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Environmental Technology Verification Program

Advanced Monitoring Systems Center

Quality Assurance Project Plan for Verification of Sediment Ecotoxicity Assessment Ring (SEA Ring)

Verification of the Sediment Ecotoxicity Assessment Ring

Draft

May 16, 2012

Version 1

Prepared by

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SECTION A

PROJECT MANAGEMENT

A1 VENDOR APPROVAL PAGE

ETV Advanced Monitoring Systems Center

Quality Assurance Project Plan for Verification of
the Sediment Ecotoxicity Assessment Ring

Draft

May 16, 2012

APPROVAL:

Name _____

Date _____

Notice

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A3 ACRONYMS AND ABBREVIATIONS

%D	percent difference
ADQ	audit of data quality
AMS	Advanced Monitoring Systems
ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
CAB	Cellulose Acetate Butyrate
cc	cubic centimeter
CCV	continuing calibration verification
CETIS	Comprehensive Environmental Toxicity Information System
COC	chain-of-custody
Cu	copper
DO	dissolved oxygen
DQI	data quality indicator
EPA	U.S. Environmental Protection Agency
ERDC	Engineer Research Development Center
ESTCP	Environmental Security Technology Verification Program
ETV	Environmental Technology Verification
GC	gas chromatography
HDPE	high density polyethylene
ICAL	initial calibration
ICP-MS	inductively coupled plasma mass spectrometry
ICV	initial calibration verification
LC50	median lethal concentration
LCS	laboratory control sample
LRB	laboratory record book
MS	Metals Contaminated Sediment
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PE	performance evaluation
ppb	parts per billion
ppm	parts per million
ppt	parts per thousand
PSNS	Puget Sound Naval Shipyard
QA	quality assurance
QAO	quality assurance officer

QAPP	quality assurance project plan
QC	quality control
QMP	Quality Management Plan
RMO	Records Management Office
SEA Ring	Sediment Ecotoxicity Assessment Ring
SED	surficial sediment
SOP	Standard Operating Procedure
SPAWAR	Space and Naval Warfare
SSC	SPAWAR Systems Center
SWI	sediment water interface
TOC	total organic carbon
TSA	technical systems audit
UHMWPE	Ultra-high molecular weight polyethylene
USACE	U.S. Army Corps of Engineers
VTC	verification test coordinator
WC	water column
YB	Yaquina Bay, OR

A4 DISTRIBUTION LIST

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A5 VERIFICATION TEST ORGANIZATION

The verification test will be conducted under the U.S. Environmental Protection Agency (EPA) Environmental Technology Verification (ETV) Program. It will be performed by Battelle, which is managing the ETV Advanced Monitoring Systems (AMS) Center through a cooperative agreement with EPA. The scope of the AMS Center covers verification of monitoring technologies for contaminants and natural species in air, water, soil and sediments. This verification test will evaluate an in-situ field sampling technology that determines the toxicity of contaminants in the sediment and water column (WC), and sediment-water interface on benthic and WC organisms.

The objective of the verification is to test the efficacy and ability of the Sediment Ecotoxicity Assessment Ring (SEA Ring) to evaluate the toxicity of contaminants in the sediment, at the sediment-water interface, and WC to organisms that live in those respective environments. The SEA Ring will improve the assessment of exposure and response at Department of Defense contaminated sediment and surface water sites to assist in making accurate and informed management decisions, particularly with respect to assessment of sediment remedy effectiveness and time-varying exposures. Although the SEA Ring is used in the field, the verification testing will focus on the ability of the SEA Ring to provide comparable data (using quantitative and qualitative criteria) to traditional EPA and American Society for Testing and Materials (ASTM)-approved laboratory methods under controlled laboratory conditions. The performance parameters for this test are *repeatability, comparability and reproducibility* as well as a number of operational factors defined in Section B.

The performance of the SEA Ring will be based on comparison with data obtained from EPA and ASTM methods for determining the toxicity of contaminated sediment and whole effluents. Both the SEA Ring exposures and the traditional laboratory exposures will be conducted in the laboratory. The test methods will follow those described in standard guidance documents (EPA and USACE, 1998; ASTM, 2000; ASTM, 2010). Over approximately a two-month time period, all exposures will be conducted at the Navy's Space and Naval Warfare (SPAWAR) Systems Center (SSC) Pacific Bioassay Laboratory, San Diego, an Environmental Laboratory Accreditation Program certified laboratory. An external laboratory, the U.S. Army Corps of Engineers (USACE) Engineer Research Development Center (ERDC), Vicksburg, MS, will be utilized for verification of sediment and tissue concentrations from relevant test samples. The subject technology is concurrently being evaluated in a project sponsored

by the Environmental Security Technology Verification Program (ESTCP) Project ER-201130 titled "Demonstration and Commercialization of the Sediment Ecosystem Assessment Protocol".

The day to day operations of this verification test will be coordinated and supervised by Battelle, with the participation of the SEA Ring technology representative (SPAWAR). Battelle will conduct laboratory testing of the SEA Ring technology at the SPAWAR Systems Center in San Diego, CA. SPAWAR will provide the SEA Ring technology for testing and replicating multiple deployments of the technology, and train Battelle staff on its use. Battelle staff and SPAWAR will operate the technology during verification testing.

The organization chart in Figure 1 identifies the responsibilities of the organizations and individuals associated with the verification test. Roles and responsibilities are defined further below. Quality assurance (QA) oversight will be provided by the Battelle Quality Manager, and also by the EPA AMS Center Quality Manager, at EPA's discretion.

A5.1 Battelle's Test Program Roles and Responsibility

Dr. Ramona Darlington is the AMS Center's Verification Test Coordinator (VTC) for this test. In this role, Dr. Darlington will have overall responsibility for ensuring that the technical, scheduling, and cost goals established for the verification test are met. Specifically, Dr. Darlington will:

- Serve as the primary point of contact with SPAWAR;
- Prepare the draft quality assurance project plan (QAPP), verification report, and verification statement;
- Revise the draft QAPP, verification report, and verification statement in response to reviewers' comments;
- Assemble a team of qualified technical staff to conduct the verification test;
- Establish a budget for the verification test and manage staff to ensure the budget is not exceeded;
- Coordinate with the technology representative for provision of its technology for testing;
- Coordinate with SPAWAR personnel for laboratory testing;
- Direct the team in performing the verification test in accordance with this QAPP;

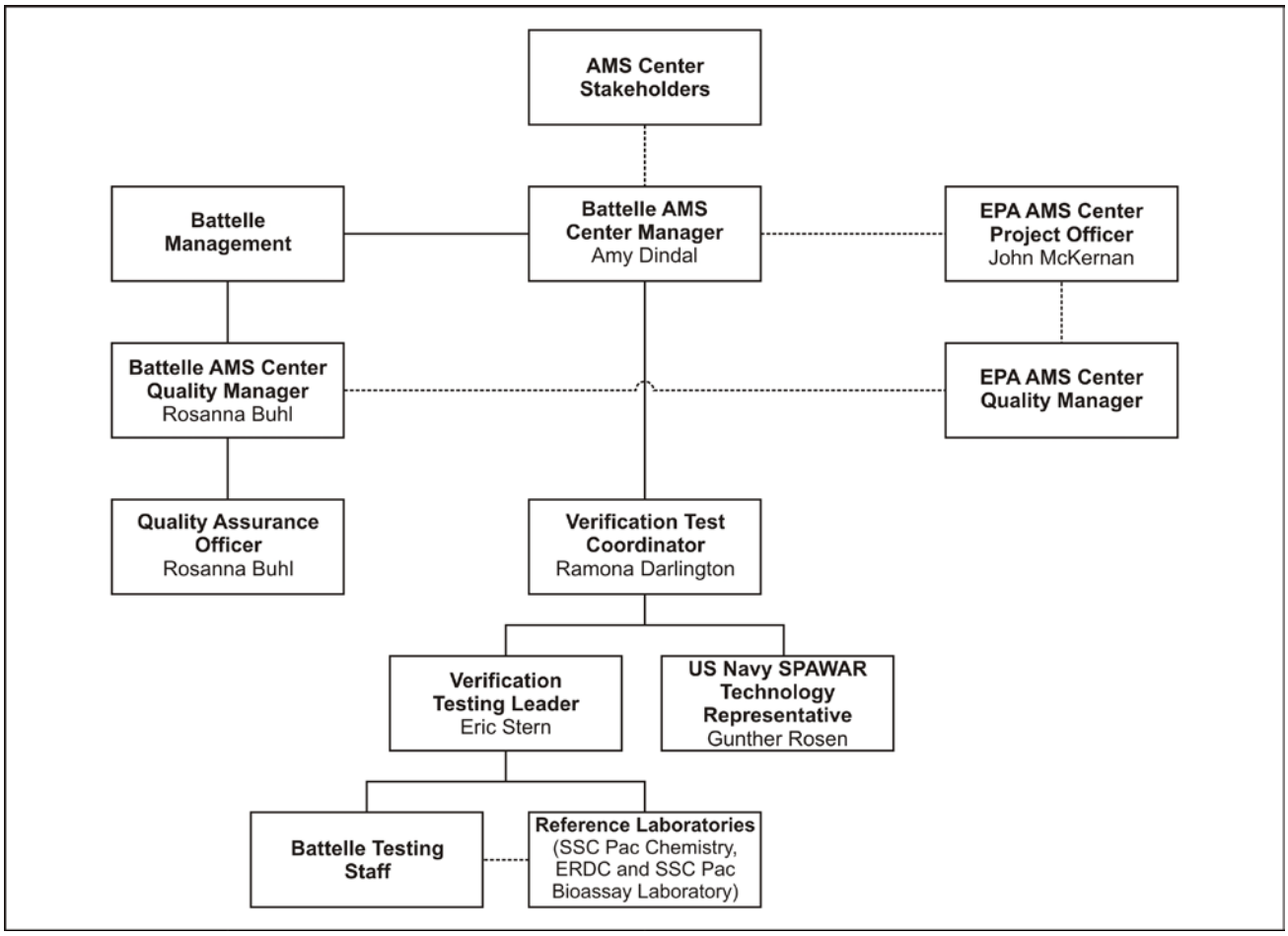

Figure 1. Organizational Chart

- Hold a kick-off meeting approximately one week prior to the start of the verification test to review the technical, logistical, and administrative critical paths of the verification test. Responsibility for each aspect of the verification test will be established by the VTC;
- Ensure that all quality procedures specified in this EPA Quality Level III QAPP and in the AMS Center Quality Management Plan (Battelle, 2011) are followed;
- Ensure that confidentiality of sensitive technology information is maintained;
- Assist SPAWAR as needed during verification testing;
- Become familiar with the operation of the technology through instruction by SPAWAR;
- Prepare a deviation report for any departure from the QAPP during the verification, obtain the requisite EPA approvals, and distribute the approved report as specified in the AMS Center Quality Management Plan (QMP);

- Respond to any challenges raised in assessment reports, audits, or from test staff observations, and institute corrective actions as necessary; and
- Coordinate distribution of the final QAPP, verification reports, and verification statements.

Ms. Amy Dindal is Battelle's Manager for the AMS Center. As such, Ms. Dindal will oversee the various stages of verification testing. Ms. Dindal will:

- Review the draft and final QAPP;
- Attend the verification test kick-off meeting;
- Review the draft and final verification report and verification statement;
- Ensure that necessary Battelle resources, including staff and facilities, are committed to the verification test;
- Maintain communication with EPA's technical and quality managers; and
- Issue a stop work order if Battelle or EPA QA staff discovers adverse or non-consistent findings that are derived from technology failure or physical deployment conditions that will compromise test results.

Technical staff from Battelle, including Mr. Eric Stern, will support Dr. Darlington in planning and conducting the verification test. The responsibilities of the technical staff will be to:

- Assist Dr. Darlington (VTC) in preparing the QAPP;
- Review the draft and final QAPP;
- Attend the verification test kick-off meeting;
- Ensure that confidentiality of sensitive vendor information is maintained;
- Support Dr. Darlington in responding to issues raised in assessment reports and audits; and
- Review the draft and final verification reports and verification statements.

Ms. Rosanna Buhl is Battelle's QA Manager for the AMS Center. Ms. Buhl will:

- Review the draft and final QAPP;
- Delegate to other Battelle quality staff any Quality Assurance Officer (QAO) responsibilities assigned below as needed to meet project schedules;
- Review and approve QAPPs, QAPP amendments, deviations and audit reports;
- Work with the VTC and Battelle's AMS Center Manager to resolve data quality concerns and disputes; and

- Recommend a stop work order if audits indicate that data quality or safety is being compromised.

Ms. Buhl will also be the QAO for this test. In this capacity she will:

- Attend the verification test kick-off meeting and lead the discussion of the QA elements of the meeting checklist;
- Prior to the start of verification testing, verify the presence of applicable training records, including any training on test equipment/technologies;
- Conduct a technical systems audit (TSA) at least once during the verification test;
- Conduct audits to verify data quality;
- Prepare and distribute an audit report for each audit;
- Verify that audit responses for each audit finding and observation are appropriate and that corrective action has been implemented effectively;
- Communicate to the VTC and/or technical staff the need for immediate corrective action if an audit identifies QAPP deviations or practices that threaten data quality;
- Provide a summary of the QA/quality control (QC) activities and results for the verification reports;
- Review the draft and final verification report and verification statement; and
- Communicate data quality concerns to the VTC.

A5.2 Technology Representative

The technology representative is US Navy SPAWAR. Mr. Gunther Rosen is the Navy's representative and point of contact. The technology was developed and patented by SPAWAR and the University of Michigan. A commercial technology vendor, Zebra-Tech, Ltd., is supporting SPAWAR in an effort (funded by ESTCP) towards commercialization and standardization of the hardware and approach, respectively. As part of the ESTCP project technology transition goals, the verified prototype of the technology will ultimately be made commercially available through Zebra-Tech, or another vendor, depending on who pursues licensing rights. The responsibilities of the technology representative are:

- Review and provide comments on the draft QAPP;
- Accept (by signature) the final QAPP prior to test initiation;
- Participate in the kick-off meeting for the verification test;

- Provide two SEA Ring technologies to carry out comparative analysis during the verification test;
- Supply instructions on the use of the technology, and written consent for test staff to carry out verification testing; and
- Review and provide comments on the draft verification report and verification statement for their respective technology.

A5.3 EPA

EPA's responsibilities in the AMS Center are based on the requirements stated in the *Environmental Technology Verification Program Quality Management Plan* (EPA, 2008). The roles of specific EPA staff are as follows.

The EPA's AMS Center Quality Manager will:

- Review the draft QAPP;
- Perform one external TSA during the verification test, at EPA's discretion;
- Notify the EPA AMS Center Project Officer of the need for a stop work order if the external audit indicates that data quality is being compromised;
- Prepare and distribute an assessment report summarizing results of any external audits; and
- Review draft verification report and verification statement.

Dr. John McKernan is EPA's Project Officer for the AMS Center. Dr. McKernan will:

- Review the draft QAPP;
- Approve the final QAPP;
- Review and approve deviations to the approved final QAPP;
- Appoint a delegate to review and approve deviations to the approved final QAPP in his absence, in order that testing progress will not be delayed;
- Review the first day of data from the verification test and provide immediate comments if concerns are identified;
- Review the draft verification report and verification statement;
- Oversee the EPA review process for the QAPP, verification report, and verification statement; and

- Coordinate the submission of verification reports and verification statements for final EPA approval.

A5.4 Verification Test Stakeholders

This QAPP and the verification report and verification statement based on testing described in this document will be reviewed by experts in the fields related to aquatic sediment toxicity and bioaccumulation (bioassay) sampling and testing. The following experts have been providing input to this QAPP and have agreed to provide a peer review.

- Marc Greenberg, PhD – EPA Environmental Response Team, Edison, NJ
- Guilherme Lotufo, PhD – ERDC, Vicksburg, MS
- Darrin Greenstein – Southern California Coastal Water Research Project, Costa Mesa, CA

The responsibilities of verification test stakeholders and/or peer reviewers include:

- Participate in technical panel discussions (when available) to provide input to the test design;
- Review and provide input to the draft QAPP; and
- Review and provide input to the verification report/verification statement.

In addition, this technology category was reviewed with the broader AMS Center Stakeholder Committees during the regular stakeholder teleconferences. Toxicity testing has been a long-standing priority area for the AMS Center, with verifications and/or protocols completed in the areas of drinking water, wastewater, and soil toxicity. This sediment toxicity technology verification was discussed with the EPA Project Officer in May 2011.

A5.5 Reference Laboratories

Two reference laboratories will be utilized for verification of test exposures and/or bioaccumulated concentrations of selected contaminant classes. The responsibilities of the reference laboratories for this verification test include:

- Acknowledging receipt of samples and completing the chain-of-custody (COC) forms for the samples;
- Analyzing all samples for copper (Cu) (SPAWAR) or polychlorinated biphenyl (PCB) congeners (ERDC);
- Providing analysis results and supporting laboratory documentation within 30 days of receipt

of samples; and

- Providing documentation as requested (such as Standard Operating Procedures [SOPs]) for an independent TSA of laboratory procedures.

The SSC Pacific Chemistry Laboratory will analyze seawater samples to verify control and spiked samples for Cu. The SSC Pacific Laboratory technical point of contact for Cu measurements will be Brandon Swope. He is responsible for providing SOPs and appropriate QA reporting for the verification test. In lieu of participating in the performance evaluation (PE) audit, the SSC Laboratory will provide results from its two most recent Cu PE samples to the Battelle Quality Manager. SOPs will be obtained and reviewed from the external laboratory.

The USACE, ERDC Environmental Chemistry Lab, in Vicksburg, MS, will analyze sediment and tissue samples from the technology representative for PCB congener measurements. Ms. Patricia Tuminello will be the point of contact at ERDC. She is responsible for providing SOPs and appropriate QA reporting for the verification test. The ERDC laboratory will participate in a PE audit (see Section C1.1) since the laboratory is not accredited.

A6 BACKGROUND

A6.1 Technology Need

The ETV Program's AMS Center conducts third-party performance testing of commercially available technologies that detect or monitor natural species or contaminants in air, water, soil, and sediment. The purpose of ETV is to provide objective and quality assured performance data on environmental technologies so that users, developers, regulators, and consultants can make informed decisions about purchasing and applying these technologies. Stakeholder committees of buyers and users of such technologies recommend technology categories, and technologies within those categories become priorities for testing. Among the technology categories recommended for testing are toxicity testing technologies, including sediment and aqueous toxicity for assessment of environmental quality in marine, freshwater and estuarine systems.

Traditionally, the bioavailability and toxicity of contaminated sediments or water samples are assessed on grab or composite samples collected in the field and tested in a laboratory. In the laboratory, test organisms are added to site sediment or water samples in beakers and exposed under controlled conditions (e.g., temperature, pH, salinity, photoperiod, feeding regime, aeration) for a specified time period (e.g., EPA, 1994a; EPA, 2000; ASTM, 2000; ASTM, 2010). This laboratory-based method of assessing sediment quality, although widely used and well established, does not necessarily represent the true in-situ exposure and effects to organisms in the field. This is especially true when the source of contamination is ephemeral, meaning exposure varies over time and with ambient conditions. Another challenge with laboratory testing is that sediment sample manipulation removes the natural vertical contaminant stratification, which in turn alters the exposure to test organisms. Such manipulation may also result in alteration of the contaminant bioavailability through processes including degradation, volatilization, and redox changes. Sediment samples removed from the field undergo physical and chemical changes which change the bioavailability and toxicity of the contaminants and may lead to misleading results in the laboratory and subsequent difficulty in program decision making.

In addition, laboratory tests may overestimate toxicity from sediment-associated contaminants due to buildup of contaminant concentrations in the overlying water as toxicants desorb from the sediment into the WC. In aqueous exposures, laboratory tests may also misrepresent actual exposure in the field when static exposures are used as a means of assessing the potential for adverse effects of a time-varying

stressor (e.g., stormwater runoff, combined sewer overflow, etc.). The limitations of standard laboratory toxicity testing and chemical analyses lead to potentially inappropriate and costly management decisions.

A6.2 SEA Ring Technology Description

The SEA Ring (U.S. Patent No. 8,011,239) is an integrated, versatile, field tested, toxicity and bioavailability assessment device. Figures 2 and 3 show top and side views of the patented, first generation version of the SEA Ring technology. The second generation model (Figure 4) will be the version used in this ETV verification. The second generation system is the commercialized version of the prototype, which was designed to be more user-friendly, more autonomous, and more rigorous to withstand environmental conditions over exposure time. The unit consists of 10 cylindrical chambers fixed to a circular ultra-high molecular weight polyethylene (UHMWPE) platform. The top end of each chamber is fitted with an integrated, multifunctional cap. The cap includes both overlying water intake and outlet ports, and an organism delivery port (opening for an optional modified plastic 30 cubic centimeter [cc] syringe). The intake port connects to a peristaltic pump that is housed in the center of the device and powered by rechargeable batteries stored in a separate housing underneath the pump. The pump is programmable to provide chamber water volume exchange at a rate (range ~6 to >25 turnovers per day) desired for the site- or project-specific preferences.

The SEA Ring was designed to evaluate toxicity in the WC, sediment water interface (SWI), and/or surficial sediment (SED; Figure 3). The SED chambers are open on the bottom, are 10 inches in length, 2.75 inches in diameter, and extend 5 inches below the base of the system. Small sediment dwelling organisms can be introduced into the SED chambers through the organism delivery port built into the cap with a modified 30 cc plastic syringe. The syringe is plugged with a silicone stopper inside the test chamber to retain the organisms until desired release. For larger organisms a ½ inch stainless steel mesh is integrated into the bottom opening of the exposure chamber, allowing organisms to be preloaded prior to deployment. The WC and SWI chambers are 5 inches in length, 2.75 inches in diameter, and have a closed bottom. The bottom consists of a solid plastic polyethylene cap or mesh insert for water quality chambers. Organisms for the WC and SWI tests can be loaded in the laboratory or in the field immediately prior to deployment. In the center of the circular platform there is a custom-built peristaltic pump and battery. These components are fully encased and water tight. The intake to the test chambers is located on top of the cap. Each inlet is directly connected to the pump through individual tubes that pass over the pump roller. As the pump rotor turns, compressing and releasing pressure on the tubing, ambient water from the surrounding area is circulated through each chamber. A water quality sensor or

passive sampler can also be attached to one of the chambers (Figure 3). Water quality sensors are used to measure a variety of physical parameters including pH, temperature, depth, salinity, conductivity, and dissolved oxygen (DO) from inside the exposure chambers.

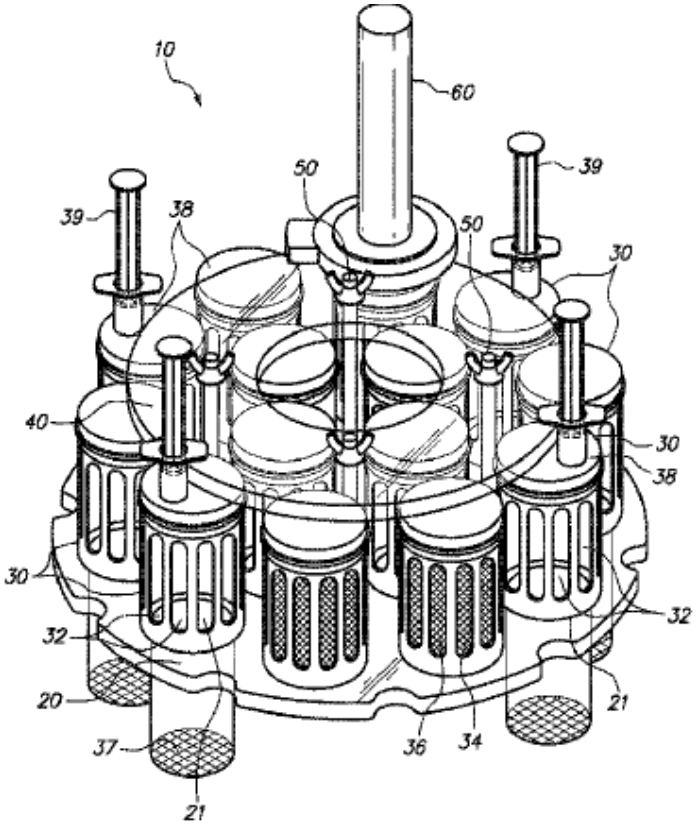

Figure 2. Schematic of SEA Ring Technology
(U.S. Patent Number 7,758,813)

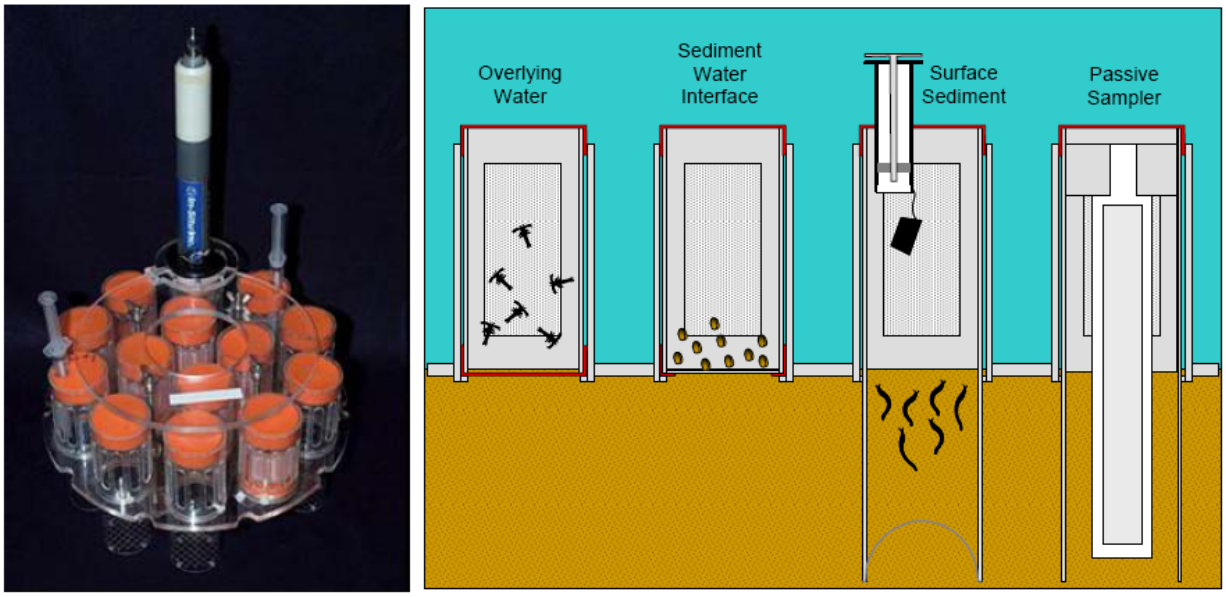

Figure 3. Multiple Lines of Evidence Use of SEA Ring Technology

Figure 4. Second Generation SEA Ring Device (left). Field Evaluation in Beach Deployment (right)

A7 VERIFICATION TEST DESCRIPTION AND SCHEDULE

The purpose of the test is to generate performance data on an innovative in-situ field technology for assessing contaminated sediment and WC toxicity and bioaccumulation potential using indigenous organisms. The ease of use and comparability of the technology to EPA and ASTM methods will be evaluated utilizing multiple species, varied sediment types, and chemicals often identified as contaminants of concern (e.g., metals such as Cu and organics such as PCBs) in the near-shore aquatic environment. The data generated from this verification test are intended to provide technology users with information on its performance in controlled laboratory settings prior to its use in the field.

A7.1 Verification Test Description

The purpose of this QAPP is to specify procedures for verification testing of the SEA Ring to assess contaminated sediment and WC toxicity to aquatic and benthic organisms. The primary evaluation will assess survival, growth, and bioaccumulation of contaminants in aquatic and benthic organisms exposed in the SEA Ring compared to responses achieved in the laboratory using standard ASTM and EPA methods. In performing the verification test, Battelle will follow the technical and QA procedures specified in this QAPP, and will comply with the data quality requirements in the AMS Center QMP (Battelle, 2011).

The SEA Ring tests will be evaluated on the following performance parameters, described in detail in Section B:

- Repeatability;
- Comparability;
- Reproducibility; and
- Operational factors (qualitative assessment).

Operational parameters including ease of use, training and sustainability (sampling time, waste produced, and the amount of protective equipment required by the individual operating the technology) will also be evaluated by Battelle staff. More details on the test design are provided in Section B.1.3.

Testing will be conducted in the laboratory over a two-month period by Battelle staff with support from the technology representative. SEA Ring and concurrent bench-top tests following the EPA and ASTM methods will be set up and evaluated in the SSC Pacific Bioassay Laboratory. With the exception of PCB

congener analyses in sediment and tissue by USACE ERDC Chemistry Laboratory, all analyses will be performed at the SSC Pacific Bioassay Laboratory.

Subsequent to verification testing, Battelle will prepare one Verification Report for the laboratory evaluations. The report will describe the SEA Ring performance on assessing sediment and WC toxicity to aquatic and benthic organisms.

QA procedures include a TSA and two audits of data quality (ADQs), (details provided in Section A7.1). The Battelle QAO or her designee will perform the TSA. The first data set will be delivered within 30 days of test initiation. Un-audited data will include the disclaimer *have not been reviewed by Battelle QA Manager*. The first ADQ will review the first data set delivered. The second ADQ will assess the remainder of the data, the draft report, and the verification statement as described in Section C.

A7.2 Verification Test Schedule

Laboratory testing of the SEA Ring is scheduled to begin in November 2012 and will be initiated upon final EPA and technology representative approval of this QAPP. Testing will occur over approximately a two-month period. Data will be evaluated and the verification report and verification statement will be drafted. It is anticipated that the final EPA-approved verification report and verification statement will be completed by September 2013.

A7.3 Verification Location

Laboratory testing will be conducted at the SSC Pacific Bioassay Laboratory, San Diego, CA. This laboratory is equipped to perform sediment and aqueous toxicity testing in a controlled environment and reduces the costs of shipping the technology to the Battelle laboratory in Columbus, Ohio.

A8 QUALITY OBJECTIVES

This verification test is designed to evaluate the performance of the SEA Ring for determining the bioavailability and toxicity of contaminants in water and sediments on aquatic and benthic organisms. This verification will vary sediment type, organism and toxicity endpoint type, and contaminant concentration in the SEA Ring device under controlled and repeatable test conditions. Parallel standard bench-top tests will be conducted. Both the SEA Ring and bench-top tests will follow EPA and ASTM testing methods, with minor modifications as necessary. Any deviations from protocols referenced will be thoroughly documented on bench datasheets and in the final report. The test conditions and quality indicators for this verification test lie in the performance parameters and the QC samples. Data quality indicators (DQIs) ensure that the verification tests provide suitable data for a robust evaluation of performance. DQIs have been established for organism age and water quality. The DQIs were established to ensure that data used to support the SEA Ring technology tests are of sufficient quality. Acceptance criteria for the DQIs and QC samples are detailed in the Tables 1 through 5, and are specific to each test species.

Table 1. Toxicity Test Methodology and QA/QC Requirements for Water Column Toxicity Tests Using the Mysid Shrimp *Americamysis bahia*

Test organism	Mysid shrimp - *Americamysis bahia*
Test organism source	Aquatic BioSystems – Laboratory culture (Fort Collins, CO)
Test organism age at initiation	3-5 days post-hatch; less than or equal to 24-h range in age (required)
Test duration; endpoint	96-hour; survival
Test solution renewal	80% volume renewal one time (48 hours)
Feeding	*Artemia* nauplii, twice daily
Test chamber	0.5-L plastic cup (laboratory); 5 inch cellulose acetate butyrate (CAB) core tube (SEA Ring)
Test solution volume	Approximately 500 mL (laboratory and SEA Ring)
Test temperature	20 ± 1°C test-wide mean, 20 ± 3°C instantaneous
Dilution water	Filtered (0.45 µm) natural seawater collected from near the mouth of San Diego Bay at SSC Pacific Laboratory
Salinity	32 ± 2% ppt
Test concentrations	Lab control, 100, 200, 400 µg/L Cu
Number of organisms/chamber	10
Number of replicates	5
Photoperiod	16 hours light/8 hours dark., ambient laboratory lighting
Aeration	None, unless DO < 4 mg/L
Test Protocol	EPA-821-R-02-012 (EPA, 2002a)
Test acceptability objective	≥ 90 % mean survival in natural seawater control
Reference toxicant	Copper sulfate (Standard EPA laboratory method only); five concentrations (3 replicates each)

Table 2. Toxicity Test Methodology and QA/QC Requirements for Water Column Toxicity Tests Using Topsmelt *Atherinops affinis*

Test organism	Topsmelt – *Atherinops affinis*
Test organism source	Aquatic BioSystems - Laboratory culture (Fort Collins, CO)
Test organism age at initiation	9-15 days post-hatch
Test duration; endpoint	96-hour; survival
Test solution renewal	80% volume renewal at 48 hours
Feeding	*Artemia nauplii,* twice daily
Test chamber	0.5-L plastic cup (laboratory); 5 inch CAB core tube (SEA Ring)
Test solution volume	Approximately 500 mL (laboratory and SEA Ring)
Test temperature	20 ± 1°C test-wide mean, 20 ± 3°C instantaneous
Salinity	32 ± 2% ppt
Dilution water	Filtered (0.45 µm) natural seawater collected from near the mouth of San Diego Bay at SSC Pacific Laboratory
Test concentrations	Lab control, 100, 200, 400 µg/L Cu
Number of organisms/chamber	10
Number of replicates	5
Photoperiod	16 hours light/8 hours dark, ambient laboratory lighting
Aeration	None, unless D.O. < 4 mg/L
Test Protocol	EPA-821-R-02-012 (EPA, 2002a)
Test acceptability objective	≥ 90 % mean survival in natural seawater control
Reference toxicant	Copper sulfate (standard EPA lab method only); 96 hours, 48-hr renewal/five concentrations (3 replicates each)

Table 3. Toxicity Test Methodology and QA/QC Requirements for Solid-Phase Toxicity Tests Using the Marine Amphipod *Eohaustorius estuarius*

Test organism	Marine Amphipod – *Eohaustorius estuarius*
Test organism source	Northwest Aquatic Sciences (Newport, OR)
Test organism age at initiation	NA - Field collected (3-5 mm adult)
Control sediment source	Sediment from amphipod collection site, Yaquina Bay, OR (YB)
Test duration; endpoint	10 days; survival
Test solution renewal	None
Feeding	None
Test chamber	1-L glass jar (lab), 10 inch CAB core tube (SEA Ring)
Test sediment depth	2 cm (lab and SEA Ring)
Overlying water volume	750 ml (lab and SEA Ring) natural seawater
Test temperature	18 ± 1ºC test-wide mean, 18 ± 3ºC instantaneous
Overlying water	Filtered (0.45 µm) natural seawater collected from near the mouth of San Diego Bay at SSC Pacific Laboratory
Salinity	32 ± 2% ppt
Test concentrations	Undiluted sediment sieved to < 2.0 mm
Number of organisms/chamber	20
Number of replicates	5 (lab and SEA Ring)
Photoperiod	Continuous light (24 hr), ambient laboratory lighting
Aeration	Laboratory filtered air, continuous (1-2 bubbles per second delivered through a Pasteur pipette in laboratory beaker, 1-2 bubbles per second from three Pasteur pipettes in SEA Ring Chemtainer (outside exposure chambers)
Test Protocol	EPA 600-R-94-025 (EPA, 1994a)
Test acceptability objective	≥ 90 percent mean survival in control
Reference toxicant	Cadmium chloride (standard EPA lab method only); 96-h water only exposure

Table 4. Toxicity Test Methodology and QA/QC Requirements for Solid-Phase Toxicity and Bioaccumulation Tests Using the Marine Polychaete *Neanthes arenaceodentata*

Test organism	Marine polychaete, *Neanthes arenaceodentata*
Test organism source	Dr. Mary Ann Rempel Hester, Aquatic Toxicity Support, Inc. (Bremerton, WA)
Test organism age at initiation	2 weeks
Control sediment source	Sediment from the amphipod collection site, Yaquina Bay, OR (YB)
Test duration; endpoint(s)	28 days; survival and growth
Test solution renewal	Twice-weekly (laboratory jar/SEA Ring Chemtainer)
Feeding	1 ml of flake food slurry twice weekly after test solution renewal (slurry comprised of 100 mL seawater: 1 g Tetramin® fish feed)
Test chamber	1-L glass jar (lab), 10 inch CAB core tube (SEA Ring)
Sediment depth	2 cm
Overlying water volume	750 ml
Test temperature	18 ± 1°C test-wide mean, 18 ± 3°C instantaneous
Overlying water	Filtered (0.45 µm) natural seawater collected from near the mouth of San Diego Bay at SSC Pacific Laboratory
Salinity	32 ± 2% ppt
Test concentrations	Undiluted sediment sieved to < 2.0 mm
Number of organisms/chamber	20
Number of replicates	5
Photoperiod	16 hours light/8 hours dark, ambient laboratory lighting
Aeration	Laboratory filtered air, continuous (1-2 bubbles per second delivered through a Pasteur pipette in laboratory beaker, 1-2 bubbles per second from three Pasteur pipettes in SEA Ring Chemtainer (outside exposure chambers)
Test Protocol	ASTM 2000 E1611-00
Test acceptability objective	≥ 90 percent mean survival in control
Reference toxicant	Copper Sulfate (standard ASTM laboratory method only); 96-hr water only exposure

Table 5. Test Methodology and QA/QC Requirements for 28-Day Bioaccumulation Tests Using the Marine Clam *Macoma nasuta*

Test organisms	Marine clam *Macoma nasuta*
Test organism source	Brezina & Associates (Dillon Beach, CA)
Test organism age at initiation	~1" Small Adult (field collected)
Control sediment source	Sediment collected from clam collection site, Dillon Beach, CA (DB)
Test duration	28 days, + 24-hr depuration period
Test solution renewal	Three-times weekly with clean seawater
Feeding	None
Test chamber	5 1-L glass beakers in 10 gallon aquarium (lab); 5 1-L CAB core tubes in Chemtainer (SEA Ring)
Sediment depth	5 cm (lab and SEA Ring chambers)
Overlying water volume	Approximately 750 mL (laboratory and SEA Ring)
Test temperature	18 ± 3 °C instantaneous
Overlying water	Filtered (0.45 µm) natural seawater (salinity 32-34 ppt) collected from near the mouth of San Diego Bay at SSC Pacific Laboratory
Salinity	32 ± 2% ppt
Test concentrations	Undiluted sediment sieved to <2.0 mm
Number of organisms/chamber	3
Number of replicates	5
Photoperiod	16 hours light/8 hours dark, ambient laboratory lighting
Aeration	Laboratory filtered air, continuous (1-2 bubbles per second delivered through a Pasteur pipette in laboratory beaker, 1-2 bubbles per second from three Pasteur pipettes in SEA Ring Chemtainer (outside exposure chambers)
Test Protocol	EPA 503/8-91/001, ASTM E-1688-10
Test acceptability objective	≥ 90 percent mean survival in controls
Reference toxicant	None

A9 SPECIAL TRAINING/CERTIFICATION

Documentation of training related to technology testing, field testing, data analysis, and reporting is maintained for all Battelle technical staff in training files at their respective locations. SPAWAR staff will receive training in documentation and records management procedures required for ETV testing during the kick-off meeting. The Battelle Quality Manager will verify the presence of appropriate training records prior to the start of testing. Battelle and EPA staff involved in this verification will be specifically trained on the operation of the SEA Ring technology. Training in the use of the SEA Ring will be conducted by the technology representative. Battelle will document this training with a consent form, signed and dated by the technology vendor, which states which Battelle technical staff have been trained to use the technology and can train other staff to do so as well. In the event that other staff members are required to use the technology, they will be trained by either the operators that were trained by the technology representative or the technology representative.

A10 DOCUMENTATION AND RECORDS

The documents for this verification test will include the QAPP, vendor instructions, reference methods, verification reports, verification statements, and audit reports. The project records will include laboratory record books (LRBs) and data collection forms, supporting laboratory records, training records, electronic files (both raw data and spreadsheets), and QA audit files. Section B10 summarizes data management for the test and the types of data to be recorded. Documentation of Battelle staff training by the technology representative and copies of other project specific training will also be included in the project files. All of these records will be maintained by the SPAWAR point of contact during the test, and will be transferred to permanent storage at Battelle's Records Management Office (RMO) at the conclusion of the verification test. All Battelle LRBs are stored indefinitely with the project files by Battelle's RMO. Section B10 further details the data management practices and responsibilities.

All data generated during this project will be recorded directly, promptly, and legibly in ink. All data entries will be dated on the date of entry, and signed or initialed by the person entering the data. Any changes in entries will be made so as not to obscure the original entry, will be dated and signed or initialed at the time of the change and will indicate the reason for the change. Project specific data forms will be developed prior to testing to ensure that all critical information is documented in real time. The draft forms will be provided to the Battelle QA Manager for review.

SECTION B

MEASUREMENT AND DATA ACQUISITION

B1 EXPERIMENTAL DESIGN

This QAPP addresses the verification of the SEA Ring through laboratory testing. Specifically, the SEA Ring will be evaluated for the following performance parameters:

- Repeatability;
- Comparability;
- Intra-unit reproducibility; and
- Operational factors.

The verification test will be conducted in the laboratory over a period of two months. Prior to initiation of the SEA Ring verification test, sediment samples will be collected for use in the experiment and testing organisms obtained from vendors. Collection records will include the collection date and location, collector and storage conditions. Test organism records will include the source, date and location of collection (if collected) or age (if cultured), and holding and acclimation conditions.

B1.1 Test Procedures

The following sections describe the test procedures that will be used to evaluate each of the performance parameters listed above. Cost information will be provided by the technology vendor (i.e., price of technology, operation and maintenance cost). The performance parameters are defined in detail in Tables 1 through 5. Figure 5 illustrates the sediment test design variables, and the WC test design is shown in Figure 6.

B1.1.1 Sediment and Water Sources. Three different types of sediment will be used in the ETV verification of the SEA Ring. The laboratory water used by SSC Pacific Laboratory is 0.45 µm filtered seawater collected from near the mouth of San Diego Bay on an incoming high tide, and has been used successfully for a number of years to conduct toxicity testing that regularly meets test acceptability criteria for a number of different standardized laboratory tests. The laboratory seawater will be used as the overlying water for sediment tests and as the dilution water for aqueous tests.

