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Acute Toxicity Testing and Culture Methods for Calanoid Copepods in Water Column (Elutriate) Toxicity Evaluations

Lauren K. Rabalais, Jennifer G. Laird, Alan J. Kennedy,
John D. Farrar, Guilherme R. Lotufo,
and James M. Biedenbach

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Abstract

Dredged material must be physically, chemically, and toxicologically evaluated according to the Marine Protection Research and Sanctuaries Act (MPRSA 40 CFR Part 227) before placement in open waters. The MPRSA requires bioassay testing of a marine organism representing zooplankton to evaluate the potential for water column impacts. Currently, commonly used standard methods include testing of echinoderm and bivalve embryos. However, those organisms are only planktonic during their larval stage. Truly planktonic marine invertebrate species such as copepods, cladocerans, and rotifers are more relevant as they inhabit the water column during their entire life cycle. Thus, they better represent zooplankton and satisfy the MPRSA zooplankton requirement. Standard methods for copepods, cladocerans and rotifers are available, but are not specific to 48-hour dredged material elutriate toxicity testing. This report provides guidance for acute elutriate toxicity testing method for calanoid copepods. Also included are refined culturing methods for acquiring viable juveniles (7–11 day old) for testing. The methods within consider two species of calanoid copepods (*Acartia tonsa* and *Pseudodiaptomus pelagicus*) but may be applicable to others.

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Preface

This research was funded by the Dredging Operations and Environmental Research (DOER) program under DOER-16-09, “Alternative Zooplankton Species for Elutriate Toxicity Testing.” The technical monitor was Mr. J. Daniel Farrar, CEERD-EP-R.

The work was performed by the Environmental Processes and Risk Branch (CEERD-EPR) of the Environmental Processes Division (CEERD-EP), U.S. Army Engineer Research and Development Center - Environmental Laboratory (ERDC-EL). At the time of publication, Dr. William Nelson was Branch Chief, CEERD, Mr. Warren Lorentz was Chief, CEERD-EP, Dr. David Moore was the Focus Area Lead, CEERD-EP-R, Dr. Todd Bridges was the Program Manager, CEERD-EM-D, and Dr. Al Cofrancesco was the Technical Director for CEERD-EM-W. The Deputy Director of ERDC-EL was Dr. Jack Davis and the Director was Dr. Ilker Adiguzel.

COL Bryan S. Green was Commander of ERDC, and Dr. David W. Pittman was the Director.

Acronyms and Abbreviations

ASTM	American Society for Testing and Materials
DM	Dredged Material
DoD	Department of Defense
EL	Environmental Laboratory
ERDC	Engineer Research Development Center
ISO	International Organization for Standardization
L	Liter
LC ₅₀	Lethal concentration at which a median effect on survival/lethality is modeled when half the population is killed during an exposure.
LOEC	Lowest observed effect concentration is the lowest concentration of a substance that is statistically significantly compared to the control (treatment with no exposure to the substance.)
mL	Milliliter
MPRSA	Marine Protection Research and Sanctuaries Act
NOEC	No observed effects concentration is the highest concentration of a substance that is not statistically significantly different relative to the control (treatment with no exposure to the substance).
OECD	Organisation for Economic Co-operation and Development
Ppt(%)	Parts per Thousand
RO	Reverse Osmosis (Water)
RW	Reconstituted Water
SOP	Standard Operating Procedure
USACE	U.S. Army Corps of Engineers

USEPA	U.S. Environmental Protection Agency
16L:8D	Refers to a light cycle of sixteen hours of light and eight hours of darkness.

1 Introduction

The toxicity testing and culturing methods described herein for calanoid copepods were developed to fulfill the regulatory need for a testing protocol to toxicologically evaluate dredged material elutriates using truly marine zooplanktonic organisms. This acute 48-hour testing protocol was developed for use of calanoid copepods *Acartia tonsa* and *Pseudodiaptomus pelagicus*, although it may be applicable to other copepod species. Establishment of this method was needed to support dredge material evaluations for the U.S. Army Corps of Engineers (USACE).

