution water or RW before

placing into a clean, pre-rinsed, pre-labeled replicate test chamber containing dilution water or RW as appropriate for the remainder of the test period.

2. Another option if screen tubes are not available is to test the amphipods in replicate test chambers without the inner screens and after the pulsed exposure gently pour the contents of each replicate test chamber through a 25 µm Nitex mesh screen, rinse with dilution water or RW, and then rinse the amphipods into a clean pre-labeled replicate test chamber with lab dilution water or RW as appropriate.
3. The amphipods (with or without screen tubes) can be rinsed into a separate set of labeled beakers with dilution water prepared ahead of time, or by placing them back in the same container after a quick rinse with dilution water. A team of two is suggested for the second method approach. For both methods the dilution water must be acclimated to the same test temperature (23 ±1°C) prior to the transfer.
4. As a quality control measure to ensure that the transfer methods do not negatively impact the organisms, ensure that a lab control that will undergo the transfer as well.
5. Prepare new exposure chambers containing culture water. Measure and record physical parameters (temperature, conductivity, DO, pH); this is termed the Tinitial measurements following the pulsed exposure.
6. Measure and record physical parameters (temperature, conductivity, DO, pH) in surrogate water quality container from existing solutions; this is termed the Tfinal measurements following the pulsed exposure.

D. Test Termination

The *Hyalella* acute test is terminated 96±2 hours following the initiation of the test.

1. Measure and record physical parameters (temperature, conductivity, DO, pH) (refer to randomization table to determine "A" replicate for each treatment/concentration).
2. Make final mortality observations and record.
3. Terminate tests by pouring contents of beakers through a sieve into a secondary container. Surviving amphipods should be sacrificed by freezing or other humane methods.

E. Analyzing Data

Using CETIS or other appropriate statistical software package, enter data retrieved from the Excel files to determine the LC_{50}, LOEC, NOEC, or other desired statistical endpoint depending on the goal of the study .

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2. TEST CONDITIONS AND ACCEPTABILITY CRITERIA FOR PULSED EXPOSURE TOXICITY TESTING

2.1 CHRONIC EMBRYO-LARVAL DEVELOPMENT TOXICITY TEST WITH PURPLE SEA URCHINS

Table 1 shows test specifications for conducting the Embryo-larval development test using the purple sea urchin, *Strongylocentrotus purpuratus*.

Table 1. Purple Sea Urchin Embryo-Larval Development Toxicity Test Specifications

Subject	Detail
Test organism	*Strongylocentrotus purpuratus*
Test endpoints	Embryo Development Rate (Proportion Normal)
Test solution renewal	At pre-determined pulsed exposure durations
Feeding	None
Test chamber size/type	400 mL polyethylene (HDPE) tri-pour containers, 16 – 20 oz disposable clear plastic drinking cups, or other similar container specific to the needs of the project. All test replicates to include polycarbonate screen tubes with 25 µm mesh to hold the embryos and facilitate transfer to clean water after the pulsed exposure
Test solution volume	250 mL
Test temperature	15 ± 1°C
Test salinity	34 ± 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	250 eggs, appropriate sperm density to provide > 90% fertilization success (determined in a pre-test trial)
No. of replicates	5 recommended (4 minimum)
Dilution water	Uncontaminated 0.45 µm filtered natural seawater, artificial seawater (Crystal Sea® (Forty Fathoms) sea salts recommended), or hypersaline brine prepared from natural 0.45 µm filtered natural seawater (32 ± 2 ppt)
Test concentrations	Project-specific. Effluent monitoring under NPDES Permits often requires 5 concentrations and a control (e.g. 0, 6.25, 12.5, 25, 50, & 100%). Many stormwater permits require a test of the 100% concentration only. Receiving waters: 100% receiving water and a control
Test duration	Pulsed Exposures: (e.g. 6, 12, or 26 hours) to test solution followed by transfer to clean ASW, 0.45 µm FSW, or RW; 96-hour total test duration
Test acceptability criteria	≥ 80% normal development in surviving controls; < 25% Minimum Significant Difference (MSD)
Test protocol	Modified from EPA 600/R-95/136 (USEPA 1995); ASTM E1563-98 (2012)

2.2 ACUTE SURVIVAL TOXICITY TEST WITH JUVENILE MYSID SHRIMP

Table 2 shows test specifications for conducting the acute toxicity test conducted with mysid shrimp, *Americamysis bahia*.