Control Sediment (YB or DB): Sediment from Yaquina Bay, OR (referred to as YB) will be used as the control sediment for testing with *E. estuarius* and *N. arenaceodentata*. Yaquina Bay sand is commonly used as a negative control in West Coast marine sediment toxicity testing. This sand will be obtained from Northwestern Aquatic Sciences (Newport, OR), which also collects *E. estuarius* from the same location for sediment toxicity testing. Sediment from Dillon Beach, CA (referred to as DB) will be used as the control sediment for *M. nasuta*. This sediment is from the clam collection site and is more organically rich and more suitable for *M. nasuta*.

Metals Contaminated Sediment (MS): A *fine-grained* marine sediment from an undisclosed (proprietary) site, contaminated primarily with Cu, zinc, and lead (referred to as MS) will be used for toxicity testing only. Chemical analysis of this sediment will be performed as part of the test design.

PCB Contaminated Sediment (PSNS): A medium-fine grained field sediment from the Puget Sound Naval Shipyard in Bremerton, WA (referred to as PSNS) that is contaminated with numerous classes of chemicals will be used for both toxicity and bioaccumulation testing. With the exception of PCBs, concentrations of other contaminants in this sediment are not expected to be at toxic levels. Historical data on the chemical profile of this material will be obtained. In addition, PCB, total organic carbon (TOC), and grain size analysis of this sediment will be performed as part of the test design.

The MS and PSNS sediments are already in storage (4 ± 2 °C, in the dark) at the SSC Pacific Laboratory. Before being introduced into the test chambers, the sediments will be re-homogenized and sieved to < 2.0 mm to remove shell hash and other indigenous material from interfering with the laboratory bioassays. The solids content (percent solids), initial TOC concentration, and percentage of silt and clay sized particles will be measured by ERDC.

Laboratory Dilution Water: The laboratory dilution water used by SSC Pacific Laboratory is 0.45 µm filtered seawater collected from near the mouth of San Diego Bay on an incoming high tide, and has been used for a number of years in successful toxicity testing that meets test acceptability criteria for a number of different standardized laboratory tests. The laboratory dilution water will be used as the overlying water for sediment tests and as the dilution water for aqueous tests.

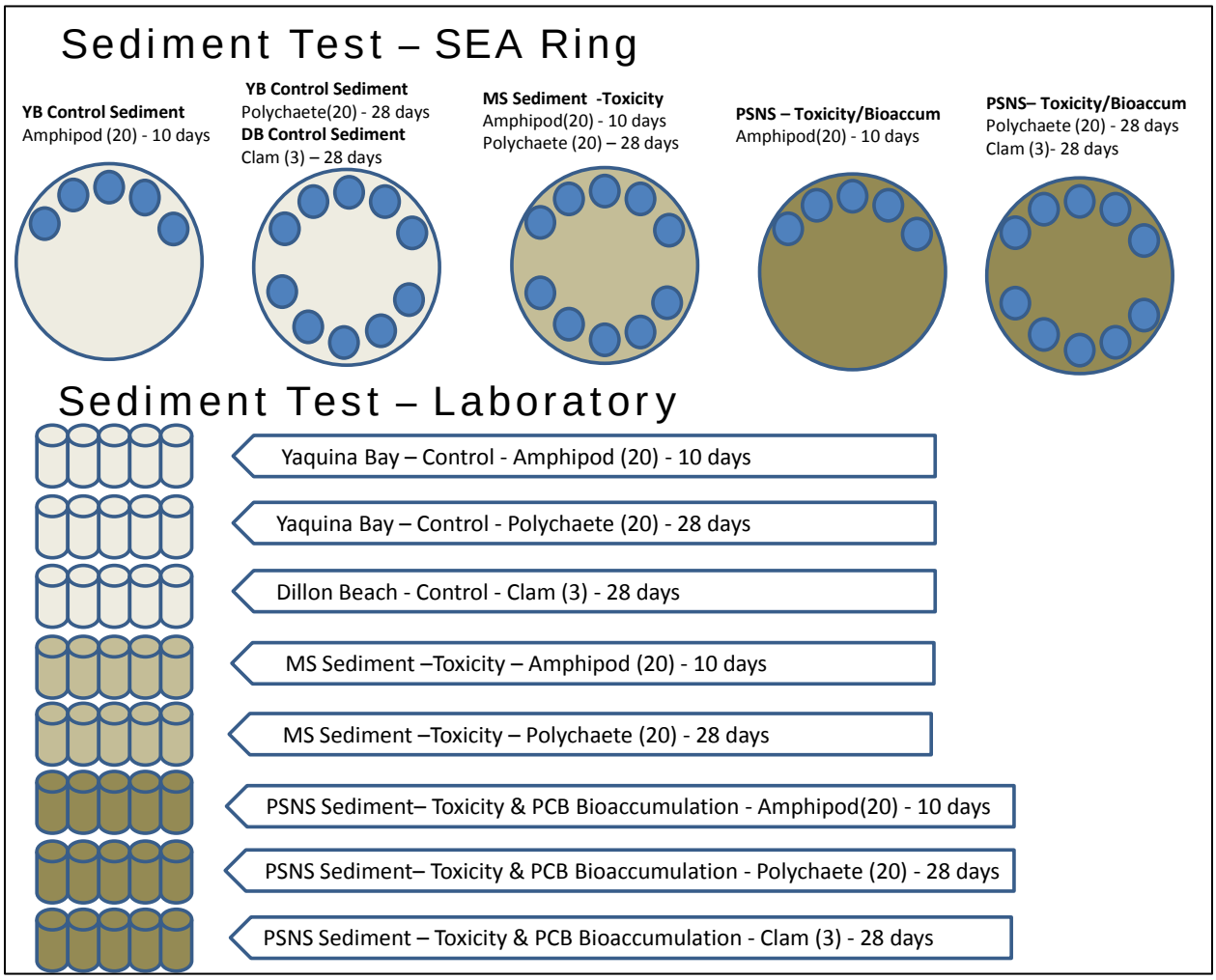

Figure 5. Overview of Sediment Toxicity and Bioaccumulation Testing Approach with Both SEA Ring and Standard Laboratory Tests (Number of test organisms per replicate in parentheses).

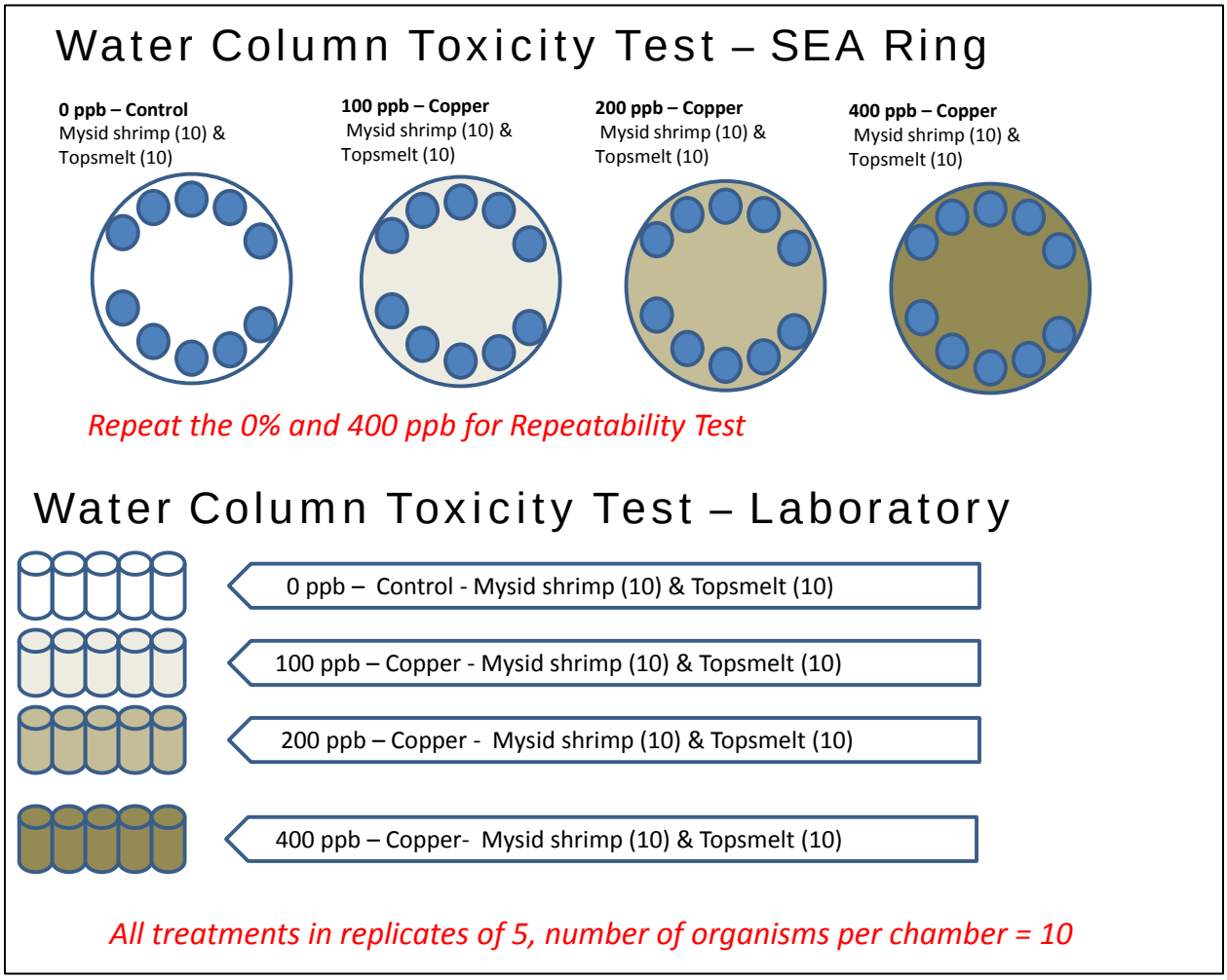

Figure 6. Overview of Water Column Toxicity Testing Approach with Both SEA Ring and Standard Laboratory Tests
(Number of test organisms per replicate is in parentheses)

Copper Spiking for Water Column Tests: Laboratory dilution water will be spiked with three concentrations of Cu, bracketing the expected median lethal concentration (LC50) for each of the two WC tests species. Concentrations of Cu to be tested are 100, 200, and 400 parts per billion (ppb) as Cu. The appropriate amount of Cu will be added to laboratory dilution water using a 1,000 parts per million (ppm) verified stock solution made from reagent grade copper sulfate ($CuSO_4$). Organisms will be loaded into one of four SEA Rings as depicted in Figure 6 and each ring will be exposed to a different Cu concentration.

B1.1.2 Benthic and Aquatic Organism Collection. Depending on availability, up to five different types of organisms will be used in this ETV verification test. For sediment tests, three organisms will be

used: a free burrowing deposit feeder (the marine amphipod, *Eohaustorius estuarius*), a deposit feeding tube building organism (the marine polychaete worm, *Neanthes arenaceodentata*), and a facultative filter feeding clam (the bent-nosed clam, *Macoma nasuta*). Survivors from each test species will be analyzed for PCB congeners from the PSNS sediment treatments, following test termination and a depuration period (overnight) in uncontaminated seawater. All replicates from one organism will be analyzed for PCB congeners in the YB control sediment. Only one organism will be analyzed because it is expected that there will be no PCB congener detections in the control sediment organisms.

Two common west coast marine test organisms will be used for the WC tests depending on their availability: *Americamysis bahia* (mysid shrimp) and *Atherinops affinis* (Pacific topsmelt). An alternative vertebrate species, the inland silverside minnow *Menidia beryllina*, may be used should topsmelt not be available.

The age/size and source information for the proposed test organisms is provided in Tables 1 through 5. All test organisms will be acclimated to laboratory exposure conditions at the SSC Pacific Laboratory for 1 to 5 days prior to use, depending on species. Acclimation time will be taken into account when the animals are ordered so that they will be within the acceptable age at the time of test initiation. During the acclimation period, water quality measurements of temperature, salinity, DO, and pH will be recorded daily. Laboratory SOPs for water quality monitoring and frequency are provided in Appendix E. Mortality of animals during holding should be no greater than 10% for all organism batches to ensure high quality organisms are being used.

B1.1.3 SEA Ring Preparation and Operation

Preparation- The SEA Ring hardware will be cleaned in a dilute (2%) detergent (Liquinox) overnight, followed by conditioning in uncontaminated, filtered laboratory seawater, and a final soak in flowing deionized water. Disposable parts (pump tubing, bottom end caps, and inner exposure chambers) will be replaced. SEA Rings will be placed into appropriate Chemtainers, and tested to ensure the pump and water quality sensor is functioning properly by connecting to a laptop uploaded with appropriate sensing software.

Initiation and Operation- The SEA Ring will be placed in a Chemtainer with enough water to be completely submerged. The water in the Chemtainer outside of the SEA Ring will be aerated continuously at a rate of one to two bubbles per second using trickle flow aeration in both the sediment

and water toxicity tests. This will allow delivery of aerated water to the exposure chambers as the water is pumped from the Chemtainer.

The required amount of sediment and/or clean seawater or Cu-spiked seawater water (Tables 1 through 5) will then be added to each exposure chamber, followed by securing of the top chamber caps, and initiation of the pump. The pump will be set to the desired turnover rate (approximately 10 exchanges between the inner exposure chamber and the water in the Chemtainer per day). For sediment tests, sediment will be allowed to settle overnight prior to organism addition. For WC tests, organisms will be added within 3 hours of addition of samples to the test chambers. Organisms will be arbitrarily selected and added through the organism delivery port in the chamber caps.

Replacement of the overlying water in both water and sediment tests will occur at the same frequency as the concurrent traditional laboratory methods according to the test method summaries in Tables 1 through 5. Approximately 80% of the water will be replaced on water renewal days. Although feeding may not take place in field exposures depending on species, organisms will be fed in laboratory trials according to test conditions found in Tables 1 through 5 to ensure that any mortality is not as a result of lack of food. Any required feeding will occur through the organism delivery port of each exposure chamber.

B1.2 Laboratory SEA Ring Test

The primary objective of the laboratory test is to evaluate the ability of the SEA Ring to provide comparable data (using quantitative and qualitative criteria) to traditional EPA and ASTM-approved laboratory methods under controlled laboratory conditions. It should be noted, however, that actual application of the SEA Ring device in situ is not expected to necessarily produce the same results as laboratory tests due to reasons already stated earlier in other sections of this document. For the purposes of this comparison, SEA Rings will be contained in the laboratory in containers using test conditions and experimental designs that are similar to those used in traditional laboratory toxicity and bioaccumulation tests. The containers are 17 gallon high density polyethylene (HDPE) containers (Chemtainer Industries, Inc.), frequently used to transport the SEA Rings to field sites (Figure 7).

Both sediment toxicity and WC toxicity tests will be conducted. The objective of WC toxicity tests is to determine the potential impact of dissolved and suspended contaminants on test organisms in the WC. The objective of benthic toxicity tests is to determine the potential impact of whole sediment exposure on benthic organisms.

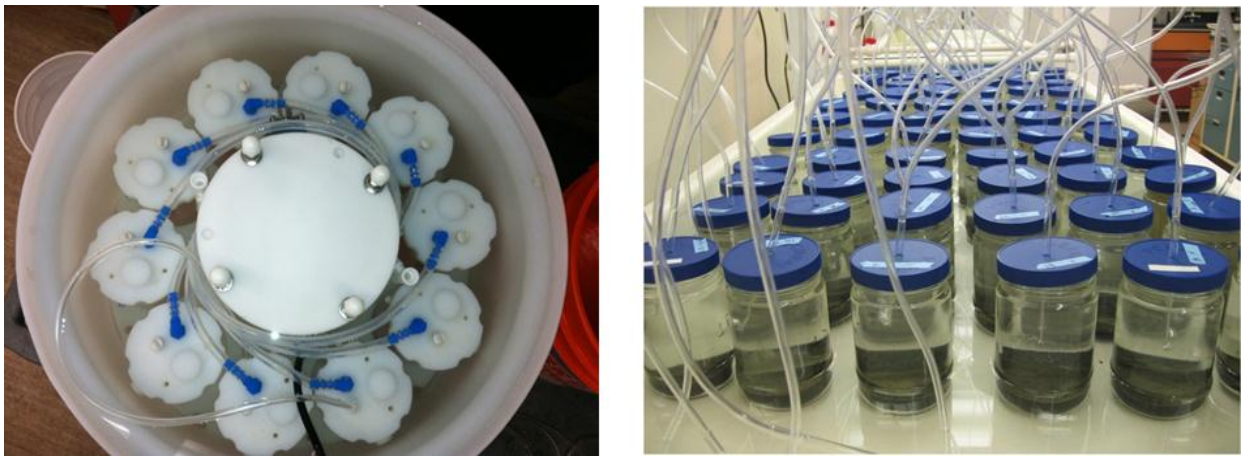

Figure 7. The SEA Ring verification testing will be conducted in 17-gallon HDPE containers (Chem-Tainer Industries; left), with concurrent standardized laboratory testing using glass beakers such as those shown at right

B1.2.1 Repeatability (Replicate Variability). Variability in biological response will be evaluated among the five replicate exposure chambers in the SEA Ring to provide a measure of repeatability within a single trial. This measure of repeatability will be assessed by quantifying biological responses at the end of the exposure period (survival of all species tested and growth of polychaetes). A control will consist of uncontaminated sediment from YB for comparison.

Sediment toxicity repeatability test - Two different organisms will be tested for the sediment toxicity repeatability test: the marine amphipod *Eohaustorius estuarius* and the marine polychaete *Neanthes arenaceodentata*. Three sediment types will be tested: 1) a sandy control sediment from Yaquina Bay, OR, where the amphipods are collected (YB); 2) a fine-grained metals contaminated sediment (MS) that has previously been shown to be toxic to the proposed test species; and 3) a medium-fine grained moderately contaminated from Puget Sound Naval Shipyard in Bremerton, WA (PSNS). This third sediment contains numerous classes of chemicals (e.g., metals, polycyclic aromatic hydrocarbons [PAHs], PCBs), but is not expected to be toxic to the species tested for this verification study based on prior studies. The exposure period for the sediment toxicity tests will be 10 days for the amphipod test (acute) and 28 days for the polychaete test (chronic). Survivorship of both species will be evaluated at the end of the exposure period. Growth of polychaetes will also be measured. Details of the test are provided in Table 6. Five replicate chambers with 20 organisms per replicate will be tested for each species. The reference toxicant for the solid phase sediment toxicity tests will be cadmium chloride ($CdCl_2$) for the amphipod and copper sulfate ($CuSO_4$) for the polychaete. The reference toxicant tests (performed as

Table 6. Summary of Tests and Testing Frequency

Performance Parameter	Objective	Endpoint	Comparison Based On	Testing Frequency	Minimum number replicates
Repeatability	Determine the repeatability among five replicates within one SEA Ring	1) Organism survival[1], or survival and growth[2] 2) Bioaccumulation of contaminant within organism tissue[3]	Survival, growth, and bioaccumulation of contaminants in organisms among five replicates within one SEA Ring	1) Survival in WC tests with four contaminant concentrations (including a control) and two test species. 2) Survival (and growth) in sediment tests with three sediment types including a control and up to three test species. 3) Bioaccumulation in sediment toxicity test of two test species, two sediment types including a control. Five replicates in each case.	Survival = 25 Growth = 5 Bioaccumulation = 15
Comparability	Determine the ability of the SEA Ring to measure toxicity of benthic and aquatic organisms compared to EPA/ASTM methods under the same conditions	1) Organism survival and growth 2) Bioaccumulation of contaminant within organism tissue	Survival (and growth), and bioaccumulation of contaminants in organisms in the SEA Ring compared to the bench scale EPA and ASTM methods	Survival in WC tests with four contaminant concentrations (including a control) and two test species. Survival and growth in sediment tests of three sediment types including a control and up to three test species. Both WC and sediment toxicity tests will be conducted in SEA Ring and in laboratory tests. Five replicates of each treatment.	Survival = 50 Growth = 110 Bioaccumulation = 30
Reproducibility	Determine the reproducibility among three different SEA Rings tested under the same contaminant concentrations and organisms	Organism survival	Survival of WC test organisms in the SEA Ring	WC tests of one toxic Cu concentration (400 ppb) and one control, both with two test species. Five replicates of each. Total of six SEA Rings required.	Survival = 40

[1]Survival will be determined in all species: mysid, topsmelt, amphipod, polychaete, and clam.
[2]Growth will be determined for one species only, the polychaete.
[3]Bioaccumulation of PCBs will be determined in amphipods, polychaetes, and clams.

standard lab exposures only) will be conducted in water only for 96 hours, with five concentrations (three replicates each), but otherwise follow the same testing conditions summarized for the relevant test organisms.

Water Column toxicity repeatability test - Survival of two organisms will be evaluated for the WC toxicity test, *Americamysis bahia* (mysid shrimp) and *Atherinops affinis* (Pacific topsmelt) under a range of Cu concentrations. This test will also include five replicate chambers for each exposure concentration and a clean seawater control (e.g., laboratory water used to acclimate test organisms) with 10 organisms in each replicate. The exposure period for the WC toxicity tests will be 96 hours, standard for acute exposures for these organisms (EPA, 2002a). The details of these tests are presented in Tables 6 and 7. Reference toxicant tests will be conducted following standard EPA methods using five dilutions of copper sulfate in the lab concurrent to the limited Cu exposures in the SEA Ring. The three concentrations of Cu tested in the SEA Ring will, however, allow for direct comparison to results in the standard reference toxicant test.

Sediment bioaccumulation repeatability test – Bioaccumulation of total PCBs (as a sum of detected congeners) will be evaluated in amphipods, polychaetes, and clams exposed to PSNS sediments in both the SEA Ring and laboratory exposures. Exposure periods for the different species are shown in Figure 6. Each test treatment will consist of five replicates, with amphipod and polychaete chambers containing 20 organisms, and clam chambers containing three organisms. Organisms from three of the replicates will be purged in clean seawater overnight and analyzed for PCB concentrations. The remaining two replicates will be purged and frozen/archived. Tissues will be analyzed by ERDC as described in Section B4.2.

B1.2.2 Comparability. Comparisons between results obtained from tests in the SEA Ring and traditional EPA and ASTM laboratory methods will be evaluated under controlled laboratory conditions as described in Section B1.2.1. Comparability will be evaluated between responses (survival, growth, and bioaccumulation) obtained in the standard laboratory exposures. Since both exposures will occur under controlled laboratory conditions, results should be similar with a goal of ± 20% for this assessment.

Sediment toxicity comparability test – The sediment comparability test will be conducted concurrently with the repeatability test, using the results derived from the approach described in Section B1.2.1.

WC toxicity comparability test – The WC comparability test will be conducted concurrently with the repeatability test, using the results derived from the approach described in Section B1.2.1.

Sediment bioaccumulation comparability test – The bioaccumulation comparability test will be conducted concurrently with the repeatability test, using the results derived from the approach described in Section B1.2.1.

B1.2.3 Reproducibility. To determine if different SEA Rings are capable of producing the same results, reproducibility among three different SEA Rings will be evaluated under the same environmental test conditions (i.e., the same environment, contaminants and test species). The reproducibility test will utilize the same conditions used in the repeatability and comparability tests. This evaluation will be conducted using the WC toxicity tests only (described in Section B1.1.2) using a single concentration of Cu (400 μg/L). This test will be conducted concurrently with the same batch of test organisms, Cu stock solutions, dilution water batch, and test conditions to minimize these potential confounding factors. Mean responses will be derived for each SEA Ring with a goal of less than 20% difference in mean response between all three, and no statistical difference among the three SEA Rings tested.

B1.3 EPA/ASTM Method Laboratory Comparability Tests

Water column toxicity bench scale test – Pre-cleaned 500 mL plastic or 1 L glass chambers will be prepared by washing with 2% dilute detergent (Liquinox), rinsing five times with tap water, placing in a clean 10% HNO_3 acid bath for a minimum of 4 h, followed by rinsing with acetone and five subsequent rinses with deionized water. The final step consists of a thorough flushing with deionized water. Salinity for marine/estuarine organisms will be kept stable within ± 2 parts per thousand (ppt) of the target 32 ppt; temperature will be stable within ± 1°C throughout the exposure period. DO concentration will be kept above a minimum threshold of 4 mg/L as feasible with the current methods described. The water quality parameters (DO, salinity, pH and temperature) will be measured daily throughout the experiment in a surrogate laboratory beaker.

Three concentrations of the Cu spiked seawater will be tested: 100 ppb (sublethal), 200 ppb (possibly lethal), and 400 ppb (likely lethal) with five replicates for each concentration. Five replicates of a negative (uncontaminated seawater) control will also be tested. The same organisms and same number of organisms used in the SEA Ring will be used in the laboratory test. The test chambers will be capped and

placed in an incubator or recirculating water bath held under constant conditions for 96 hours. Survival will be assessed at the end of the exposure.

Sediment toxicity bench-scale test – Tests will be conducted in 1 L containers that have been washed with detergent (2% Liquinox), rinsed with acetone, five times with tap water, placed in a clean 10% nitric acid bath for a minimum of 4 h, rinsed five times with deionized water, soaked in filtered, uncontaminated seawater, and then thoroughly flushed with either distilled or deionized water. The final step consists of a thorough flushing with deionized water. Salinity for marine/estuarine organisms will be kept stable within ± 2 ppt of the target 32 ppt; temperature will be stable within ± 1°C throughout the exposure period. DO concentration will be kept above a minimum threshold of 4 mg/L as feasible with the current methods described. The water quality parameters (DO, salinity, pH and temperature) will be measured daily throughout the experiment in a surrogate laboratory beaker. The test sediments will be thoroughly homogenized and press-sieved (< 2.0 mm) to remove any naturally occurring benthic organisms. Sediment will be allowed to settle overnight before introducing the organisms.

Flow rate: Per Tables 1 through 5, laboratory exposures will be conducted as static (amphipod) or static-renewal (mysid, topsmelt, polychaete, clam) tests. The 17-gallon Chemtainers holding the SEA Rings will follow the same renewal rate of the concurrent laboratory tests. It should be noted that although the SEA Ring's on-board pump will be programmed to circulate the overlying water within the Chemtainer (i.e., between the SEA Ring exposure chamber replicates and the water inside the Chemtainer outside the replicates), there will be no actual replacement of the water from the system until the renewal is conducted per the relevant laboratory-based protocol. It is possible that the circulation of the overlying water between the outside and inside of the SEA Ring exposure chambers could result in a different exposure to the samples than the standard laboratory tests, but this difference is expected to be minimal.

Other observations: During the exposure period, daily records will be kept of observable test species' mortality, emergence of infaunal organisms, formation of tubes or burrows, and any other or unusual behavior. Daily records of water quality (e.g., DO, salinity temperature, and pH) will be recorded in one of the test replicates. Water quality within SEA Rings will also include continuous water quality sensing within one replicate chamber for each treatment using a Troll 9500 (In Situ, Inc.) datasonde (Figure 8). Ammonia concentration will be determined in the overlying water at test initiation and test end for each test type.

Figure 8. A Troll 9500 datasonde (In Situ, Inc.) will be used to continuously measure and record water quality parameters in one of the SEA Ring exposure chambers associated with each treatment type

B1.4 Operational Factors

The operational factors to be evaluated include the training required to operate the SEA Ring. The technology representative will train one Battelle staff member on the use of the SEA Ring. The Battelle staff member, as well as the technology representative, will individually use the SEA Ring during the tests. The Battelle staff member will then document the ease of training and use of the SEA Ring. The SEA Ring will also be compared to the EPA/ASTM approved method in terms of its practicality, implementation and sustainability (i.e., the sampling time, waste produced, and the amount of protective equipment required by the individual operating the technology). This will be evaluated visually by the Battelle staff member and recorded. Examples of information to be recorded include (1) effort during training, (2) ease of preparation of site and technology for use, (3) actual use and repair of the technology, (4) cost associated with maintenance and repair of the technology, (5) overall convenience of the technology, (6) safety issues when using the technology, (7) number of samples that can be tested per day, and (8) clarity of the technology representative's instructions. Battelle will summarize these observations to aid in describing the technology performance in the Technology Verification Report.

B1.5 Supporting Analyses

Several supporting measurements will be performed by SPAWAR during testing. Table 7 summarizes the measurements, equipment and analytical methods or SOP.

Table 7. Test Methods and Equipment

Parameter	EPA Reference Test Method and Equipment	SEA Ring Method and Equipment
Temperature	Oakton pH 11 meter	Troll 9500 Datasonde (In Situ, Inc.)
Dissolved oxygen	Orion 830A D.O. Meter	Troll 9500 Datasonde (In Situ, Inc.)
pH	Oakton pH 11 Meter	Troll 9500 Datasonde (In Situ, Inc.)
Salinity	Orion A+ conductivity meter	Troll 9500 Datasonde (In Situ, Inc.)
% solids	EPA 1311	
Total Organic Carbon (sediment)	Modified Corp Eng. 81 and EPA 9060 procedures	
Silt and clay content	ASTM Method D422-63	
PCB Congeners (sediment)	Extraction: EPA Method 3545 Analysis: EPA Method 8082B	
PCB Congeners (tissue)	Extraction: Jones et al. (2006) Analysis: EPA Method 8082B	
Copper (seawater)	EPA Method 6020	
Ammonia (overlying water)	HACH Method 10031	

B1.6 Statistical Analysis

Sediment toxicity data: A total of six test groups (two organisms, and three test sediment types) including a reference sediment group (controls) will be assessed. Each group will be assessed in replicates of five. General descriptive characteristics will be provided in the form of n, mode, mean, standard deviation, median, minimum and maximum for continuous measures (test conditions, initial number of organisms, concentration of contaminants, and the number and percent of organisms surviving in each of the replicate chambers at the test) (EPA, 2002b).

Mean mortality in the control sediment of less than 10% will indicate acceptability of the test (organisms are not affected by stressors other than the contaminants being tested) (EPA, 1994, 2002a). For comparison purposes, the distribution of the proportion of surviving organisms and the homogeneity of variances will be examined. If the data do not satisfy the assumptions of normality and constant variance, they will be transformed using the arcsine/square root transformation or any other transformation that increases normality and stabilizes the variance, such as the log transformation. The primary comparisons of the number of organisms surviving between the replicates within a SEA Ring and between SEA Rings will be performed using the analysis of variance (ANOVA) and the Dunnett's test (each test versus control) or other suitable multiple comparison method. A secondary comparison of the number of organisms surviving in all the test groups combined with that in the reference sediment (uncontaminated)

will be performed using the t-test. A non-parametric test such as Kruskal-Wallis test may also be explored on the untransformed data. Test results that are significantly different than the controls will be determined using these statistical tests.

The LC50, defined as the concentration at which 50% lethality occurs, will be calculated for the reference toxicant tests. The statistical package CETIS (Comprehensive Environmental Toxicity Information System) to calculate the LC50. The LC50s will also be compared to historical data available at SPAWAR and Nautilus to see if sensitivity of the test species/method is similar to that historically observed under controlled laboratory conditions.

Water column toxicity data: Test groups (two organisms, three Cu concentrations) and a clean seawater group (control) will be assessed. Each group will be assessed in replicates of five (ASTM, 2008; EPA, 2002a). General descriptive characteristics will be provided in the form of n, mode, mean, standard deviation, median, minimum and maximum for continuous measures (test conditions, initial number of organisms, concentration of contaminants, and the number and percent of organisms surviving in each of the replicate chambers at the test).

A mean mortality in the control group of less than 10% will indicate acceptability of the test (organisms are not affected by stressors other than the contaminants being tested) (EPA, 1994, 2002a). For comparison purposes, the distribution of the proportion of surviving organisms and the homogeneity of variances will be examined. If the data do not satisfy the assumptions of normality and constant variance, they will be transformed using the arcsine/square root transformation or any other transformation that increases normality and stabilizes the variance, such as the log transformation. The primary comparisons of the number of organisms surviving between the groups will be performed using the ANOVA and the Dunnett's test (each test versus control) or other suitable multiple comparison method. The test group with the highest Cu concentration will be compared to the control group. A secondary comparison of the number of organisms surviving in all the test groups combined with that in the control group will be performed using the t-test. A non-parametric test such as Kruskal-Wallis test may also be explored on the untransformed data. Test results that are significantly different than the controls will be determined using these statistical tests.

The LC50 will be calculated for the standard laboratory reference toxicant tests, as well as the concurrent Cu dilutions series conducted in the SEA Rings.

Bioaccumulation test data: PCB concentrations will be measured in tissues from organisms exposed to sediment from YB/DB controls and PSNS.

Student t-tests will be used to compare the differences between the groups ($\alpha = 0.05$) in order to determine whether organisms exposed in the SEA Rings bioaccumulated PCBs differently than in the laboratory tests. Dunnett's test may be used to compare individual test groups with the reference sediment group.

Repeatability: Repeatability, assessed as replicate variability in this case, will be evaluated for the sediment toxicity, WC toxicity and bioaccumulation tests.

The outcome (the number of organisms surviving in each of the replicate chambers at the end of the test period or the bioaccumulation) will be calculated overall across all test groups and within each test group (one of two organisms, and one of three sediment types) using descriptive statistics.

Precision will be evaluated using the standard deviation and the standard error of the sample mean (se), calculated as the sample standard deviation ( ) divided by the square root of the sample size (n):

$$= / \sqrt{\ }$$

The smaller the *se*, the greater the precision.

The coefficient of variation ( ) will be calculated as the percentage of the sample standard deviation ( ) divided by the sample mean ($\bar{x}$):

$$= \frac{}{\bar{x}} \ 100$$

Similar measurements will be conducted for the organisms in the reference sediment (uncontaminated) and will be considered a measure of stability of the SEA Ring device. A *CV* of less than 25% will be a goal.

Differences in the outcome between the groups within the same SEA Ring will be explored using ANOVA and the Tukey method. The number of organisms surviving (or the uptake of contaminants in

case of the bioaccumulation test) in each test group will be compared to that in the control group using ANOVA and the Dunnett's test or other suitable multiple comparison method. A non-parametric test such as Kruskal-Wallis test may also be explored on the untransformed data.

Comparability: The purpose of this analysis is to ensure that the SEA Ring will provide comparable data to the traditional EPA/ASTM methods under controlled laboratory conditions. Thus, the concurrently conducted traditional EPA/ASTM methods will be considered the *gold* standard in this analysis.

Comparability will be assessed for the same tests used to evaluate replicate variability. The general analytical approach will be to compare the difference between all test groups with the corresponding traditional EPA methods, followed by between group comparisons.

For the sediment and WC toxicity tests, the overall difference in the number of organisms surviving in the SEA Ring will be compared to that observed using traditional EPA methods. Comparisons will be performed using the t-test or a non-parametric analog as discussed above for two sample comparisons. Between group differences (with more than two groups) will be explored using ANOVA and the Dunnett's test.

Uptake of contaminants in tissues during the bioaccumulation exposures conducted in the SEA Ring will be compared to that obtained following the traditional EPA/ASTM methods using the t-test or a non-parametric analog.

Other tests may be conducted as appropriate. For example, within the sediment toxicity and WC toxicity tests, each test group result may be standardized by the corresponding control and that standardized result compared to the standardized result obtained using the traditional EPA methods.

Deviation of the sediment toxicity and WC toxicity test results from the traditional EPA methods may be assessed in terms of bias. Bias will be calculated as average percent difference (%D) of each of the sediment toxicity, WC toxicity and bioaccumulation test results from the traditional EPA methods both overall and within each test group, as shown below:

$$\%D = \frac{1}{k}\sum_{j=1}^{k}\frac{\bar{x} - X}{X}_{j} 100$$

where *k* is the number of valid comparisons, and $\bar{x}$ is the sample mean and X is the mean of the traditional EPA methods.

Reproducibility: The purpose of this analysis is to determine if different SEA Ring units have a similar performance under controlled experimental conditions. At least three different SEA Rings will be compared under the same experimental conditions (same environment, contaminant concentration and test organism). The test will be conducted for the WC toxicity tests only (described in Section B1.1.2), and will be conducted concurrently with the same batch of test organisms, the highest Cu stock solution, dilution water batch, and test conditions to minimize these as potential confounding factors.

The general analytic approach will be to compare the results among all SEA Rings deployed. The overall difference in the number of surviving organisms will be compared among the SEA Rings using ANOVA and between group differences will be explored using multiple comparison tests.

B2 SAMPLING METHOD REQUIREMENTS

B2.1 Toxicity Test Breakdown – Collection of Test Organisms

The exposure chambers in the SEA Ring are held in place with a retaining pin. Upon completion of the exposure period, the retaining pin is removed and the chamber freed from the chamber holder. Test organisms from both SEA Ring exposure chambers and laboratory beakers will be recovered by sieving sediment through a 500 µm mesh sieve, which will retain the survivors.

B2.2 Collection and Analysis of Tissue Samples

At the conclusion of each sediment toxicity test, organisms will be recovered from the sediment with a 500 µm mesh size stainless steel sieve, enumerated, and transferred to clean seawater to purge ingested sediment overnight. Whole amphipods and polychaetes, and soft body portions from clams from each replicate will then be quickly rinsed in deionized water, weighed (for wet weight/growth assessment), and frozen (-20 °C) in 2 mL plastic micro-centrifuge vials until chemical analysis.

B2.3 Collection and Analysis of Water and Sediment Samples

The concentration of Cu in WC toxicity tests will be confirmed through quantitative analysis. Water samples of each Cu test concentration (control, 100 ppb, 200 ppb, and 400 ppb) will be collected for analysis. Samples will be collected using trace metals techniques (Method EPA 6020) in 500 mL HDPE or fluorocarbon bottles acidified with HNO_3 to pH < 2. Samples will be stored at 0 to 4ºC for up to 6 months, and shipped to the laboratory under COC.

The concentration of PCBs in sediment toxicity and bioaccumulation tests (YB/DB and PSNS sediments) will be confirmed through quantitative analysis. Prior to dispensing the homogenized sediments to test chambers, a 500 g sample will be collected into a wide-mouth glass with a Teflon®-lined lid and chilled to 0 to 4ºC. Sediment will be extracted using EPA SW846, Method 3545, and analyzed using Method 8082B.

B3 SAMPLE HANDLING AND CUSTODY REQUIREMENTS

B3.1 Handling of Aquatic Organisms

All test organisms will be acquired from commercial vendors from either laboratory culture or field collection, and shipped overnight to the SSC Pacific Laboratory. Upon arrival, all test organisms will immediately commence acclimation to laboratory test water quality conditions. Organisms will be observed for abnormal behavior and mortality prior to use in tests. A mortality rate of 5% will be used as a threshold for organism quality prior to use in verification testing.

Organism handling will follow laboratory or above-mentioned procedures for addition to the SEA Ring apparatus. Following the appropriate exposure duration, all organisms from the bioaccumulation tests will be purged in uncontaminated SSC Pacific Laboratory seawater overnight, weighed, and frozen in preparation for shipment to ERDC. A subsample of organisms will also be frozen at the beginning of the test without any exposure to assess time zero concentrations, if needed.

B3.2 Sample Custody

Sample custody will be maintained for all water, sediment, and tissue samples. Each sample will have a unique project identification number. This identification number will be recorded on a sample collection form along with the other information specified on the form. After the labeled sample containers are inspected, the sample custodian will complete the analysis request on the COC form. The COC form will include details about the sample, such as the time, date, location, and person collecting the sample. The COC form will track sample release from the sampling location to the testing laboratory. The COC form will be signed by the person relinquishing samples once that person has verified that the COC form is accurate. Samples will be sent to the appropriate laboratory via Federal Express Next or Second Day Service (or equivalent service).

The COC procedures emphasize careful documentation of constant secure custody of samples during the laboratory, transport, and analytical stages of project. The sample custodian (and alternate) responsible for the proper COC during this project is:

Sample Custodian:

Gunther Rosen
SPAWAR Systems Center Pacific

53475 Strothe Rd., Bldg. 111, Rm 216
San Diego, CA 92152
Tel: (619) 553-0886
Cell: (619) 890-9692
E-mail: gunther.rosen@navy.mil

Alternate custodian:

Marienne Colvin
SPAWAR Systems Center Pacific
53475 Strothe Rd., Bldg. 111
San Diego, CA 92152
Tel: (619) 553-5615
Cell: (858) 349-2926
E-mail: marine.colvin.ctr@navy.mil

B3.3 Sample Receipt

The laboratory's sample clerk will examine the shipping container and each sample cassette or sample container to verify sample numbers and check for any evidence of damage or tampering. The COC form will be checked for completeness and signed and dated to document receipt. Any changes will be recorded on the original COC form and then the form will be forwarded to the VTC. The sample clerk will log in all samples and assign a unique laboratory sample identification number to each sample and sample set. COC procedures will be maintained in the analytical laboratory.

B4 ANALYTICAL METHOD REQUIREMENTS

B4.1 Water Analysis

Cu analysis will be conducted at the SSC Pacific Laboratory. Samples associated with the WC testing will be analyzed for Cu in duplicate. Cu concentrations in the exposure water will be verified using a Perkin Elmer ELAN DRC II ICP-MS. The lab will use EPA Method 6020 for quantification. Actual detection limits will be determined by the laboratory and the method used to calculate them will be reported with the test data. Duplicate samples as well as spike samples will be measured as a QA/QC measure. The SSC Pacific Laboratory technical point of contact for Cu measurements will be Brandon Swope (brandon.swope@navy.mil). He will provide SOPs and appropriate QA reporting for the verification test.