This document describes methods to (1) culture copepods for the purpose of bioassay testing, (2) conduct acute toxicity test, and (3) generate acceptable, reliable, and repeatable toxicological endpoints.

1.1 Background

The dredging of ports and harbors is necessary to maintain navigable waterways. Dredged material must be managed and evaluated to determine if it is suitable for open water placement, beneficial use, or another management strategy. Open water placement of dredged material into the oceans is regulated under the Marine Protection Research and Sanctuaries Act (MPRSA, *40 CFR Part 227*). Generally, recommended species for elutriate toxicity tests are fish, invertebrates, and zooplankton (MPRSA *40 CFR Part 227.27*; USEPA/USACE 1991). The MPRSA requires a representative from each of the three following animal groups be tested: fish, invertebrate, and (zoo) plankton. Currently, there are published, standardized testing methods by the American Society for Testing and Materials (ASTM) for echinoderm (ASTM *E1563-98 2004*) and bivalve (ASTM *E724-98 2004*) embryos and larvae, life stages that are considered planktonic. These testing methods have been incorporated into some dredging evaluations as a means to meet the zooplankton requirement in the MPRSA evaluations (ASTM *E1563-98(2004)e1*). However, echinoderms and bivalves are not planktonic for the majority of their life cycle. These organisms have a short lived planktonic stage (as embryos and larvae), before metamorphosing into benthic, epibenthic, and sessile forms. The embryonic life stage of these organisms is particularly sensitive to the non-persistent contaminants, such as ammonia, which is commonly

present at sometimes elevated concentrations in the elutriate water due to natural microbial processes in the sediment (Kennedy et al 2015, 2016). Biological effects due to ammonia may distract from assessing the effects of persistent contaminants (e.g., metals) within the elutriate water, which is of greater environmental concern.

The purpose of this document is to disseminate an elutriate toxicity bioassay method for test organisms that are planktonic for their entire life cycle to: (1) better represent the zooplankton requirement; and (2) that are sensitive to the persistent contaminants of concern. Marine calanoid copepods are a good candidate test species for zooplankton elutriate toxicity tests as they are sensitive to persistent contaminants of concern, and they live in the water column for their entire life cycle (Kennedy et al. 2016). Laboratory cultured copepods are available year round for testing, whereas echinoderms and bivalves must be field collected during times when they are gravid. Further, not all echinoderm and bivalve species are gravid year round, and thus, embryos are not readily available for testing.

According to the *Evaluation of Dredged Material Proposed for Ocean Disposal (Ocean Testing Manual)*. (USEPA/USACE 1991):

“Species characteristics to consider when designing water-column tests are:

- Comply with paragraph CFR 227.27(c)
- Are readily available year-round
- Tolerate handling and laboratory conditions
- Give consistent, reproducible response to toxicants
- Have related phylogenetically and/or by ecological requirements to species characteristic of the water column of the disposal site area in the season of the proposed disposal
- Can be readily tested as juveniles or larvae to increase sensitivity
- Are important ecologically, economically, and/or recreationally.”

Therefore, copepods are better suited for zooplankton elutriate toxicity testing due to their ecological relevance, laboratory rearing feasibility, sensitivity to persistent chemicals, and slightly greater tolerance to

ammonia (Kennedy et al. 2016). Methods are available for laboratory culturing of copepods in large numbers (van Dom et al. 2008).

Some species of calanoid copepods are commercially available, often as fish feed (Drillet et al. 2011). Currently there is a 24-hour marine test method available from the International Organization for Standardization (ISO) (ISO/FDIS 19820). Publications and standard methods are available for epibenthic harpacticoid copepods, but these are less relevant to the water column than calanoid copepods (Kennedy et al. 2016). Publications are available for acute toxicity testing of calanoid copepods. Calanoid copepods are used in ASTM E1850-04 (2012) as part of an acute testing method, but the methods are generalized to a large number of other organisms such as freshwater, marine, vertebrate, and invertebrate. It also appears that calanoid copepods tend to be more sensitive than the harpacticoid copepods when tested with various metals (Wang et al. 2007; Kennedy et al. 2016). Further research and testing was needed to determine the necessary culturing, handling, feeding, and test conditions, along with validating and standardizing the methods specific for elutriate testing.