Table 2. Mysid Shrimp Acute Survival Toxicity Test Specifications

Subject	Detail
Test organism	*Americamysis bahia* (Mysid shrimp)
Test endpoints	Survival
Test solution renewal	None
Feeding	Feed 100 newly hatched *Artemia* nauplii per shrimp twice daily, morning and evening
Test chamber size/type	400 mL polyethylene (HDPE) tri-pour containers,16 – 20 oz disposable clear plastic drinking cups, or other similar container specific to needs of the project. Recommended: include polycarbonate screen tubes with 25 µm mesh to hold amphipods and facilitate transfer to clean water after the pulsed exposure. Organisms may also be transferred after each pulse by pouring animals onto a single screen and rinsing back into clean dilution water or receiving water (RW).
Test solution volume	250 mL
Test temperature	20 ± 1°C
Test salinity	30 ± 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 recommended (4 minimum)
Dilution water	Uncontaminated 0.45 µm filtered natural seawater, artificial seawater (Crystal Sea® (Forty Fathoms) sea salts recommended), or hypersaline brine prepared from natural 0.45 µm filtered natural seawater (32 ± 2 ppt)
Test concentrations	Project-specific. Effluent monitoring under NPDES Permits often requires 5 concentrations and a control (e.g. 0, 6.25, 12.5, 25, 50, & 100%). Many stormwater permits require a test of the 100% concentration only. Receiving waters: 100% receiving water and a control
Test duration	Pulsed Exposures: (e.g. 6, 12, or 26) hours to test solution followed by transfer to clean ASW, 0.45 µm FSW, or RW; 96-hour total test duration
Test acceptability criteria	≥ 90% survival in controls
Test protocol	Modified from EPA 821/R-02/012, 2002

2.3 ACUTE SURVIVAL TOXICITY TEST WITH NEONATE WATER FLEAS

Table 3 shows test specifications for conducting the acute toxicity test conducted with the water flea, *Ceriodaphnia dubia*.

Table 3. Water Flea Acute Survival Toxicity Test Specifications

Subject	Detail
Test organism	*Ceriodaphnia dubia* (water flea)
Test endpoints	Survival
Test solution renewal	None
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; 100 µL YCT and 100 µL *Selenastrum* per replicate.
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 recommended (4 minimum).
Age of test organism	<24 hour
No. of replicates	5
Dilution water	Moderately hard water prepared in accordance with EPA protocols (i.e., 4:1 culture water prepared with Nanopure water Perrier® or EPA recipe with salts)
Test concentrations	Project-specific. Effluent monitoring under NPDES Permits often requires 5 concentrations and a control (e.g. 0, 6.25, 12.5, 25, 50, & 100%). Many stormwater permits require a test of the 100% concentration only. Receiving waters: 100% receiving water and a control
Test duration	Pulsed Exposures: (e.g. 6, 12, or 26 hours) to test solution followed by transfer to clean culture water or RW; 96-hour total test duration
Test acceptability criteria	≥ 90% survival in controls
Test protocol	Modified from EPA 821/R-02/012, 2002

2.4 ACUTE SURVIVAL TOXICITY TEST WITH THE FRESHWATER AMPHIPOD

Table 4 shows test specifications for conducting the acute toxicity test conducted with the freshwater amphipod, *Hyalella azteca*.

Table 4. Freshwater Amphipod Acute Survival Toxicity Test Specifications

Subject	Detail
Test organism	*Hyalella azteca* (amphipod)
Test endpoints	Survival
Test solution renewal	None
Feeding	Feed YCT or ground TetraMin® fish food flakes iwhile holding prior to the test; feed 0.5 mL YCT or TetraMin® fish food flake solution at 48 hour during test. Add 4.0g of grounded TetrMin® fish food to 100 mL dilution water and stir vigorously while subsampling with a 1-ml disposable pipette for feeding.
Test chamber size/type	400 mL polyethylene (HDPE) tri-pour containers,16-20 oz disposable clear plastic drinking cups, or other similar container specific to needs of the project. Reccomended: include polycarbonate screen tubes with 25 µm mesh to hold amphipods and facilitate transfer to clean water after the pulsed exposure. Organisms may also be transferred after each pulse by pouring animals onto a single screen and rinsing back into clean dilution water or receiving water (RW).
Test solution volume	250 mL
Test temperature	23 ± 1°C
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 amphipods per chamber	5
Age of test organism	7 – 14 days; 1-2 day range in age
No. of replicates	5 recommended (4 minimum)
Dilution water	Moderately hard water prepared in accordance with EPA protocols (i.e., 4:1 culture water prepared with Nanopure water and Perrier® or EPA recipe with salts)
Test concentrations	Project-specific. Effluent monitoring under NPDES Permits often requires 5 concentrations and a control (e.g. 0, 6.25, 12.5, 25, 50, & 100%). Many stormwater permits require a test of the 100% concentration only. Receiving waters: 100% receiving water and a control
Test duration	Pulsed Exposures: (e.g. 6, 12, or 26 hours) to test solution followed by transfer to clean culture wate or RW; 96-hour total test duration
Test acceptability criteria	≥ 90% survival in controls (required)
Test protocol	Modified from EPA 821/R-02/012, 2002

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14. ABSTRACT

This document describes standard operating procedures for conducting pulsed exposure toxicity testing for evaluation of episodic and/or ephemeral discharges such as those associated with stormwater runoff. These methods are slight modifications to existing whole effluent toxicity (WET) testing protocols designed for continuous flow discharges. Test methods included herein are the chronic toxicity test using the purple sea urchin (*Strongylocentrotus purpuratus*) and acute toxicity tests using mysid shrimp (*Americamysis bahia*), water fleas (*Ceriodaphnia dubia*) and the freshwater amphipod (*Hyalella azteca*).

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