The contact information for the SSC Pacific Laboratory representative is:
Brandon Swope
SPAWAR SSC Pac Chemistry Laboratory
53560 Hull Street
San Diego, CA 92152-5001

B4.2 Sediment and Tissue Analysis

PCB congeners will be analyzed in both sediment and tissues of relevant tests. Following the appropriate exposure duration, all necessary organisms will be purged in uncontaminated (SSC Pacific Laboratory dilution water) seawater overnight, weighed, and frozen in preparation for shipment to ERDC. ERDC will be responsible for analyzing the samples for PCB congeners. The 18 National Oceanic and Atmospheric Administration Status & Trend Congeners will be quantified for this test: PCBs 8, 18, 28, 52, 44, 66, 101, 118, 153, 105, 138, 187, 128, 180, 170, 195, 206, and 209. The handling of the sediment and tissue samples by ERDC is outlined in its SOP. Sediment samples will be extracted using pressurized fluid extraction (EPA Method 3545), and analyzed using gas chromatography (GC) following EPA Method 8082B. Reporting limits for PCB congeners in sediment are expected to be <0.6 µg/kg dry wt. Tissue analysis will be conducted using a micro-extraction technique for use with small masses (150-500 mg wet weight; Jones et al., 2006). Tissue extracts will be analyzed for PCB congeners by GC (EPA Method 8082B). Reporting limits for tissue are expected to be less than 2 µg/kg on a wet weight basis. Sediment and tissue PCB concentrations will be expressed as the sum of all detected PCB congeners, or

as the sum of PCB homologs. The actual detection limits and the method used to calculate them will be reported with the test data.

Three tissue samples for each species will be analyzed for both laboratory and SEA Ring exposures, providing QA of the measurement and sufficient data with which to make statistical comparisons between the laboratory and SEA Ring exposure methods. Ms. Patricia Tuminello will be the point of contact at ERDC. She will provide SOPs and appropriate QA reporting for the verification test.

The contact information for the SSC Pacific ERDC Chemistry Laboratory representative is:
Patricia Tuminello
USACE ERDC Chemistry Laboratory
3909 Halls Ferry Road
Vicksburg, MS 39180-6199

B4.3 Tissue Lipid Analysis

Polychaete lipid concentrations will be analyzed by the ERDC toxicology laboratory with a spectrophotometer at 490 nm following homogenization and chloroform/methanol extraction, and calibrated using stock solutions of soybean oil according to Van Handel (1985).

The contact information for the USACE ERDC Environmental Laboratory Risk Assessment Branch representative is:
Dr. Jacob Stanley
3909 Halls Ferry Road
Vicksburg, MS 39180-6199
jacob.k.stanley@us.army.mil

B4.4 Instrument Calibration Requirements

The inductively coupled plasma mass spectrometry (ICP-MS) calibration requirements are presented below. If criteria are not met, analysis will stop, corrective action taken, the instrument recalibrated, and samples not bracketed by a passing initial calibration (ICAL) or continuing calibration verification (CCV) reanalyzed:

- For copper measurements using ICP-MS a multi-point (no less than five) calibration curve will be generated using Perkin Elmer multi-element solution 3 (Part No. N9300233) diluted

with 1N optima grade nitric acid. The standard curve is rejected if the R^2 value is less than 0.995. The range of the calibration curve is constructed based on the best estimate of copper concentrations being measured. If a measured value falls outside of the standard curve range, the sample will be re-run with a different dilution factor or using a standard curve with a greater concentration range.

- ICAL: Prior to analysis a minimum of one high standard and a calibration blank; if more than one calibration standard is used, $r \geq 0.995$.
- CCV: After every 10 field samples and at the end of the analysis sequence. All analytes within ±10% of true value.

The GC calibration requirements are presented below. If criteria are not met, analysis will stop, corrective action taken, the instrument recalibrated, and samples not bracketed by a passing ICAL or CCV reanalyzed:

- ICAL: Prior to analysis a minimum of five standard standards; $r \geq 0.995$.
- Second source calibration verification (ICV): Immediately following ICAL; all project analytes within ±20% of expected value from
- CCV: Prior to sample analysis, after every 10 samples, and at the end of the analysis sequence. All project analytes within ±20% of expected value.

B4.5 Quality Control

Laboratory QC samples will be processed with each analytical batch to demonstrate analytical control. If criteria are not met, the sample should be re-analyzed and/or re-extracted and re-analyzed. If re-analysis is not possible due to available sample mass or holding time, then the data should be reported with a "J" qualifier to indicate that the value is an estimated value, typically outside of the calibration range. This is a common EPA data qualifier used in data analysis.

The ICP-MS QC requirements for Cu analysis are presented below.

- Method blank: One per batch of ≤20 samples; no target analyte detected at > detection limit.
- Laboratory control sample (LCS): One per batch of ≤20 samples; recovery within laboratory control limits or 80 to 120%.
- Matrix spike sample: One per batch of ≤20 samples; used to assess matrix interference recovery within laboratory control limits or 25 to 145% as determined by the laboratory. If LCS passes, re-analysis is not required.

The GC QC requirements for PCB analysis are presented below.

- Method blank: One per batch of ≤20 samples; no target analyte detected at > detection limit.
- LCS: One per batch of ≤20 samples; recovery within laboratory control limits or 25 to 145% (based on PCB congener or Aroclor)
- Matrix spike sample: One per batch of ≤20 samples; used to assess matrix interference; recovery within laboratory control limits or 25 to 145% (based on PCB congener or Aroclor). If LCS passes, re-analysis is not required.
- Surrogate recovery: One or more surrogates spiked into each sample prior to sample processing and extraction; recovery within laboratory control limits. The acceptable percent recoveries for the surrogates are: for water samples -TMX, 25 to 140% and decachlorobiphenyl, 40 to 135%; for sediment samples -TMX, 40 to 125%, decachlorobiphenyl, 50 to 125%; and for tissue samples - TMX, 45 to 125% and decachlorobiphenyl, 45 to 125%. The surrogate recoveries are defined by the laboratory based on historical experience with the extraction and analysis method in tissue. In particular, it should be noted that the tissue sample size (150 to 500 mg) is significantly less than the standard amount (30 g) and that will reduce extraction efficiency.
    - Acceptance criteria for the PE sample will be assessed as the percent recovery vs. the actual value defined by the PE supplier. The MS recovery criteria will be applied (25 to 145%) as acceptance criteria. This is well within acceptable control limits because due to interferences from the matrix itself (tissue samples) it may not be possible to obtain a clean chromatogram to accurately and specifically integrate a specific PCB peak. EPA method 8082A shows a similar range for the fish tissue Standard Reference Material: 33 to 133% recovery.

B5 QUALITY CONTROL REQUIREMENTS

QC measures are included to ensure quality data are provided by the verification test. This includes reference toxicant tests, acceptable results in control treatments, documentation that test conditions were within required conditions, sufficient replication to demonstrate repeatability and ability to detect significant differences among treatments.

B5.1 Reference Toxicant Test

Reference toxicant tests (also known as positive controls) are typically conducted concurrently with each batch of test organisms to ensure organism and laboratory technical quality. Reference toxicants for the selected test types are Cu or cadmium, depending on the species (Tables 1 through 5). Five concentrations and a control will be prepared from verified stock solutions consisting of $CuSO_4$ or $CdCl_2$. LC50 values generated from the dose response curves should be within two standard deviations of the running mean for the testing laboratory. The proposed concentrations for the reference toxicity tests are within the same range as that used for the WC toxicity test (100 to 400 ppb) and include an additional concentration of 800 ppb. Where insufficient data are available, LC50s should be comparable (within a factor of 2) to published values for tests conducted under the same conditions. The control charts are provided in Appendix B.

B5.2 Control Performance

Control survival is frequently used as a measure of test acceptability/QC. Where denoted in Tables 1 through 5, survival requirements will be used to assess overall QC, typically 90% survival in exposed organisms.

B5.3 Test Conditions Acceptability

Each test has specific water quality acceptability criteria, including measures for pH, temperature, salinity, and DO. These data will be recorded daily on the attached data sheets (Appendix A), and compared with the acceptable ranges shown in Tables 1 through 5. Deviations from the acceptable ranges will be considered during data interpretation. Ammonia concentration (a confounding factor in some sediment toxicity tests) will also be measured in the overlying water prior to test initiation and test end for each test type, using a HACH DR/2400 Spectrophotometer (Colorimetric Method, Method 10031). If ammonia concentrations exceed published thresholds for the test species, a renewal of the overlying water prior to organism addition will be considered and/or resulting data will be flagged prior to acceptance as

part of the verification study. It should be noted that ammonia is also considered a naturally occurring toxicant.

B5.4 Comparison to Background Tissue Levels

PCB bioaccumulation in the polychaete and clam will be used as a means of assessing repeatability within SEA Ring tests, and comparability between laboratory and SEA Ring tests. Tissue concentrations in the PSNS sediment will be compared statistically with the YB control sediment.

B6 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

When Battelle staff operate and maintain the SEA Ring undergoing testing, those activities will be performed as directed by the technology representative. Otherwise, operation and maintenance of the samplers will be the responsibility of the technology representative. The manual for the SEA Ring is provided in Appendix D.

B7 INSTRUMENT CALIBRATION AND FREQUENCY

The SEA Ring will be cleaned as specified above, disposable parts replaced, batteries charged, and tested for proper function prior to test initiation. Prior to and during (daily) the test, SEA Ring pumping operation will be verified using the on board hardware and connection to a laptop computer. Water quality monitoring, which will be recorded continuously aboard SEA Rings, will be checked several times during the exposures to ensure proper operation. All bench-top meters and probes (e.g., pH, DO, salinity and temperature) used to measure water quality in the laboratory tests will be calibrated daily.

B8 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

All materials, supplies, and consumables will be ordered by the technology vendor. Reagents and standards used by SPAWAR in preparation of analytical standards, spiking solutions, and reference toxicant tests will be reagent grade or better and used within the expiration date assigned by the manufacturer.

B9 NON-DIRECT MEASUREMENTS

Data published previously in the scientific literature will not be used to evaluate the vendor's technology during this verification test.

B10 DATA MANAGEMENT

Various types of data will be acquired and recorded electronically or manually by Battelle and vendor staff during this verification test. Table 8 summarizes the types of data to be recorded. All maintenance activities, repairs, calibrations, and operator observations relevant to the operation of the sampling systems being tested will be documented by Battelle or vendor staff in the project-specific LRB or dedicated data collection forms. During testing, raw data (records of test setup, measurements, observations, etc.) will be held by the SPAWAR point of contact. Once testing is complete, these raw data forms and records will be submitted to the VTC. Report formats will include all necessary data to allow traceability from the raw data to final results. A dedicated shared folder within the ETV AMS Center SharePoint site will be established for all project records.

Records received by or generated by any Battelle or subcontractor staff during the verification test will be reviewed by a Battelle staff member within 5 days of receipt or generation, respectively, before the records are used to calculate, evaluate, or report verification results. If a Battelle staff member generated the record, this review will be performed by a Battelle technical staff member involved in the verification test, but not the staff member who originally received or generated the record. The review will be documented by the person performing the review by adding their initials and date to the hard copy of the record being reviewed. In addition, any calculations performed by Battelle will be spot-checked by Battelle technical staff to ensure that calculations are performed correctly. Some of the checks that will be performed include:

- QC samples and calibration standards were analyzed according to the QAPP and the acceptance criteria were met. Corrective action for exceedances was taken;
- 100% hand-entered and/or manually calculated data were checked for accuracy;
- Calculations performed by software are verified at a frequency sufficient to ensure that the formulas are correct, appropriate, and consistent;
- For each cut and paste function, the first and last data value was verified versus the source data;
- Data are reported in the units specified in the QAPP;
- Results of QC samples are reported; and
- Any statistical calculations described in this QAPP.

Battelle will provide technology test data and associated reference data (including records; data sheets; notebook records) from the first day of testing within one day of receipt to EPA and the vendor for simultaneous review. The goal of this data delivery schedule is prompt identification and resolution of any data collection or recording issues. These data will be labeled as preliminary and will not have had a QA review before their release.

Table 8. Summary of Data Recording Process

Data to Be Recorded	Where Recorded	How Often Recorded	By Whom	Disposition of Data
Dates, times, and details of test events	ETV LRBs, test forms	Real time data recording throughout testing	Battelle staff	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
SEA Ring operating conditions, maintenance, down time, etc.	ETV LRBs, or electronically	When performed	Technology Representative and Battelle staff	Incorporated in verification report as necessary
Cu concentration in water and PCB concentration in sediment	Obtained from laboratory	After each sampling event	Battelle Staff	Converted to spreadsheet for statistical analysis and comparisons
Water quality parameters	Read electronically from instrument and recorded in laboratory notebook	Initially and daily	Technology Representative and Battelle staff	Converted to spreadsheet for statistical analysis and comparisons
Final dry weight of polychaetes	Obtained from laboratory	After each sampling event	Technology Representative	Converted to spreadsheet for statistical analysis and comparisons
Number of surviving organisms	Obtained from laboratory	After each sampling event	Technology Representative	Converted to spreadsheet for statistical analysis and comparisons
PCB concentration in tissue samples	Obtained from laboratory	After each sampling event	Technology Representative	Converted to spreadsheets for statistical analysis and comparisons

SECTION C

ASSESSMENT AND OVERSIGHT

C1 ASSESSMENT AND RESPONSE ACTIONS

Every effort will be made in this verification test to anticipate and resolve potential problems before the quality of performance is compromised. One of the major objectives of this QAPP is to establish mechanisms necessary to ensure this. The procedures described in this QAPP, which is peer reviewed by a panel of outside experts, implemented by the technical staff and monitored by the VTC, will provide information on data quality on a day-to-day basis. The responsibility for interpreting the results of these checks and resolving any potential problems resides with the VTC. Technical staff has the responsibility to identify problems that could affect data quality or the ability to use the data. Any problems that are identified will be reported to the VTC, who will work with the Battelle Quality Manager to resolve any issues. Action will be taken to control the problem, identify a solution to the problem, minimize losses, and correct data, where possible. Independent of any EPA QA activities, Battelle will be responsible for ensuring that the audits described below are conducted as part of this verification test.

C1.1 Performance Evaluation Audit

PE audits provide an independent assessment of the accuracy of laboratory analyses. For the ERDC laboratory, which is analyzing PCB congeners in sediment and tissue samples, a PCB congener standard reference material will be obtained from the National Institute of Standards and Technology and sent to the ERDC laboratory for analysis. The PE sample will be a blind, independent standard reference material supplied to the laboratory by Battelle. The range of potential congeners will encompass the congeners of interest; however, the actual congeners are blind so that both false positives and false negatives can be assessed. The acceptance criteria will be based on the actual concentrations which are blind at this time. Battelle will evaluate whether the laboratory has passed or failed the PE. The results of the PE sample will be reported to Battelle and EPA management. If the laboratory PE results are not acceptable, the laboratory will be informed as to whether the results are biased high or low. Corrective action will include an examination by the laboratory of instrument, sample handling, and sample analysis procedures. A second PE will be supplied once the laboratory feels its analytical system is in control. Sample analysis will not begin until PE results are acceptable. Alternatively, another laboratory will be identified. Routine analysis will not be initiated until the laboratory demonstrates the ability to analyze the sample with acceptable results.

C1.2 Technical Systems Audits

The Battelle QAO or delegate will perform a TSA during testing at SPAWAR. The purpose of the audit is to ensure that the verification test is being performed in accordance with the AMS Center QMP and this QAPP. The reference laboratories are not expected to be assessed during a separate TSA, provided acceptable performance on the PE audits. The TSA may be designated to an independent person by providing a checklist to be completed on site. During the TSA, the Battelle QAO or designee will compare actual test procedures to those specified or referenced in this plan and review data acquisition and handling procedures. A project-specific checklist based on the QAPP requirements will be prepared to guide the TSA, which will include a review of the test and analytical procedures, use of the SEA Ring technology and general testing conditions and review of test records and documentation. The Battelle QAO will also check data acquisition procedures, and may confer with the vendor staff. The Battelle QAO will prepare an initial TSA report and submit the report to the EPA Quality Manager (with no corrective actions documented) and VTC within 10 business days after completion of the audit. A copy of the final TSA report (with corrective actions documented) will be provided to the EPA AMS Center Project Officer and Quality Manager within 20 business days after completion of the audit. At EPA's discretion, EPA QA staff may also conduct an independent on-site TSA during the verification test. The TSA findings will be communicated to technical staff at the time of the audit and will be documented in a TSA report.

C1.3 Data Quality Audits

The Battelle QAO, or designee, will audit at least 10% of the sample results acquired in the verification test and 100% of the calibration and QC data versus the QAPP requirements. Two ADQs will be conducted for this project: The first will be conducted on the data set delivered within 30 days of test initiation. The ADQ will be completed within 10 business days of receipt using a project-specific checklist. The second ADQ will assess the remainder of the data, the draft report, and the verification statement. During these audits, the Battelle QAO, or designee, will trace the data from initial acquisition through reduction and statistical comparisons, to final reporting. All calculations performed on the data undergoing the ADQ will be checked. Data must undergo a 100% validation and verification by technical staff (i.e., VTC, or designee) before it will be assessed as part of the data quality audit. All QC data and all calculations performed on the data undergoing the audit will be checked by the Battelle QAO. Results of each ADQ will be documented using the checklist and reported to the VTC and EPA within 10 business days after completion of the audit. These reports will not include documented corrective actions. The completed ADQs with corrective actions documented will be provided to EPA within 10 business

days of receipt from the VTC. A final ADQ that assesses overall data quality, including accuracy and completeness of the technical report, will be prepared as a narrative and distributed to the VTC and EPA within 10 business days of completion of the audit.

C1.4 QA/QC Reporting

Each assessment and audit will be documented in accordance with Section 3.3.4 of the AMS Center QMP. The results of all audits will be submitted to EPA within 10 business days as noted above. Assessment reports will include the following:

- Identification of any adverse findings or potential problems;
- Recommendations for resolving problems (If the QA audit identifies a technical issue, the VTC or Battelle AMS Center Manager will be consulted to determine the appropriate corrective action);
- Response to adverse findings or potential problems;
- Confirmation that solutions have been implemented and are effective; and
- Citation of any noteworthy practices that may be of use to others.

C2 REPORTS TO MANAGEMENT

During the laboratory evaluation, any QAPP deviations will be reported immediately to EPA. The Battelle Quality Manager and/or VTC, during the course of any assessment or audit, will identify to the technical staff performing experimental activities any immediate corrective action that should be taken. A summary of the required assessments and audits, including a listing of responsibilities and reporting timeframes, is included in Table 9. If serious quality problems exist, the Battelle Quality Manager will notify the AMS Center Manager, who is authorized to stop work. Once the assessment reports have been prepared, the VTC will ensure that a response is provided for each adverse finding or potential problem and will implement any necessary follow-up corrective action. The Battelle Quality Manager will ensure that follow-up corrective action has been taken. The QAPP and final report are reviewed by the EPA AMS Center Quality Manager and the EPA AMS Center Project Officer. Upon final review and approval, both documents will then be posted on the ETV Web site (www.epa.gov/etv).

Table 9. Summary of Assessment Reports[(a)]

Assessment	Prepared By	Report Submission Timeframe	Submitted To
TSA	Battelle	10 business days after TSA is complete	EPA ETV AMS Center
ADQ	Battelle	ADQ will be completed within 10 business days after receipt of the initial data batch and then after all data for a phase is submitted	EPA ETV AMS Center
PE	Battelle	10 business days after receiving results of PE samples	EPA ETV AMS Center

(a) Any QA checklists prepared to guide audits will be provided with the audit report.

SECTION D

DATA VALIDATION AND USABILITY

D1 DATA REVIEW, VERIFICATION, AND VALIDATION REQUIREMENTS

The key data review and data verification requirements for this test are stated in Section B10 of this QAPP. In general, the data review requirements specify that data generated during this test will be reviewed by a Battelle technical staff member within 5 days of generation of the data. The reviewer will be familiar with the technical aspects of the verification test but will not be the person who generated the data. This process will serve both as the data review and the data verification, and will ensure that the data have been recorded, transmitted and processed properly. Furthermore, this process will ensure that the monitoring systems data were collected under appropriate testing.

The data validation requirements for this test involve an assessment of the quality of the data relative to the DQI (organism age and water quality) and QC results for this test referenced in Tables 1 through 5. Any deficiencies in these data will be flagged and excluded from any statistical comparisons to the SEA Ring being tested, unless these deviations are accompanied by descriptions of their potential impacts on the data quality.

D2 VERIFICATION AND VALIDATION METHODS

Data verification is conducted as part of the data review as described in Section B10 of this QAPP. A visual inspection of handwritten data will be conducted to ensure that all entries were properly recorded or transcribed, and that any erroneous entries were properly noted (i.e., single line through the entry, with an error code, such as *wn* for wrong number, and the initials of the recorder and date of entry). Instrument parameters and laboratory data collected during the test will be inspected to ensure proper transfer from the data-logging system. All calculations used to transform the data will be reviewed to ensure the accuracy and the appropriateness of the calculations. Calculations performed manually will be reviewed and repeated using a handheld calculator or commercial software (e.g., Excel). Calculations performed using standard commercial office software (e.g., Excel) will be reviewed by inspection of the equations used for the calculations and verification of selected calculations by handheld calculator. Calculations performed using specialized commercial software (i.e., for analytical instrumentation) will be reviewed by inspection and, when feasible, verified by handheld calculator, or standard commercial office software.

To ensure that the data generated from this test meet the goals of the test, a number of data validation procedures will be performed. Sections B and C of this QAPP provided a description of the validation safeguards employed for this verification test. Data validation efforts include the completion of QC activities and the performance of a TSA as described in Section C. The data from this test will be evaluated relative to the measurement DQIs described in Section A8 of this QAPP. Data failing to meet these criteria will be flagged in the dataset and not used for evaluation of the SEA Ring, unless these deviations are accompanied by descriptions of their potential impacts on the data quality.
An ADQ will be conducted by the Battelle Quality Manager to ensure that data review, verification, and validation procedures were completed, and to ensure the overall quality of the data.

The PE sample will be used as verification that the laboratory analytical system is in control to correctly identify and quantify the PCB congeners of interest.

D3 RECONCILIATION WITH USER REQUIREMENTS

The purpose of this verification test is to evaluate the performance of the SEA Ring in situ technology relative to standard laboratory-based EPA/ASTM Methods for evaluating sediment and WC toxicity to aquatic and benthic organisms. To meet the requirements of the user community, input on the tests described in this QAPP has been provided by external experts. Additional performance data regarding operational characteristics of the SEA Ring will be collected by verification test personnel. To meet the requirements of the user community, these data will include thorough documentation of the performance of the samplers during the verification test. The data review, verification, and validation procedures described above will ensure that data meeting these requirements are accurately presented in the verification reports generated from this test, and will ensure that data not meeting these requirements will be appropriately flagged and discussed in the verification reports.

SECTION E

REFERENCES

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EPA. 2000. Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR Part 136). United States Environmental Protection Agency. Office of Water (4303). EPA 821-B-00-004. July.

EPA. 2002a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms" Fifth Edition. EPA 821/R-02/012, October.

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APPENDIX A

TEST DATA SHEETS

10-Day Marine Sediment Bioassay
Static Conditions

Water Quality Measurements

Client: _____ **Test Species:** *E. estuarius*

Sample ID: _____ **Start Date/Time:** _____

End Date/Time: _____

Test Day	Salinity (ppt)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH (units)	Technician Initials	Comments
0						
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

QC Check: _____ **Final Review:** _____

28-Day Marine Sediment Bioassay
Static-Renewal Conditions

Water Quality Measurements

Client: _____ **Test Species:** _____

Sample ID: _____ **Start Date/Time:** _____

End Date/Time: _____

Test Day	Salinity (ppt)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH (units)	Fed	Water Change	Technician Initials	Comments
0								
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								
25								
26								
27								
28								

QC Check: _____ **Final Review:** _____

Marine Acute Bioassay
Static-Renewal Conditions

Water Quality Measurements
& Test Organism Survival

Project: _____ **Test Species:** _____
Sample ID: _____ **Start Date/Time:** _____
Test No.: _____ **End Date/Time:** _____

	Tech Initials				
	0	24	48	72	96
Counts:					
Readings:					
Dilutions made by:					

Concentration ppb	Rep	Number of Live Organisms					Salinity (ppt)					Temperature (°C)					Dissolved Oxygen (mg/L)					pH (units)				
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
Lab Control	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
50	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
100	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
200	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
400	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
800	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
	A								i					i					i					i		
	B								f					f					f					f		
	C																									
	D																									

Initial Counts QC'd by: _____

Animal Source/Date Received: _____ **Age at Initiation:** _____

	Feeding Times				
	0	24	48	72	96
AM:					
PM:					

Comments: i = initial reading in fresh test solution, f = final reading in test chamber prior to renewal

Organisms fed prior to initiation, circle one (y / n)

Tests aerated? Circle one (y / n) if yes, sample ID(s): Duration:

Aeration source:

QC Check: _____ **Final Review:** _____

Marine Chronic Bioassay **Water Quality Measurements**

Project: _____ Test Species: _____
Sample ID: _____ Start Date/Time: _____
Test No.: _____ End Date/Time: _____

Concentration (%)	Salinity (ppt)			Temperature (°C)			Dissolved Oxygen (mg/L)			pH (pH units)		
	0	24	48	0	24	48	0	24	48	0	24	48
Lab Control												
Brine Control												
6.25												
12.5												
25												
50												

		0	24	48
Technician Initials:	WQ Readings:			
	Dilutions made by:			

Animal Source/Date Received: _____

Comments: 0 hrs: _____
24 hrs: _____
48 hrs: _____

QC Check: _____ Final Review: _____

Marine Chronic Bioassay **Water Quality Measurements**

Project: _____ **Test Species:** *S. purpuratus*

Sample ID: _____ **Start Date/Time:** _____

Test No.: _____ **End Date/Time:** _____

Concentration %	Salinity (ppt)					Temperature (°C)					Dissolved Oxygen (mg/L)					pH (pH units)				
	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96

		0	24	48	72	96
Technician Initials:	**WQ Readings:**					
	Dilutions made by:					

Animal Source/Date Received: _____

Comments:

0 hrs: _____

24 hrs: _____

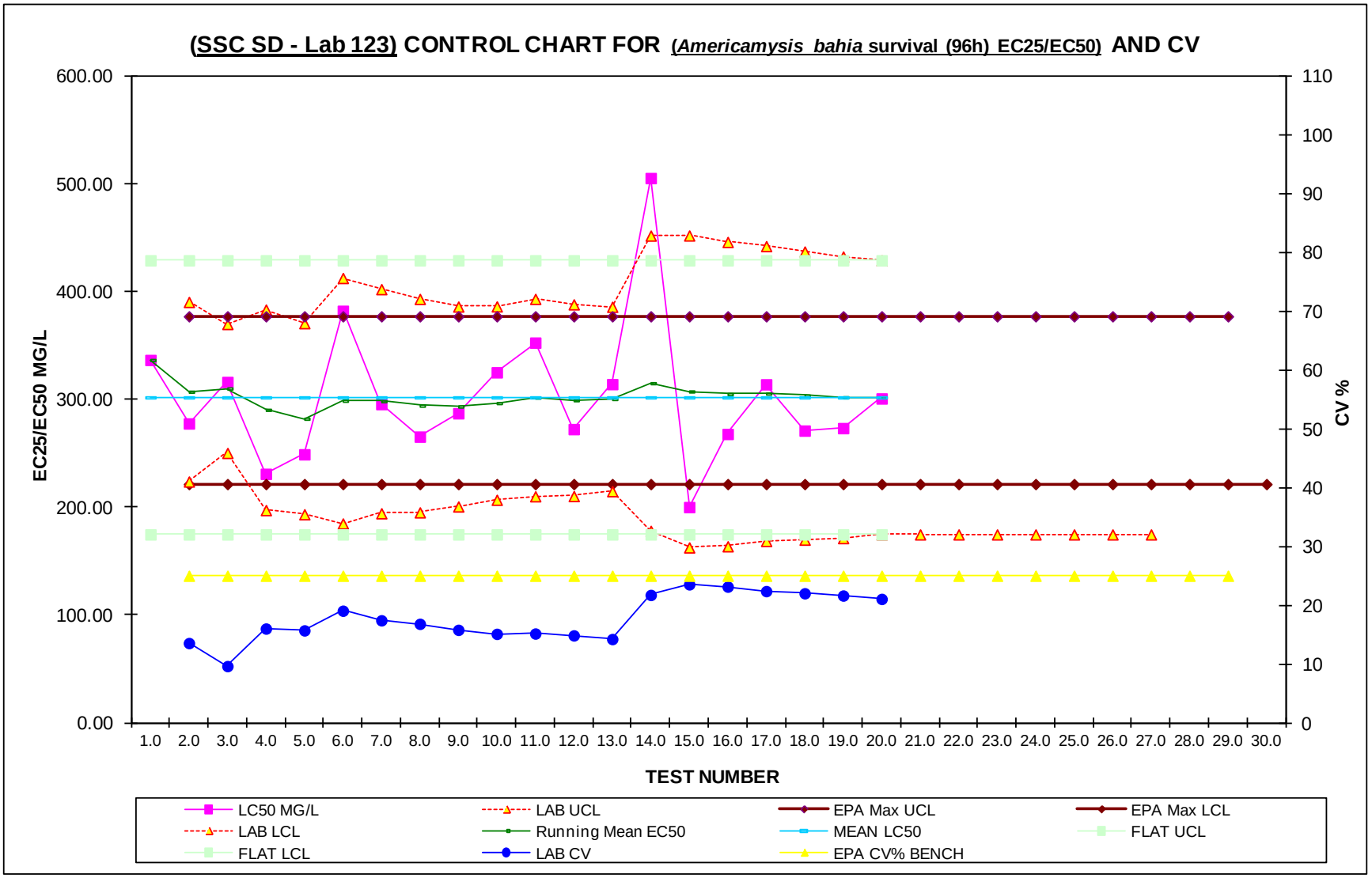
48 hrs: _____

72 hrs: _____

QC Check: _____ **Final Review:** _____

APPENDIX B

CONTROL CHARTS


(SSC SD - Lab 123) CONTROL CHART FOR (Americamysis bahia survival (96h) EC25/EC50) AND CV
EC25/EC50 MG/L
CV %
TEST NUMBER
600.00
500.00
400.00
300.00
200.00
100.00
0.00
110
100
90
80
70
60
50
40
30
20
10
0
1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0 22.0 23.0 24.0 25.0 26.0 27.0 28.0 29.0 30.0
LC50 MG/L
LAB UCL
EPA Max UCL
EPA Max LCL
LAB LCL
Running Mean EC50
MEAN LC50
FLAT UCL
FLAT LCL
LAB CV
EPA CV% BENCH

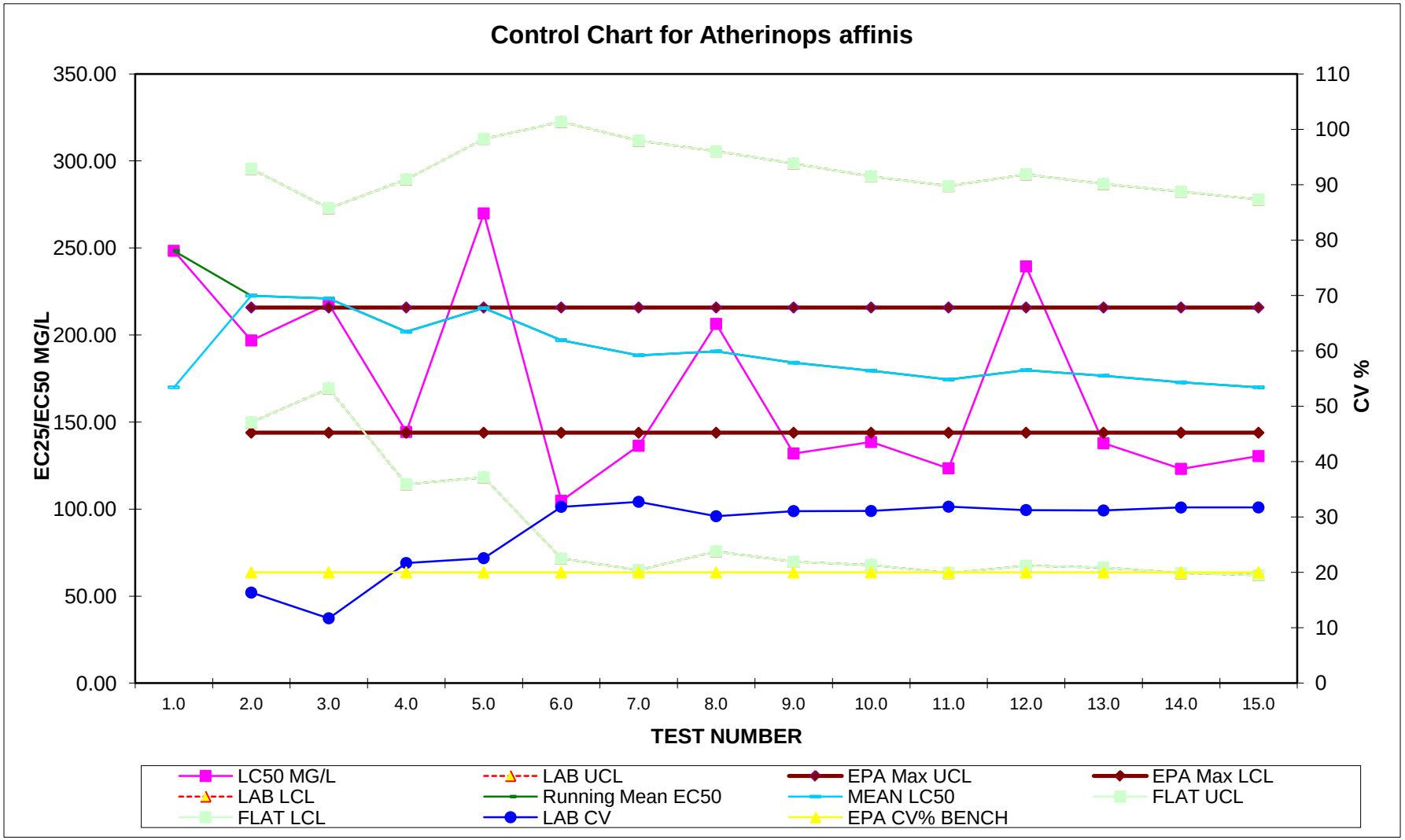

Control Chart for Atherinops affinis
EC25/EC50 MG/L
CV %
TEST NUMBER
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
110
100
90
80
70
60
50
40
30
20
10
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
LC50 MG/L
LAB UCL
EPA Max UCL
EPA Max LCL
LAB LCL
Running Mean EC50
MEAN LC50
FLAT UCL
FLAT LCL
LAB CV
EPA CV% BENCH

Table B-1. 96 hr Reference Toxicity Test Data

Species & Endpoint	Test Period	LC_{50} or EC_{50} (mg/L Cd or µg/L Cu)	Historical mean ± 2 SD (mg/L Cd or µg/L Cu)	95% Lower Confidence Limit	95% Upper Confidence Limit	CV (%)
E. estuarius 96-hr survival	6/10 – 6/14/08	7.0	6.4 ± 4.8	6.2	7.8	37
E. estuarius 96-hr survival	6/17 – 6/21/08	7.9	6.1± 4.3	6.6	9.5	35.2
M. galloprovincialis 48-hr development	6/6 - 6/8/08	8.9	6.5 ± 4.1	4.1	10.6	31.5
M. galloprovincialis 48-hr development	6/12 – 6/14/08	10	6.7 ± 4.3	4.3	10.9	32
S. purpuratus Fertilization	6/13/2008	20.5	18.8 ± 15.5	19.5	21.6	41.2

APPENDIX C

CHAIN OF CUSTODY FORMS

SPAWAR
Systems Center
San Diego

ENVIRONMENTAL SCIENCES AND
APPLIED SYSTEMS BRANCH, CODE 71750
53605 HULL STREET
SAN DIEGO, CA 92152-5000

Chain of Custody Record

Date: _____

Page _____ of _____

Project Title/Project Number:																					
Remarks/Air Bill:																					
Sampler(s): (Signature)																					
Tel:	***Fax:***	***Email:***																			
Special Instructions:																					
Field Sample Identification	***Date***	***Time***	***Matrix***	***Type***	***Temp (°C)***																
Relinquished by: (Signature)			***Received by: (Signature)***									***Date:***						***Time:***			
Relinquished by: (Signature)			***Received by: (Signature)***									***Date:***						***Time:***			

APPENDIX D

SEA RING MANUAL

ZEBRA-TECH LTD
www.zebra-tech.co.nz

SEA Rings

Operation Manual

Version 1.0

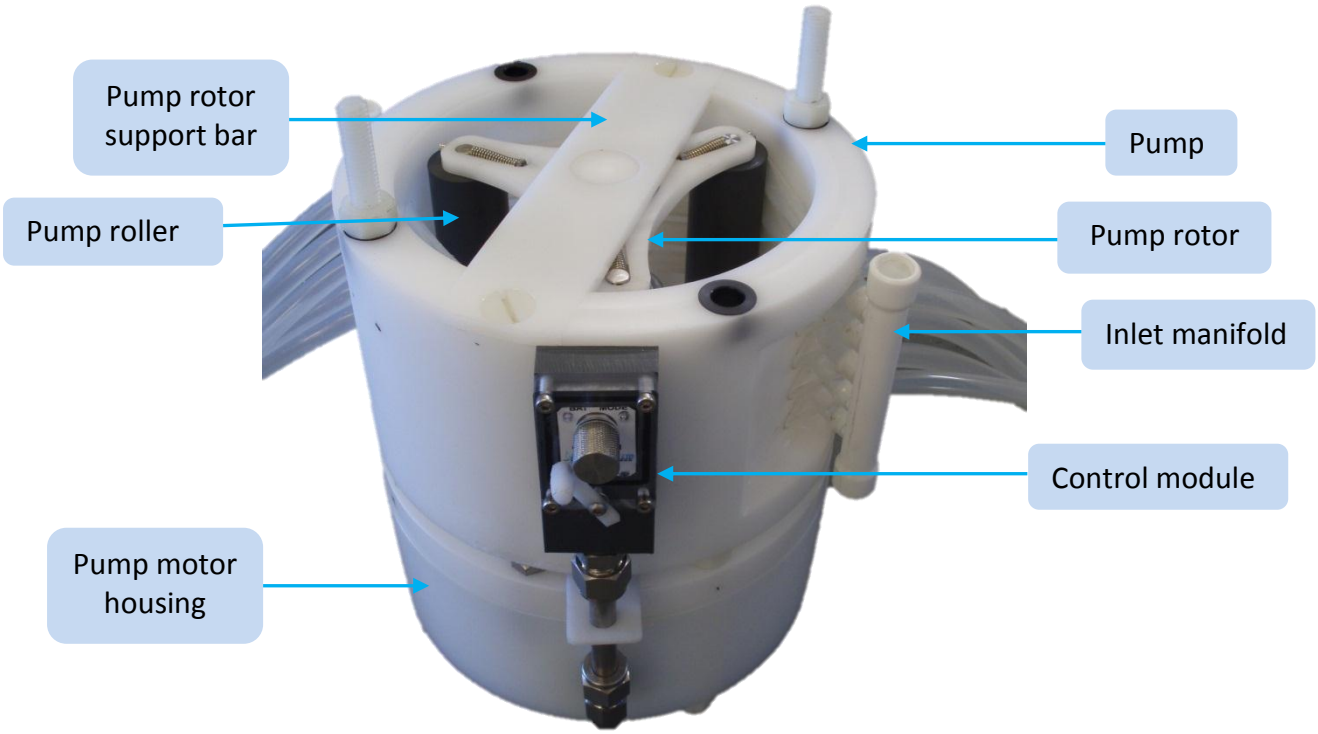
Contents

1. Overview

Pump

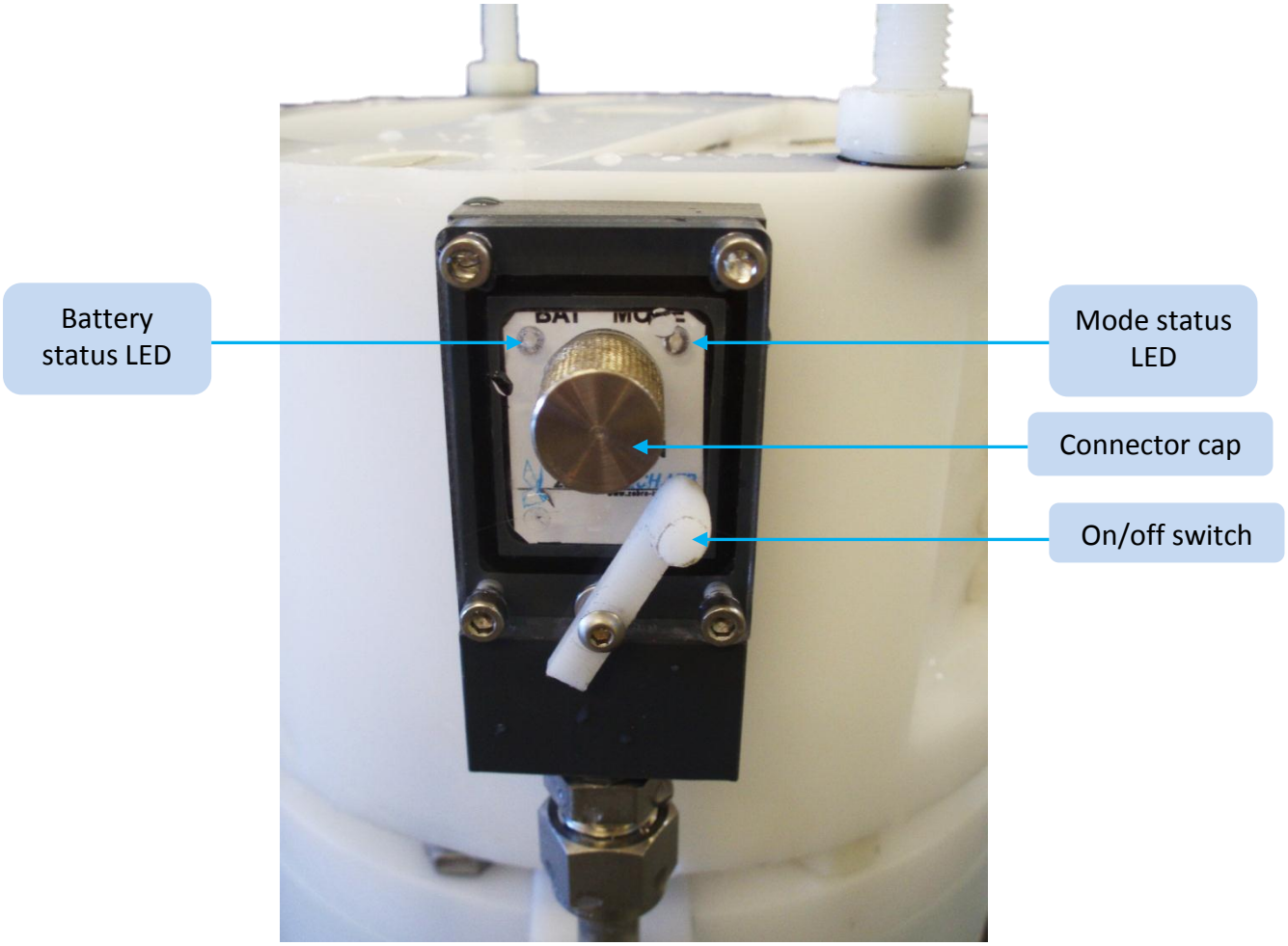
The pump consists of a pump motor housing and a pump housing.
The pump motor housing contains the pump motor, control electronics, and battery pack.