The use of copepods as test organisms for use in elutriate evaluations is mentioned in at least one Regional Implementation Agreement (USEPA / USACE 2003), but methodological guidance for testing is not provided. Planktonic copepods are not typically used in testing, nor are they considered as a test species in national (USEPA/USACE 1991, 1998) and regional (USEPA/USACE 2008) guidance. Therefore, detailed guidance for conducting acute elutriate toxicity testing using calanoid copepods was needed and are provided in this document.

1.2 Objectives

The objectives of this document were to:

1. Describe the need for additional test zooplankton species for marine, dredged material elutriate toxicity testing
2. Suggest an alternative copepod species
3. Disseminate culturing methods for the copepod species
4. Disseminate toxicity testing methods for the copepod species

1.3 Approach

This study was designed to provide more appropriate water column evaluation test methods to satisfy MPRSA dredged material evaluation requirements, emphasize relevant ecology, and protection from persistent contaminants. The research was organized into three tasks. The first task identified candidate zooplankton species, methods and standards that are more appropriate for use in dredged material (DM) evaluations than current practice. The second task involved obtaining test species in the laboratory, developing and improving upon culture methods and to build toxicity reference value control charts. The third task is in progress to generate high quality metals, ammonia, and phenanthrene sensitivity data for copepods.

1.4 Scope

The scope of this project was to research and develop a reliable testing method for marine elutriate toxicity testing using zooplankton calanoid copepods. Research was conducted to determine the best method for culturing, feeding, handling, and testing conditions for the copepods. It is important to have a testing method for the calanoid copepods for marine dredged material evaluations performed under the MPRSA because they are holozooplankton, meaning they remain planktonic for their entire life cycle (Kennedy et al. 2016). Routine testing of holozooplankton is needed to satisfy MPRSA requirements for dredging evaluations to determine if materials are suitable for open water placement. Currently, standard methods are available for testing echinoderm and bivalve embryos; however, those organisms are only planktonic during their larval stage. Calanoid copepods are relatively sensitive to contaminants of concern but less sensitive to confounding factors, such as handling, physical particle effects and non-persistent contaminants (Kennedy et al. 2016). This document is needed to inform dredge material program managers, regulators, and testing facilities as well as private bioassay labs and commercial vendors of bioassay organisms of the availability of copepod species and test methods for evaluations. The researched procedural modifications and methods that were developed for handling and elutriate toxicity testing using the calanoid copepods are described within.

2 Terminology

2.1 Related Documents

The following standard toxicity test method and dredging evaluation guidance documents are relevant to the readers of this report as additional resources for toxicity test methods using copepods.