!	Warning: The pump has a very powerful motor that can cause personal harm. Keep fingers away from the pump rotor and always switch off before removing the pump cover plates.

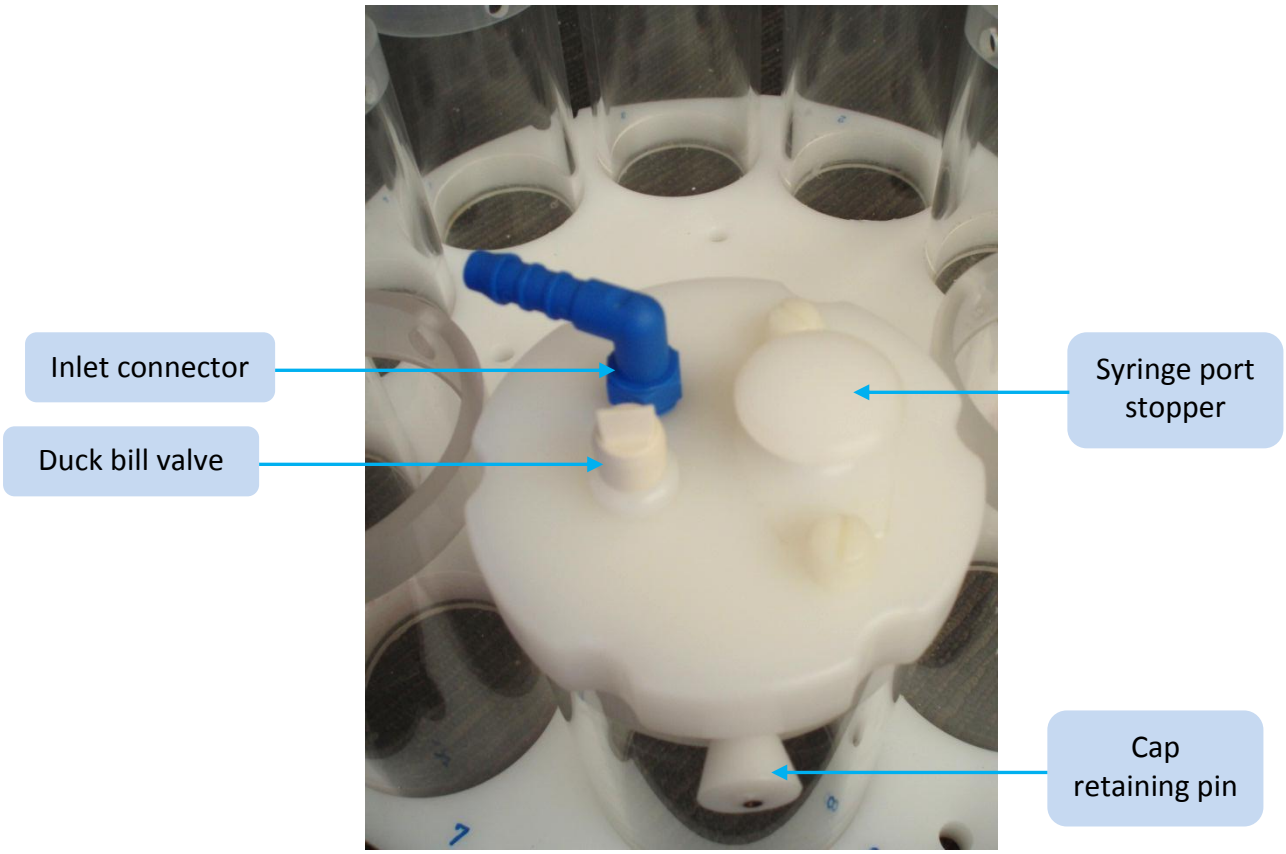


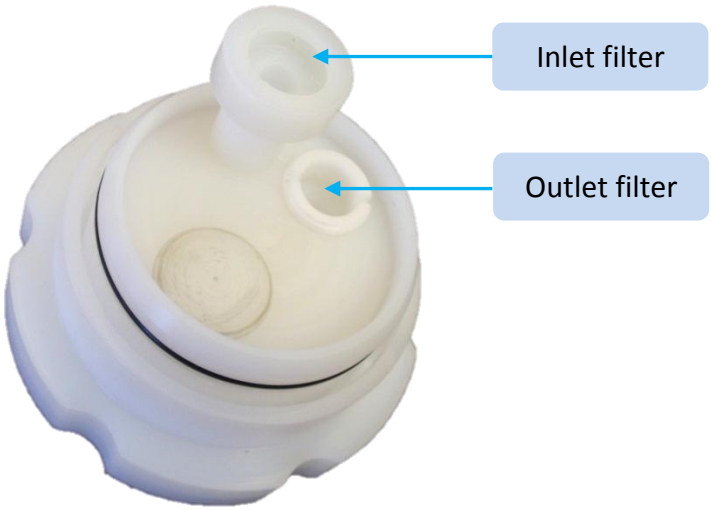
Control module

The control module features 2 status indicator LED's, an on/off switch, and the charging/communication connector.



Chamber cap


Inlet connector
Duck bill valve
Syringe port stopper
Cap retaining pin


Inlet filter
Outlet filter

2. Software Installation

The SEA Rings are supplied with a USB flash drive. This contains the SEA Rings communication software installation package. Double clicking this should launch the installer. When upgrading to a more recent version, the previous version does not need to be removed prior to installation.

The latest software is available for the Zebra-Tech web site;

http://www.zebra-tech.co.nz/downloads

3. Charging

The SEA Rings have an on-board Metal Hydride battery pack. The pack can be re-charged using the supplied charger. Allow 24 hours for a full charge.

The charger model number is the Universal charger, part number BPNC112900. This can be powered from an AC adaptor. The AC adaptor can be obtained from Radio Shack, part number 273-318, with an adaptor plug, part number 273-344. (Note: Align “tip” on the adaptor plug with “+” on the charger).

After disconnecting the charger, do not replace the coms connector cap on the SEA Rings for 1 hour. This enables any gas discharged by the battery pack to vent through the coms connector.

Metal Hydride batteries self-discharge at a rate of around 1% per day. Always charge the SEA Rings as close to the deployment date as possible.

4. On-Off Switch

The SEA Rings control module has an On-Off switch. In the off position, the SEA Rings pump will not operate, although the SEA Rings will still communicate with a PC whilst in the “off” position.

When the switch is turned on, if the start time/date has not been reached, the SEA Rings will sleep until the start time/date rolls over. The first flush then occurs after the flush interval has expired.

If the start time/date has expired when the switch is turned on, the first flush occurs after the flush interval has expired.

5. Status Indicator LED's

The SEA Rings control module has 2 status indicator LED's, that blink every 15 seconds.

Battery status indicator:

LED Blink Sequence:	Status Description:
One flash	Ok
Two flashes	Low battery warning (< 7.3 volts)
Three flashes	Low battery shutdown (6.5 volts)

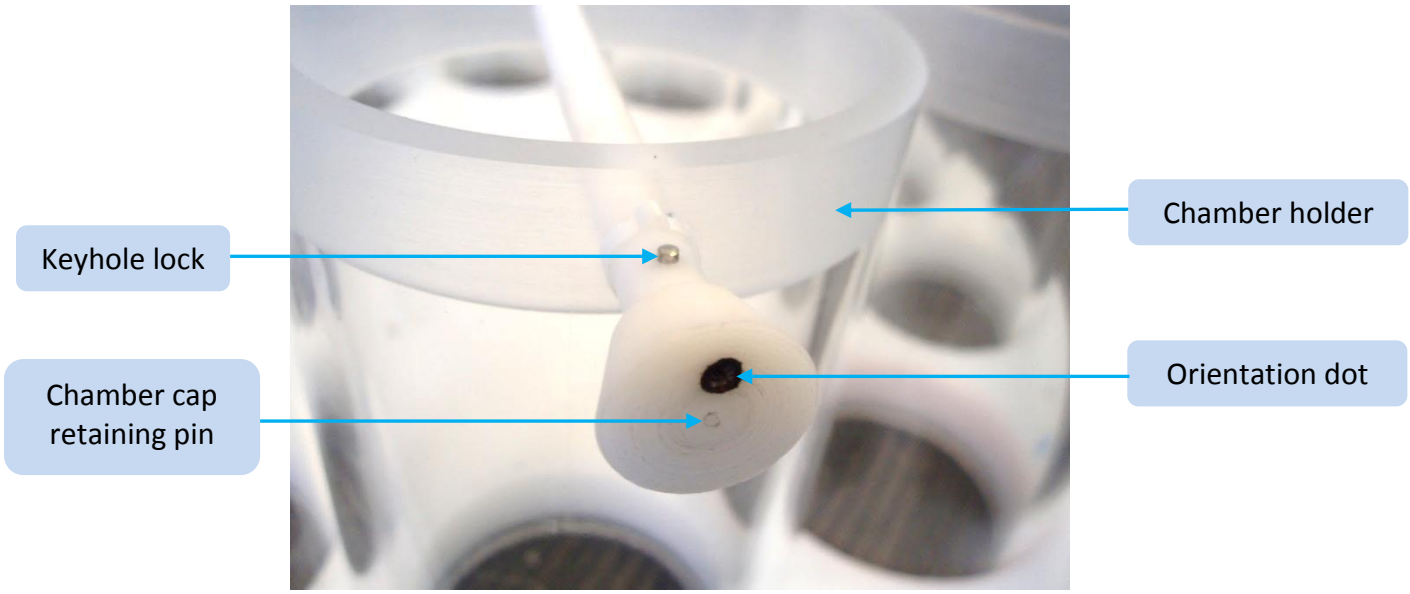
Operation mode indicator:

LED Blink Sequence:	Status Description:
One flash	Off
Two flashes	Delayed start countdown
Three flashes	Operational

6. Operation

Chamber cap removal

The chamber caps are secured in the Chamber Holder with a retaining pin. The retaining pin is secured by a keyhole style locking mechanism. To remove the retaining pin, rotate it so that the black dot is uppermost. The pin can then be pulled out of the chamber holder.



Software

Ensure the SEA Rings are charged. Connect the coms cable to the SEA Rings and a USB port on the PC. Start the SEA Rings communication application. Provided the SEA Rings are correctly connected and operational, the min window should open (Figure 1).

Figure 1: SEA Rings application main program window

Test Pump

Pressing this button switches on the pump. The pump remains on until the button is pressed again, or the SEA Rings application is closed.

Offload

The Offload button downloads data from the SEA Rings to a user selected file on the PC. The file format is ASCII, comma separated, and can be opened in Excel.

The data in the SEA Rings is stored in non-volatile memory. If the battery goes flat, data is not lost.

Upload Settings

Once the operating parameters have been set, they are sent to the SEA Rings by pressing the “Upload settings” button.

Delete Data

Data can be deleted off the SEA Rings using the “Delete data button”.

Set Time

The current time and date of the SEA Rings can be synchronised with the PC time and date. The SEA Rings time will be reset if the battery goes completely flat.

Chamber Flush Duration

This field is the number of minutes that the pump will be operating for each flush cycle.

Chamber flush interval

This field is the number of minutes that the pump is not operating between flush cycles.

Voltage

This field indicates the battery voltage. Around 9 volts is fully charged, 7.5 volts is mid-charge, and 6.5 volts is flat. If the battery voltage drops lower than 6.5 volts, the SEA Rings will cease functioning, and enter a low power shutdown mode. The pump will not operate until the batteries have been recharged.

Memory status

This is the percentage of the memory used.

7. Datafile

SEA Rings serial number: 1231
PC download time 13/01/2012 15:30

Start	15:17	13/1/2012	8.4	5
Stop	15:18	13/1/2012	8.4	4
Start	15:19	13/1/2012	8.4	5
Stop	15:20	13/1/2012	8.4	5

The fields are:
Start/stop, time (HH:MM), date (MM: DD: YY), battery voltage, number of pump revolutions.

8. Serial Debug

The SEA Rings can be optionally supplied with a wet pluggable connector on the side of the pump housing. This can be used to monitor the pump operation in a laboratory test situation.

To display the serial debug, connect the cable onto a PC and start a terminal emulator, such as “Term”, which is included on the Zebra-Tech USB flash drive. Set the serial port to the appropriate number and set the baud rate to 19200. The parity is None, data bits 8, stop bits 1.

Whenever the pump starts or stops, the time and date will be displayed, together with the number of pump revolutions.

When the serial debug cable is disconnected, the dummy connector MUST be fitted to protect the connector.

Never connect both the serial debug cable and the main coms cable onto SEA Rings at the same time.

Fitting Exposure Chambers

The exposure chambers can be made out of Butyrate tube. The size is 2.75 OD x 2.625 ID.

The tube can be sourced from K-Mac Plastics, 3821 Clay Ave SW, Wyoming, Michigan 49548,
Tel: 616-406-0671.

The part number is KM-2340 - CAB- Hollow Tubes- Clear- Tenite- 2.75 OD x 2.625 ID.

A cross hole for the chamber cap pin needs to be drilled through the tube:

1. Install the exposure chamber onto the Chamber Jig, available from Zebra-Tech. Alternatively fit the chamber onto a chamber cap.
2. Using a 9mm drill, drill the cross hole through the walls of the tube, using the cap or jig as the guide.
3. De-burr the 2 holes, particularly the internal side of the holes.

9. Servicing

Changing the pump tubing

The SEA Rings use around 3m of silicone tube, 8mm ID x 10mm OD. When replacing the tube, replace all the tubes using tube from a single roll. This ensures the tube wall thickness will be consistent. If tubes with inconsistent wall thickness are used, the pump performance maybe compromised.

To change the peristaltic pump tubing:

1. Switch off the SEA Rings.
2. Disconnect the pump tubing from the tube connectors on the chamber caps, and the inlet manifold.
3. Remove the 4 nuts around the top of the pump, and lift off the pump cover plate.
4. Lift the pump housing off the pump motor housing.
5. Unscrew the 2 slotted nylon countersunk screws and remove the pump rotor support bar.
6. Gently ease the pump rotor up, out of the pump housing.
7. Remove the old pump tubing from the pump housing.
8. Replace the pump rotor and the rotor support bar.
9. Systematically thread the new pump tube pieces into the pump housing, rotating the pump rotor to aid insertion.
10. Connect the tubes onto the corresponding port on the inlet manifold. Manually rotate the pump rotor to ensure the tubing is correctly positioned.
11. Hook the outlet end of the pump tubes onto the connector on the corresponding chamber caps.
12. Replace the pump onto the pump motor housing ensuring the drive train engages correctly.
13. Switch on the SEA Rings, and using the setup application, test run the pump, checking the tubes remain correctly positioned. Stop the pump.
14. Replace the pump cover plate.

O Rings

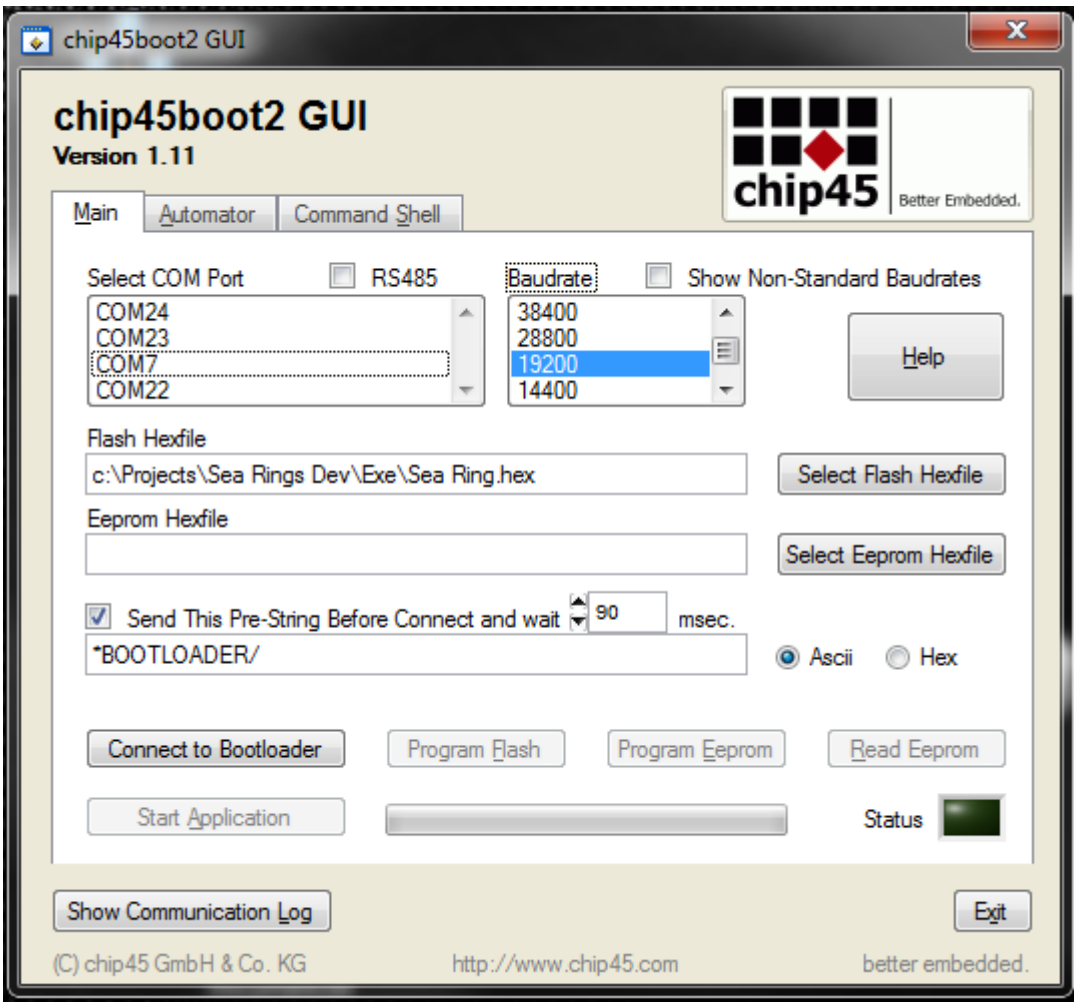
Chamber Cap: 2 3/8” x 3/32” Nitrile (Optionally silicone)

Syringe port: 15/16” x 3/32” Nitrile (Optionally silicone)

10. Firmware Upgrade

The firmware inside the SEA Rings can be updated using the boot-loader application provided on the Zebra-Tech USB flash drive. Consult Zebra-Tech prior to performing a boot-load.

1. Ensure SEA Rings application is closed. Connect the SEA Rings to the coms cable and plug the cable into the PC.
2. Start the boot-loader application.



11. Connector Pin Outs

Charging/Communication Cable:

Pin number:	Function:
1	Charge
2	Ground
3	PC Transmit
4	PC Receive

Optional Serial Debug Connector:

Pin number:	Function:
1	Ground
2	PC Receive

APPENDIX E

STANDARD OPERATING PROCEDURES

Systems Center
PACIFIC

Standard Operating Procedures
November 10, 2011

For
SSC Pacific Bioassay Laboratory
Bldg. 111 Rm. 116

53475 Strothe Road
Bldg. 111 Room 116
San Diego, CA 92152-5000
619-553-0886 · 619-553-2766

Table of Contents

Section **Page**

1.0 PROCEDURES FOR CONDUCTING TOXICITY TESTS

1.1 BIVALVE EMBRYO-LARVAL DEVELOPMENT TEST

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method estimates the chronic toxicity of effluent and receiving waters to the embryos and larvae of bivalve molluscs. The test endpoint is normal shell development and should also include mortality[1].

II. **NECESSARY MATERIALS AND SUPPLIES**

- Brillo pads – to clean exterior of mussels
- Beakers- 400-600ml - with dilution water held at 15°C for mussels once spawning is induced, and 1 L beaker for egg solution.
- Plastic holding tanks – 3 to 6L
- 1ppt copper stock solution- for reference toxicant test
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions.
- pH meter – for measuring test solutions
- Dissolved oxygen meter – for measuring test solutions
- Refractometer – for determining salinity of test solutions
- Thermometer – digital or laboratory grade
- Test chambers – 20ml glass scintillation vials and caps – pre-conditioned in dilution water.
- Colored labeling tape
- Dilution water – natural seawater or hypersaline brine made from natural seawater and diluted with deionized water
- Light microscope and slides
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Volumetric flasks- Class A, borosilicate glass or non-toxic plastic labware, 250ml 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
- Inverted microscope – for inspecting gametes and counting embryos and larvae.
- Counter, two unit, 0-999 – for recording counts of embryos and larvae
- 80 μm screen
- 54 μm screen
- 37 μm screen
- 22 μm screen
- Hemocytometer- for counting sperm
- Data sheets

III. **METHODS**

A. OBTAINING AND HOLDING ORGANISMS

1. Purchase mussels or oysters from Carlsbad Aquafarm and hold in tanks (cold room) with raw flowing seawater (ideal conditioning temperature is 12 – 14 °C) until they are needed for testing. Holding and conditioning tanks should be drained and sprayed with fresh water at least once weekly to prevent accumulation of organic matter and bacteria. Dead animals should be removed daily.

2. Clean the exterior of approximately 50 mussels or oysters with a brillo pad and filtered seawater. Hang remaining mussels or oysters off research pier or place in cold room tanks, depending on space availability and testing requirements.

B. SPAWNING AND FERTILIZATION

1. Place approximately 10-15 mussels/oysters in a single layer at the bottom of the spawning chambers (3 or 6 L plastic holding tanks). Plug sink and fill with hot water. Place 2 L Erlenmeyer flasks filled with dilution water into the hot water. Remove flask when temperature reaches 25-30 °C, or approximately 10 °C above the holding temperature. Pour enough of the 25-30 °C water on mussels/oysters so that they are covered completely. When individuals begin to spawn, remove from the holding tank and place each in a separate beaker at testing 15-20 °C) temperature in filtered seawater. During spawning, a sub-sample of gametes from each beaker should be observed for quality under the microscope and then labeled "eggs" or "sperm".

2. If no animals spawn within 30 minutes, the water should be returned to conditioning temperature (15 °C for mussels, 20 °C for oysters) for 15 minutes and the stimulation process repeated. In addition to heat treatment, mussels can be injected in the posterior adductor muscle with 1.0 ml of 0.5 M KCl. When individuals begin to release gametes, immediately isolate in a 200-300 ml glass beaker with filtered dilution water held at 15 ºC in the incubator.

3. Eggs should be passed through an 80-µm screen. The eggs will pass through the screen and debris retained. Pool quality eggs from different females together in a 1 L beaker, and fill with dilution water to approximately the 600 ml mark. Poor quality eggs will be vacuolated, small, or abnormal in shape. The concentration of the egg stock can be determined by counting a 1 ml sample at 400X. The pooled egg density should be adjusted to 5,000 to 8,000 eggs/ml before adding sperm.

4. Sperm should be passed through a 37-µm screen to remove feces and other material. Sperm will pass through the screen while debris will be retained. Sperm counts can be made with a hemacytometer. Sperm should be added so there are 10^5 to 10^7 sperm/ml in the final mixture.

5. Add sperm to beaker with eggs. Hold at 15 °C, gently mixing solution with a stirring rod every few minutes.

6. After fertilization (10 to 15 minutes), pass the embryo suspension through a 54-µm screen to remove debris. Excess sperm, bacteria, and protozoans should be removed by pouring embryos onto a 22-µm screen, washing delicately with dilution water, then backwashing into a suitable container with dilution water. Adjust embryo density to about 1500 to 3000 embryos/ml. Maintain the resulting mussel embryo suspension at 15 °C, and oyster embryo solution at 20 °C. Keep embryos suspended by stirring frequently, and begin test within 4 hours.

C. CONDUCTING THE TEST

1. About 1 hour after fertilization, a 1 ml sample should be placed in a Sedgwick-Rafter cell and the number of embryos developing to a 2-cell stage or beyond counted.

2. In addition to test materials or effluents, a reference toxicant test with copper should be conducted. Copper concentrations should include 0, 2.9, 4.1, 5.9, 8.4, 17.2, 25 ppb Cu for mussels. Concentrations should include 0, 4.1, 5.9, 8.4, 17.2, 25, 35 ppb Cu for oysters. Allow these solutions to equilibrate for at least one hour before testing. *Please refer to the *"Bivalve embryo development data sheet"* [1] for calculations.

3. Within 4 h of fertilization, distribute embryos to test containers already containing 10 ml of test solution in a random order using an automatic pipette. Be sure to keep embryo suspension well mixed. This is achieved by use of a perforated plunger or gently alternating between swirling and back and forth motions of the flask. **The concentration of embryos in the test solutions should be about 20 embryos/ml.** This typically requires an addition of 100 µl of a 2000 embryo/ml suspension.

4. Cap or cover (with acrylic plates) scintillation vials to prevent evaporation. Keep vials in an incubator at 15 °C (mussels) or 20 °C (oysters) on a 12 hr light/12 hr dark cycle.

5. Initial embryo density is measured in five additional scintillation vials, which are immediately preserved by adding 1 ml of concentrated formaldehyde to each vial.

6. Water quality parameters (pH, temperature, salinity, dissolved oxygen) are measured for an additional replicate with test organisms, but not used to assess larval development, at test initiation and termination.

7. After 48 h (or up to 54 h if development in clean water controls is not complete), preserve test organisms by adding 1 ml concentrated formaldehyde and capping vials.

IV. DATA COLLECTION

A. Within 7 days, count larvae using an inverted microscope, noting normally developed (those that have achieved the D-hinge stage) vs. abnormally developed.

B. Refer to *"Protocol for counting mussel larvae"* [2] for tips on counting.

V. ANALYZING DATA

A. Using CETIS or Toxcalc, enter data retrieved from enumeration of larvae to determine the EC50, LOEC, NOEC, or other appropriate toxicity metric. Please refer to *"Protocol for Statistical Analysis of Toxicity Data*[3] *".*

B. In accordance with USEPA (2002), all Toxcalc-generated concentration-response curves will be evaluated for acceptability.

[1]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.

[2] "Bivalve embryo development data sheet" and "Protocol for counting mussel larvae" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

[3] "Protocol for Protocol for Statistical Analysis of Toxicity Data" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

1.2 ACUTE TOXICITY TEST WITH BIOLUMINESCENT DINOFLAGELLATES (QWIKLITE)

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method is used to estimate toxicity of effluent and receiving waters to dinoflagellates. When specific species of bioluminescent Dinoflagellates are exposed to toxicants, a measurable reduction in bioluminescence is observed following mechanical stimulation when compared to controls[1].

II. **NECESSARY MATERIALS AND SUPPLIES**

- 500 ml flasks to maintain dinoflagellate cultures.
- Temperature controlled light chamber (e.g. Percival Scientific Model I-35LLVL) capable of maintaining test conditions at 19 ºC with a 12h light: 12h dark photoperiod
- Test chambers – 4.5 ml clear plastic cuvettes – pre-soaked in dilution water.
- Cuvette rack
- Colored labeling tape
- Dilution water – natural seawater (i.e. Scripps) or hypersaline brine made from natural seawater
- Light microscope and slides
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Volumetric flasks- Class A, borosilicate glass or non-toxic plastic labware, 250ml for making test solutions
- 1ppt copper stock solution
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions.
- pH meter – for measuring test solutions
- Dissolved oxygen meter – for measuring test solutions
- Refractometer – for determining salinity of test solutions
- Thermometer – digital or laboratory grade
- Wash bottles – for reagent water, dilution water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes
- Qwiklite testing equipment – either SeaLite or NRaD testing units
- Data sheets
- Black felt or box to cover dinoflagellates while testing
- Red light for minimal illumination for experimenter during testing

III. METHODS

A. DETERMINING CULTURE DENSITIES

1. Select a culture that is 1 to 2 weeks old.

2. To identify a healthy culture, look for a flask with a high density of cells (high density ensures less culture needed, which lowers the chance of EDTA and trace metals from interfering with the test), low levels of debris, and one that illuminates brightly when agitated (in a dark room).

3. After ensuring the culture is well homogenized, pipette a 20 µl aliquot onto a slide and count cells. Cultures are homogenized by gentle mixing, alternating between swirling and side to side, and back and forth movement of the flask. If cells are moving too fast on the slide, add a drop of formalin. Repeat 2 more times with additional 20 µl aliquots.

4. To determine the density in cells/ml, take the mean count of the three 20 µl aliquots and use the following formula:

(X cells/20 µl) x (1000 µl/ml) = _____ cells/ml, where X= mean of 3 aliquots

IV. CONDUCTING THE TESTS

A. Calculate the volume of culture to add to each flask (reference toxicant or effluent dilution) using the following formula:

$$c_1v_1=c_2v_2$$

For example, if your culture has 2000 cells/ml, your desired cell concentration is 100 cells/ml and the total desired volume of each flask is 50ml;

(2000cells/ml) (X) = (100cells/ml)(50 ml)
X= 2.5 ml of culture be added to 47.5 ml of solution.

B. Prepare reference toxicant dilutions using the table described in *"protocol for reference toxicant dilutions*[2]*"*. Prepare effluent dilutions as well. Allow solutions to calibrate for a least one half-hour.

C. Add calculated volume of cell culture to test solutions, remembering to agitate culture adequately so cells are evenly distributed. Pipet from the same place in the flask each time.

D. Pipette five replicates of 3 ml test solution for each concentration into cuvettes, swirling test solution every three replicates.

E. Store cuvettes in 19 °C incubator for 24 hrs.

V. DATA COLLECTION

A. Turn the lights off in the laboratory, gently remove test cuvettes from incubator- approximately 4 hours after initiation of the dark phase (typically between 11 am – 2 pm, depending on how lights are set in incubator).

B. Be very gentle. Do not shake, bump or swing cuvettes around (this will cause them to illuminate and lose potential light productivity).

C. Carefully take each cuvette and place into SeaLite or SPAWAR testing unit, press appropriate start button and record data onto pre-printed data sheet. Be sure to keep cuvettes covered with black felt as they await reading of light output. If using the SeaLite unit, the "QwikLite" software it uses can record data automatically, but will not take into account randomization of cuvette readings, which is recommended.

D. After measuring bioluminescence in each cuvette, empty solution into beaker and discard cuvette.

VI. ANALYZING DATA

A. QwikLite software will compute statistics (e.g. EC50 values where appropriate).

B. If SPAWAR unit is being used, PMT counts can be entered into an MS Excel spreadsheet called "*NRaD analysis*[3]".

C. After opening the Excel file, enter PMT counts into appropriate cells. Calculations will be made automatically.

D. If an EC50 value is desired, it can be determined with CETIS or ToxCalc software. Alternatively, the "Toolkit" program found on the LAB 116 computer desktop can be used to determine an EC50 by linear interpolation.

[1] Modified from ASTM Standard Guide for Conducting Toxicity Tests With Bioluminescent Dinoflagellates. Designation E 1924 – 97
[2] Protocol for reference toxicant dilutions can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs
[3] Nard analysis can be found in the sub-directory: C:\WINDOWS\Desktop\Laboratory 116\Sealite and NRaD.

1.3 REFERENCE TOXICANT TEST WITH MARINE AMPHIPODS

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method estimates the acute (96 h) toxicity of a reference toxicant to amphipods using 3-5 mm individuals, in a 96 h, water only, non-renewal exposure. Reference toxicant tests are used to evaluate quality of the test organisms[1].

II. **NECESSARY MATERIALS AND SUPPLIES**

- Plastic holding tanks – 3 to 6L
- Reference toxicant solution – Ammonia stock solution
- graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions
- pH meter – for measuring test solutions
- Dissolved oxygen meter – for measuring test solutions
- Refractometer – for determining salinity of test solutions
- Thermometer – digital or laboratory grade
- Dissolved Ammonia meter and probe - (prepare 24 hours in advance)
- Test chambers – 1 L glass beakers or jars with lids
- Colored labeling tape
- Dilution water – natural seawater or hypersaline brine made from natural seawater and diluted with deionized water
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Beakers- Class A, borosilicate glass or non-toxic plastic labware, 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
- Siphon tubes – for acclimation water changes
- Pasteur pipettes – for collection of amphipods
- Light box – for examining organisms
- Glass dishes for counting and transferring amphipods

III. METHODS

A. OBTAINING, FEEDING AND HOLDING ORGANISMS

1. Amphipods should be ordered within a week and at least three days prior to testing date to allow for acclimation to testing conditions. Approximately 20% more amphipods than needed for the test should be ordered.

2. Acclimation rates to test salinity and temperature should not exceed 3 °C and 3‰ per 24 hours.

3. Determine arriving temperature, pH, and salinity.

4. Transfer amphipods to a large plastic container with sediment at the bottom in a 15 °C temperature controlled room, incubator, or water bath. A squirt bottle filled with filtered seawater can be used to help get amphipods off plastic bags or containers and into the holding tanks. Remove dead by siphoning out of tank with a small rubber hose.

5. Each day prior to distribution of amphipods into beakers/jars, remove any dead, record physical parameters (temp, pH, salinity, D.O.), perform a 50% water change with seawater of the appropriate salinity and 15 °C seawater.

B. CONDUCTING THE TEST

Day 0 (Hour 0)

1. Mix up the appropriate salinity seawater with the appropriate amount of reference toxicant (e.g. cadmium, ammonia). Add 750 mL to each of at least 2 replicates per concentration.

2. Record water quality parameters (temperature, salinity, and D.O.) from one replicate of each treatment on Day 0 and Day 4 of test.

3. Sieve amphipods from holding tray and place in a smaller plastic or glass container with test seawater. Fill glass dishes with approximately 150 mL of test seawater. Select healthy and active individuals with a transfer pipette and distribute in batches of 10 to glass dishes. The number of amphipods in each dish should be verified by recounting before adding to test chambers. Add one dish of 10 to each replicate.

4. Cover chambers with an opaque material or place in a dark room or enclosure and hold at 15°C.

Day 1 (Hour 24)

1. Measure and record temperature in one test chamber from each treatment every day thereafter.

2. Note and remove any mortalities.

3. Lights must remain off or chambers must remain in the dark during the entire exposure.

C. TEST TERMINATION

Day 4 (Hour 96)

1. Count surviving amphipods and record. Amphipods will occasionally "play dead". Look for movement in the pleopods (back legs).

IV. ANALYZING DATA

Using CETIS or Toxcalc 5.0, enter data retrieved from the survival endpoint to determine the LC50 or other relevant toxicity metric. Please refer to "*Protocol for Statistical Analysis of Toxicity Data*" [2].

[1]Modified from "U.S. EPA Methods for Assessing the Toxicity of Sediment-associated Contaminants with Estuarine and Marine Amphipods" June 1994 EPA 600/R-94/025

[2] "Protocol for Statistical Analysis of Toxicity Data" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and log

1.4 SEDIMENT TOXICITY TEST WITH MARINE AMPHIPODS

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method estimates the acute (10 day) toxicity of whole sediment to amphipods using 3-5 mm individuals, in 10 d non-renewal exposures. Amphipods are intimately associated with sediment by nature of their burrowing or tube-dwelling and feeding habits, thus making them suitable species for sediment toxicity testing[1].

II. **NECESSARY MATERIALS AND SUPPLIES**

- Plastic holding tanks – 3 to 6L
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions
- pH meter – for measuring test solutions
- Dissolved oxygen meter and probe – for measuring test solutions
- Dissolved Ammonia meter and probe - (prepare 24 hours in advance)
- Refractometer – for determining salinity of test solutions
- Thermometer – digital or laboratory grade
- Test chambers – 1 L glass beakers or jars with lids
- Colored labeling tape
- Dilution water – natural seawater or hypersaline brine made from natural seawater and diluted with deionized water
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Beakers- Class A, borosilicate glass or non-toxic plastic labware, 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
- Siphon tubes – for acclimation water changes
- Pasteur pipettes – for collection of amphipods
- Light box – for examining organisms
- Glass dishes for counting and transferring amphipods
- Turbulence reducer – to prevent disturbance of sediment when adding overlying water
- Air grid and filter
- Plastic tubing
- 1 mm sieves
- Plastic buckets – for sieving sediment
- Spatulas - nylon, fluorocarbon or polyethylene

III. **METHODS**

A. OBTAINING, FEEDING AND HOLDING ORGANISMS

1. Amphipods should be ordered within a week and at least three days prior to testing date to allow for acclimation to testing conditions. Approximately 20% more amphipods than needed for the test should be ordered.

2. Acclimation rates to test salinity and temperature should not exceed 3 °C and 5‰ per 24 hours.

3. Determine arriving temperature, pH, and salinity.

4. Transfer amphipods to a large plastic container with sediment at the bottom in a 15 °C temperature controlled room, incubator, or water bath. A squirt bottle filled with filtered seawater can be used to help get amphipods off plastic bags or containers and into the holding tanks. Remove dead by siphoning out of tank with a small rubber hose.

5. Each day prior to distribution of amphipods into beakers/jars, remove any dead, record physical parameters (temp, pH, salinity, D.O.), perform a 50% water change with seawater of the appropriate salinity and 15 °C seawater.

B. CONDUCTING THE TEST

1. TEST PREPARATION
   Day -1

   a. Sediments should be stored at 4°C and be tested within two weeks after collection[1]. Press-sieving (1mm) all sediments (including control and reference) should be performed if there is concern about the presence of predatory organisms, large debris, or organisms taxonomically similar to the test species. Ensure that nearly all sediment is pressed through sieve to prevent composition change in sediment. Wash sieves between samples with acetone sparingly, then rinse well with deionized water. Also rinse spatulas, spoons and other utensils between samples.

   b. Take note of sediment homogeneity and grain size.

   c. Add 2 cm of homogenized sediment to each beaker/jar. Settle the sediment by either tapping the side of the test chamber against the hand or smoothing with a nylon, fluorocarbon or polyethylene spatula.

   d. Add 750 mL of 20‰ seawater to each replicate. To minimize disruption of sediment as seawater is added, use a turbulence reducer. Position the turbulence reducer just above the sediment surface and raised slowly as seawater is added.

   e. Cover all replicates and ensure gentle (approx. 100 bubbles/minute) aeration.

2. ADDITION OF AMPHIPODS
   Day 0

   f. Measure and record physical parameters (temp., salinity, DO, pH, ammonia) for overlying water in one replicate. Pour off overlying water of the same replicate and remove sediment for centrifugation to make Day 0 pore water measurements if required.

   g. Sieve amphipods from sediment in holding tray and place in a smaller plastic or glass container with test seawater. Fill glass dishes with approximately 150 mL of test seawater. Select healthy and active individuals with a transfer pipette and distribute in batches of 10 to glass dishes. The number of amphipods in each dish should be verified by recounting before adding to test chambers. Add two dishes of 10 to each replicate (20 animals total). Be sure to select dishes randomly.

   h. After addition of animals, examine beakers/jars for animals that have been injured or stressed. These individuals will not burrow into sediment and should be removed and replaced. *Eohaustorius estuarius* generally burrows in 5 – 10 minutes. Record the number of amphipods that are replaced.

3. TEST MAINTENANCE
   Days 1-10

   a. On Day 1, salinity, pH, D.O., and temperature from overlying water should be measured from a replicate of each treatment every day thereafter.
   b. Note and remove any mortalities.
   c. Check aeration in each chamber.
   d. Lights must remain on during the entire exposure.

C. TEST TERMINATION

1. On Day 10, measure water quality (salinity, pH, D.O. and temperature) and ammonia from overlying and pore water (if needed) from one replicate of each treatment.

2. Pour approximately half of the overlying water over a 1 mm sieve. Use remaining water to loosen sediment by swirling gently. Place the 1 mm sieve over a plastic bucket and pour sediment onto sieve. Using a spray bottle with seawater of the appropriate salinity, wash sediment through sieve.

3. Transfer amphipods into a counting dish. Be very careful not to leave any amphipods on the sieve during this process.

4. Count surviving amphipods and record. Amphipods will occasionally "play dead". Look for movement in the pleopods (back legs).

VI. ANALYZING DATA

A. Two sample comparisons can be done using a t-test to detect a significant departure from the control and each treatment. *Please refer to "*Protocol for Statistical Analysis of Toxicity Data*"[2].

[1]Modified from "U.S. EPA Methods for Assessing the Toxicity of Sediment-associated Contaminants with Estuarine and Marine Amphipods" June 1994 EPA 600/R-94/025

[2] Protocol for Statistical Analysis of Toxicity Data can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

1.5 EMBRYO-LARVAL DEVELOPMENT TEST WITH SAND DOLLARS

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY 116
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method estimates the chronic toxicity of effluent and receiving waters to the embryos and larvae of sand dollars (*Dendraster excentricus*). The test endpoint is normal larval development and may include mortality.

II. **NECESSARY MATERIALS AND SUPPLIES**

- Refractometer – for determining salinity
- Thermometers – glass or electric, laboratory grade for measuring water temperatures
- DO and pH meters – for routine physical and chemical measurements
- Balance – Analytical, capable of accurately weighing to 0.0001g.
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions.
- Volumetric flasks – Class A, borosilicate glass or non-toxic plastic labware, 100-1000ml for making test solutions.
- Plastic holding tanks – 3 to 6L
- Test chambers – 20ml glass scintillation vials and caps – pre-soaked in dilution water.
- Colored labeling tape
- Dilution water – natural seawater or hypersaline brine made from natural seawater and diluted with deionized water
- Biological microscope and slides
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Wash bottles – for reagent water, dilution water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes
- Inverted microscope – for inspecting gametes and counting embryos and larvae.
- Counter, two unit, 0-999 – for recording counts of embryos and larvae
- Beakers, 5-10ml borosilicate glass for collecting sperm from sand dollars
- Beakers, 1,000 ml for rinsing and settling sea urchin eggs.
- Vortex mixer – to mix sea urchin semen in tubes prior to sampling.
- Hemocytometer – for counting sperm
- Siphon hose – for removing water from settled eggs

III. METHODS

A. OBTAINING AND HOLDING ORGANISMS

1. Obtain ripe sand dollars from an uncontaminated subtidal area (i.e. mouth of Mission Bay) on morning of test setup and hold in tanks (e.g. cold room) with raw flowing seawater (target holding/conditioning temperature is 10 – 14 °C) until they are needed for testing. Holding and conditioning tanks should be drained and sprayed with fresh water at least once weekly to prevent accumulation of organic matter and bacteria. Dead animals should be removed daily.

B. SPAWNING AND FERTILIZATION

1. Pour 20-30 ml seawater into 100 ml beakers for females and 25 ml in 25-50 ml beakers for males and place in 15 °C incubator.

2. Carefully place sand dollars in a container lined with moist paper towels.

3. Inject 0.5 ml of 0.5 M KCl into oral cavity of each sand dollar, cleaning needle with hot water between injections if sex of sand dollar is not known to prevent cross contamination. Record injection time on data sheet.

4. Swirl sand dollar for a few seconds then place back on moist paper towels.

5. When gametes begin to shed, note time, and separate sexes. Place males onto 25-50 ml beakers and females onto 100 ml beakers both oral side up. Spray eggs and sperm of sand dollar into beaker with a wash-bottle. It is optimal to obtain gametes from at least 3 spawning individuals of each sex.

6. After confirming good motility of each sperm sample under the microscope, combine equal quantities from up to four males, and store in refrigerator or on ice until use within 4 h.

7. Observe egg quality under the microscope for each spawning female. Pool quality eggs (i.e. normal size, regular shaped and absence of germinal vesicle) into a 100 ml or 250 ml graduated cylinder, bring volume up and cover with parafilm and keep at 15 °C.

8. Confirm fertilization success by placing a drop of eggs onto a well slide with a small amount of sperm. Check for fertilization membrane. If no fertilization membrane present isolate new eggs.

9. To determine the egg density the egg stock will need to be diluted. **Always cut pipette tip so that it is at least 2 mm wide.** First, label two scint vials A and B, then fill each with 9 ml of filtered seawater. Next, add 1ml of the concentrated egg stock to vial A invert gently several times, then add 1ml of vial A to vial B. Count 1 ml from vial B in a Sedgewick-Rafter counting cell. If the count is less than 30, count Vial A. Vial A represents a 1:10 dilution and vial B represents a 1:100 dilution.

10. Using the volume of concentrated egg stock determined by the equation on the Egg/Sperm count page, prepare egg stock in dilution water at the final target concentration of 1000 eggs/ml. Check prepared solution by counting eggs again.

11. Recommended sperm to egg ratio for fertilization is 500:1.tests.

C. SPERM DILUTION

Note: If able to decant overlying water the final sand dollar sperm density is usually between 2x10^9 and 2x10^10 sperm/ml.

See the Protocol for Counting Sperm with a Hemocytometer for instructions on how to count sperm. With experience, the amount of sperm required for successful fertilization can be estimated, avoiding the need for precise cell counts.

D. FERTILIZATION

Add calculated volume of sperm dilution to the egg dilution for a 500 sperm:1egg ratio and mix gently. Wait 10 minutes and check for fertilization. If fertilization is not at least 90%, add a second volume of sperm dilution, wait 10 minutes and re-check. If fertilization is still not 90%, 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.

IV. CONDUCTING THE TEST

A. REFERENCE TOXICANT TESTS

1. Prepare reference toxicant stock and dilutions. Make up a 1 ppm stock using 200 µl of 1 ppt copper solution in 199.8 mL dilution water. Make dilutions according to species sensitivity and add 10 mL of each concentration to scintillation vials. In general, five replicates per treatment are used, plus one additional vial for water chemistry. Cover and place in 15 °C to equilibrate for at least 30 minutes.

2. New, seawater leached scintillation vials containing 10 mL of test solution are pre-cooled to 15 °C. To each vial, inject 0.25 mL fertilized eggs. It is important to be sure that the eggs are homogenized during additions. 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.

3. The embryos should be incubated for 72 hours in the test chambers at 15 °C at ambient light level (16 h light and 8 h dark). If controls have not achieved the pluteus stage after 72 h, the exposure can be extended up to 96 hours.

4. Terminate test by addition of 1mL of concentrated Formaldehyde and record the time.

B. EFFLUENTS, RECEIVING WATERS, AND OTHER SAMPLES

1. Test should begin within 36 hours of sample collection (USEPA 2002).

2. Prepare sample dilutions. Add 10 mL of each concentration to scintillation vials. In general, five replicates per treatment are used, plus one additional vial for water chemistry. Cover and place in 15 °C chamber to equilibrate.

3. Once scintillation vials have reached 15 °C, add embryos using a cut pipette tip. 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.

4. The embryos should be incubated for 72 hours in the test chambers at 15 °C and at ambient laboratory light levels (16 h light and 8 h dark). If controls have not achieved the pluteus stage, the exposure can be extended up to 96 hours.

5. Terminate test by addition of 1ml of concentrated Formaldehyde. Record the time.

6. Additional notes:

   - Additional vials with site samples may need to be collected for water quality and chemistry.
   - Be sure to adequately homogenize sample before addition to vials and before taking water quality measurements.
   - If samples are salted up with hypersaline brine, be sure to incorporate a brine control.

V. DATA COLLECTION

A. Observe embryos within one week of preservation. For each test replicate, the proportion of normal to abnormal larvae will be determined. Please refer to *"Protocol for counting larvae with an inverted microscope*[2]*".*

VI. ANALYZING DATA

A. Using CETIS or Toxcalc, enter data retrieved from counting to determine the EC50, LOEC, NOEC, or other appropriate toxicity metric. Please refer to *"Protocol for Statistical Analysis of Toxicity Data*[3]*".*

B. In accordance with USEPA (2002), all Toxcalc-generated concentration-response curves will be evaluated for acceptability.

[1]Modified from "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.

[2] "Protocol for Counting larvae with an inverted microscope" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

[3] "Protocol for Statistical Analysis of Toxicity Data" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

1.6 EMBRYO-LARVAL DEVELOPMENT TEST WITH PURPLE SEA URCHINS

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method estimates the chronic toxicity of effluent and receiving waters, pore water, and other seawater samples to the embryos and larvae of echinoderms (the sea urchin *Stronglyocentrotus purpuratus*) relative to control or reference samples. The test endpoint is normal larval development and may include mortality.

II. **NECESSARY MATERIALS AND SUPPLIES**

- Refractometer – for determining salinity
- Thermometers – glass or electric, laboratory grade for measuring water temperatures
- D.O. and pH meters – for routine physical and chemical measurements
- Balance – Analytical, capable of accurately weighing to 0.0001g.
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions.
- Volumetric flasks – Class A, borosilicate glass or non-toxic plastic labware, 100-1000ml for making test solutions.
- Plastic holding tanks – 3 to 6L
- Test chambers – 20ml glass scintillation vials and caps – pre-soaked in dilution water.
- Colored labeling tape
- Dilution water – natural seawater or hypersaline brine made from natural seawater and diluted with deionized water
- Biological microscope and slides
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Wash bottles – for reagent water, dilution water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes
- Inverted microscope – for inspecting gametes and counting embryos and larvae.
- Counter, two unit, 0-999 – for recording counts of embryos and larvae
- Beakers, 5-10ml borosilicate glass for collecting sperm from sand dollars
- Beakers, 1,000 ml for rinsing and settling sea urchin eggs.
- Vortex mixer – to mix sea urchin semen in tubes prior to sampling.
- Hemocytometer – for counting sperm
- Siphon hose – for removing water from settled eggs
- Sieves – 80 μm, 20 μm and 25 μm

III. **METHODS**

A. OBTAINING AND HOLDING ORGANISMS

1. Obtain ripe sea urchins from an uncontaminated subtidal area (e.g. mouth of Mission Bay) on morning of test setup and hold in tanks (e.g. cold room) with raw flowing seawater (target holding/conditioning temperature is 12 – 14 °C) until they are needed for testing. Kelp should be added to tanks as a food supply. Holding and conditioning tanks should be drained and sprayed with fresh water at least once weekly to prevent accumulation of organic matter and bacteria. Dead animals should be removed daily.

B. SPAWNING

1. Pour 0.45 μm filtered seawater into 100 mL beakers for females and place in 15 °C incubator. Smaller (e.g. 25-50 mL) beakers can be used for males.

2. Carefully remove urchins from holding tanks to prevent damage to tube-feet, and place in a container lined with moist paper towels to prevent reattachment.

3. Inject 0.5 mL of 0.5 M KCl into soft periostomal membrane of each urchin, rinsing the needle with hot water between injections if sex of urchins is not known to prevent cross contamination. Record injection time on data sheet.

4. Swirl urchin for a few seconds, then place onto the beakers, oral side down.

5. When gametes begin to shed, note time, and separate sexes. Let females shed eggs into seawater-filled beakers oral side down. It is optimal to obtain at least 3 spawning individuals from each sex.

6. Collect sperm from each male in 25-50 mL beakers, with minimal dilution. After confirming good motility of each sperm sample under the microscope, combine equal quantities from three to four males and use within 4 h.

7. Observe egg quality under the microscope for each spawning female. Pool quality eggs (i.e. normal size, regular shaped and absence of germinal vesicles) into a 1 L beaker. Pass eggs through an 80 μm mesh screen (the eggs will pass through and debris is retained on screen).

8. Pass sperm stock through a 25 μm mesh screen (sperm will pass through and debris are retained on screen).

9. Confirm fertilization success by placing a drop of eggs onto a well slide with a small amount of sperm. Check for fertilization membrane. If no fertilization membrane is present, isolate new eggs.

10. To determine the egg density, the egg stock will need to be diluted. **Always cut pipette tip so that it is 2 mm wide to prevent damage to eggs.** First, label two scint vials A and B, then fill each with 9 mL of filtered seawater. Next, add 1 mL of the egg stock to vial A invert

gently several times, then add 1ml of vial A to vial B. Count 1 mL from vial B in a Sedgewick-Rafter counting cell. If the count is less than 30, count Vial A. Vial A represents a 1:10 dilution and vial B represents a 1:100 dilution.

11. Using the volume of concentrated egg stock determined by the equation on the Egg/Sperm count page, dilute to 20-50 eggs / mL for fertilization.

C. FERTILIZATION

1. Add sperm to the diluted egg stock at 15 °C. 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 hemacytometer (see Protocol for Counting Sperm with a Hemocytometer). With experience, precise sperm counts are not necessary (sperm should make diluted egg stock very slightly cloudy). Wait 10-15 minutes and check for complete fertilization. If fertilization is not at least 90%, add a second volume of sperm stock, wait 10 minutes and re-check. If fertilization is still not 90%, test must be restarted with different gametes.

2. After adequate fertilization has been achieved, gently pour embryo stock over a 20 μm mesh screen to remove any excess sperm and debris (embryos will be retained on screen while sperm and debris will pass through). Gently rinse embryos on screen with filtered seawater.

3. Re-concentrate embryo stock solution to desired density (e.g. 2000 embryos / mL).

IV. CONDUCTING THE TEST

7. REFERENCE TOXICANT TESTS

   a. Prepare reference toxicant stock and dilutions. Make up a 1 ppm sub-stock using 200 μL of 1 ppt Copper stock in 199.8 mL dilution water. Make dilutions according to species sensitivity and add 10 mL of each concentration to scintillation vials. In general, five replicates per treatment are used, plus one additional vial for water chemistry. Cover and place in 15 °C to equilibrate for at least 30 minutes.

   b. Scintillation vials containing 10 mL of each test concentration should have been pre-cooled to 15 °C. To each vial, add 100 μL embryos being sure that the 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.

   c. The embryos should be incubated for at least 72 hours in the test chambers at 15 °C at ambient laboratory light levels (16 h light and 8 h dark). If controls have not achieved the pluteus stage by 72 hours, the exposure can be extended up to 96 hours.

   d. Terminate test by addition of 1 mL of concentrated Formaldehyde. Record the time.

8. EFFLUENTS, RECEIVING WATERS, AND OTHER SAMPLES

e. Test should begin within 36 hours of sample collection (USEPA 2002).

f. Prepare sample dilutions. Add 10 mL of each concentration to scintillation vials. In general, five replicates per treatment are used, plus one additional vial for water chemistry. Cover and place in 15 °C to equilibrate.

g. Once scintillation vials have reached 15 °C, add embryos using a cut pipette tip. Be sure that embryo stock is always homogenized during the additions. 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.

h. The embryos should be incubated for 72 hours in the test chambers at 15 °C and at ambient laboratory light levels (16 h light and 8 h dark). If controls have not achieved the pluteus stage, the exposure can be extended up to 96 hours.

i. Terminate test by addition of 1ml of concentrated Formaldehyde. Record the time.

- Additional vials with site samples may need to be collected for water quality and chemistry.
- Be sure to adequately homogenize sample before addition to vials and before taking water quality measurements.
- If samples are salted up with brine addition, be sure to incorporate a brine control.

V. DATA COLLECTION

A. Observe embryos within one week of preservation. For each test replicate, the proportion of normal to abnormal larvae will be determined. Please refer to *"Protocol for counting larvae with an inverted microscope"*.

VI. ANALYZING DATA

A. Using CETIS or Toxcalc, enter data retrieved from counting to determine the EC50, LOEC, NOEC, or other appropriate toxicity metric. Please refer to *"Protocol for Statistical Analysis of Toxicity Data*[3]*"*.

B. In accordance with USEPA (2002), all Toxcalc-generated concentration-response curves will be evaluated for acceptability.

[1] Modified from "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.

[2] "Protocol for Counting larvae with an inverted microscope" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

[3] "Protocol for Statistical Analysis of Toxicity Data" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

1.7 ACUTE TOXICITY TEST WITH JUVENILE MYSID SHRIMP

Testing Facility: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method estimates the acute (96 h) toxicity of effluents and receiving waters to the mysid using three to five day old juveniles, in a 96 h static-renewal exposure[1]. Mysids are exposed to effluent samples via dilution series experiments (typically 5 concentrations plus a control). Receiving water tests are conducted using undiluted receiving water alongside a negative control.

II. **NECESSARY MATERIALS AND SUPPLIES**

- Plastic holding tanks – 3 to 6L
- Reference toxicant solution - 1ppt copper stock solution
- graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions
- pH meter – for measuring test solutions
- Dissolved oxygen meter – for measuring test solutions
- Refractometer – for determining salinity of test solutions
- Thermometer – digital or laboratory grade
- Test chambers – 300ml glass beakers
- Watch glasses – for covering test chambers
- Colored labeling tape
- Dilution water – natural seawater or hypersaline brine made from natural seawater and diluted with deionized water
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Beakers- Class A, borosilicate glass or non-toxic plastic labware, 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*, culture unit
- Separatory funnels, 2L – two for culturing *Artemia*
- Siphon tubes (Tygon tubing) – for test solution renewal
- Light box – for examining organisms
- Parafilm

III. METHODS

A. OBTAINING, HOLDING AND FEEDING ORGANISMS

1. Mysids should be 1-3 days old at time of shipping, so that they will be approximately 3-5 days old at start of 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 h at 20 °C. Please refer to "*Protocol for preparing Artemia nauplii*"[2].

3. Upon receipt of mysids, open plastic bag and determine arrival temperature, pH, D.O. and salinity. Record values on the "Organism Arrival Log" sheet.

4. Provide gentle aeration by placing an airstone 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 filtered (0.45 μm) 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 34 ‰), adjust by no more than 2 ‰ per day.

7. Collect newly hatched *Artemia* nauplii. Pipette nauplii so that each mysid receives about 100 nauplii per day.

8. Each day prior to distribution of mysids into beakers, remove any dead, record physical parameters (temp, pH, salinity, DO), perform a 50% water change with filtered 20 °C seawater of the appropriate salinity, and feed.

B. TEST SETUP

1. Test should begin within 36 hours of sample collection (USEPA 2002).

2. Randomly distribute 10 larvae to each 300 ml glass beaker, using a 5 ml plastic pipette with the lowest 0.5 cm cut off to prevent injury. Be sure beaker has a few ml of filtered seawater to cushion entry. It is generally easiest to track and count mysids with the holding tank and beakers on a light table.

3. After all required beakers have been filled with test solutions, mark them either with numbers from a randomization chart or with the test concentration and replicate (e.g. A, B, or C). If using random numbers, ensure that identification of each number is written down and stored in a safe place for referral after mortality assessment. Test results will be meaningless if you don't know what the exposure was!

4. Feed all replicates with *Artemia*.

C. TEST MAINTENANCE

Day 0 (Hour 0)

1. Measure and record physical parameters (temp., salinity, DO, pH, ammonia) for all samples and concentrations.

2. Make up dilutions. Dilutions for samples lower in salinity than desired are generally "salted up", or adjusted to the testing salinity with synthetic sea salt (Crystal Sea MarineMix, Bioassay Grade). The copper reference dilutions are made from clean, filtered 0.45-µm seawater (i.e. Scripps) and a 5 ppm copper solution prepared in filtered seawater on the day of test setup (from 1ppt master stock solution).

3. Siphon off as much water from beakers as possible without stressing the mysids. Replenish with 200 ml of the appropriate dilution just prepared. Siphon and replenish one beaker before moving on to next one to reduce stress on mysids.

4. Measure and record physical parameters from one replicate from each test solutions or test concentration. If D.O. 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).

5. Be sure test organisms have been fed.

DAY 1 (Hour 24)

1. Measure and record physical parameters.
2. Note and remove any mortalities.
3. Feed mysids with *Artemia* nauplii.

DAY 2 (Hour 48)

1. Measure and record physical parameters.
2. Note and remove any mortalities.
3. Prepare fresh dilutions as on Day 0 using same effluent sample.

4. Siphon off all but approx. 10% of sample, and replenish beakers with appropriate dilution that was just prepared.
5. Feed mysids with Artemia nauplii.

DAY 3 (Hour 72)

1. Measure and record physical parameters.
2. Note and remove any mortalities.
3. Feed mysids with *Artemia* nauplii.

DAY 4 (Hour 96)

1. Measure and record physical parameters.
2. Make final mortality observations and record.
3. Terminate tests by pouring contents of beakers through a sieve into sink. Surviving mysids should be sacrificed by freezing or other humane methods.

IV. ANALYZING DATA

Using CETIS or Toxcalc, enter mortality data obtained from the test at 48- and/or 96-hour exposure periods to determine the LC50, LOEC, NOEC, or other relevant toxicity metrics. Please refer to "*Protocol for Statistical Analysis of Toxicity Data*"[3].

In accordance with USEPA (2002), all Toxcalc-generated concentration-response curves will be evaluated for acceptability.

[1] Modified from "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.

[2] "Protocol for preparing Artemia nauplii" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

[3] "Protocol for Statistical Analysis of Toxicity Data" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

1.8 ACUTE TOXICITY TEST WITH TOPSMELT LARVAE

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method estimates the acute (96 h) toxicity of effluents and receiving waters to topsmelt (*Atherinops affinis*) larvae static- renewal exposure. Topsmelt are exposed to effluent samples via dilution series experiments (typically 5 concentrations plus a control). Receiving water tests are typically conducted using full strength (100%) sample, and are compared with control performance.

II. **NECESSARY MATERIALS AND SUPPLIES**

- Plastic holding tanks – 3 to 6L
- Reference toxicant solution - 1ppt copper stock solution
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions
- pH meter – for measuring test solutions
- Dissolved oxygen meter – for measuring test solutions
- refractometer – for determining salinity of test solutions
- Thermometer – digital or laboratory grade
- Test chambers – 400ml glass beakers
- Watch glasses – for covering test chambers
- Colored labeling tape
- Dilution water – natural seawater or hypersaline brine made from natural seawater and diluted with deionized water
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Beakers- Class A, borosilicate glass or non-toxic plastic labware, 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*, culture unit
- Separatory funnels, 2L – two for culturing *Artemia*
- Siphon tubes – for test solution renewal
- Light box – for examining organisms
- Parafilm

III. **METHODS**

A. OBTAINING HOLDING AND FEEDING ORGANISMS

1. Fish should be approximately 7-10 days old at time of shipping, so that they will fall within the 9-15 day requirement for conducting the test.

2. Prepare *Artemia* culture so that there are freshly hatched nauplii available when the fish arrive. Hatching takes 24-36 h at 20 °C. Please refer to "*Protocol for preparing Artemia nauplii*"[2].

3. Upon receipt of fish, open plastic bag and determine arriving temperature, pH, salinity, and D.O.

4. Provide gentle aeration by inserting an airstone into the bag.

5. Transfer fish to one or two large plastic holding tanks (6 L) in a 20 °C temperature controlled room, incubator, or water bath. One easy way to transfer from the bag is to place the open plastic bag in plastic holding tank and cut open bottom of bag with a razor blade. Gently pull up on bag, releasing fish into the container. A squirt bottle filled with filtered seawater can be used to retrieve any fish adhered to the bag. Remove dead by siphoning out of tank with a small rubber hose.

6. Perform a ~50% water change with filtered (0.45 μm) seawater adjusted to 20 °C. Be sure seawater is within 2 ‰ and 2 °C of the arriving conditions. If the salinity is below desired, adjust by no more than 2 ‰ per day.

7. Collect newly hatched *Artemia* nauplii. Pipette nauplii so that each fish larva receives about 40 nauplii at each feeding. Feed two times a day.

8. Each day prior to distribution of fish into beakers, remove any dead, record physical parameters (temp, pH, salinity, DO), perform ~50% water change with filtered 20 °C seawater, and feed.

B. TEST SETUP

1. Test should begin within 36 hours of sample collection (USEPA 2002).

2. Randomly distribute 5 larvae to each 400 ml glass beaker using a 5 ml plastic pipette with the lower 0.5 cm cut off to prevent injury. Be sure beaker has a few ml of filtered seawater to cushion entry. Fill to 200 ml marking on beaker with test solution. It is generally easiest to track and count fish with holding tank and beakers on a light table (there is one in Rm. 116).

3. After all required beakers have been filled, mark them either with numbers from a randomization chart or with the test concentration and replicate (i.e. A, B, C, or D). If using random numbers, ensure that identification of each number is written down and stored in a safe place for referral after mortality assessment. Test results will be meaningless if you don't know what the exposure was!

4. Distribute *Artemia* to all replicates.

C. TEST MAINTENANCE

***DAY 0* (Hour 0)**

1. Measure and record physical parameters (temp., salinity, DO, pH, ammonia) for all samples and test concentrations.

2. Make up dilutions. 800 mL of each test concentration will be required to fill four replicates. The copper reference dilutions are made from clean, filtered 0.45-µm seawater (i.e. Scripps) and a 5 ppm copper stock solution prepared in filtered seawater on the day of test setup (from 1ppt stock solution).

3. Siphon off as much water from beakers as possible without stressing fish. Replenish with 200 ml of the appropriate dilution just prepared. Siphon and replenish one beaker before moving on to next one to reduce stress on fish.

4. Measure and record physical parameters for one replicate from each test concentration or test sample. If D.O. 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).

5. Be sure test organisms are fed two times a day.

***DAY 1* (Hour 24)**

1. Measure and record physical parameters.
2. Note and remove any mortalities.
3. Feed two times as usual (morning and evening).

***DAY 2* (Hour 48)**

1. Measure and record physical parameters.
2. Note and remove any mortalities.
3. Prepare fresh dilutions as on Day 0 using same effluent sample.
4. Siphon off all but approx. 25 ml of sample, and replenish beakers with appropriate dilution that was just prepared.
5. Feed two times as usual (morning and evening).

DAY 3 (Hour 72)

1. Measure and record physical parameters.
2. Note and remove any mortalities.
3. Feed two times as usual (morning and evening).

DAY 4 (Hour 96)

1. Measure and record physical parameters.
2. Make final mortality observations and record.
3. Terminate tests by pouring contents of beakers through a sieve into sink. Surviving fish should be sacrificed by freezing or other humane methods.

IV. ANALYZING DATA

Using CETIS or Toxcalc, enter mortality data obtained from the test at 48- and/or 96-hour exposure periods to determine the LC50, LOEC, NOEC, or other relevant toxicity metrics. Please refer to "*Protocol for Statistical Analysis of Toxicity Data*"[3].

In accordance with USEPA (2002), all Toxcalc-generated concentration-response curves will be evaluated for acceptability.

[1] Modified from "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.

[2] "Protocol for preparing Artemia nauplii" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and logs

[3] "Protocol for Statistical Analysis of Toxicity Data" can be found in the sub-directory : C:\WINDOWS\Desktop\Laboratory 116\Protocols and log

2.0 TEST CONDITIONS AND ACCEPTABILITY CRITERIA

2.1 BIVALVE EMBRYO-LARVAL DEVELOPMENT TEST (CHRONIC)

Oyster (*Crassostrea gigas*) or Mussel (*Mytilus galloprovincialis*)

Test Type	static-nonrenewal
Salinity	30 ± 2 ppt
Temperature	20 ± 1 °C (oysters) and 15 or 18 ± 1 °C (mussels)
Light quality	ambient laboratory illumination
Light intensity	10-20 µE/m^2/s (Ambient laboratory levels)
Photoperiod	16 h light/ 8 h darkness
Test Chamber type/size	20 ml
Test solution volume	10 ml
No. Larvae/test chamber	150-300
No. of replicate chambers/concentration	4 or 5
Dilution water	Uncontaminated 1µm filtered natural seawater or hypersaline brine prepared from natural seawater
Test Concentrations	Effluent: Minimum of 5 and a control; 0, 6.25,12.5, 25, 50, 100% Copper Ref. Tox.; 0, 4.1, 8.4, 12, 17.2, 24, 35 ppb Receiving waters: 100% receiving water and a control.
Dilution factor	Effluents: ≥ 0.5 Receiving waters: ≥ 0.5
Test Duration	48h
Test acceptability criteria	≥ 70% survival in controls (oysters), and ≥ 50% survival in (Mussels); ≥ 90% normal development of shell with surviving controls. MSD of <25%.
Endpoint measured	Survival and normal shell development

Criteria from EPA's Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters To West Coast Marine and Estuarine Organisms. EPA/600/R-95/136. August 1995

2.2 SEDIMENT-WATER INTERFACE (SWI) TOXICITY TEST WITH BIVALVE EMBRYOS

For mussel (*Mytilus galloprovincialis*)

Test Type	static-nonrenewal
Salinity	30 ± 2 ppt
Temperature	15 or 18 ± 1 °C
Light quality	ambient laboratory illumination
Light intensity	10-20 µE/m^2/s (Ambient laboratory levels)
Photoperiod	16 h light/ 8 h darkness
Test Chamber type/size	Polycarbonate tubing with polyethylene mesh
Test solution volume	300-500 ml
No. Larvae/test chamber	150-300
No. of replicate chambers/concentration	4 or 5
Dilution water	Uncontaminated 1µm filtered natural seawater or hypersaline brine prepared from natural seawater
Test Concentrations	Copper Ref. Tox.; 0, 4.1, 5.9, 8.4, 12, 17.2, 24, 35 ppb Receiving waters: 100% receiving water and a control.
Dilution factor	Receiving waters: None or ≥ 0.5
Test Duration	48h
Test acceptability criteria	≥ 70% normal survival
Endpoint measured	Survival and normal shell development

Criteria from EPA's Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters To West Coast Marine and Estuarine Organisms. EPA/600/R-95/136. August 1995

2.3 MARINE AMPHIPOD REFERENCE TOXICITY TEST

For *Eohaustorius estuarius* or *Rhepoxynius abronius*

Test Type	Water-only test
Salinity	20 ppt (*E. estuarius*); 30 ppt (*R. abronius*), ± 1 ppt
Temperature	15 ± 1 °C
Light quality	Chambers should be kept in dark or covered with opaque material
Photoperiod	24 hours dark : 0 hours light
Test Chamber type/size	1 L glass beaker or jar with ~10 cm I.D.
Test solution volume	750 mL (minimum)
No. of organisms/chamber	10 (minimum) / chamber
No. of replicate chambers/concentration	1 minimum : 2 recommended
Dilution water	Uncontaminated sand filtered natural seawater or hypersaline brine prepared from natural seawater
Test Concentrations	Ammonia: 0, 37.5, 75, 150, 300, 600 mg/L for *E. estuarius* Ammonia: 0, 18.75, 37.5, 75, 15, 300 mg/L for *R. abronius* Cadmium: 0, 1.5, 3, 6, 12 mg/L for *E. estuarius* Cadmium: 0, 0.125, 0.25, 0.5, 1, 2 mg/L for *R. abronius* Control and at least 5 test concentrations (0.5 dilution factor)
Aeration	Recommended; but not necessary if >90% D.O. saturation can be achieved without aeration
Test Duration	96 hours
Test acceptability criteria	minimum mean control survival ≥ 90% Survival
Endpoint measured	Survival

Criteria from EPA's Methods for Assessing the Toxicity of Sediment-associated Contaminants with Estuarine and Marine Amphipods. (EPA/600/R-94/025 June 1994)

2.4 SEDIMENT TOXICITY TEST WITH MARINE AMPHIPODS (ACUTE)

For *Eohaustorius estuarius* or *Rhepoxynius abronius*

Test Type	whole sediment toxicity test - static non-renewal
Salinity	20 ppt (*E. estuarius*) ± 1 ppt; 30 ppt (*R. abronius*) ± 1 ppt
Temperature	15 ± 1 °C
Light quality	wide-spectrum fluorescent lights
Light intensity	50-1000 lux
Photoperiod	24 hours light : 0 hours dark
Test Chamber type/size	1 L glass beaker or jar with ~10 cm I.D.
Test solution volume	2cm sediment : 750 mL overlying water
No. of organisms/chamber	20 / chamber
No. of replicate chambers/concentration	at least 4, with at least one additional for chemistry
Dilution water	Uncontaminated sand filtered natural seawater or hypersaline brine prepared from natural seawater
Aeration	Recommended; but not necessary if >90% D.O. saturation can be achieved without aeration
Test Duration	10 day
Test acceptability criteria	minimum mean control survival ≥ 90% survival
Endpoint measured	Survival

Criteria from EPA's Methods for Assessing the Toxicity of Sediment-associated Contaminants with Estuarine and Marine Amphipods. (EPA/600/R-94/025 June 1994)

2.5 BIOLUMINESCENCE INHIBITION TEST (QWIKLITE) WITH DINOFLAGELLATES

For *Ceratocorys horrida*

Test Type	static, non-renewal
Salinity	34 ± 2 ppt
Temperature	19 ± 1 °C
Light quality	ambient laboratory illumination
Light intensity	10-20 $\mu E/m^2/s$ (Ambient laboratory levels)
Photoperiod	12 h light/ 12 h darkness
Test Chamber type/size	4.5 ml cuvettes
Test solution volume	3 ml / replicate
Age of test organism	12-20 days
	50-100 cells/ml
No. of replicate chambers/concentration	at least 4
Dilution water	Uncontaminated 1µm filtered natural seawater or hypersaline brine prepared from natural seawater
Test Concentrations	Effluent: Minimum of 5 and a control; 0, 6.25,12.5, 25, 50, 100% Copper Ref. Tox.; 0, 15.6, 31.3, 62.5, 125, 250 ppb Receiving waters: 100% receiving water and a control.
Dilution factor	Effluents: ≥ 0.5 Receiving waters: None or ≥ 0.5
Test Duration	24 hours
Test acceptability criteria	at least 10^6 PMT counts in controls
Endpoint measured	bioluminescence inhibition

Modified from ASTM Standard Guide for Conducting Toxicity Tests With Bioluminescent Dinoflagellates. Designation E 1924 - 97.

2.6 ECHINODERM EMBRYO-LARVAL DEVELOPMENT TEST (CHRONIC)

For *Strongylocentrotus purpuratus* or *Dendraster excentricus*

Test Type	static non-renewal
Salinity	34 ppt ± 2 ppt
Temperature	15 ± 1 °C
Light quality	ambient laboratory illumination
Light intensity	10-20 μE/m^2/s (Ambient laboratory levels)
Photoperiod	16 h light: 8 h darkness
Test Chamber type/size	20 ml Scintillation vials
Test solution volume	10 ml
No. of replicate chambers/concentration	at least 4
Dilution water	Uncontaminated 1μm filtered natural seawater or hypersaline brine prepared from natural seawater
Test Concentrations	Effluent: Minimum of 5 and a control; 0, 6.25,12.5, 25, 50, 100% Copper Ref. Tox.; 0, 4.1, 8.4, 12, 17.2, 24, 35 ppb Receiving waters: 100% receiving water and a control.
Dilution factor	Effluents: ≥ 0.5 Receiving waters: 100% and a control
Test Duration	72-96 h
Test acceptability criteria	at least 80% Normal development in the controls (USEPA 1995); MSD <25%
Endpoint measured	Normal development; mortality can be included

2.7 MYSID SHRIMP SURVIVAL TEST (ACUTE)

For *Americamysis bahia*

Test Type	static-renewal
Salinity	5-34 (± 2 ppt)
Temperature	20 ± 1 °C
Light quality	ambient laboratory illumination
Light intensity	10-20 μE/m^2/s (Ambient laboratory levels)
Photoperiod	16 h light/ 8 h darkness
Test Chamber type/size	300 ml
Test solution volume	200 ml/replicate
Renewal of test solutions	48 hour minimum
Age of test organism	1-5 days; 24-h range in age
No. Larvae/test chamber	10
No. of replicate chambers/concentration	minimum, 2 for effluent tests. minimum, 4 for receiving water tests
Source of food	Newly hatched *Artemia* nauplii (less than 24 h old)
Feeding regime	Feed 40 nauplii per larvae twice daily, morning and night
Cleaning	cleaning not required
Aeration	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers.
Dilution water	Uncontaminated 1μm filtered natural seawater or hypersaline brine prepared from natural seawater
Test Concentrations	Effluent: Minimum of 5 and a control; 0, 6.25,12.5,25,50,100% Copper Ref. Tox.; 0, 94, 127, 169, 225, 300, 350 ppb Receiving waters: 100% receiving water and a control.
Dilution factor	Effluents: ≥ 0.5 Receiving waters: None or ≥ 0.5
Test Duration	Acute: 96 h; Chronic: 7 d
Test acceptability criteria	90% or greater survival in controls
Sample volume required	2L per renewal
Endpoint measured	Effluents: Survival (e.g. LC50) Receiving waters: Survival (Significant Difference from control)

2.8 TOPSMELT LARVAL SURVIVAL TEST (ACUTE)

For *Atherinops affinis*

Test Type	static-renewal
Salinity	15 - 34 ppt (± 2 ppt of the selected test salinity)
Temperature	21 ± 1 °C
Light quality	ambient laboratory illumination
Light intensity	10-20 μE/m^2/s (Ambient laboratory levels)
Photoperiod	16 h light / 8 h darkness
Test Chamber type/size	400 ml
Test solution volume	200 ml / replicate
Renewal of test solutions	48 hour minimum
Age of test organism	9-15 days post-hatch
No. Larvae/test chamber	10
No. of replicate chambers/concentration	minimum, 2 for effluent tests. minimum, 4 for receiving water tests
Source of food	Newly hatched *Artemia* nauplii
Feeding regime	Feed 40 nauplii per larvae twice daily, morning and night
Cleaning	cleaning not required
Aeration	None, unless D.O. concentration falls below 4.0 mg/L, then aerate chambers. Rate should be less than 100 bubbles/min.
Dilution water	Uncontaminated 1μm filtered natural seawater or hypersaline brine prepared from natural seawater
Test Concentrations	Effluent: Minimum of 5 and a control; 0, 6.25,12.5,25,50,100% Copper Ref. Tox.; 0, 56, 100, 180, 320 ppb Receiving waters: 100% receiving water and a control.
Dilution factor	Effluents: ≥ 0.5 Receiving waters: None or ≥ 0.5
Test Duration	Acute: 96 h, Chronic: 7d
Test acceptability criteria	90% or > survival in controls
Sample volume required	2L per day
Endpoint measured	Effluents: Survival (LC50) Receiving waters: Survival (Significant Difference from control)

3.0 PROCEDURES FOR EQUIPMENT

3.1 PROTOCOL FOR AUTOCLAVE

- The autoclave is a device used for sterilizing objects by exposing them to steam at above atmospheric pressure (and thus at a temperature above the normal boiling point of water)[1].

- The Autoclave is found in the Rm 127, down the hall from the Bioassay Lab.

- Turn water valve on (orange valve located on the wall to the left of the autoclave). On position is vertical, while the off position is horizontal.

1. Switch main power on by pulling the lever down (lever located on the wall to the right and behind the autoclave).
2. Place glassware and stoppers in metal tray inside autoclave.
3. Close autoclave door and turn handle to the right until tightened (locking mechanism will move into place).
4. Set Sterilizing dial to 20 minutes (Located on right hand side of autoclave).
5. Set Exhaust dial to 10 minutes (Located below sterilizing dial).
6. Turn red on/off switch to regular (switch located on autoclave below door).
7. Turn power switch to the "on" position.
8. Buzzer will indicate when cycle is finished*.
9. Check that chamber pressure gauges are at zero before opening autoclave door.
10. Open autoclave door slowly to release steam.
11. Glassware may be HOT!! Use protective gloves when removing glassware.
12. Leave autoclave door slightly ajar.
13. Turn all switches back to the off positions.
14. Turn light off.

* As of July 2004, the sterilization timer is broken. Please take note of sterilization time in order to manually switch off.

[1] http://www.webster-dictionary.net/definition/Autoclave

3.2 CALIBRATION AND USE OF THE ORION 720A ISE METER/ AMMONIA PROBE

I. STORAGE OF AMMONIA PROBE

A. **Between measurements**, keep tip immersed in a 10^{-3} or 10 ppm standard with ISA added. For low-level measurements, keep tip in pH 4 buffer between measurements.

B. For **overnight or week-long storage**, place electrode tip in a 0.1 M or 1000 ppm standard w/o ISA.

C. **For storage over a week**, disassemble completely and rinse the inner body, outer body, and bottom cap with D.I. water. Dry and reassemble electrode without filling solution or membrane.

II. PROBE CALIBRATION

A. Calibration must be performed every time meter is used.

B. Plug in meter.

C. Look on back of meter to see which input the electrode is plugged in to. Make sure this is the number on the prompt line. If not, press the **2nd** key, and then **channel** repeatedly until selected input is displayed.

D. Using the 0.1M NH_4^+ standard, prepare concentrations that bracket the expected sample range and differ in concentration by a factor of ten.

Example: if sample concentration is estimated to range from 0-50 ppm, make up 3 calibration solutions such as 0.1 ppm, 10 ppm, 100 ppm. These bracket the range and differ by a factor of ten from each other. Since the 0.1 M NH_4^+ standard is equivalent to 1700 ppm, use $c_1v_1=c_2v_2$ to solve for dilutions;

For the **0.1ppm** solution: (1700 ppm)(v_1)=(0.1 ppm)(1000 mL)
(v_1)= .058 mL or 58 µL of 1700 ppm in 999.94 mL of deionized water

For the **10 ppm** solution: (1700 ppm)(v_1)=(10 ppm)(100 mL)
(v_1)= 0.588 mL or 588 µL of 1700 ppm in 99.41 mL of deionized water

E. Measure 25 mL of the more dilute standard into a 30 mL beaker.

F. Add 0.5 mL ISA and stir thoroughly.

G. Select concentration mode by pressing **mode** until **"CON"** is displayed. Then press **1st – Calibration**, when asked enter number of standards to be measured and press **2 or 3** depending on how many standards are being used**,** then **yes.**

H. Rinse electrode with deionized water then blot dry.

I. Place electrode in beaker and stir moderately.

J. When "READY ENTER VALUE" is displayed on prompt line, enter value of standard and press yes.
K. Example: For the 0.1 ppm dilution, type 0.100 on keypad and press yes.

L. The meter automatically switches to standard 2. Rinse electrode and add 0.5 mL of ISA to the second standard.

M. Place electrode in beaker and stir moderately.

N. When "READY ENTER VALUE" is displayed, once again enter value of 2nd standard and press yes.

O. Repeat steps 8-10 for the 3rd standard.

P. The electrode slope is then calculated and displayed. Slope should be within the range of –54 to –60.

III. MEASUREMENTS

A. After calibration meter will automatically proceed to "MEASURE" mode.

B. Place electrode into sample, when **"RDY"** is displayed and meter beeps, record sample results.

IV. NH_4^+ PROBE TROUBLESHOOTING

1. Membrane life can be anywhere from one week to several months. If there are any dark spots or discoloration on the membrane it needs to be changed. Follow instructions on page 4 of the **Orion Ammonia electrode instruction manual**.

2. Obtaining the slope (slope= Δin mV/ tenfold Δ in concentration) provides the best means for checking electrode operation. (**page 6 of electrode instruction manual)**

    1. Place 100 mL of DI water in a 150 mL beaker.
    2. Add 2 mL of ISA and stir.
    3. Set the function switch to mV mode.
    4. Rinse electrode with deionized water and place in solution.
    5. Pipet 1mL of 0.1 M NH4 into beaker and record mV's when reading is stable.
    6. Next, pipet 10 mL of 0.1 M NH4 solution into **the same** beaker. Stir thoroughly and measure mV reading when stable.
    7. The difference between the first and second reading should be between **–54 to –60 mV/decade**.

3. If electrode slope is low during operation, check electrode inner glass body. **(page 24 of electrode instruction manual)**

    1. This is done by first soaking the inner glass body in filling solution for at least two hours, if it has been dry.

2. Next, rinse inner-body with deionized water and immerse in 200 mL of pH 7 buffer w/ 0.1 M NaCl added. Assure that reference element is covered, stir and record stable mV reading.
3. Rinse in DI water and immerse in 200 mL of pH 4.0 buffer with 0.1M NaCl added.
4. Watch the change in meter readings carefully. The reading should change 100 mV in less than 30 seconds after immersion.
5. After 3-4 minutes the reading should stabilize, the difference between the pH 7 and pH 4 should be greater than 150 mV.

V. METER TROUBLESHOOTING

A. The **set up and self-test** should be performed on the meter. Easy to follow instructions are on page 6 of the **Orion manual for the 720A meter.**

B. Next, run the **checkout** procedure on page 10 of the same book mentioned in the previous step. If you receive any error codes, go to the back of the manual for further troubleshooting tips.