- American Society for Testing and Materials (ASTM). 2012. Standard guide for conducting renewal microplate-based life-cycle toxicity tests with a marine meiobenthic copepod. Designation E2317-04. West Conshohocken, PA: ASTM International.
- American Society for Testing and Materials (ASTM). 2012. Standard guide for selection of resident species as test organisms for aquatic and sediment toxicity tests. Designation: E1850 – 04. West Conshohocken, PA: ASTM International.
- American Society for Testing and Materials (ASTM). 1979. A chronic toxicity test with the marine copepod *Acartia tonsa*, ASTM STP667. West Conshohocken, PA: ASTM International.
- International Organization for Standardization (ISO). 1999. Water quality determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea). International Standard ISO/DIS14669. International Organization for Standardization, Geneva, Switzerland.
- International Organization for Standardization (ISO). 2015. Water quality calanoid copepod early-life stage test with *Acartia tonsa*. ISO 16778:2015. International Organization for Standardization, Geneva, Switzerland.
- Organisation for Economic Co-operation and Development (OECD). 2007. *Validation report of the full life-cycle test with the harpacticoid copepods Nitocra spinipes and Amphiascus tenuiremis and the calanoid copepod Acartia tonsa – Phase 1. Series on testing and assessment*. 79. ENV/JM/ MONO (2007) 26. Brussels, Belgium.
- Rose A., A. M. Carruthers, J. Stauber, R. Lim, and S. Blcokwell. 2006. Development of an acute toxicity test with the marine copepod *Acartia sinjiensis*. *Australasian Journal of Ecotoxicology* 12:73–81.
- USEPA/USACE. 1991. *Evaluation of dredged material proposed for ocean disposal (Ocean Testing Manual)*. EPA 503/8–91/001. Washington, DC: U.S. Environmental Protection Agency.

- USEPA/USACE. 1998. *Evaluation of material proposed for discharge to waters of the U.S. – testing manual (inland testing manual)*. EPA/823/B-98/004. Washington, DC: U.S. Environmental Protection Agency.

2.2 Definitions

- Acute toxicity test - short term testing used to determine lethality effects when exposed to a substance (typically a 48–96-hour exposure).
- Bioassay - laboratory organism exposure used to determine the effects of a potentially harmful substance.
- Control – an experiment that does not involve exposure to the test substance to which statistical comparisons are made. Usually involves assessing test subjects in their clean, culture water for the same duration as the substance concentrations.
- Copepodites – any of the five stages (twelve total) in the life cycle prior to becoming a sexually mature adult.
- Dredging evaluation – evaluation of sediment that has been removed from rivers or harbor for toxicity purposes.
- Immobilization - when an organism is unable to swim/move after gentle agitation with a transfer pipette but may still be technically alive.
- Juveniles – age range of 7–11 days old, also operationally defined herein as copepods retained with mesh sizes of 212–105 microns.
- Nauplii – first stage of the larvae copepod, which has an unsegmented body with three pairs of appendages.
- Survival- endpoint assessed as the number or percentage of living organisms at the end of a bioassay.

3 Materials and Apparatus

3.1 Materials needed for culturing

- Vincon Vinyl Tubing for airline (1/4" outside diameter)
- 5-gallon bucket
- Air stone - glass bonded silica
- Natural seawater or 30% artificial seawater (Crystal Sea Marine Mix (bioassay formula), Baltimore, Maryland or similar) made in reverse osmosis water, deionized water, or other ultrapure water.
- Environmental chamber at 25° C (preferred)* – lower temperatures will reduce the reproduction of organisms.
- T-*Isocrysis* with *Chaetoceros* (80%/20%) algae for feeding.
- Reverse osmosis water.
- Sieve sizes:
 - 212 micron nylon mesh – adults are retained on this screen.
 - 105 micron nylon mesh – copepodites are retained on this screen.
 - 44 micron nylon mesh – nauplii are retained on this screen while eggs pass through.
- 3–5 L Plastic pitcher for holding sieved copepodites 24 hours prior to testing.

3.2 Materials needed for 48-hour acute testing

- Data sheets for survivability, samples, and parameters (temperature, pH, D.O., and salinity).
- Glass crystalizing dishes (Kimble Chase, 50mm x 35mm).
- Natural seawater or 30% artificial seawater (Crystal Sea Marine Mix (bioassay formula), Baltimore, Maryland or similar) made in reverse osmosis water, deionized water, or other ultrapure water.
- Large glass culture bowls.
- Wide bore glass pipette (5.55 mm internal diameter) with rubber bulb.
- Environmental chamber to maintain temperature at 25° C (± 1), recommended, or 20° C (± 1).
- 10mL pipette and tips.

* While culturing at 20° C is possible, lower reproductive output is possible. If bioassay testing is to be performed at 20° C, it is recommended that copepods be cultured at 25° C, followed by gradual acclimation to 20° C.