3.3 CALIBRATION AND USE OF THE ACCUMET PH METER

Calibration should be performed at least once daily and about every hour (once measurements begin) for more accurate results since the electrode's slope and zero potential may change over time.

Note: Meter should be on Channel A for pH readings. It should be on Channel A already, but if it is not, press "Channel" until it reads "A" only.

I. PROBE CALIBRATION

A. Press "Standardize" and select "2" to clear existing standards.

B. Press "Standardize" again and select "1" to add the first standard.

C. Obtain fresh yellow pH 7.0 buffer solution and pour about 15 mL into 20 mL scintillation vial and add a small magnetic stir bar.

D. When the *Buffer Value* screen appears type in "7.0" and press Enter.

E. The *Prepare Buffer/Standard* screen will appear.

F. Remove electrode from pH 4.0 or 7.0 storing solution, rinse with deionized water, and blot dry.

G. Place pH electrode inside 7.0 buffer solution with magnetic stir plate on low.

H. Press Enter.

I. When accurate reading of buffer value is displayed, press the enter key to manually accept the reading.

J. Repeat these steps for the second standard (pH 10.0).

K. When accurate reading of buffer value is displayed, press the enter key to manually accept the reading.

L. Press "Slope/Efficiency" button and ensure efficiency is 100 ± 2%.

M. If efficiency is poor, recalibrate with fresh standards.

II. MEASUREMENTS

A. Rinse electrode with deionized water.

B. Blot dry electrode with a KimWipe.

C. Immerse electrode in sample, stirring gently (i.e. with magnetic stirrer).

D. Wait for pH reading to stabilize, and record value. The electrode also provides the temperature, if needed.

E. Rinse electrode with deionized water between samples.

F. Store rinsed electrode in pH 4.0 or 7.0 storage solution when finished.

3.4 CALIBRATION AND USE OF THE ORION (MODEL 840) DISSOLVED OXYGEN PROBE

Calibration should be performed at least once daily and about every hour (once measurements begin) for more accurate results.

I. BEFORE USE

A. Be sure sponge in calibration sleeve is saturated with distilled or deionized water.

B. If probe has been disconnected from instrument or silver anode has been cleaned, it must be reconnected and allowed to polarize for 20 to 50 minutes before use.

II. CALIBRATION

A. Turn meter on by depressing "On/Off" button.

B. Depress and hold down "Mode" button until display cursor is at "Cal". As long as the Mode key is depressed, the display will cycle. *Be sure calibration sleeve is completely covering probe and the probe is lying flat on lab counter during calibration steps.

C. Depress quickly and release the Mode key. The word "SAL" will appear on display and then the salinity will appear. Adjust as necessary with up and down arrows.

D. After correct salinity is entered, quickly depress the mode key again and three dashes (---) should appear on the display.

E. After a few moments, the slope of the electrode/membrane will be displayed. It should read between 0.7 and 1.2. If it does not, see Troubleshooting below.

III. SAMPLE MEASUREMENTS

A. Remove calibration sleeve. You are now ready to make D.O. measurements.

B. Depress Mode key to choose mg/L or %.

C. Immerse probe in sample, making sure the stainless steel thermistor is submerged.

D. Stir slowly so that flow rate past the membrane is approximately 15 cm/sec.

E. Take reading when the value on the display is stable. Also record temperature from display window next to D.O. value.

F. Rinse in deionized water and return to calibration sleeve when finished.

IV. TROUBLESHOOTING

A. Slope out of range or an error message "E1" indicates electrolyte may need replacement, electrode cap is old, or electrodes need cleaning.

B. To replace electrolyte and cap, first disconnect probe from instrument.

C. Unscrew and discard old membrane cap.

D. Rinse electrode assembly with distilled water.

E. Moisten inside of new membrane with a few drops of electrolyte from the Probe Service Kit that should be on the counter adjacent to the meter.

F. Completely fill membrane cap with electrolyte.

G. Holding probe at a slant, with the flat surfaced vent on top, insert electrode assembly vertically into the new membrane cap and tighten cap quickly. Excess electrolyte will be expelled through vent. If air bubbles are in the membrane cap, repeat procedure.

H. Plug probe back into instrument and allow approximately 20 minutes for repolarization.

I. Calibrate as before. If the slope still does not fall within range still, the electrode may need cleaning. Refer to "Cleaning the Electrode" section of Orion 840 Instruction Manual.

3.5 MEASUREING AMMONIA WITH THE HACH DR/2400 SPECTROPHOTOMETER

I. GETTING STARTED

A. Turn power on with the blue power on/off key on the far left side of the spectrophotometer.

B. The main menu will appear, if screen is hard to read turn on the backlight with the button that has a light bulb symbol on it.

C. To select an operator, go to **Instrument Setup** and press **Operator ID**. Either select your initials or enter new.

D. At the main menu select either **Hach Programs** or **Favorite Programs**, which contains frequently used programs.

II. MEASURING NITROGEN AS AMMONIA (SALICYLATE METHOD, 385N OR 8155)

A. Select program **385N** and press **Start**.

B. Fill a round 10 mL sample cell to the 10 mL mark with deionized water (this is the blank).

C. Fill another round 10 ml sample cell to the 10 mL mark with sample.

D. Add the contents of one **Ammonia Salicylate** powder pillow to each cell. Stopper and shake to dissolve the powder.

E. Touch the **timer icon**. Touch **OK** to start a 3 minute reaction period.

F. When the timer beeps, add one **Ammonia Cyanurate** reagent powder pillow to each cell. Stopper and shake to dissolve reagent.

G. Touch the **timer icon**. Touch **OK** to start a 15 minute reaction period. A green color will develop if ammonia nitrogen is present.

H. When the timer beeps, wipe blank with a Kimwipe to remove fingerprints and place in cell holder.

I. Touch **Zero**, display will show 0.00 mg/L.

J. Wipe the sample with a Kimwipe and place into holder.

K. Touch **Read**. Results will appear in mg/L – NH_3-N (unionized). To read in NH_3 or NH_4^+ go to **Options**. Select **Chemical Form** and select desired form.

L. When finished touch **Return** and sample value will be converted.

III. PREPARING AN AMMONIA NITROGEN STANDARD

A. Prepare a 0.20 mg/L ammonia nitrogen standard solution.

B. Dilute 2.00 Ml Ammonia Nitrogen Standard Solution (10 mg/L) to 100 mL with deionized water.

C. To adjust the calibration curve using the reading obtained with the 0.20 mg/L standard solution, touch **Options** on the program menu.

D. Touch **Standard Adjust**. Touch **On**. Touch **Adjust** to accept the displayed concentration.

3.6 BARNSTEAD E PURE WATER PURIFICATION SYSTEM

The Barnstead E-Pure water purification system is used to produce deionized (reagent) water with a resistivity of as high as 18.2 megohms/cm and TOC content of less than 10 ppb. The unit is located on the wall near the fume hood in Rm 244. The following steps should be taken to obtain E-Pure water:

A. Open orange-colored water valve every morning.

B. Turn pump on and monitor water resistivity.

C. When water has reached desired resistivity, open draw off valve to get water. Close draw off valve.

D. Leave pump on.

E. At end of day, turn pump off & close water valve.

F. Cartridges are replaced on an as needed basis by Ignacio Rivera.

3.7 CALIBRATION AND USE OF THE ORION APLUS (105A+) BASIC CONDUCTIVITY METER

Choose standards that bracket expected sample values. Brackish water and seawater range from 1-100 milli-Siemens. The meter will shut off automatically after 20 minutes of non-use. To turn this feature off, depress the mode button while turning the meter on. A low-pitched beep should indicate that auto shutoff has been disabled. This feature will be reactivated when the meter is shut off and turned on again.

III. PROBE CALIBRATION

A. To turn on the meter, press the "on" button.

B. Disable the temperature compensation by depressing the "setup" button. Change the number located at the bottom of the screen to 0.0 by pressing the "down arrow (▼)" button.

C. Press the "mode" button to return to measurement screen. Press "cal" button to initiate calibration. The last cell constant used will appear on display.

D. Immerse conductivity cell in the standard. Agitate solution slightly to remove air bubbles from probe.

E. Enter the cell constant printed on the cell cable (1.00), the decimal point can be moved by pressing the up or down arrows. Press "yes" to accept cell constant value.

F. Meter will return to measurement mode, compare the displayed value with the standard at its specified temperature value (see tables included with manual).

G. If the correct standard value is not displayed, calculate the cell constant adjustment factor using the following formula:

Q = Standard value / Displayed Value

H. Multiply the initial cell constant (1.00) by Q. This is the new cell constant.

I. Repeat steps C-G.

J. If Displayed Value is still different from the Standard Value, calculate Q again (step G) and multiply the derived Q by the previous cell constant.

K. Repeat steps C-G until the Standard Value and Displayed Value are the same.

Example:

Initial Cell Constant = 1.00
1st Standard Value = 9.288 (@ 21.4 ºC)
1st Displayed Value = 9.01
Q = 9.288/9.01 = 1.0308 = new cell constant

2nd Standard Value = 9.233 (@ 21.2 ºC)
2nd Displayed Value = 9.29
Q = 9.233/9.29 = 0.9938
Multiply 1.0308 (last cell constant) X 0.9938 (newest cell constant) = 1.0244
3rd Standard Value = 9.215 (@ 21.1 ºC)
3rd Displayed Value = 9.21
Because the Standard and Displayed Values are the same, the instrument is calibrated.

IV. MEASUREMENTS

A. Rinse conductivity cell with deionized water.

B. Blot cell with a Kimwipe.

C. Immerse cell in sample and agitate gently to remove air bubbles.

D. Press the "mode" button to move between conductivity and salinity.

E. Allow reading to stabilize.

F. For storage overnight or longer, conductivity cell should be clean and dry. While in use, the cell can remain in deionized or seawater.

V. TROUBLESHOOTING

A. Check battery, 9V battery required and calibrate after battery change.

B. Run a self-test.

1. Disconnect the conductivity cell (probe).

2. Press and hold the "yes" button while pressing the "on/off" button to turn meter on.

3. This will cause the meter to perform an electronic hardware diagnostics test.

4. After test "7", a "0" will appear on display.

5. Press each key on meter, each key must be pressed within four seconds of the previous key.

6. After test "7", the meter's display will read "test 8" and turn off.

7. An operator assistance code will be displayed if any errors are found.

8. See troubleshooting guide in manual for further instruction.

3.8 CALIBRATION AND USE OF THE ORION (MODEL 830A) PORTABLE DISSOLVED OXYGEN PROBE

Calibration should be performed at least once daily.

I. BEFORE USE

A. Be sure sponge in calibration sleeve is saturated with deionized water.

B. If the meter has been off for longer than 72 hours, it will need to re-polarize for 60 minutes.

C. Probe storage: for short-term storage (overnight or between measurements), probe should remain plugged into meter and kept in the moist sleeve. For long-term storage, probe should be disconnected from meter, membrane cap should be removed, and probe should be stored cleaned and dry.

II. CALIBRATION

A. Turn meter on by pressing the “power” button.

B. To change salinity, simultaneously depress the “cal” and “power” button. Pressing the “cal” button allows you to scroll through configuration options. After changing salinity to the value of your sample using the up and down arrows, press “meas” key to return to measure mode.

C. Press the “cal” button to enter the calibration mode. Press the “cal” button a second time to begin calibration. Be sure calibration sleeve is completely covering probe and the probe is lying flat on the lab counter during calibration steps.

D. A range value will appear. If the slope is out of the required range of 60-120%, “error” is displayed.

E. To abort calibration, press the “meas” button at any time.

F. The display screen features a “Stat face” that resembles a smiley face. It provides information on the electrode condition. If a sad face appears, calibration may be needed.

III. SAMPLE MEASUREMENTS

A. Remove calibration sleeve.

B. To change the measurement mode (ie. mg/L or % saturation), simultaneously press the “cal” and “power” button. Pressing the “cal” button allows you to scroll through configuration options. After choosing appropriate measurement, press “meas” key to return to measure mode.

C. Immerse probe in sample, making sure the stainless steel thermistor is submerged.

D. Take measurement when the value on the display is stable, if Auto-read is on (indicated by an "A" on the right hand side of the screen), the "A" will stop flashing when the reading is stable. The temperature can also be recorded from the display window below the D.O. value.

E. Rinse probe in deionized water and return to calibration sleeve when finished.

IV. TROUBLESHOOTING & MAINTENANCE

A. The display screen features a "Stat face" that resembles a smiley face. It provides information on the electrode condition (slope, response time, etc.). Deterioration of electrode condition is shown first by a straight face and then by the frowning of "Stat face." An improvement can only take place after calibration.

B. The display screen also features what looks like a bulb with flashing lines coming from it. This symbol is an indication of electrode response time. Response time can become sluggish due to aging, lack of maintenance, or membrane tearing and fouling.

C. To replace electrolyte and membrane cap, first disconnect probe from instrument.

D. Unscrew and discard old membrane cap.

E. Fill the new membrane cap half way with electrolyte solution (Polarographic DO Probe Electrolyte 080514).

F. Holding probe at a slant, insert electrode assembly vertically into the new membrane cap and tighten cap quickly. Excess electrolyte will be expelled through vent. If air bubbles are in the membrane cap, repeat procedure.

G. Plug probe back into instrument and allow approximately 25 minutes for repolarization.

H. Calibrate. If the slope still does not fall within the required range, the electrode may need cleaning. Use polishing paper to clean probe then repeat steps E-H.

I. Please consult manual for further troubleshooting and definitions of error messages.

3.9 PERCIVAL SCIENTIFIC 136LL INCUBATOR

I. LIGHTING

A. To enter the Lights Menu, press the "LIGHTS" key. To navigate through the menu, use the up and down arrow keys.

B. When the display reads "Light 1," press "ENTER." Switch the setting to "ON" or "OFF" using the arrow keys. Press "ENTER" to accept the setting.

C. Use the arrow keys to scroll until the display reads "Light 2." Switch the setting to "ON" or "OFF" using the arrow keys. Press "ENTER" to accept the setting.

D. To exit the Lights Menu, press the "LIGHTS" key.

II. TEMPERATURE MENU

A. To enter the Temperature Menu, press the "TEMP/ALARM" key. To navigate through the menu, use the up and down arrow keys.

B. To set the temperature manually,
   1. Press "ENTER" when the display reads "Manual Temp Set Pt."
   2. The temperature reading will begin to flash. Use the up and down arrows to change the set point to the desired temperature.
   3. Press "ENTER" to accept value.

C. To set the temperature high safety setting,
   1. Press "ENTER" when the display reads "Safety High Alarm."
   2. The temperature will begin to flash. Use the up and down arrows to change the display to the desired temperature, which is recommended to be 3°C above the highest programmed temperature. When the temperature gets higher than this value, a safety alarm will be triggered and the incubator will shut down its control functions.
   3. Press "ENTER" to accept value.

D. To set the temperature low safety setting,
   1. Press "ENTER" when the display reads "Safety Low Alarm."
   2. The temperature will begin to flash. Use the up and down arrows to change the display to the desired temperature, which is recommended to be 3°C below the lowest programmed temperature. When the temperature gets lower than this value, a safety alarm will be triggered and the incubator will shut down its control functions.
   3. Press "ENTER" to accept value.

E. To exit the Temperature Menu, press the "TEMP/ALARM" key.

III. 96 STEP PROGRAM SETUP

A. Press the "PROG" key.

B. Use the up and down arrow keys to select "Enter/Edit 96 Step" and press "ENTER."

C. If a program has not been entered, a message will be given that there are no steps in the profile. If a program has been entered, press the "PROG" key, select "Delete All Steps," and press "ENTER."

D. To add the first step,
   1. Press the "PROG" key, select "Add Step," and press "ENTER."
   2. Press the "TIME" key. Use the up and down arrows to change the time.
   3. Press the "TEMP/ALARM" key. Use the up and down arrows to change the temperature.
   4. Press the "LIGHTS" key. Use the up and down arrows to change the lighting so that "1" represents on and "0" represents off.
   5. Press "ENTER" and verify that no settings are flashing.

E. To add the second step,
   1. Press the "PROG" key, select "Add Step," and press "ENTER."
   2. Follow steps D2-5.

IV. RUN 96 STEP PROGRAM

A. Press the "PROG" key

B. Use the up and down arrow keys to select "Run 96 Step" and press "ENTER."

V. SAMPLE 96 STEP PROGRAM

Below is a sample 96 Step Program for a 16 hour light: 8 hour dark cycle.

Step 1	9:00 am 15.0 C	LT:11

Step 2	1:00 am 15.0 C	LT:00

3.10 CALIBRATION AND USE OF THE OAKTON PH 11 METER

Calibration should be performed at least once daily for more accurate results.

VI. PROBE CALIBRATION

N. Make sure that the MODE on the meter is set to measure pH, as indicated in the upper right corner of the display.

O. Remove the probe from the electrode storage bottle, rinse with deionized water, and shake dry.

P. Place the electrode in pH 4.0 buffer solution and stir.

Q. Press CAL/MEAS. The CAL indicator will be shown.

R. When the measured pH value is stable, press the HOLD/ENTER key to confirm calibration.

S. Rinse the electrode with deionized water and shake dry. Place the electrode in pH 7.0 buffer solution and stir.

T. Repeat steps D and E.

U. Rinse the electrode with deionized water and shake dry. Place the electrode in pH 10.0 buffer solution and stir.

V. Repeat steps D and E

W. When finished calibrating, the meter will automatically return to Measurement mode. If this does not occur, press CAL/MEAS to return manually.

X. In Measurement mode, perform a calibration check using pH 7.0 buffer solution. If the measured value is not within the required range, change buffer solutions and repeat calibration.

VII. MEASUREMENTS

G. Rinse the electrode with deionized water and shake dry.

H. Immerse the electrode in sample and stir gently.

I. Wait for the pH reading to stabilize and record the value. The electrode will also provide a temperature reading, if needed.

J. Rinse the electrode with deionized water between samples.

K. Store rinsed electrode in electrode storage bottle when finished.

4.0 STANDARD OPERATING PROCEDURES- MISCELLANEOUS

4.1 GLASSWARE AND PLASTICWARE CLEANING

I. NEW PLASTICWARE

Rinse new plasticware with sample dilution water before use.

II. NEW GLASSWARE

New glassware must be soaked overnight in 10% acid, then rinse well in deionized water and seawater.

III. NON-DISPOSABLE SAMPLE CONTAINERS, TEST VESSELS, PUMPS, TANKS AND OTHER EQUIPMENT

Any equipment coming in contact with samples must be washed to remove surface contaminants as described below:

1. Rinse with tap water several times.
2. Soak in tap water and 10% Liquinox or other detergent for at least 15 minutes, then scrub with brush.
3. Rinse in tap water several times.
4. Rinse in 10% Nitric (HNO_3) or hydrochloric (HCl) acid to remove scales, metals, and bases. 10% = 10 mL concentrated acid + 90 mL deionized water.
5. Rinse several times in deionized water.
6. If organic toxicant used, rinse once with pesticide grade acetone in fume hood.
7. Rinse three times with deionized water.

IV. SEDIMENT-WATER INTERFACE TUBES

A. AFTER USE

1. Soak in 10% Citranox or RBS for 24 hours.
2. Scrub screen surface and tube gently with brush and rinse 2-3 times in tap water.
3. Dip screen tubes in 10% nitric acid for 5-10 seconds.
4. Rinse thoroughly in deionized water.

B. PRIOR TO NEXT USE

1. Soak in seawater for 24 hours.
2. Rinse 3 times in deionized water.

V. CARBOYS

B. SEMI-ANNUALLY

1. Soak in ~2% nitric acid solution for approximately one week, check that pH of acid solution is below 2.00.
2. Rinse 3 times with deionized water.
3. When refilling with Scripps water, rinse the inside, nozzle and outside of the carboy 2-3 times with seawater before refilling. Avoid touching the metal nozzle of the hose inside the carboy.

VI. SEDIMENT CORE TUBES

A. AFTER USE

1. Soak in 10% Citranox or RBS for 24 hours.
2. Scrub tube gently with brush and rinse 2-3 times in tap water.
3. Dip core tubes in 10% nitric acid for 5-10 seconds.
4. Rinse thoroughly in deionized water.

B. PRIOR TO NEXT USE

1. Soak in seawater for 24 hours.
2. Rinse 2-3 times in deionized water.

VII. EMBRYO/LARVAL IN-SITU DRUMS

A. AFTER USE

1. Remove plastic screws from ends.
2. Soak in 10% Citranox or RBS for 24 hours.
3. Scrub screens very gently with brush and rinse 2-3 times in tap water.
4. Dip drums in 10% nitric acid for 5-10 seconds.
5. Rinse thoroughly in deionized water.

B. PRIOR TO NEXT USE

1. Soak in seawater for 24 hours.
2. Rinse 2-3 times in deionized water.

VIII. DINOFLAGELLATE FLASKS

A. AFTER USE

1. Soak in 10% Citranox or RBS for 24 hours.

PROTOCOL FOR CLEANING GLASSWARE/PLASTICWARE (Cont'd)

2. Scrub with brush and rinse 2-3 times in tap water.
3. Place in 10% nitric acid for 5-10 seconds.
4. Rinse thoroughly (2-5 times) in deionized water.

B. PRIOR TO NEXT USE

1. Sterilize in autoclave (see protocol for using autoclave[1])

IMPORTANT: All glassware **must** be soaked overnight in dilution water prior to use in each test.

4.2 RECEIVING AND HOLDING TEST ORGANISMS

This protocol is intended for receiving and holding of mysid shrimp (*Americamysis bahia*), topsmelt larvae (*Atherinops affinis*), and inland silversides (*Menidia beryllina*):

1. Upon arrival, check temperature before placing into aquarium/holding tank (6 L or 22 L). Test organisms should not be subjected to changes of more than 3 °C in water temperature or 3 ppt salinity in any 12-hour period.

2. In order to acclimate animals, place shipping bag in clean aquarium/holding tank for at least 60 minutes. After initial water quality measurements are taken, the top of the bag should be propped open and water should be gently aerated. A small amount of food may be added if the animals do not appear stressed.

3. After temperature in the shipping bag has approached appropriate holding temperature (depending on test method), remove the shipping bag and add filtered seawater to the holding tank.

4. **Mysids:** gently siphon mysids into holding tank using a wide-bore pipette and tygon tubing. As mysid:water ratio in the shipping bag decreases, siphon out the excess water into a clean beaker. When all of the mysids have been transferred, rinse the bag with filtered sea water and check for mysids that may have stuck to the sides of the bag, also check the excess water that was siphoned off into the clean beaker. Loading rate for mysids should not exceed 20 mysids per liter.

   Fish Larvae: Carefully siphon off extra water from the travel bag in order to concentrate fish larvae. Gently pour larvae into clean holding tank. Be sure not to transfer any fish that died during shipment. When bag level gets low, individually pipette larvae into holding tanks using a wide-bore pipette. Loading rate for fish should not exceed 0.4 g fish per liter.

5. Gently aerate each holding tank with a small airstone.

6. Animals should be fed newly hatched *Artemia* nauplii liberally.

7. Check temperature frequently to make sure it is maintained at appropriate holding temperature ± 2 °C. If temperature is not maintained in range, organisms should be held an additional day prior to testing. Organisms should be acclimated for at least 2 days prior to testing.

8. Ensure that the photoperiod to be used during testing is being used during acclimation.

9. Renew holding water every other day or renew one half of the water every day. This depends on the amount of fecal matter and density of animals in the holding tank. All fecal matter, dead, etc. should be siphoned daily.

PROCEDURE FOR RECEIVING/HOLDING TEST ORGANISMS *(Americamysis bahia, Atherinops affinis, and Menidia beryllina) Cont'd*

10. If the organisms need to acclimate to the testing salinity, mix filtered sea water with the appropriate amount of deionized water to obtain the desired salinity (do not adjust salinity more than 3ppt in a 12-hr period) during water changes.

11. The following should be recorded during the holding period:

    a. Condition of the organisms upon arrival and every day thereafter.
    b. Temperature in holding tanks
    c. Frequency of water change and siphoning
    d. Dissolved oxygen level in holding tanks
    e. Frequency and approximate quantity of feeding
    f. General appearance of water (cloudy, clear, etc.) and organisms (active, dead, etc.)

12. Before disposal, any surviving test organisms are killed, generally by concentrating into a container and freezing. Under no circumstances are test organisms ever released to the wild or used more than once for testing.

4.3 MAINTAINING DINOFLAGELLATE CULTURES

FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. OBJECTIVE: To maintain organism health and propagation, dinoflagellate cultures need to be split about every two weeks to so that they do not become too dense. Species being maintained include*; Lingulodinium polyedrum, Ceratacorys horrida, Pyrocystis noctiluca, Gonyaulax grindleyii, Pyrocycstis lunula, and Pyrocystis fusiformis.*

II. NECESSARY MATERIALS AND SUPPLIES

- Beakers- 1 Liter, Class A, borosilicate glass– 3 or 4 for media
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Watch glasses – for covering 1 L beakers
- Colored labeling tape
- Stock solutions A, B and C[1]
- Erlenmeyer flasks – 5-10 acid washed and autoclaved for split cultures
- Glass microscope slides – for examining dinoflagellate species
- Light biological Microscope
- Microwave
- Foam stoppers – to stopper flasks with culture

III. METHODS

**Be aware of cross contamination! Wear gloves at all times, not allowing anything that will come in contact with the inside of the flasks to touch countertops – use different pipette tips for each culture, etc.

A. ONE DAY PRIOR TO CULUTRE SPLIT

1. Sterilize all flasks and stoppers in the autoclave[2].

2. Filter 2 to 3 (depending on how many cultures you will split) liters of seawater (collected from the cold room in bldg. 111) with 0.22 µm filter paper.

3. To each 1 Liter glass beaker add:
   - Stock A – 15 mL
   - Stock B – 1 mL
   - Stock C – 0.5 mL

4. Add 1 L of filtered seawater to each beaker.

PROTOCOL FOR MAINTAINING DINOFLAGELLATE CULTURES OF SEVERAL SPECIES *Cont'd*

5. Heat in microwave for 25 minutes. Include a small beaker filled with deionized water for possible evaporation.
6. Allow media to cool to room temperature (about 20 °C) overnight.

B. DAY OF CULTURE SPLIT

1. Remove a 25 mL aliquot from each beaker of media and take pH, salinity and temperature. Salinity should be approximately 34 ‰, pH from 8.0-8.1 and temperature range should be 19 °C ± 2 °C.

2. Record data on the dinoflagellate log.

3. Choosing dinoflagellate stocks to split

    a. Turn off the lights and return to the incubator.
    b. Swirl each culture and move brightest (most dense) cultures to the front row.
    c. Remove all flasks in front row, making sure that one of each six cultures is selected.
    d. Depending on the culture density remove .020 mL to 1 mL and view under the microscope to determine viability, presence of motility (for some species) and density.

4. Label all new flasks with current date and species name and strain (if applicable).

5. Rinse each sterilized flask with approximately 50 mL of medium.

6. Add approximately 50 mL into flask (to cushion entry of dinoflagellates).

7. For high density cultures (i.e. L. polyedrum) add 100 mL of culture in new flask then bring up to 250 mL line with media.

8. For low-density cultures (P. noctiluca and C. horrida) add 125 mL of culture in new flask and then bring up to the 250 mL line with media.

9. Additionally, bring source flask up to the 250 mL mark with new media.

10. Always assure that incubator is functioning at 19 °C when replacing cultures.

[1] Please refer to "PROTOCOL FOR PREPARATION OF ENRICHED SEAWATER MEDIUM" located on: C:\Documents and Settings\zacharia\Desktop\Laboratory 116\Protocols and logs

[2] Please refer to "PROTOCOL FOR AUTOCLAVE" located on: C:\Documents and Settings\zacharia\Desktop\Laboratory 116\Protocols and logs

4.4 PREPARATION OF ENRICHED SEAWATER MEDIUM (ESM)[1]

FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method allows one to prepare stock solutions that serve as growth medium for algae, diatoms and dinoflagellates.

II. **NECESSARY MATERIALS AND SUPPLIES**

- Polycarbonate bottles – (3) 1 Liter
- Deionized water
- Graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 50-1000ml for making test solutions
- Colored labeling tape
- Pipets, automatic – adjustable, to cover a range of 0.01 to 5 ml and pipette tips
- Calculator
- Wash bottles – for topping off graduated cylinders
- Analytical toploading scale – for measuring chemicals

III. **METHODS**

A. MICRONUTRIENT STOCK SOLUTION (A)

1. For Cultures:

To a 1 L polycarbonate bottle, add 1 L deionized water and the following chemicals in the order listed:

$FeCl_3 \cdot 6H_2O$ – 0.072 g
$MnCl_2 \cdot 4H_2O$ – 0.144 g
$ZnSO_4 \cdot 7H_2O$ – 0.045 g
$CuSO_4 \cdot 5H_2O$ – 0.157 **mg (see below)**
$CoCl_2 \cdot 6H_2O$ – 0.404 **mg (see below)**
H_3BO_3 – 1.140 g
Na_2EDTA – 1.0 g

Note: Analytical scales will not accurately measure some chemicals required in amounts below 1 mg. To obtain these amounts of chemical accurately, see example below;

For $CuSO_4 \cdot 5H_2O$ – 0.157 **mg**, measure 0.157 g of chemical, and add to volumetric flask (100 mL). Fill flask to line and invert several times. Use a 100 µL aliquot to obtain correct amount of chemical. If using a different volumetric flask follow example calculation:
for $CoCl_2 \cdot 6H_2O$ – 0.404 **mg** and a 200 mL flask; 0.404 mg / 100 µL x 1 ml /1000 µL = 4.04mg / 1 mL and if we are using trying to create 200 mL of this solution, we need 4.04mg/1mL x200= 808 mg of chemical in 200 mL of deionized water, use 100 µL of this solution for the correct amount of chemical. Check calculation: 808mg/200mL x 0.100mL= 0.404 mg.

PROTOCOL FOR PREPARATION OF ENRICHED SEAWATER MEDIUM (ESM) ***Cont'd***

2. For Bioassays:

Follow the same procedure, but omit copper ($CuSO_4 \cdot 5H_2O$) and add only .05 g Na_2EDTA instead of 1.0 g.

B. MACRONUTRIENT STOCK SOLUTION (B)

1. For Diatoms (i.e. *Skeletonema costatum*):

To a 1 L polycarbonate bottle, add 1 L deionized water and the following chemicals in the order listed:

- K_3PO_4 – 3.0 g
- $NaNO_3$ – 50.0 g
- $NaSiO_3 \cdot 9H_2O$ – 20.0 g

2. For Dinoflagellates:

Do not add any $NaSiO_3 \cdot 9H_2O$.

C. VITAMIN STOCK SOLUTION (C)

To a 1 L polycarbonate bottle, add 1 L deionized water and the following chemicals in the order listed:

- Thiamine hydrochloride – 500 **mg**
- Biotin – 0.1 mg
- B12 – 1.0 **mg**

To renew dinoflagellate cultures, stock solutions are added to a sterile container containing natural seawater that has been filtered through a 0.22 μm membrane filter in the following proportions:

Stock A: 15 mL / L of medium
Stock B: 1 mL / L of medium
Stock C: 0.5 mL / L of medium

Adjust to pH 8.0 ± 0.1 with NaOH or HCl.

Store excess medium in the dark at approximately 4 °C until use.

[1] modified from 1995 ASTM vol.11.05, section E 1218, p.581-2

4.5 HATCHING BRINE SHRIMP AND THEIR USE AS TEST ORGANISM FOOD

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** Brine shrimp (*Artemia spp*) are the preferred and most convenient food for Mysids (*Americamysis bahia*) and Topsmelt (*Atherinops affinis*) for whole effluent toxicity testing and holding/acclimation.

II. **NECESSARY MATERIALS AND SUPPLIES**

- Separatory Funnels – (2), 2-Liter capacity
- Air pump
- Plastic tubing – to provide aeration in separatory funnels
- Glass Pasteur pipettes
- Flashlight
- Dark Material – to aid in collection of brine shrimp
- Brine Shrimp (*Artemia*) cysts

Note: EPA suggests use of Brazilian or Colombian brine shrimp cysts. These can be purchased from Aquarium Products, 180L Penrod Ct., Glen Burnie, MD 21061. Other suppliers are on p. 28 of EPA/600/R-95/136.

III. **METHODS**

1. Add 1 L of seawater to a 2-L separatory funnel, or equivalent.

2. Add 10 mL or 1-2 grams of *Artemia* cysts to the separatory funnel and aerate for 24 hours at 27 °C. Actual hatching time will vary with temperature and strain.

3. After 24 hours, remove the air supply from the separatory funnel. Cover funnel with a dark cloth or paper towel while directing the beam of a flashlight through the bottom of the funnel for 5-10 minutes. *Artemia* are phototactic, and will concentrate at the bottom of the funnel. Do not leave concentrated nauplii at bottom for more than 10 minutes without aeration, or they will die.

4. Drain the nauplii into a funnel fitted with a <150 μm Nitex or stainless steel screen, and gently rinse with seawater.

5. Gently spray nauplii into a beaker and fill until desired concentration is reached.

6. Approximately 40-50 nauplii per feeding per test organism is targeted for most tests. In order to feed 10 organisms, this requires 200 μl of a suspension with a density of 2000 nauplii/ml. This concentration can be achieved by dilution or concentration of nauplii following cell counts under a light microscope. For test protocols using 5 organisms per beaker, 100 μl of the suspension would be used.

4.6 HYPERSALINE BRINE AND ARTIFICIAL SEA SALT USE

I. OBJECTIVE

Since many effluents entering marine and estuarine systems have little measurable salinity, salinity adjustment may be necessary for tests with marine/estuarine organisms. It is important to maintain an essentially constant salinity across all treatments. Two methods are available to adjust salinity – artificial sea salts and hypersaline brine. Some test methods (e.g. embryo tests, QwikLite) may require use of HSB due to toxicity associated with artificial salting.

II. MAKING HYPERSALINE BRINE:

A. Collect seawater on an incoming tide and pour through a 10 μm filter.

B. Store 4 L of filtered seawater in a carboy that has a bottom valve.

C. Freeze for approximately 6 hours at –10 °C to –20 °C.

D. Remove hypersaline water from container, leaving behind ice (primarily freshwater).

E. Check salinity and pH, adjust if necessary (salinity should never exceed 100 ppt).

F. Filter through a 1 μm filter.

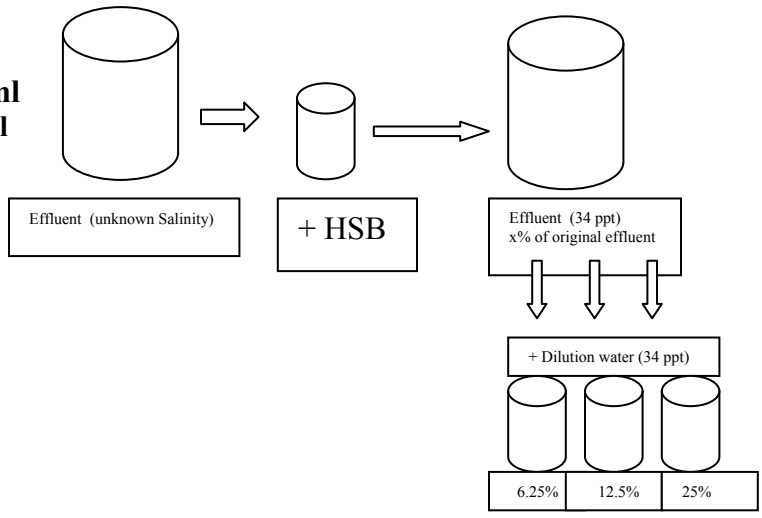
G. Cap, label, date and store in the dark at 4 °C.

III. DILUTIONS WITH HYPERSALINE BRINE (SEE BRINE DILUTION WORKSHEET):

Several dilutions of effluent are needed in a definitve test. The highest test concentration will include a combination of effluent and hypersaline brine. The concentration of the highest test concentration will depend on how much brine is required. If the target salinity is 34 ppt, diluting to make different test concentrations must be done with dilution water that is also 34ppt. Use the following equation to determine the volume of brine to be added to effluent.

VB = VE x (34 – SE) / (SB – 34), where

VB = volume of brine to be added in **ml**
VE = volume of effluent to be added in **ml**
SE = salinity of the effluent (**ppt**)
SB = salinity of the brine (**ppt**)



For example, if the brine is 68 ppt (SB) and the effluent is 2 ppt (SE), to 1 L effluent, you would add 1000 ml x (34-2) / (68-34) = 941.18mL brine to make a 34 ppt effluent solution (51.5% effluent).

Serial dilution with dilution water (baseline water) can then be used to achieve other effluent concentrations (i.e. 6.25%, 12.5%, 25%, 50%).

**Check pH of all solutions, and adjust appropriately by adding dropwise, dilute hydrochloric acid or sodium hydroxide.*

IV. BRINE CONTROLS

Brine controls should contain the quantity of brine used in the highest effluent concentration. First, D.I. water should be adjusted to the salinity of effluent using dilution water (34 ppt) therefore the same amount of brine can be added to the control as the effluent. The amount of reagent water (D.I. water + dilution water) (VE) added to the brine controls can be determined by the following equation:

VE = VB x (SB-34) / (34-SE)

V. ARTIFICIAL SALT

A. For every 1ppt increase in salinity desired, add 1g/L of artificial salt (Crystal Sea Marine Mix) directly to effluent. Underestimation is good practice, so that the sample does not get over-salted.

B. Dissolve salt by use of magnetic stirrers.

C. If undissolved salt remains, decant effluent into another flask, leaving behind the particulates.

D. The pH of the effluent may be a little different from natural seawater, but it is generally not adjusted.

4.7 REFERENCE TOXICANT TEST DILUTIONS

I. OBJECTIVE

Reference toxicant tests provide an indication of the sensitivity of the test organisms and the performance of the testing laboratory. A dilution factor of 0.5 or greater is generally used. Below are concentrations generally used for various tests used by the Bioassay Lab and general procedures for preparing test dilutions.

Species	Toxicant	Test Endpoint	**Concentrations (µg/L, ppb)**	**Test Duration**
Atherinops affinis	$CuS0_4$	Survival: LC50	0, 50, 100, 200, 400	96 h
Menidia beryllina	$CuS0_4$	Survival: LC50	0, 50, 100, 200, 400	96 h
Americamysis bahia	$CuS0_4$	Survival: LC50	0, 25, 50, 100, 200, 400	96 h
Mytilus edulis	$CuS0_4$	Normal shell development EC50	0, 4.1, 5.9, 8.4, 12, 17.2, 24	48 h
Crassostrea gigas	$CuS0_4$	Normal shell development EC50	0, 4.1, 5.9, 8.4, 12, 17.2, 24	48 h
Strongylocentrotus purpuratus	$CuS0_4$	Normal larval development EC 50	0, 5.8, 8.4, 12, 17.2, 24, 35	72 h
Dendraster excentricus	$CuS0_4$	Normal larval development EC 50	0, 4.1, 8.4, 12, 17.2, 24, 35	72 h
Ceratocorys horrida	$CuS0_4$	Photon emission: EC50	0, 15.6, 31.3, 62.5, 125, 250	24 h
Eohaustorius estuarius	$CdCl_2$	Survival/ reburial		96 h
Rhepoxynius abronius	$CdCl_2$	Survival/ reburial		96 h

II. MAKING REFERENCE TOXICANT STOCK SOLUTIONS

A 1 ppt Cu solution is made on an annual basis (or as needed) and stored in the RM 116 refrigerator. The solution is made as follows (other stock solutions are made similarly, with the appropriate weight of solid material substituted for the ones shown here):

A. Obtain reagent grade $CuSO_4 \bullet 5H_2O$ crystals from chemical storage area in RM 116.
B. Weigh out 0.982g $CuSO_4 \bullet 5H_2O$ on Sartorius balance in Rm 115.
C. Add to 250 ml E-pure (deionized) water in clean polycarbonate bottle.

D. Label bottle with estimated concentration (e.g. 1 ppt), date, and analyst initials.
E. Have solution analyzed by STGFAA.
F. Label the bottle with the measured concentration and date, and record on log sheet.

III. MAKING SUB-STOCK FOR USE IN DILUTIONS

A. The day of the test, make up 200 mL of **1 ppm** (or other relevant concentration) **Cu** stock solution by pipetting 0.2 mL of the 1ppt stock in 199.8 mL filtered seawater. This volume is adequate for most types of testing.

B. Store at testing temperature until use.

IV. MAKING TEST DILUTIONS

A. Construct a table or use log sheet with pre-constructed table similar to the one below

Col 1	**Col 2**	**Col 3**	**Col 4**	**Col 5**
Concentration (ppb)	**mL 1 ppm Cu stock in seawater**	**mL filtered seawater (diluent)**	**mL dinoflagellate culture**	**Total mL**
Control	0	48	2	50
15.63	0.78	47.22	2	50
31.25	1.56	46.44	2	50
62.5	3.13	44.87	2	50
125	6.25	41.75	2	50
250	12.5	35.5	2	50

Where,

Column 1 is determined by the specific test endpoint and species.
Column 2 is determined by the equation: $\mathbf{C_1V_1 = C_2V_2}$

Example: (1ppm =1000 ppb) x (V_1) = (15.625 ppb) x (50 ml), V_1**= 0.78ml**

where: C_1= concentration of stock solution (1ppm)
V_1= unknown volume of stock to be added
C_2= desired concentration in dilution flask (e.g. 15.625 ppb)
V_2= volume of dilution flask (e.g. 50 ml)

Column 3 is determined by subtracting ml of Cu stock and ml of culture added.

Example: Col 3 = Col 5- (Col 4 + Col 2)

Column 4 is determined by concentration of culture needed for specific test (e.g. QwikLite).

Column 5 is determined by how many replicates are in each treatment. Excess should be made so water chemistry can easily be measured.

Example: if each treatment has 3 replicates each containing 20ml total, at least 110 ml of solution should be made. 3 replicates x 20 ml= 60 ml + 50 ml for water quality measurements.