- Sieve sizes:
 - 212 micron nylon mesh – adults are retained on this screen.
 - 105 micron nylon mesh – copepodites are retained on this screen.
 - 44 micron nylon mesh – nauplii are retained on this screen while eggs pass through.
- Plastic Squirt bottles.
- Dissection microscope.
- 1/8 in. thick acrylic sheet for cover over the test.
- Light table.

3.3 Materials needed for bioassay termination

- Data sheets for survivability, samples, and parameters (temperature, pH, D.O., and salinity).
- Beakers for composite water quality measurements.
- Containers for obtaining samples.
- 5 mL Pipette and tips for sample collection.
- Wide bore glass pipette (5.55 mm internal diameter) with rubber bulb.
- Dissection Microscope.
- Light table.

4 Procedure

4.1 Culture Methods

4.1.1 Transfer of shipped copepods (if applicable)

Upon arrival, all organisms are transferred to five-gallon cylindrical container (e.g., high density polyethylene bucket) filled with approximately 4.5 gallons of fresh 30% seawater at 25° C with a 16L:8D photoperiod. If the organisms are received at a temperature higher or lower than 25° C, allow them to acclimate to temperature first by placing the plastic bags the organisms arrived in into the 5-gallon bucket at chamber temperature for two hours, then transfer to the bucket by gently pouring from the bag.

Distribution of nauplii into culture chamber for optimal growth (e.g., an estimated density of one thousand nauplii per one five-gallon bucket). Aeration should be light enough to not harm copepods but rigorous enough to keep the algae suspended in the water column.

4.1.2 Water exchanges

During the acclimation phase (first 1–4 days after receiving organisms), no water exchanges are performed. Copepods are fed daily (see below for recommended ration).

After the acclimation phase, 75%–100% water changes are performed once per week by siphoning the copepods out of the 5-gallon bucket and onto a 30 micron sieve. All eggs and ages of copepods are retained on this sieve.

Transfer all copepods from 30 micron sieve to a pitcher.

Once the five-gallon bucket is emptied, clean out and replace with fresh 30% seawater. Once the temperature stabilized to 25° C (± 1), pour the pitcher of sieved copepods into the five-gallon bucket. Ensure that the organisms have sufficient aeration and food. Aeration should be light enough to keep the algae suspended in the water column.

4.1.3 Feeding during culturing

The cultures should be fed approximately 10^7 algal cells (which are harvested approximately ten days after the cell density has reached its maximum) to reach a total of 1.25×10^7 cells/L. A photospectrometer may be used to screen for the proper algae density based on color intensity. Feeding should occur daily to ensure the organisms are not underfed. Do not allow the water column to become clear of food (i.e., outside the above absorbance range), there should be food present at all times.

The cultures are continuously renewed while being cultured in the five-gallon buckets.

Observations were made, with male and female *Pseudodiaptomus* spp. beginning to reproduce by the male coupling to the posterior end of the female. Female copepods will then have egg sacs attached to their posterior end until they hatch, producing nauplii.

4.2 Bioassay method

An overview of the test method is provided in Table 2 (test method parameters) and Table 3 (test maintenance activities).

Day one (i.e., one day prior to toxicity test initiation).

- The copepods are sieved into a pitcher (or other similar container). This allows a period of time for any injured copepods to be excluded from use in the bioassay.
- Copepods are sieved to separate them into size classes. They are siphoned from their culture bucket into stacked sieves. The top sieve is 212 microns and the bottom sieve is 105 microns. Adult copepods will be retained on the top sieve and the copepodites will be retained on the bottom sieve, all nauplii and eggs will pass through the bottom sieve. This will allow the desired size and age of copepods to be obtained for testing.
- Transfer the copepods with a squirt bottle containing 30% ($\pm 10\%$) seawater and retain copepods on the 105 micron sieve and transfer into a pitcher by gently indirectly spraying across the sieve. Never directly spray onto the copepods to avoid injury to the organisms. Place the pitcher on very light aeration and ensure there is sufficient food available in the pitcher.

- Create test exposure (elutriate) water for the bioassays as needed for experiment specifications for the contaminant of concern, on the day of testing. Note that according to standard guidance, elutriates have a hold time of ≤ 24 hours following preparation.
- Guidance on elutriate water preparation can be found in guidance documents (USEPA / USACE 1991, 1998, 2008; Kennedy et al 2015). Briefly, four parts dredging site water is thoroughly mixed with one part sediment for thirty minutes followed by a sixty-minute settling period.
- Test water volume requirement. The volume of elutriate required for all bioassays (multiple species) must be determined prior to elutriate preparation. According to guidance cited above, typical elutriate toxicity tests under MPRSA involve three (sometimes four) dilutions of the elutriate, 100% elutriate, 50% elutriate, 10% elutriate (and sometimes 1% elutriate). The 100% elutriate is as prepared (undiluted elutriate). General guidance (USEPA / USACE 1991, 1998) also recommends five replicates per concentration. Elutriate dilutions can be prepared using a disposal site water or approved natural or laboratory reconstituted water by individual mixing or by serial dilution. See Table 1 below for details on the specific volume requirements for a single copepod test.

Table 1. Summary of concentrations and volume minimum volume requirements.

Elutriate concentration	Minimum volume requirement per chamber (mL)	Number of replicates (recommended)	Total volume for concentration	Volume of elutriate needed (mL)
100% elutriate	30	5	150	150
50% elutriate	30	5	150	75
10% elutriate	30	5	150	15
1% elutriate†	30	5	150	1.5
Minimum elutriate volume				241.5

† recommended if statistically significant effects are expected at the 10% elutriate concentration.

Addition of copepodites that are retained on 105 micron nylon mesh screen but pass through 212 micron nylon mesh screen (approximately 7-11 days old).

- The copepods should be fed two hours prior to the addition to the test. The feeding rations are the same as when culturing with a 1.25×10^7 cells/L of *T-Isocrysis* with *Chaetoceros*. Food should not be added to the test water to avoid interactions with chemicals.
- Test treatments may consist of three or more substance concentrations or elutriate dilutions that are tested alongside a performance control (30% seawater as the dilution water and the laboratory performance control).
- The copepods are gently poured, without causing bubbles or turbulence, from the pitcher into a culture bowl, containing 30% seawater, for addition to the test. Any copepods remaining in the pitcher that were not used or contaminated for testing purposes can be added back into the culture. Any unused copepods remaining in the culture bowl must be thrown out due to possible contamination. Any copepods stuck to the sides of containers or injured individuals should not be used in testing.
- Five copepods are added using a wide bore pipette to each crystallizing dish with 30 mL of test solution or control water. This may be performed over a light table for better observation. If more than five organisms are transferred to the test vessel, the extras must be removed and discarded or transferred to another replicate of the same treatment. When adding organisms, the amount of water transferred must not exceed 10% of the original test volume so the test substance is not diluted. The amount of water added to the test during the addition of organisms should be minimized.
- The test is conducted at 25° C (± 1), or 20° C (± 1), in an environmentally controlled chamber with a light cycle of 16L:8D for 48 hours.
- There are no water exchanges performed during the test, organisms are not fed, and crystallization dishes do not need aeration.
- All crystallizing dishes should be placed in a tray and a sheet of acrylic (1/8 in. thick) is placed on top of the crystallizing dishes to reduce evaporation.

4.3 Analysis: Endpoints and data recording

4.3.1 Acceptability criteria

For acute copepod tests to be acceptable, the criteria listed in Table 2 must be obtained. Survival in the laboratory control must be $\geq 80\%$. In addition, water quality should be within specified ranges throughout the test, temperature (20 or $25 \pm 1^\circ$ C), dissolved oxygen ($\geq 40\%$ saturation,

≥ 4.00 mg/L), pH (6.5 – 9.0), salinity (± 10% variability within each treatment). Finally, it is recommended that a reference toxicity test be performed, with potassium chloride (KCl), and that the resulting EC/LC₅₀ be within two standard deviations within laboratory control charts.