B. After dilutions have been made, cover with parafilm.

C. Wait 1-2 hours for equilibration before exposure to test organisms.

4.8 ACQUISITION, REDUCTION, AND REPORTING OF DATA

I. MANUAL DATA REDUCTION

A. Precisely measure and record all readings and output.

B. Calculate final results using select suitable formulas and programs. (e.g. unionized ammonia or proper statistical tests).

C. Manually enter at least one sample calculation onto data sheet or notebook.

D. Double check recorded data when transferred into forms or spreadsheets.

E. Compare raw data entries with summaries and results to assure accurate initial data entry.

All raw data must be retained as a part of the study records. These records must be identified with the following information: date; sample ID; analyst or operator; species identification, and instrument operating conditions (if applicable). Raw data is stored in a filing system maintained in the Bioassay Laboratory.

II. COMPUTER DATA REDUCTION

A. Ascertain that all data used in final calculations are entered accurately: mortality, number normal, number alive, water quality reporting, etc.

B. Record appropriate and accurate information concerning sample identification, date; sample ID; analyst or operator; species identification, and instrument operating conditions (if applicable).

C. Identify analysis in the "Test ID Log" notebook and assign a test number that can be cross-referenced.

D. Calculate results using appropriate computer software and analyses.

E. Manually enter at least one sample calculation onto data sheet or notebook.

F. Properly interpret the computer output.

G. Attach all relevant test material (raw data, summaries, analyses, and reports) and place in appropriate binder or filing system.

4.9 RECORDING AND HANDLING DATA

I. **OBJECTIVE:** To provide guidelines on recording data in notebooks, forms and any other media to ensure legibility, accuracy, validity and clarity.

II. **GUIDELINES**

A. All entries should be made legible.

B. All entries should be made with a black or blue ballpoint pen.

C. Use initials or name to indicate the originator of entries.

D. All blank cells with no data should contain a short slash or horizontal dash.

E. Abbreviations should not be used unless they are for chemical names (i.e. NaCl for Sodium Chloride).

F. Cross out errors with a single line and note initials.

III. **NOTETAKING**

A. Notes should be recorded in a laboratory notebook. Include date, person(s) responsible, project name, and signatures.

B. A generic note page can be found on the laptop in room 116 at: *C:\Documents and Settings\zacharia\Desktop\Laboratory 116 titled "Note page".*

C. Fill in the top of the page where there is space for the date (month day and year) and the notetaker's name.

D. Record any observations such as experimental procedure, equipment, materials and calculations.

E. Attach note page with all other relevant test data and file into a binder or folder with corresponding project.

IV. RECORDING DATA

Standard units are used among organizations to ensure consistency. When appropriate, the mean and standard deviation should be reported.

pH	pH units
Salinity	ppt or ‰
Temperature	° C

Dissolved Oxygen mg/L
Ammonia % unionized NH_3
Sulfide H_2S mg/L

V. DATA MANAGEMENT

A. The analyst shall internally review data by checking for completeness and accuracy. The analyst will verify:

1. Analyses are within the calibration curve range
2. QC samples meet acceptance criteria
3. Data meets quality objectives
4. Calculations are performed correctly

B. When entering data into an electronic format, analyst shall use a hardcopy to compare with entries or use a double entry technique.

C. Data entered should be backed up as frequently as needed.

D. Any data that is analyzed with a computer program such as Toxcalc 5.0 or Microsoft Excel should be verified with randomly chosen hand calculations. Provide calculations on original sheet and initial.

VI. HANDLING SUSPECT AND ERRONEOUS DATA

A. If suspect data is identified during review, it should be examined further.

B. Document investigation of suspect data.

C. If necessary, report erroneous data to laboratory director for corrective action.

Please refer to the document titled “Corrective Action” which can be found at: *C:\Documents and Settings\zacharia\Desktop\Laboratory 116\Protocols and logs\QA Manual Documents*

4.10 STATISTICAL ANALYSIS OF DATA

I. OBJECTIVE: To statistically analyze data for determination of NOEC/LOECs via hypothesis tests and/or LC50s using point estimation techniques.

II. METHODS

ACUTE TOXICITY ANALYSIS

A. Fill out data tables using data sheets specific to test method.

B. Enter data into ToxCalc 5.0 – found in the Windows XP Programs Menu.

C. ToxCalc automatically runs Shapiro-Wilk's test for normality and Bartlett's test for equality of variance. If data is not normally distributed, perform an arc-sin square root transformation.

D. Run Hypothesis test function in ToxCalc

Determining which Hypothesis test to use:

1. Equal # of reps & data is normal – Dunnet's Multiple Comparison test

2. Equal reps & data in non-normal – Steel's Many –One Rank test (only if there are at least 4 replicates per treatment).

3. Unequal reps & data is normal – T-test with a Bonferroni Adjustment

4. Unequal replicates & data is non-normal – Wilcoxon Rank Sum test

E. Results of a hypothesis test are expressed in terms of the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC).

F. Use the Maximum likelihood probit for the point estimation. If data do not fit this method, use the Trimmed Spearman-Karber method. Linear Interpolation is used if neither of the former methods can be performed based on the test data. Results of the point estimate techniques are expressed as EC, IC, or LC values.

G. Verify that all data was entered into database correctly and save.

H. Print spreadsheets with data output and graphs for future reference.

CHRONIC TOXICITY ANALYSIS

SURVIVAL ENDPOINT

A. Fill out data tables using data sheets specific to test method.

B. Calculate proportion surviving each day.

C. Toxcalc will automatically run Shapiro-Wilk's test for normality and Bartlett's test for equality of variance on data. If data is not normally distributed, perform an arc-sin square root transformation.

D. Run Hypothesis test function in ToxCalc

Determining which Hypothesis test to use:

1. Equal # of reps & data is normal – Dunnet's Multiple Comparison test

2. Equal reps & data in non-normal – Steel's Many –One Rank test (only if there are at least 4 replicates per treatment).

3. Unequal reps & data is normal – T-test with a Bonferroni Adjustment

4. Unequal reps & data is non-normal – Wilcoxon Rank Sum test

E. Results of a hypothesis test are expressed in terms of the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC).

F. Use the Maximum likelihood probit for the point estimation. If data do not fit this method, use the Trimmed Spearman-Karber method. Linear Interpolation is used if neither of the former methods can be performed based on the test data. Results of the point estimate techniques are expressed as EC, IC, or LC values.

G. Verify that all data was entered into database correctly and save.

H. Print spreadsheets with data output and graphs for future reference.

GROWTH ENDPOINT

A. Follow the same procedures as used from survival data.

B. Concentrations with 100% mortality and concentrations with significant mortality are not included in growth analysis.

C. Graph the mean growth values for each concentration with ranges.

D. Summarize any other information indicating toxicity.

4.11 HAZARDOUS MATERIAL STORAGE, DISPOSAL AND SAFETY INFORMATION

I. **OBJECTIVE**: This document provides guidelines for the lifecycle management of hazardous materials (HM) and hazardous wastes at the Space and Naval Warfare Systems Center San Diego (SSC-SD).

What is a hazardous material?
Any material that, because of its quantity, concentration, physical or chemical characteristics, poses a present or potential health hazard to human health and safety or to the environment[1].

How to identify a hazardous material:
Look on the label of the original container; if the material is hazardous it will usually indicate this. If there are any uncertainties refer to the Material Safety Data Sheet (MSDS) located in room 116.

II. PURCHASING HAZARDOUS MATERIALS

A. Please refer to the Hazardous Materials Information notebook located in room 116 for specific purchasing instructions.

B. When any new chemicals are received (either hazardous or non-hazardous) notify the Safety office at X3-3873.

III. STORAGE AND MANAGEMENT OF HAZARDOUS MATERIALS

A. LABELING OF HAZARDOUS MATERIAL

All hazardous material must be labeled with:

Original Containers
1. Chemical name(s) or common name(s)
2. Manufacturer's name and address
3. Chemical hazards (flammable, corrosive, etc.)
4. HSMS barcode label (contact Safety office if missing)

Secondary Containers
1. Chemical name or common name
2. Manufacturer's name and address
3. Chemical hazards (flammable, corrosive, etc.)
4. Date that the container was filled
5. Name of the owner of the HM

B. Periodically inspect HM to ensure that there aren't any leaks and labels are intact.

C. Give useable HM that is no longer needed to the HazMin Center, Bldg. 116, for reissue.

In addition, all hazardous materials must have a MSDS on site where they are stored (electronic access is acceptable). MSDS's in room 116 are kept in a black notebook located near the fume hood in the southeast corner of the room. Please refer to the hazardous materials information notebook located in room 116 for MSDS explanations.

IV. HANDLING HAZARDOUS MATERIALS

A. CATEGORIES OF HAZARDS

1. *Flammables*
   These are substances that have a flash point (The lowest temperature at which the vapor of a combustible liquid can be made to ignite momentarily in air) below 100 °F.
   a. most liquids volatile, generating flammable vapors
   b. vapors are irritating or toxic
   c. skin or eye contact can be irritating

   examples: hexane, benzene, methanol, acetone, most paints, propane

2. *Halogenated Solvents*
   Solvents containing a halogen such as chlorine, fluorine, bromine, or iodine
   a. non-flammable
   b. volatile
   c. vapors are irritating or toxic
   d. skin or eye contact can cause burns

   examples: trichloromethane, carbon tetrafluroide
   Organic solvent management – concentrated wastes (essentially pure or as water mixtures that have flashpoints below 140 °F) must not be discharged into the sewer, these include alcohols, ketones, and solvents immiscible with water.

3. *Corrosives*
   Acidic or caustic materials that can cause irreversible alterations to human skin tissue
   a. Generally liquid, but may be granular or powdered solids
   b. skin or eye contact can cause *severe* burns
   c. skin contact with hydrofluoric acid may be fatal
   d. vapors irritating to eyes, skin and mucous membranes and are generally toxic

   examples: nitric acid, acetic acid, ammonia, potassium hydroxide

4. *Toxics*
   Lethal dose higher than limits designated by OSHA
   a. May be solid, liquid or gas
   b. May exhibit flammability or corrosivity

   examples: heavy metals (cadmium, lead, mercury), toluene, oils and greases, adhesives, detergents, paints

5. *Compressed Gases*
   Gases stored at pressures above one atmosphere (1 atm)
   a. May be toxic (chlorine, ammonia)
   b. May be flammable (propane, oxygen)
   c. May be inert (nitrogen, argon, helium)
   d. May be cryogenic (liquid nitrogen)

6. *Oxidizers*
   Materials that readily contribute oxygen to a reaction or combustion
   a. Unstable and reactive
   b. May be flammable
   c. Are corrosive
   d. May be explosive
   e. skin or eye contact can cause burns
   f. fumes from reaction, decomposition or fire may be toxic and cause irritation to skin and eyes.

   examples: hydrogen peroxide, benzoyl peroxide, t-butyl peracetate

7. *Water Reactives*
   Materials that react violently when exposed to water
   a. Can react with moisture in air
   b. Can react with oxygen containing liquids such as alcohols and ketones
   c. Difficult to extinguish if ignited
   d. May be explosive

   examples: lithium, sodium, sodium hydroxide, magnesium nitride, calcium carbide, nitric acid

8. *Pyrophorics*
   Materials that spontaneously combust with exposure to air
   a. May be liquid or powder
   b. May have violent reactions
   c. Skin contact may cause burns
   d. Fumes from fire or reaction may be irritating or toxic
   e. Extinguished fire may re-ignite

   examples: diethyl zinc, trimethyl aluminum, elemental phosphorus

9. *Explosives*
   Materials that release a tremendous amount of energy in the form of heat, light, and expanding pressure in a very short period of time
   a. May be sensitive to shock or heat
   b. Many are flammable
   c. Explosions can produce projectiles or pressure shock waves

   examples: TNT, picric acid, nitroglycerine, ammonium nitrate

B. PROTECTION FROM HAZARDS

1. Eliminate the possibility of exposure through material substitution
2. Use engineering controls, such as fume hoods, to eliminate exposure
3. Use personal protective equipment (gloves, goggles and aprons)

V. HAZARDOUS WASTE DISPOSAL

Hazardous waste is any discarded, excess or spilled material that is solid, liquid or gas and meets the definition of a hazardous material[1]. It is either **Characteristic** (toxic, reactive, ignitable or corrosive) or **Listed** (appears on a specific EPA or state list).

The following are prohibited sewer discharges:

- Flammable or explosive substances – flashpoint < 140 °F
- Corrosives – pH <5.0 or >12.5
- Hazardous Wastes
- Trucked pollutants (from offsite)
- Substances that may obstruct flow (solid or viscous)
- Odorous wastes
- Uncontaminated ground, storm and surface water
- Sludge
- Heated wastestreams >150 °F
- Radioactive wastes
- Greases and oils (that will cause interference or pass through treatment system to ocean)

A batch discharge request may be made to discharge small and large quantities of wastewater by contacting Brett Radsliff (code 20384) at X3-1437.

A. DETERMINE IF WASTE IS HAZARDOUS

1. If waste needs to be analyzed, contact Mary Anne Flanagan at X3-6363.

B. DISPOSAL

1. Schedule a pick up by calling X3-7464 as soon as possible after generation.
   a. Complete all paperwork prior to pickup
      - HW Disposal Request Form (Required for all HW)
      - HW Profile Sheet (not req'd for materials in original containers)
      - Copy of MSDS's
   b. Forms available in Hazardous Materials Information notebook or are accessible on line at: https://iweb.spawar.navy.mil/services/sti/publications/inst/forms/

2. If you need to accumulate HW

a. Contact Mary Anne Flanagan at X3-6363, so she can visit work site and determine which type of accumulation you require.
b. She will complete an accumulation area designation form.
c. Two methods of HW accumulation;
    i. *Satellite Accumulation* (accumulate for no longer than 9 months, 55 gallon limit, under control of designated operator)
    ii. *Standard Hazardous Waste Accumulation* (accumulate for no longer than 45 days, no volume restriction, under the control of code's representative)
d. Both methods require analyst to fill out a HW label and affix to containers
e. When ready to turn in HW, call X3-7464 and complete all paperwork prior to pickup:
    i. HW Disposal Request Form (Required for all HW)
    ii. HW Profile Sheet (not required for materials in original containers)
    iii. Copy of MSDS's
f. Forms available in Hazardous Materials Information notebook or are accessible on line at:
    i. https://iweb.spawar.navy.mil/services/sti/publications/inst/forms/
g. If Disposal Containers are needed contact Louie Don or Rudie at X3-7464.

3. OTHER HW DISPOSAL METHODS

h. Fluorescent light tubes – call the HW Office, X3-7464, and they will pick them up.
i. Printer toner cartridges recycling – Bldg. 116 A-33 Wing 6, Ground Floor. Each cartridge must be in original box and re-sealed.
j. Batteries – from pagers, phones, flashlights, clocks, etc. are collected in a bucket located in Bldg. 111, 2nd floor, northeast corner.
k. Glass – If broken, dispose of in a broken glass container, to obtain more glass disposal boxes, call Joel Baumbaugh at X3-5030.

VI. HAZARDOUS MATERIALS SPILLS

A. If material spill is unknown, a large spill, a danger to personnel, or too large to contain or clean up
    1. evacuate the area
    2. report to X9-911 (Federal Fire Department) and X3-5024

B. If spill is safe and type of spill is known
    1. Contain the spill using proper protective equipment and spill kit material
    2. Identify hazards through use of the MSDS
    3. Absorb the spill with appropriate spill pads or absorbent material
    4. Bag and Tag spill and material used
    5. Call X3-5024 for disposal coordination

Report all spills to the Safety and Environmental Office X3-5024.

[1] Protocol derived from SSC-SD Document 4110.1

4.12 COUNTING SPERM WITH A HEMOCYTOMETER

Preparation

1. Mix sperm by agitating the tube with a vortexer. Add about 0.025 ml of sperm to a 100 ml beaker containing 50 ml of 15 °C dilution water and stir with a Pasteur pipette. Cover and keep at 15 °C, use within 1.5 hours.

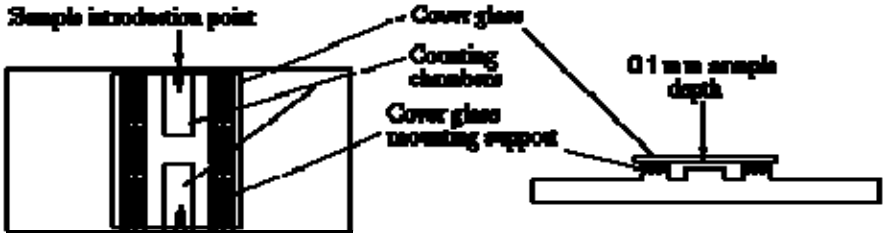
2. Slowly withdraw a subsample of semen (i.e. 0.5 ml), dispense it into a 1% glacial acetic acid solution (killing solution) in an Erlenmeyer flask (i.e. 5 ml of 10% acetic acid in 45 ml of filtered seawater). Rinse residual semen from pipet several times by filling and emptying into flask. Cover flask with parafilm and mix thoroughly by repeated inversion. *(0.5 ml of semen in 50 ml of 1% acetic acid is a 100-fold dilution (50 / .05 = 100)).

3. Transfer well-mixed acetic acid/sperm samples to a hemocytometer and wait 15 minutes to settle before counting.

Cell Counting

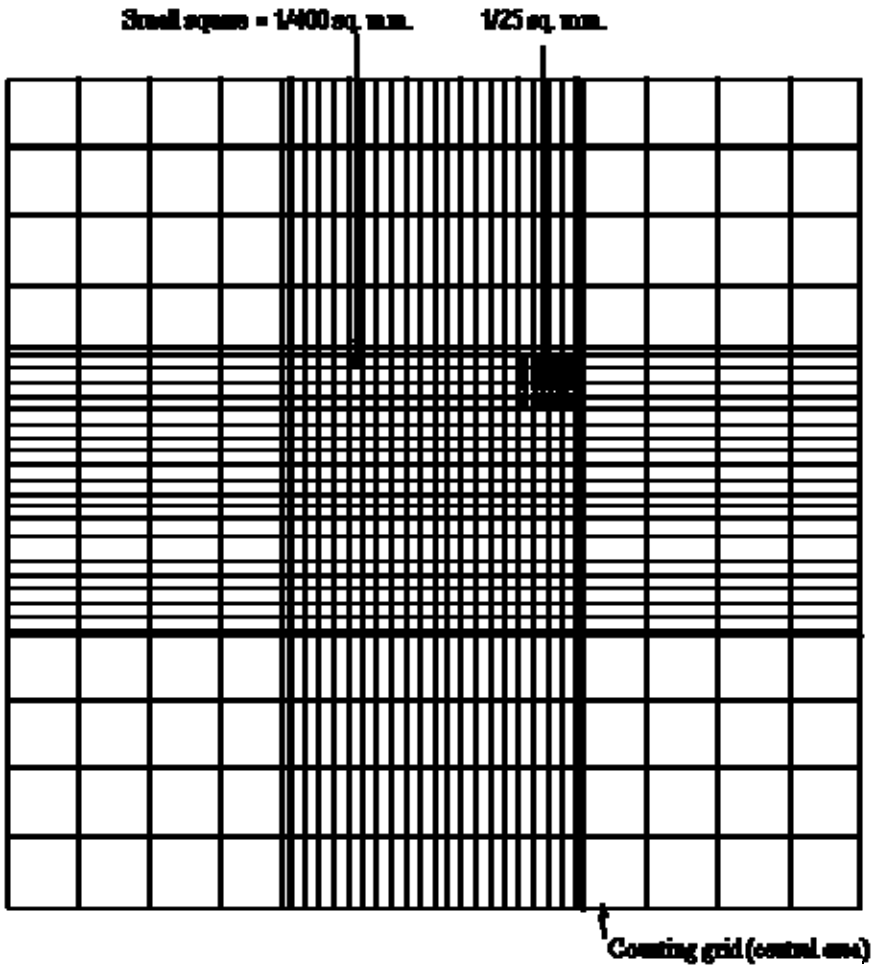
(Adapted from: http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html)

A device used for cell counting is called a counting chamber. The most widely used type of chamber is called a hemocytometer, since it was originally designed for performing blood cell counts.



To prepare the counting chamber the mirror-like polished surface is carefully cleaned with lens paper. The coverslip is also cleaned. Coverslips for counting chambers are specially made and are thicker than those for conventional microscopy, since they must be heavy enough to overcome the surface tension of a drop of liquid. The coverslip is placed over the counting surface prior to putting on the cell suspension. The suspension is introduced into one of the V-shaped wells with a pasteur or other type of pipet. The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered. The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low power.

PROTOCOL FOR COUNTING SPERM WITH A HEMOCYTOMETER *cont'd*



One entire grid on standard hemocytometers with Neubauer rulings can be seen at 40x (4x objective). The main divisions separate the grid into 9 large squares (like a tic-tac-toe grid). Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Thus the entire counting grid lies under a volume of 0.9 mm-cubed.

Cell suspensions should be dilute enough so that the cells do not overlap each other on the grid, and should be uniformly distributed. To perform the count, determine the magnification needed to recognize the desired cell type. Now systematically count the cells in selected squares so that the total count is 100 cells or so (number of cells needed for a statistically significant count). For large cells this may mean counting the four large corner squares and the middle one. For a dense suspension of small cells you may wish to count the cells in the four 1/25 sq. mm corners plus the middle square in the central square. Always decide on a specific counting pattern to avoid bias. For cells that overlap a ruling, count a cell as "in" if it overlaps the top or right ruling, and "out" if it overlaps the bottom or left ruling.

To get the final count in cells/ml, first divide the total count by 0.1 (chamber depth) then divide the result by the total surface area counted. For example if you counted 125 cells in each of the four large corner squares plus the middle, divide 125 by 0.1, then divide the result by 5 mm-squared, which is the total area counted (each large square is 1 mm-squared). 125/ 0.1 = 1250.

PROTOCOL FOR COUNTING SPERM WITH A HEMOCYTOMETER *cont'd*

1250/5 = 250 cells/mm-cubed. There are 1000 mm-cubed per ml, so you calculate 250,000 cells/ml. Sometimes you will need to dilute a cell suspension to get the cell density low enough for counting. In that case you will need to multiply your final count by the dilution factor.

Using the equation on the egg and sperm count sheet, determine concentration of sperm. Using either table 5 in EPA manual or C1V1=C2V2, determine volume of concentrated sperm stock needed to make a 500:1 solution.

4.13 COUNTING MUSSEL/OYSTER LARVAE USING AN INVERTED MICROSCOPE

TESTING FACILITY: SPAWAR SYSTEMS CENTER
BIOASSAY LABORATORY (RM 116)
CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

I. **OBJECTIVE:** This method allows for estimation of the chronic toxicity of effluent and receiving waters to the embryos and larvae of bivalve mollusks. After larvae have been exposed to test solutions for the specified amount of time they should be preserved in 1.0 mL of 37% (concentrated) buffered formalin so that each sample has a final formalin concentration of 4%. Larvae should ideally be examined within one week of preservation.

II. **NECESSARY MATERIALS AND SUPPLIES**

- Inverted microscope – for inspecting gametes and counting embryos and larvae (Located in room 152, Bldg. 111)
- Counter, two unit, 0-999 – for recording counts of embryos and larvae
- Data record sheets

III. **METHODS**

A. Turn on microscope and adjust lamp to reasonable brightness.

B. Ensure that the magnification is set to 40X (4x objective and 10x oculars).

C. Carefully unscrew cap and place vial on the center of the mechanical stage. If vial has been shaken at all, contents must be allowed to settle before counting.

D. Using the mechanical stage, begin at the upper left corner of one end of the vial and rotate the stage so that the field of view moves downward as you count all larvae, scoring them as normal or abnormal. When you've come to the bottom edge of the vial, focus on an embryo or particle that lies at the edge of the field of view and move stage so that the particle has moved from one end to the other (i.e. right to left), bringing in view only larvae that have not been counted. Count that field of view, and repeat this procedure until the entire vial has been counted.

E. Use the fine focus to view larvae that may be at different depths near the bottom of the vial. This is particularly important around the edges of the vial, where objects can appear distorted. It is important to count all larvae in the vials, so take time ensuring that this is done correctly.

IV. **DISTINGUISHING BETWEEN NORMAL AND ABNORMAL LARVAE**

A. Larvae that were live before preservation with completely developed D-hinged shells should be marked as normal. Larvae that appear slightly deformed, but have achieved the

D-hinge stage should still be counted as normal unless they are not clearly D-shaped. If the shells are empty, they are considered dead and this should be noted as abnormal.

B. Mortality will be assessed by comparing the number of normal and abnormal larvae in the test vials with a set of initial embryo vials that were preserved at the beginning of the test.

5.0 LOGS AND DATA SHEETS

5.1 ECHINODERM EMBRYO-LARVAL DEVELOPMENT TEST – WATER QUALITY DATA

WATER QUALITY DATA – 96 Hour Echinoderm Embryo Development Test

Marine Chronic Bioassay **Water Quality Measurements**

Project: _____ **Test Species:** *S. purpuratus*

Sample ID: _____ **Start Date/Time:** _____

Test No.: _____ **End Date/Time:** _____

Concentration %	Salinity (ppt)					Temperature (°C)					Dissolved Oxygen (mg/L)					pH (pH units)				
	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96

	0	24	48	72	96
Technician Initials: WQ Readings:					
Dilutions made by:					

Animal Source/Date Received: _____

Comments: **0 hrs:** _____

24 hrs: _____

48 hrs: _____

72 hrs: _____

QC Check: _____ **Final Review:** _____

5.2 BIVALVE EMBRYO-LARVAL DEVELOPMENT TEST – WATER QUALITY DATA

WATER QUALITY DATA – 48 Hour Bivalve Embryo Development Test

Marine Chronic Bioassay **Water Quality Measurements**

Project: _____ Test Species: _____

Sample ID: _____ Start Date/Time: _____

Test No.: _____ End Date/Time: _____

Concentration (%)	Salinity (ppt)			Temperature (°C)			Dissolved Oxygen (mg/L)			pH (pH units)		
	0	24	48	0	24	48	0	24	48	0	24	48
Lab Control												
Brine Control												
6.25												
12.5												
25												
50												

	0	24	48
WQ Readings:			
Dilutions made by:			

Technician Initials:

Animal Source/Date Received: _____

Comments: 0 hrs: _____

24 hrs: _____

48 hrs: _____

QC Check: _____ Final Review: _____

5.3 EMBRYO-LARVAL DEVELOPMENT TEST CALCULATIONS

Embryo-Larval Development Test – SPAWNING CHECKLIST & CALCULATIONS

Batch ID: _____ Spawn/Test Date: _____ Test Species _____
Analyst: _____

Task	Time
Spawning Inducement Initiated	
Spawning Begins	
Females/Males Isolated in Incubator	
Fertilization Initiated	
Fertilzation Terminated/eggs rinsed	
Embryo Counts	
Embryo addition to vials	

Embryo Counts:
Embryo Stock #1: ___, ___, ___ Mean = ___/___ uL * 1000 uL/mL = _____ cells/mL

Embryo Stock #2: ___, ___, ___ Mean = ___/___ uL * 1000 uL/mL = _____ cells/mL

Embryo Stock #3: ___, ___, ___ Mean = ___/___ uL * 1000 uL/mL = _____ cells/mL

Adjust selected embryo stock to 2000 embryos/ml. Confirm density:

Selected Stock : ___, ___, ___ Mean = ___/___ uL * 1000 uL/mL = _____ cells/mL

Add 100 µl of 2000 embryo/ml stock to obtain 20 embryos/ml in test vials.

Notes: _____
_____
_____
_____
_____
_____
_____
_____
_____
_____
_____
_____

5.4 EMBRYO-LARVAE DEVELOPMENT TEST RESULTS RAW DATA SHEET

Embryo Larval Bioassay **96-Hour Development**

Project: _____ **Test Species:** _____

Sample ID: _____ **Start Date:** _____

Test No.: _____ **End Date:** _____

Random #	Number Counted	Number Normal	Technician Initials
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			
33			
34			
35			

QC Check: _____ **Final Review:** _____

Embryo Larval Bioassay **48-hour Development**

Project: _____ **Test Species:** _____

Sample ID: _____ **Start Date:** _____

End Date: _____

Random #	Number Normal	Number Abnormal	Technician Initials
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			
33			
34			
35			

QC Check: _____ **Final Review:** _____

5.5 DINOFLAGELLATE PMT COUNT SHEET FOR COPPER REFERENCE TOXICANT TEST

Qwiklite (Sealite) Data for *Ceratocorys horrida* - Copper Reference Toxicant Test

DATE: _____

TEST ID: _____ **TOXCALC TEST ID:** _____

Backround noise

Rep	PMT count
1	
2	
3	
4	

Mean: _____

Control -

Rep	PMT count
1	
2	
3	
4	
5	

Mean: _____

Conc. **0 ppb**

Rep	PMT count
1	
2	
3	
4	
5	

Mean: _____

Conc. **25 ppb**

Rep	PMT count
1	
2	
3	
4	
5	

Mean: _____

Conc. **50 ppb**

Rep	PMT count
1	
2	
3	
4	
5	

Mean: _____

Conc. **100 ppb**

Rep	PMT count
1	
2	
3	
4	
5	

Mean: _____

Conc. **200 ppb**

Rep	PMT count
1	
2	
3	
4	
5	

Mean: _____

Conc. **400 ppb**

Rep	PMT count
1	
2	
3	
4	
5	

Mean: _____

5.6 DINOFLAGELLATE PMT COUNT SHEET

Qwiklite (Sealite) Data for *Ceratocorys horrida*

DATE: ____________________

TEST ID: ____________________ **TOXCALC TEST ID:** ____________________

Backround noise

Rep	PMT count
1	
2	
3	
4	

Mean: ____________________

Control -

Rep	PMT count
1	
2	
3	
4	
5	

Mean: ____________________

Conc/Sample ID:

Rep	PMT count
1	
2	
3	
4	
5	

Mean: ____________________

Conc/Sample ID:

Rep	PMT count
1	
2	
3	
4	
5	

Mean: ____________________

Conc/Sample ID:

Rep	PMT count
1	
2	
3	
4	
5	

Mean: ____________________

Conc/Sample ID:

Rep	PMT count
1	
2	
3	
4	

Mean: ____________________

Conc/Sample ID:

Rep	PMT count
1	
2	
3	
4	
5	

Mean: ____________________

Conc/Sample ID:

Rep	PMT count
1	
2	
3	
4	
5	

Mean: ____________________

Conc/Sample ID:

Rep	PMT count
1	
2	
3	
4	
5	

Mean: ____________________

Conc/Sample ID:

Rep	PMT count
1	
2	
3	
4	
5	

Mean: ____________________

DINOFLAGELLATE PMT COUNT ANALYSIS

SPAWAR - C. horrida 24 hour exposure test **Date**

Conc. (%)	PMT Counts	Mean PMT Counts	SD	CV (%)	% control	Normalized SD
0						
		#DIV/0!	#DIV/0!	#DIV/0!	100	#DIV/0!
15.625						
		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
31.25						
		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
62.5						
		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
125						
		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
250						
		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

5.7 NEANTHES 28 DAY WATER CHEMISTRY DATA SHEET

28-Day Marine Sediment Bioassay
Static-Renewal Conditions

Water Quality Measurements

Client: _____ **Test Species:** _____

Sample ID: _____ **Start Date/Time:** _____

End Date/Time: _____

Test Day	Salinity (ppt)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH (units)	Fed	Water Change	Technician Initials	Comments
0								
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								
25								
26								
27								
28								

QC Check: _____ **Final Review:** _____

5.8 NEANTHES SURVIVAL DATA SHEET

Marine Sediment Bioassay **Organism Survival**

Client: _____ **Test Species:** *N. arenaceodentata*

Project ID: _____ **Start Date/Time:** _____

End Date/Time: _____

Sample ID	Initial No.	No. Recovered	Pan Weight (mg)	Pan + Org. Weight (mg) WET	Pan + Org. Weight (mg) DRY	Technician Initials

QC Check: _____ **Final Review:** _____

5.9 AMPHIPOD 10 DAY WATER CHEMISTRY DATA SHEET

10-Day Marine Sediment Bioassay **Water Quality Measurements**
Static Conditions

Client: _____ **Test Species:** *E. estuarius*

Sample ID: _____ **Start Date/Time:** _____

End Date/Time: _____

Test Day	Salinity (ppt)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH (units)	Technician Initials	Comments
0						
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

QC Check: _____ **Final Review:** _____

5.10 AMPHIPOD SURVIVAL DATA SHEET

Marine Sediment Bioassay — Organism Survival

Client: _____ **Test Species:** *E. estuarius*

Project ID: _____ **Start Date/Time:** _____

End Date/Time: _____

Sample ID	Initial No.	No. Recovered	Technician Initials

QC Check: _____ **Final Review:** _____

5.11 ACUTE FISH/MYSID SURVIVAL SHEET

ACUTE FISH / MYSID SURVIVAL AND WATER QUALITY DATA

Marine Acute Bioassay
Static-Renewal Conditions

Water Quality Measurements
& Test Organism Survival

Project: _____ **Test Species:** _____
Sample ID: _____ **Start Date/Time:** _____
Test No.: _____ **End Date/Time:** _____

	Tech Initials				
	0	24	48	72	96
Counts:					
Readings:					
Dilutions made by:					

Concentration _ppb_	Rep	Number of Live Organisms					Salinity (ppt)					Temperature (°C)					Dissolved Oxygen (mg/L)					pH (units)				
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
Lab Control	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
50	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
100	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
200	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
400	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
800	A	5							i					i					i					i		
	B	5							f					f					f					f		
	C	5																								
	D	5																								
	A								i					i					i					i		
	B								f					f					f					f		
	C																									
	D																									

Initial Counts QC'd by: _____

Animal Source/Date Received: _____ **Age at Initiation:** _____

	Feeding Times				
	0	24	48	72	96
AM:					
PM:					

Comments: i = initial reading in fresh test solution, f = final reading in test chamber prior to renewal

Organisms fed prior to initiation, circle one (y / n)

Tests aerated? Circle one (y / n) if yes, sample ID(s): Duration:

Aeration source:

QC Check: _____ **Final Review:** _____

5.12 DINOFLAGELLATE MAINTENANCE LOG

Dinoflagellate Maintenance Log

Date	Media ID	Salinity (ppt)	pH	Temp (°C)	Date of next media split	Date of Culture Being Split (check mark indicates that culture has been split at that date)	Normal Temp. and Light Regime? (18°C -12h light/12h dark)
						❑ L. polyedrum ______________ ❑ C. horrida ______________ ❑ P. noctiluca ______________ ❑ G. gridleyii ______________ ❑ P. lunula ______________ ❑ P. fusiformis ______________	❑ Yes ❑ No
						❑ L. polyedrum ______________ ❑ C. horrida ______________ ❑ P. noctiluca ______________ ❑ G. gridleyii ______________ ❑ P. lunula ______________ ❑ P. fusiformis ______________	❑ Yes ❑ No
						❑ L. polyedrum ______________ ❑ C. horrida ______________ ❑ P. noctiluca ______________ ❑ G. gridleyii ______________ ❑ P. lunula ______________ ❑ P. fusiformis ______________	❑ Yes ❑ No
						❑ L. polyedrum ______________ ❑ C. horrida ______________ ❑ P. noctiluca ______________ ❑ G. gridleyii ______________ ❑ P. lunula ______________ ❑ P. fusiformis ______________	❑ Yes ❑ No
						❑ L. polyedrum ______________ ❑ C. horrida ______________ ❑ P. noctiluca ______________ ❑ G. gridleyii ______________ ❑ P. lunula ______________ ❑ P. fusiformis ______________	❑ Yes ❑ No
						❑ L. polyedrum ______________ ❑ C. horrida ______________ ❑ P. noctiluca ______________ ❑ G. gridleyii ______________ ❑ P. lunula ______________ ❑ P. fusiformis ______________	❑ Yes ❑ No
						❑ L. polyedrum ______________ ❑ C. horrida ______________ ❑ P. noctiluca ______________ ❑ G. gridleyii ______________ ❑ P. lunula ______________ ❑ P. fusiformis ______________	❑ Yes ❑ No

5.13 Brine Dilution Worksheet

Marine Chronic Bioassay **Brine Dilution Worksheet**

Project: _____ **Analyst:** _____

Sample ID: _____ **Test Date:** _____

_____ **Test Type:** _____

Salinity of Effluent _____

Salinity of Brine _____ **Date of Brine used:** _____

Target Salinity _____ **Alkalinity of Brine Control:** _____ mg/L as CaCO3

Test Dilution Volume _____

Effluent Brine Control

Salinity Adjustment Factor:
(TS - SE)/(SB - TS) = _____
TS = target salinity
SE = salinity of effluent
SB = salinity of brine

Concentration %	Effluent Volume (ml)	Salinity Adjustment Factor	Brine Volume (ml)	Dilute to: (ml)
Control	NA	NA	NA	200
6.25	12.5			200
12.5	25.0			200
25	50.0			200
50	100.0			200
				200
	DI Volume			
Brine Control			0.0	200

Total Brine Volume Required (ml): **0.0**

QC Check: _____ **Final Review:** _____

Brine Dilution Worksheet Summary

STEP 1: Calculate the Effluent Salinity Adjustment Factor

Salinity Adjustment Factor: $\frac{\textbf{TS - SE}}{\textbf{SB - TS}}$

TS = target salinity
SE = salinity of effluent
SB = salinity of brine

Ex: $\frac{\textbf{34 - 10}}{\textbf{71 - 34}} = \frac{\textbf{24}}{\textbf{37}} = \textbf{0.65}$

Concentration %	Effluent Volume (mL)	Salinity Adjustment	Brine Volume (mL)	Dilute to to: (mL)
Control	NA	NA	NA	500
6.25	31.3	0.65	20.3	500
12.5	62.5	0.65	40.6	500
25	125	0.65	81.2	500
50	250	0.65	162.5	500
61*	303	0.65	197	500
	DI Volume			
Brine Control	214	0.92	197	500

STEP 2: Calculate Effluent Volumes
Multiply 500 by each conc. in decimal form
Ex: 500 x 0.0625 = **31.3**

STEP 3: Calculate Brine Volumes
Multiply the effluent volumes by the salinity adjustment
Ex: 31.3 x 0.65 = **20.3**

STEP 4: Determine the Highest Obtainable Test Concentration *
Divide 500 by 1 + the salinity adjustment
Ex: 500/1.65 = **303**
Then divide 303 by 500 to determine a %
303/500 = **61%**

STEP 5: Calculate the Brine Control Salinity Adjustment Factor

Brine Control Calculation: $\frac{\textbf{TS - 0}}{\textbf{SB - TS}}$ Ex: $\frac{\textbf{34 - 0}}{\textbf{71 - 34}} = \textbf{0.92}$

STEP 6: Calculate the DI Volume Required for the Brine Control
Divide the highest brine volume from above by the brine control salinity adjustment factor

Ex: $\frac{\textbf{197}}{\textbf{0.92}} = \textbf{214}$

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SPAWAR

Systems Center
PACIFIC

Bioassay Laboratory
Quality Assurance Manual
Version V
May 11, 2011

Approvals:

Laboratory Director_____ **Date:**_____
Gunther Rosen

Code 71750 Branch Head_____ **Date:**_____
D. Bart Chadwick

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Bldg. 111 Room 116
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Table of Contents

INTRODUCTION

Space and Naval Warfare Systems Center Pacific (SSC Pacific) is responsible for development of the technology to collect, transmit, process, display and, most critically, manage information essential to U.S. Navy operations. The mission of the Environmental Sciences and Applied Systems Branch (Code 71750) at SSC Pacific is to provide cost effective technology for Navy environmental compliance and restoration through ecological risk assessment and restoration research, sediment characterization and management technology development, and environmental sensor and instrument development. The use of both standardized and innovative bioassays for evaluating effluents, receiving water, sediments, and other environmental samples have been a critical component to research within the branch for a number of years. The potential for toxicity data generated by the Bioassay Laboratory to be used in modification of Navy discharge permits as well as uses towards other regulatory issues led to the effort to obtain certification by the state of California's Environmental Laboratory Accreditation Program (ELAP) and by the State of Washington Department of Ecology.

Code 71750 consists of approximately 40 personnel, and is made up of biologists, chemists, oceanographers, and engineers, two-thirds of which have advanced degrees with a general emphasis in environmental science. Typically, a small number of the staff are directly involved in studies requiring toxicity testing, and the Bioassay Laboratory itself is generally run by two to three people, the laboratory director and one to two analysts, due to the relatively small scale of the projects being conducted. The Bioassay Laboratory at SSC-Pacific is not a production laboratory, yet is dedicated to producing results of the highest quality.

This manual presents the Bioassay Laboratory's quality assurance plan. It includes laboratory procedures with emphasis on Quality Assurance/Quality Control (QA/QC) requirements based on EPA guidelines for aquatic bioassays, specifically whole effluent toxicity (WET) testing as intended for compliance with National Pollution Discharge Elimination System (NPDES) permits. This manual is intended for Bioassay Lab staff and any other relevant parties interested in understanding the laboratory's approach to quality assurance.

1.0 LABORATORY ORGANIZATION & PERSONNEL RESPONSIBILITIES

1.1 LABORATORY ORGANIZATION

TESTING FACILITY: SSC PACIFIC
BIOASSAY LABORATORY (RM 116), CODE 71750
53475 STROTHE RD.
BLDG. 111
SAN DIEGO, CA 92152

Space and Naval Warfare Systems Center Pacific (SSC-Pacific) is responsible for development of the technology to collect, transmit, process, display and, most critically, manage information essential to U.S. Navy operations. The mission of the Environmental Sciences and Applied Systems Branch (Code 71750) at SSC-Pacific is to provide cost effective technology for Navy environmental compliance and restoration through ecological risk assessment and restoration research, sediment characterization and management technology development, and environmental sensor and instrument development. The use of both standardized and innovative bioassays for evaluating effluents, receiving water, sediments, and other environmental samples have been a critical component to research within the branch for a number of years. The potential for use of toxicity data generated by the Bioassay Laboratory to modify Navy discharge permits and make other regulatory changes led to application for certification by the state of California's Environmental Laboratory Accreditation Program (ELAP) and by the State of Washington Department of Ecology.