Table 2. Summary of copepod elutriate toxicity test method, presented to be consistent with USEPA / USACE (1998).

Test species	<i>Acartia tonsa</i> & <i>Pseudodiaptomus pelagicus</i>
Test type	Static, non-renewal.
Test duration	48 hours.
Temperature (°C)	25 ± 1 (recommended) or 20 ± 1
Salinity	25–30% (± 10%)
Light quality	Ambient laboratory
Light intensity	10–20 uE/m ² /s (50–100 ft-c).
Photoperiod	16L/8D
Test chamber size	40 mL
Test solution volume	30 mL
Renewal of test solutions	None
Age of test organisms	7–11 days (±1 day range) juveniles (mesh size 212–105 microns).
Number of organisms per chamber	5
Number of replicate chambers per concentration	5
Number or organisms per concentration	50
Feeding regime	2 hours prior to test (1.25 x 10 ⁷ cells/L <i>T-Isocrysis</i> with <i>Chaetoceros</i>). No feeding during test.
Test chamber cleaning	None
Test solution aeration	None
Dilution water	Disposal site water, approved natural sea water, or approved laboratory reconstituted water.
Test concentrations	100%, 50%, 10% (1%, if needed to determine a no effect concentration).
Endpoint	Survival, immobilization.
Sampling and sample holding requirements	<eight weeks (sediment); elutriates are to be used within 24 hours of preparation.
Salinity (‰)	25–30
Elutriate Sample volume required	0.2 L per site.
Test acceptability criterion	≥ 80% survival in control.†
Aeration	Only if needed to maintain acceptable dissolved oxygen (>40% saturation).

† This requirement may be re-evaluated.

Table 3. Summary of general test maintenance during the bioassay. Day 1 is the day prior to initiation of the bioassay (day 0).

Test day	Activity
-1	Sieve out the appropriate sized copepodites and place in pitcher overnight- on air and fed
0	Prepare elutriate and test dilutions. Measure salinity, temperature, pH, D.O. and ammonia of testing solution. Transfer five copepods into each test chamber.
1	Perform a 24-hour survival count on each test chamber. In some cases this may not be possible due to turbidity.
2	Terminate test by obtaining a survival count on each test chamber, and measure salinity, temperature, pH, D.O. and ammonia of each testing solution.

4.3.2 Survival

Survival in each crystalizing dish is recorded daily. During elutriate testing, it is possible that the elutriate water will be too turbid to see the copepods, in such cases, only a survival count at 48 hours will be possible. To do this, organisms may be gently poured into a larger volume container (i.e. culture bowl) containing seawater to dilute the elutriate color, thus, enabling observation. Copepods should not be directly poured into an empty container to avoid injury. Always ensure copepods are poured into a container that already contains seawater. A copepod is considered effectively dead if it is immobilized on the surface of the water or at the bottom of the crystalizing dish, and no movement is observed after ten seconds of stimulus (e.g., gentle swirling or pipetting). A more thorough assessment of survival involves observation of movement under a dissecting scope. Greater than, or equal to 80% survival in the laboratory performance control is required for the test to pass acceptability criteria for survival. All survival values in test treatments are summarized for each individual replicate and as means (± 1 standard deviation from the mean).

4.3.3 Statistical analysis

All endpoint data should be summarized for the control and each individual substance concentration by the mean and one standard deviation from the mean (Table 4). Minimum and maximum values may also be reported. Statistical analysis is required if mean survival in a test treatment is greater than 10% reduced relative to the control or reference, as provided in guidance for other acute survival tests (USEPA / USACE 1998). Statistical comparisons of each substance concentration to the control may be conducted as described in USEPA (2002) to determine the no observed effects concentration (NOEC), Lowest observed effect

concentration (LOEC), and LC₅₀ (survival) and other desirable toxicological endpoints. Additional statistical guidance can be found in USEPA / USACE (1991, 1998).

Table 4. Summary of control performance for multiple tests conducted using different age classes, chambers and methods. Survival data are presented as percentages, with one standard deviation from the average.

Test number	Test Species	Age class (mm)	Chamber (mL)	Survival (%)	Mean Species survival between tests	Pass rate (≥80% survival)
1	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	90% ± 0.9	94%	89%
2	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	100% ± 0		
3	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	100% ± 0		
4	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	85% ± 0.4		
5	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	90% ± 0.5		
6	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	90% ± 0.5		
7	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	100% ± 0		
8	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	95% ± 0.4		
9	<i>P. pelagicus</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	95% ± 0.4		
10	<i>A. tonsa</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	90% ± 0.5	93%	86%
11	<i>A. tonsa</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	95% ± 0.4		
12	<i>A. tonsa</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	85% ± 0.8		
13	<i>A. tonsa</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	100% ± 0		
14	<i>A. tonsa</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	95% ± 0.4		

Test number	Test Species	Age class (mm)	Chamber (mL)	Survival (%)	Mean Species survival between tests	Pass rate ($\geq 80\%$ survival)
15	<i>A. tonsa</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	100% \pm 0		
16	<i>A. tonsa</i>	Copepodites (212-105 microns)	30 mL crystalizing Dish; 25 °C	85% \pm 0.8		
17	<i>P. pelagicus</i>	Adults (> 212 microns); Natural seawater (wide pipettes used)	100 mL beakers; 20 °C	100% \pm 0	87%	33%
18	<i>P. pelagicus</i>	Adults (> 212 microns); Natural seawater (narrow pipettes used)	100 mL beakers; 20 °C	80% \pm 0		
19	<i>P. pelagicus</i>	Adults (> 212 microns); Crystal (artificial) seawater (narrow) pipettes used)	100 mL beakers; 20 °C	80% \pm 1		
20	<i>A. tonsa</i>	Adults (> 212 microns); Crystal (artificial) seawater (wide) pipettes used)	100 mL beakers; 20 °C	84% \pm 0.7	78%	0%
21	<i>A. tonsa</i>	Adults (> 212 microns); Natural seawater (wide pipettes used)	100 mL beakers; 20 °C	72% \pm 0.8		

5 Conclusion

This technical report provides a procedure for using marine copepods to meet the MPRSA requirements to test zooplankton to determine the acceptability of open water placement of dredged material. Additional work is needed to standardize this test method with recognized international standardization organizations to ensure full acceptability and incorporation of this working into routine dredging evaluations, national dredging evaluation guidance and regional implementation manuals.

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14. ABSTRACT Dredged material must be physically, chemically, and toxicologically evaluated according to the Marine Protection Research and Sanctuaries Act (MPRSA, 40 CFR Part 227) before placement in open waters. The MPRSA requires bioassay testing of a marine organism representing zooplankton to evaluate the potential for water column impacts. Currently, commonly used standard methods include testing of echinoderm and bivalve embryos. However, those organisms are only planktonic during their larval stage. Truly planktonic marine invertebrate species such as copepods, cladocerans, and rotifers are more relevant as they inhabit the water column during their entire life cycle. Thus, they better represent zooplankton and satisfy the MPRSA zooplankton requirement. Standard methods for copepods, cladocerans and rotifers are available, but are not specific to 48-hour dredged material elutriate toxicity testing. This report provides guidance for acute elutriate toxicity testing method for calanoid copepods. Also included are refined culturing methods for acquiring viable juveniles (7–11 day old) for testing. The methods within consider two species of calanoid copepods (<i>Acartia tonsa</i> and <i>Pseudodiaptomus pelagicus</i>) but may be applicable to others.					
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