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1.2 PERSONNEL RESPONSIBILITIES

The Bioassay Lab staff is responsible for conducting acute and/or chronic toxicity testing as well as sample handling, and laboratory equipment calibration and maintenance. The work performed by this group consists of acquisition, management, analysis, interpretation and presentation of toxicological data. The group utilizes several tools to manage, analyze, and present data (Toxcalc 5.0, SigmaPlot, SigmaStat, Microsoft Excel, Microsoft Word, and Microsoft PowerPoint). These analyses are reported to the appropriate principal investigator or to the project sponsor.

There is considerable overlap with respect to individual responsibilities within the toxicology group. There are two primary positions, however, including the

laboratory/technical director and one or two analysts. Additional support is provided by other members of Code 71750 where needed. The roles of these positions are briefly described below:

A) Laboratory/Technical Director

Responsibilities of this position are overseeing laboratory operations, establishing quality assurance and quality control (QA/QC) policies and enforcing them, conducting toxicity testing and data analysis, verifying the quality of the data and taking corrective action when needed, interfacing with other scientists and project sponsors, attending project-related meetings, report writing, and presentation of project results. For this position, an advanced degree in the biological sciences and several years of related experience is preferred.

B) Analyst

An analyst is responsible for sample handling, preparation, and disposal, test organism maintenance, carrying out toxicity testing, data analysis, record keeping, calibration and troubleshooting of instruments, inventory, and general implementation of QA/QC policy. This position requires at least a bachelor's degree in biological or related sciences and at least one year of related experience.

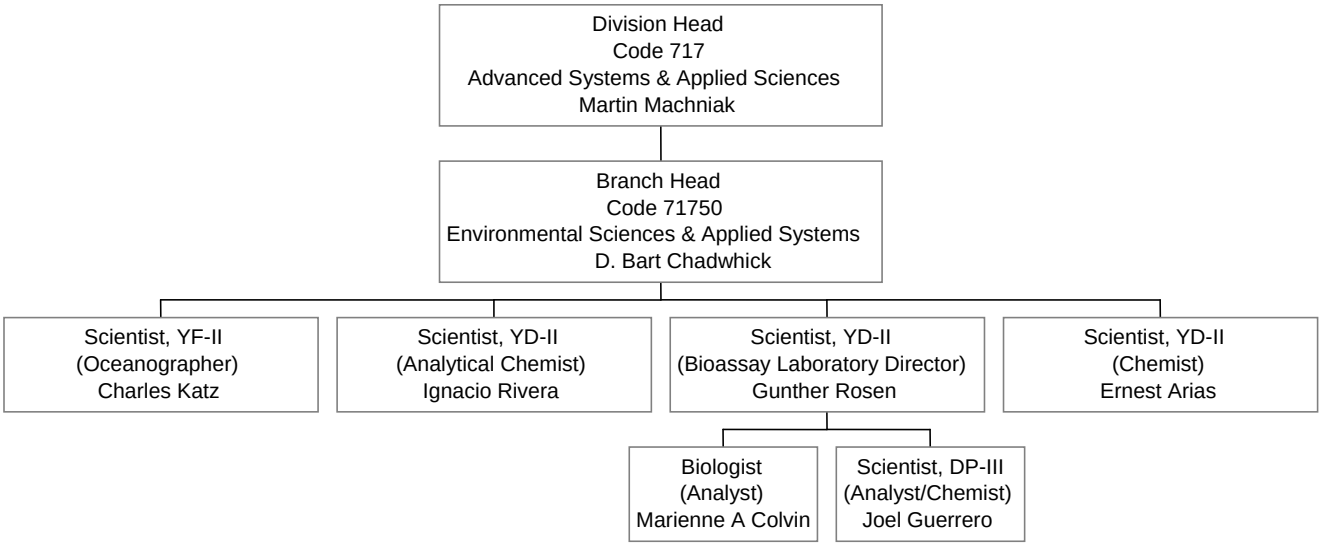
C) Additional Branch Support

There are several other scientists in the branch with extensive capabilities that assist in one way or another with the functioning of the Bioassay Lab (e.g. sampling or sample handling, chemical analysis of reference toxicant stock solutions, assistance with larger scale testing, etc.).

1.3 EXPERTISE AND PROFICIENCY

To ensure a high level of professionalism, the staff is expected to be at the forefront of scientific research in their respective field. All scientists in code 71750 have a minimum of a bachelor's degree, while approximately 67% have advanced degrees (e.g. M.S., Ph.D.). A number of resources for professional development are also available at SSC-Pacific. Employees may access SSC-Pacific's Marine Environmental Support Office (MESO) in Bldg. 111 for a large collection of technical reports and scientific journals. SSC-Pacific also has a technical library located at Topside in building 81 and the research library at Scripps Institution of Oceanography is nearby. Subscriptions to journals in the areas of toxicology and marine biology/chemistry, memberships to professional associations, internet links to scientific journals, are other avenues for increasing technical knowledge. Employees are also encouraged to attend and present at seminars and departmental and division meetings to extend communication and promote

interdepartmental organization. Career development via additional training/certification programs is encouraged. General training in laboratory safety and hazardous materials handling, storage and disposal are provided to the staff via seminars and training conducted at SSC-Pacific.



Organizational chart for the Bioassay Lab and relevant additional staff at SSC-Pacific.

2.0 FACILITIES AND EQUIPMENT

2.1 FACILITIES

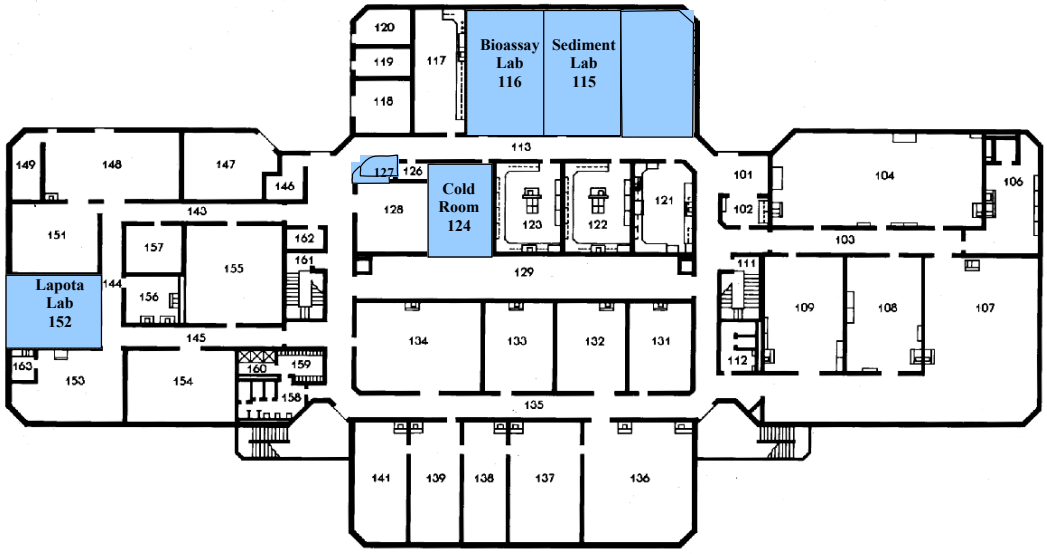
The Bioassay Laboratory is located on the first floor of Bldg. 111 at the Bayside location of SSC-Pacific's Point Loma campus, just north of the Submarine Base. Bldg. 111 is a combination of office, laboratory, and storage space with a net working space of 35,662 sq. ft. The first floor is primarily dedicated to laboratories.

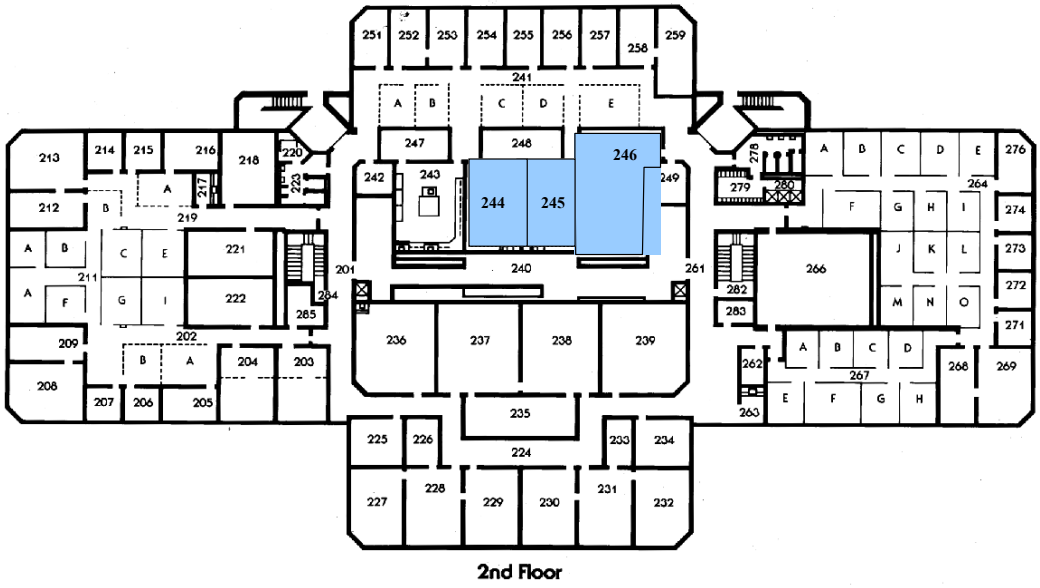
The main Bioassay Lab is located in Room 116, but a number of other labs are also utilized. A temperature controlled lab space is located across the hall in Room 124. Flow-through experiments can be conducted in the Rm 124 (also known as the "cold room") as it is plumbed to receive clean seawater from north San Diego Bay. The cold room also receives clean compressed air and has fluorescent lighting wired to a timer. Additional equipment, storage, and sample processing space can be found in the following locations (see map of Bldg. 111 on following page): 115 (counter space, balances), 127 (autoclave), 244 (E-pure water), and 246 (centrifuge).

Bldg. 111 was built in 1982 and was originally designed to facilitate naval research in marine sciences. All labs, therefore, were plumbed to receive natural seawater, deionized water, and compressed air. Natural seawater is pumped from Pier 160, located near the mouth of San Diego Bay, and passes through two sand filters before it is stored in a settling tank on the top of the building. From there, it is distributed to individual laboratories. Reagent water is provided by a water purification system that includes carbon filtration, water softener, reversed osmosis cartridge prefilters, and a UV-sterilizer to produce deionized water with a resistivity of $\geq$ 15 megohms/cm. Where required, reagent water with a resistivity of $\geq$ 18 megohms/cm is available from the Barnstead E-pure System located in Rm 244. Compressed air is dehydrated before distribution to labs, and is subsequently filtered in the Bioassay Lab and cold room with a 5-µm in-line filter to remove potential particulates, oil, or residual moisture.

Adjacent to Bldg. 111, there are three research piers bordering north San Diego Bay. Pier 169 is home to the RV/ECOS, the branch's 40-foot survey boat that is used to collect many of the samples used for bioassays. The vessel is part of the branch's Marine Environmental Survey Capability (MESC), which is houses an elaborate flow through system used to obtain a variety of water quality parameters in real-time. A 22-foot whaler is also available for use, and is launched from the boat ramp located near Pier 160. Pier 160 is a concrete pier that supports various other SSC-Pacific research vessels (e.g. Acoustic Explorer, USS Dolphin). Pier 159 is primarily dedicated to the Navy's Marine Mammal Program.

Schematics of Bldg. 111
First floor (top figure), second floor (bottom figure). Blue shaded rooms represent those housing equipment or space used by the bioassay laboratory.





Aerial photo of SSC-Pacific Bayside.

Aerial photo of piers at SSC-Pacific Bayside.

2.2 EQUIPMENT

A list of major equipment used by the Bioassay Lab is below:

Description	**Make/Model**	**Location (Rm.)**
Incubators (2, temp/light controlled)	Percival Scientific, Model I-35LL VL	116
Incubator (temp/light controlled)	Percival Scientific, Model 136LL	116
Light Microscope	Olympus/CH-2	116
Inverted Microscope	Olympus	116
Spectrophotometer	HACH/DR2400	116
UV/VIS Spectrophotometer	ThermoSpectronic/Genesys 10UV	114
Ion Selective Electrode (Ammonia)	Orion/720A	116
Conductivity Meter	Orion/105+	116
Dissolved Oxygen Meter	Orion/840	116
pH Meter	Accumet/50	116
Drying Oven	Yamato Gravity Convection Oven, Model DX-600	116
Ultracentrifuge	Beckman/L8-80M	246
Ice Maker	Scotsman	246
E-Pure Water Purification System	Barnstead 18 megohm-cm	244
Analytical Balance	Mettler PE22 top-loading, 0.1 g	115
Analytical Balance	Sartorius, 0.1 mg	115
Fume Hood	Labconco	All labs
Microtox Toxicity Analyzer	Microtox/2055	123
Fluorometer	Turner/112	116
Dinoflagellate Toxicity Analyzer	QwikLite	152
Microwave	Daewoo	116
Light Tables (2)	Porta-Trace/1618	116
Magnetic stirrers/hot plates (10)	Corning	116

Refrigerator	Whirlpool Estate TT18TKXSQ	116

3.0 QUALITY ASSURANCE OBJECTIVES

The purpose of the SSC-Pacific Bioassay Laboratory Quality Assurance Program is to ensure that the lab provides high-quality data for principal investigators, project sponsors, and other clients. The laboratory aspires to adhere to the following objectives:

- Data should be accurate in terms of agreement with reference "true" values (for water quality parameters only)
- Data should agree among individual measurements made under similar conditions
- Data should be complete in terms of the amount of valid data achieved vs. planned
- Data should be comparable to prior relevant data for evaluation and testing purposes
- Data should be representative of the overall population of database of parameter measurements
- Data should be reproducible under similar conditions at any site

These objectives are achieved by ensuring that all staff members participate in the QA/QC program, which covers all phases of the data generation, including strict compliance with SOPs for sample handling, equipment calibration and proper use, record keeping, and data handling.

*3.1 QA/QC AND TOXICITY DEFINITIONS**

Accuracy- The degree of agreement of an analytical result with the true (reference) value. Accuracy is affected by both random and systematic errors, but is sometimes used improperly to denote only systematic error (see "Bias" below). Because "true" values don't necessarily apply to toxicity testing, this term is not typically applied.

Batch- A set of consecutive determinations (analyses) made without interruption; a "run". Results are usually calculated from the same calibration curve or factor.

Bias- That part of inaccuracy of analytical results caused by systematic error.

Blank- An analysis made by the same procedure as a sample, but intended not to contain the analyte.

Calibration- Standardization of a measurement or instrument by use of another standard or instrument to adjust any variance in accuracy. The concentrations of the calibration standards should bracket the expected concentration of the test materials.

Calibration Curve- The graphical relationship between the known values, such as concentrations, of a series of calibration standards and their instrument response.

Calibration Standard- A substance or reference material used to calibrate an instrument.

Certified Reference Material (CRM)- A reference material one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body.

Chain of Custody (COC) Form- Record that documents the possession of the samples from the time of collection to receipt in the laboratory. The record generally includes: number and types of containers; mode of collection; collector; time of collection; preservation (if any); and requested analyses.

Coefficient of Variation (CV)**-** A standard statistical measure of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean. It is also called the relative standard deviation (RSD). The CV can be used as a measure of the precision within and between laboratories, or among replicates for each treatment concentration (EPA, 2000).

Control Chart- A cumulative summary chart of results from QA tests with reference materials (e.g. reference toxicants). The results of a given QA test are compared to the control chart mean value and acceptance limits (typically 95% confidence limits, i.e. mean + 2 standard deviations) or warning limits (typically 99% confidence limits, i.e. mean + 3 standard deviations).

Corrective Action- The action taken to eliminate the causes of an existing nonconformity, defect, or other undesirable situation in order to prevent recurrence.

Data Quality Objectives (DQOs)- A statement of the overall level of uncertainty that a decision maker is willing to accept in results derived from environmental data. This is qualitatively distinct from quality measurements such as precision, bias, and detection limit.

Data Validation- The process of evaluating the available data against the project DQOs to determine to what degree the objectives were met.

Detection Limit- The lowest concentration or amount of the target analyte that can be identified, measured, and reported with confidence that the analyte concentration is not a false positive value.

Effect Concentration (EC)- A point estimate of the toxicant concentration that would cause an observable adverse effect (e.g., death, immobilization, or serious incapacitation) in a given percent of the test organisms, calculated from a continuous model (e.g., Probit Model). EC25 is a point estimate of the toxicant concentration that would cause an observable adverse effect in 25 percent of the organisms (EPA, 2000).

False negative- A determination that a material is nontoxic when it is in fact toxic.

False positive- A determination of toxicity when the material is in fact nontoxic.

Holding Times- The maximum times that samples may be held prior to analysis and still be considered valid or not compromised.

Hypothesis Testing- A statistical technique (e.g. Dunnett's test) for determining whether a tested concentration is statistically different from the control. Endpoints determined from hypothesis testing are NOEC and LOEC. The two hypotheses commonly tested in WET are: Null Hypothesis (Ho)- The effluent is not toxic. Alternate hypothesis (Ha)- The effluent is toxic (EPA, 2000).

Inhibition Concentration (IC)- A point estimate of the toxicant concentration that would cause a given percent reduction in a non-lethal biological measurement (e.g., reproduction or growth), calculated from a continuous model (e.g., Interpolation Method). IC25 is a point estimate of the toxicant concentration that would cause a 25-percent reduction in a non-lethal biological measurement (EPA, 2000).

LC50 (lethal concentration, 50 percent)- The toxicant or effluent concentration that would cause death in 50% of the test organisms (EPA, 2000). The concentration is calculated from the data set using statistical or graphical models. The lower the LC50, the more toxic the chemical or effluent sample. Other LC values, e.g. the LC90 or LC5 may also be calculated to determine concentrations causing more or less mortality to the population. Note: The LC value must always be associated with the duration of exposure. Thus a 48-h LC50, 96-h LC50, etc. is calculated.

LOEC (Lowest-observed-effect-concentration)- The lowest concentration of an effluent or toxicant that results in adverse effects on the test organisms (i.e., where the values for the observed endpoints are statistically different from the control) (EPA, 2000).

Negative Control- A negative control is a part of an experiment where the experimental conditions are identical to the regular experiment except the substance being tested in not present.

NOEC (No-observed-effect-concentration)- The highest concentration of an effluent or toxicant that causes no observable adverse effects on the test organisms (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically different from the control) (EPA, 2000).

NPDES (**National Pollutant Discharge Elimination System)**- Created under the Clean Water Act. The permitting system under which point source discharges are regulated to eliminate or minimize the discharge of toxicants into surface waters. States frequently oversee their own programs which must comply with (i.e. be equally or more stringent) the national permit program.

Precision- 1) A qualitative term used to denote the scatter of results. Precision is said to improve as the scatter among results becomes smaller. Precision is usually measured as standard deviation (SD), coefficient of variation (CV), or relative percent difference (RPD). 2) A measure of the reproducibility within a data set. Precision can be measured both within a laboratory and between laboratories using the same test method and toxicant (EPA, 2000).

Quality Assurance (QA)- An integrated system of activities involving planning, quality control, quality assessment, reporting and quality improvement to ensure that a product or service meets defined standards of quality with a stated level of confidence.

Quality Control (QC)- The overall system of technical activities whose purpose is to measure and control the quality of a product or service so that it meets the need of users.

Reagent Water- Water suitable for use in making up critical reagents or for use in sensitive analytical procedures.

Reference Toxicant Test- Reference toxicants are routinely tested to demonstrate the continuing ability of the laboratory to successfully perform the tests and to evaluate the overall health and sensitivity of the test organisms over time. The coefficient of variation (CV) for the test LC50s (acute tests) or IC25s (chronic tests) provides a measure of test repeatability or precision; the lower the CV value, the less variable the results and the lower the frequency of false positive and false negative results. Individual reference test results are compared to control charts to determine acceptability.

Replicate- Each of several experimental units that are tested simultaneously using the same experimental conditions (ASTM, 2002).

Standard Curve- A plot of concentration of known analyte standards versus the instrument response to the analyte.

Toxicity Test- A procedure to determine the toxicity of a chemical or an effluent using living organisms. A toxicity test measures the degree of effect of a specific chemical or effluent on exposed test organisms (EPA, 2000).

*Unless otherwise noted, definitions were taken from EPA SW-846 Revision 1, July 1992 and Washington Department of Ecology Model Quality Assurance Model http://www.epa.gov/epaoswer/hazwaste/test/pdfs/chap1.pdf.

3.2 DATA ACCURACY, PRECISION, COMPLETENESS, REPRESENTATIVENESS, AND COMPARABILITY

1) Precision

Toxicity test precision is determined by comparison of a) the variation among laboratory replicates of individual samples, and b) reference toxicant tests. Depending on the study objectives and the test method, replication will vary. Typically, three to five replicates are analyzed for each sample/test concentration. Standard deviations or CVs can be compared with results from other studies to estimate specific test precision. Reference toxicant tests are useful because they can both verify the technical quality of the testing facility as well as the sensitivity of the test organisms. Because toxicity of reference toxicants is assumed to be constant, their use is a good indicator of precision. Resulting LC50s are plotted on control charts indicating current and past performance. If test results fall within 2 standard deviations of the running mean, the test is generally considered acceptable. Calculated CVs of the LC50 should also not exceed the 75th percentile of CVs reported nationally as reported in EPA (2000). See Section 8.2 for more information on the use of reference toxicants.

2) Accuracy

Because there is no "true" or "correct" response against which to compare toxicity test results, accuracy cannot be determined. Therefore, data quality objectives to test accuracy of toxicity tests are not available. Water quality measurements, however, are assessed for accuracy by comparing measured values of standards against known values. If they differ by more than 10% for dissolved oxygen, pH, or salinity, or by more than 30% for ammonia, corrective action will be taken.

3) Completeness

It is anticipated that all samples received will be tested. There are several factors that may affect the successful completion of testing including: a) acceptable negative control response; b) acceptable reference toxicant (positive control) tests; c) acceptable test condition variability; and d) test organism availability. For these reasons, it is important that enough sample volume be collected in case retesting is necessary. A test failure rate of approximately 20% is estimated, but with retesting a completeness rate of 95% is expected. Sample holding time may become an issue in the case of retesting, and needs to be considered by the project planner.

4) Representativeness

A number of factors determine the degree to which toxicity tests represent actual effects of typical effluent discharges. These include sampling design, sample

handling, test species and endpoint used, and exposure time. Careful consideration of how samples and what kinds of samples (e.g. grab vs. composite) are collected, therefore, is important.

5) Comparability

The use of standardized testing allows for comparability among laboratories. The use of negative controls and reference toxicant tests can be used to compare test results with other studies. Splits of samples can also be used to compare the Bioassay Lab's performance with other laboratories. Comparability should take into account variables such as species, test conditions (e.g. pH, temperature, salinity, dissolved oxygen), dilution factor, test endpoint, reference toxicant, and dilution water characteristics.

4.0 SAMPLE AND TEST ORGANISM HANDLING

4.1 RECEIVING SAMPLES

1) Upon arrival, samples should be handled with the utmost care. Samples should be received in a well-ventilated area in case leakage has occurred during shipping.

2) A **chain of custody form should be completed** and a copy given to the person who delivered the sample. The chain of custody form should contain the following information:

- Sample ID
- Sample description/quantity
- Date received
- Date collected
- Sample collector
- Location of delivery
- Analyses requirements for each sample
- Date and signatures of the delivery person and receiver

3) The **sample should be logged in** and given an identification number. The number given should be the next consecutive number on the list for SSC-Pacific Sample IDs. The following information should also be recorded in the logbook:

- Time/date that the sample was collected.
- Time/date that the sample was received.
- Temperature of sample during collection and upon arrival at lab.
- Company or organization the sample came from.
- Type of analyses that will be performed on the sample.
- Description of sample (volume, type [seawater, freshwater, sediment], preserved or frozen, compounds known or suspected to contain).
- Initials of the person who received the sample.

- Location sample was stored upon arrival.

4) Dilution or laboratory water such as Scripps Institution of Oceanography (SIO) seawater is collected at the Scripps pier in La Jolla, California by a laboratory technician. Dilution or laboratory water is logged into the "Seawater Collection Log" upon arrival and is stored in a clean carboy or a suitable HDPE container for a maximum of 14 days.

4.2 HOLDING SAMPLES

If samples are not immediately prepared for testing, they are stored at a target temperature of **4 °C** in the cold room (Bldg.111, Rm. 124) until used. Temperature of the cold room is logged daily during sample holding and should at no time fall outside the range of **0-6 °C** (USEPA 2002). Every effort should be made to initiate testing with effluent sample on the arrival day, and effluent sample-holding time should not exceed **36h**. Sediment samples must be tested within **14 days**.

4.3 SAMPLE HANDLING AND CHAIN OF CUSTODY

I. **OBJECTIVE:** Methods for tracing and transfer of samples ensure the integrity from time of collection to sample disposal. Custody of samples is defined as either having physical possession, being in a person's view after taking possession, security from tampering or holding in a place restricted to authorized personnel.

II. **METHODS**

A. Transferring Custody

1. Records shall be kept in permanent ink on a chain of custody form for receiving samples.

2. Chain of custody forms should always travel with test organisms or samples.

3. Upon arrival of samples, examine containers to detect any damage or tampering.

4. If containers are damaged, it should be noted on the chain of custody form.

5. Note the date and time on the form and sign.

B. Subdividing Samples

If samples need to be sub-divided and sent to other laboratories, this should be noted on the original chain of custody form and a new chain of custody form should be made.

C. Sample Disposal

Indicate disposal of samples, which terminates the chain of custody.

Copies of chain of custody forms shall be kept in the laboratory or with all corresponding project data.

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PACIFIC

ENVIRONMENTAL SCIENCES AND
APPLIED SYSTEMS BRANCH, CODE 71750
53605 HULL STREET
San Diego, CA 92152-5000

CHAIN OF CUSTODY RECORD

Date _____
Page _____ of _____

Project Title / Project Number:			Project Leader:
Remarks / Air Bill:			Contact:
Sampler(s): (Signature)			Contact Tel:
Tel:	Fax:	E-mail:	Requested Analyses
Special Instructions:			

Field Sample Identification	Sampling Temp. (°C)	Sampling Date	Sampling Time	Matrix													

Relinquished by: (Signature)	Received by: (Signature)	Date:	Time:
Relinquished by: (Signature)	Received by: (Signature)	Date:	Time:

4.4 ORGANISM ARRIVAL LOG

ORGANISM ARRIVAL LOG

Batch ID	Date Received	Received From	Project Title	Species	Age when shipped	Number Ordered	Organism Condition (e.g. number dead)	Initial Water Quality				Storage Location	Analyst Initials
								pH	D.O.	Temp.	Salinity		

Species

A.a. - Atherinops affinis

A.b.- Americamysis bahia

C.g. - Crassostrea gigas

C.h. - Ceratocorys horrida

M.g. - Mytilus galloprovincialis

R.a.- Rhepoxinius abronius

S.p. - Strongylocentrotus purpuratus

E.e. - Eohaustorius esturaius

M.b. - Menidia beryllina

Other: _____

4.5 SAMPLE COLLECTION RECORD

SAMPLE COLLECTION RECORD

SSC Sample ID (correspond w/ Toxcalc ID)	Sample Name	Sampling Date		Sampling Time		Collection Temp.	Water Quality on Arrival				Sample Type (e.g. Grab/Comp.)	Received By (print name)	Company or Organization	Storage Location
		Begin	End	Begin	End		pH	D.O.	Temp.	sal.				

4.6 RECEIVING/HOLDING TEST ORGANISMS

With the exception of dinoflagellates, test organisms used by the Bioassay Lab are generally not cultured on the premises; therefore, they are purchased and shipped or hand delivered by outside vendors. The fastest method of shipment is always used to prevent unnecessary stress on the test organisms. Analysts are trained in the proper procedures for receiving and maintaining test organisms. Notes are recorded for all stages, from arrival in the lab to termination and disposal of unused organisms. See "Receiving and Holding Test Organisms" SOP for details. General considerations with respect to successful maintenance of test organisms are:

- Minimum shipping time
- Shipped in aerated containers
- Immediate assessment of water quality and organism health upon arrival
- Preparation and maintenance of high quality food supply
- Daily feeding
- Acclimation to test conditions at safe rate for species
- Regular water changes
- Water quality regularly monitored
- Organisms are not overcrowded in holding tanks
- Minimization of disturbances to prevent stress

Before disposal, any surviving test organisms are humanely killed, generally by concentrating into a container and freezing. Under no circumstances are test organisms ever released to the wild or used more than once for testing.

5.0 HAZARDOUS MATERIAL (HM) STORAGE, DISPOSAL AND SAFETY CONSIDERATIONS

A hazardous material (HM) is any material that, because of its quantity, concentration, physical or chemical characteristics, poses a present or potential health hazard to human health and safety or to the environment[1]. Staff members receive an initial hazardous materials training course as well as annual refresher trainer from SSC-Pacific. It is the responsibility of the laboratory director and analyst that all hazardous materials are acquired, handled, stored, and disposed of according to policy detailed in SSC- Pacific Document 4110.1. A detailed description of the policy is available in the "Hazardous Material Storage, Disposal and General Information" SOP. A summary is provided below:

5.1 PURCHASING HAZARDOUS MATERIALS

Purchasing instructions are provided in a handbook located in Rm 116. Upon receipt of new chemicals, the Safety Office is notified (x33873).

5.2 STORAGE AND MANAGEMENT OF HAZARDOUS MATERIALS

Prior to storage, all HM needs to be labeled with name, manufacturer, hazard, barcode label, and owner, whether in original or secondary containers. HM is inspected regularly to ensure absence of leakage and that labels are intact. A notebook in Rm 116 contains Material Safety Data Sheets (MSDS) for each chemical stored in the lab.

5.3 HANDLING HAZARDOUS MATERIALS (SAFETY)

Hazardous materials are grouped into different categories including: flammables, halogenated solvents, corrosives, toxics, compressed gases, oxidizers, water reactives, pyrophorics, and explosives. All staff members are trained in the characteristics of these materials, and the safety considerations associated with working with these materials. Engineering controls such as fume hoods are used to eliminate exposure. Personal protective equipment such as gloves, goggles, respirators, and aprons are also used where necessary. Safety equipment including first aid kits, fire extinguishers, and eye wash stations are located in each lab. Emergency showers are also located in designated areas of the building.

5.4 HAZARDOUS WASTE DISPOSAL

Hazardous waste is any discarded, excess or spilled material that is solid, liquid or gas and meets the definition of a hazardous material[1]. It is either **Characteristic** (toxic, reactive, ignitable or corrosive) or **Listed** (appears on a specific EPA or state list). The staff is trained in which wastes are permitted sewer discharges, and which are not. Questionable waste can be analyzed by contacting the Safety

and Environmental Office (x36363). Otherwise, proper labels and paperwork are filled out (HW Disposal Request Form, HW Profile Sheet, copy of MSDS) and the code's hazardous waste coordinator is notified so that it can be removed during the next scheduled pick up date. Forms are available in Hazardous Materials Information notebook or are accessible on line at: https://iweb.spawar.navy.mil/services/sti/publications/inst/forms/

5.5 HAZARDOUS MATERIALS SPILLS

All staff is trained in how to respond to HM spills. Larger spills deemed a potential danger to personnel result in area evacuation and a call to the fire department (9-911). Smaller, known spills are cleaned up using proper protective equipment and spill kit materials stored in each lab. All spills are reported to the Safety and Environmental Office (x35024).

6.0 CALIBRATION, USE, AND TROUBLESHOOTING OF INSTRUMENTATION

Calibration refers to the standardization of a measurement or instrument by use of another standard or instrument to adjust any variance in accuracy. Proper calibration of equipment prior to sample measurement is the responsibility of the analyst. Calibration procedures for laboratory instrumentation are covered in the standard operating procedures (SOPs) manual.

6.1 CALIBRATION OF BASIC LABORATORY INSTRUMENTATION

A) Spectrophotometers

The HACH spectrophotometers are put through a "self-test" each time they are turned on and generally do not require calibration. However, chemical standards certified by HACH are used to verify accuracy prior to measurement of samples. If necessary, a standard curve is constructed to make corrections to samples measurements.

B) Pipettes

Pipettes are routinely checked for accuracy, and calibrated only if necessary. Checking calibration is performed by repetitively weighing aliquots of distilled water at room temperature. After weight is converted to volume, the value is compared against permitted values outlined in the equipment manual. If outside the permitted range, pipettes are recalibrated with the enclosed service tool and rechecked.

C) Balances

Balances are calibrated by outside specialists on an annual basis. If a balance has been moved or if standard Class-S calibration weights indicate there is reason to suspect that the balance is not producing accurate measurements (e.g. values vary by more than 2% of calibration weight values), this is noted and alternate balances that are calibrated correctly are used until calibration can be performed.

D) Ion electrodes

Ammonia, conductivity/salinity, pH, and dissolved oxygen electrodes are calibrated each time they are used, and recalibrated as necessary during sample measurements depending on the specific method. Refer to the SOP for each piece of equipment for specifics on their calibration.

E) Thermometers

Thermometers are compared against an NIST certified thermometer on an annual basis. If temperature readings on thermometers vary by more than 0.5°C, a correction factor may be applied to the thermometer or the thermometer will no longer be used.

F) Temperature of cold room, incubators, freezers, lab

Temperature in the main laboratory, cold room, and both incubators are continuously monitored with max/min thermometers and/or HOBO temperature data loggers. For storage of samples, cold room temperature is maintained at 4(± 2)°C, while freezers are held at –20(± 10)°C. Temperature in incubators is dependent on the test being conducted. If temperature is outside the range, the thermostat is adjusted accordingly and the deviation recorded if samples or test organisms are suspected of having been affected.

G) Fume Hood

The fume hoods in all labs are measured annually for adequate air-flow by an industrial hygienist provided by the Safety Office (POC: Gary Douglas, x35026).

6.2 LABORATORY STANDARDS

Analytical standards used for calibration and preparation of quality control samples shall be traceable to standard reference materials. Reference toxicant stock solutions are created from reagent grade chemicals, which are analyzed in-house by stabilized temperature graphite furnace atomic absorption (STGFAA) spectroscopy by direct injection. The standard reference material (SRM) CASS4 (coastal seawater) from the National Research Council of Canada is used to quantify the recovery of any preconcentration, and SRM 1643d (trace metals in water) of the National Institute of Standards & Technology is used to evaluate the precision and accuracy of the STGFAA analysis. These measurements are done by injections in triplicate for each sample, with relative standard deviation in the absorbance measured of less than 10%. Accuracy better than 15% is required for the SRM 1643d. An efficiency of ± 10% (90% to 110%) in the recovery of CASS4 is required in the case of samples that are preconcentrated following the APDC/DDDC liquid/liquid procedure. The limit of detection is determined as three times the standard deviation of the concentrations measured in blanks. Metal stock solutions are prepared in E-Pure water and stored in the dark at 4°C in polycarbonate bottles to minimize binding to wall surfaces.

7.0 CLEANING GLASSWARE/PLASTICWARE

In general, containers used to hold effluent samples are not reused because they could carry over adsorbed toxicants from one test to another. Non-disposable sample containers, test vessels, tanks, and other equipment that comes into contact with effluent are washed according to EPA protocol (EPA 1993). New plasticware or glassware not previously used by the lab is first tested to ensure that no toxic effects are associated with the container. After an absence of toxicity has been established, new plasticware is rinsed with dilution water prior to its first use, while all new glassware must be soaked in 10% acid and rinsed well with deionized and dilution water prior to its first use. A brief description of the cleaning procedure used for non-disposable containers after exposure to effluent is provided below. More details are provided in the SOP Manual.

General cleaning procedures:

1. Rinse with tap water several times.
2. Soak in tap water and 10% Liquinox or other detergent for at least 15 minutes.
3. Rinse in tap water several times.
4. Rinse in 10% Nitric (HNO_3) acid to remove scales, metals, and bases.
5. Rinse several times in deionized water.
6. Rinse once with pesticide grade acetone in fume hood.
7. Rinse three times with deionized water.

8.0 QUALITY CONTROL SAMPLES

One method for assessment of the quality of bioassay results is the evaluation of performance for quality control (QC) samples. QC samples used by the Bioassay Laboratory include negative controls and reference toxicant tests (positive controls).

8.1 NEGATIVE CONTROLS

A negative control is a part of an experiment where the experimental conditions are identical to the regular experiment except the substance being tested is not present. Negative controls are suggestive of test organism health and/or laboratory quality and are used to assess if apparent effects in experimental treatments are real. Performance of negative controls is also used to determine test acceptability as dictated by individual test methods.

Negative controls typically consist of dilution water (e.g. deionized water or filtered, natural seawater). If an experiment calls for natural seawater, it is collected in a clean carboy from the Scripps Institution of Oceanography pier in La Jolla, California. Seawater is obtained a day or two before the test and discarded no later than 14 days after collection. Depending on the objectives of the test, there may be other types of negative controls including solvent controls, synthetic salt controls, or hypersaline brine (HSB) controls. The analyst is responsible for determining which control(s) is/are relevant to a test.

8.2 REFERENCE TOXICANT TESTS

Reference toxicant tests are a means of assessing test precision. These tests are conducted concurrently with effluent samples, and employ a known toxicant known as a reference toxicant. The reference toxicant is copper for most test species used at SSC-SD. By exposing different batches of the test organism to the same concentrations of the reference toxicant in the same dilution water, under identical testing conditions, the lab can assess repeatability via comparison of LC50 or EC50 values for a given species. Values are plotted on a control chart to monitor the lab's performance over time. In general, reference toxicant test results that fall within two standard deviations above or below the running mean are an indication of acceptable performance. In addition to the mean and standard deviation, the coefficient of variation (CV) may also be used to demonstrate the lab's precision. Actual tested concentrations in reference toxicant tests are dependent on the test method due to differences in sensitivity among species and endpoints.

9.0 PREVENTIVE MAINTENANCE PROCEDURES FOR LABORATORY EQUIPMENT AND CHEMICALS

9.1 PREVENTIVE MAINTENANCE FOR EQUIPMENT

A preventive maintenance program for equipment increases laboratory efficiency, reduces the potential for inferior quality test results, and prolongs the life of essential laboratory tools. Analysts are trained in the proper use of all laboratory equipment and how to troubleshoot problems associated with their normal function. Equipment operating manuals are stored in a drawer labeled "Equipment Manuals" in Rm. 116 for easy reference. Standard Operating Procedures including troubleshooting guides are also available for commonly used equipment. When repairs required are beyond the capability of the analyst, outside vendors are contacted for repair. A maintenance record for equipment is kept in the lab. Information included in the maintenance record is provided on the following page.

9.1 Preventive Maintenance for Equipment

Equipment Description	Storage Requirements	General Maintenance	Frequency
Accumet pH/ion/conductivity meter model 50	Benchtop	Clean case with a mild detergent and damp cloth. Never use solvent.	As needed
Accumet pH Probe	Between measurements: D.I. water Overnight: pH 4.0 buffer Long-term: cotton cap over electrode	Required when troubleshooting probe.	As needed
Orion Dissolved Oxygen Meter model 840 and probe	Between measurements: put probe in sleeve moistened with D.I. water Overnight: turn meter off Long-term: remove membrane and replace when returned to service	Soak probe in silver anode cleaning solution, refill electrolyte solution, and replace membrane cap.	6 months or As needed
Orion Portable Dissolved Oxygen Meter model 830A and probe	Between measurements: D.I. water Overnight: turn meter off Long-term: remove membrane and replace when returned to service	Clean external surfaces with water and a mild detergent. Never wipe the meter with a dry cloth. Batteries – alkaline AA.	As needed
Orion Conductivity / Salinity Meter model 105A+	Between measurements: D.I. water or seawater Overnight or Long-term: clean and dry	Clean case and touchscreen with a damp cotton cloth. Do not use strong solvents. Batteries - 9 volt. Always recalibrate after battery change.	As needed
Orion 720A meter	Between measurements: 10ppm standard with ISA Overnight: place electrode tip in a 1000ppm standard without ISA Long-term: disassemble completely and rinse with D.I. water and dry	Send to Orion Technical Service for repairs.	As needed
Orion Ammonia Probe model 95-12	Between measurements: 0.1M ammonia standard Long-term: disassemble and place in storage box	Check membrane. Refill internal filling solution.	As needed

9.1 (cont.) Preventive Maintenance for Equipment

Equipment Description	Storage Requirements	General Maintenance	Frequency
Hach DR 2400 Spectrophotometer	Benchtop	Clean case and touchscreen with a damp cotton cloth. Do not use strong solvents. Batteries - 3 D-cell. Send to HACH for recertification to maintain accuracy in measurement. Troubleshooting – www.hach.com for latest information.	As needed

9.2 PREVENTIVE MAINTENANCE FOR CHEMICALS

Chemicals for laboratory functions, such as glassware cleaning (acid water baths) and reference toxicant testing (metal stock solutions), need to be properly made, maintained, and disposed of to ensure high quality test results. Analysts are trained in how to safely and accurately prepare chemical solutions. All containers holding chemicals are labeled with the contents and the date prepared. The following chemicals are tracked on a log sheet:

Reagent/ Solution	Storage
Nitric Acid bath - 10%	20 L HDPE container near rear sink
Concentrated Nitric Acid	Corrosives locker
Copper (sulfate) – 1 ppt stock	500 mL polycarbonate container in refrigerator at 4 ºC
Reagent Grade $CuSO_4 \bullet 5H_2O$ crystals	Original bottle/Room Temp
Zinc (sulfate) – 1 ppt stock	250 mL polycarbonate container in refrigerator at 4 ºC
Reagent Grade $ZnSO_4 \bullet 7H_2O$	Original bottle/Room Temp
pH Buffers	Original bottles/Room Temp
0.1 N Ammonia Standard	Original bottle/Room Temp
Ammonia ISA Solution	Original bottle/Room Temp

10.0 ACQUISITION, REDUCTION AND REPORTING OF DATA

10.1 ACQUISITION

At the beginning of a test, a test identification number is assigned in the "Test ID" log stored in Rm 116 for easy cross-referencing. The Test ID is used on all other applicable data sheets. Raw data is recorded in non-erasable ink on computer-derived data sheets. Notes are taken on computer-derived note sheets. At a minimum, all data and note sheets require the date, Sample ID, Test ID, analyst or operator, and species/method identification or water quality parameter. Standard units as defined below are always used to ensure consistency. Raw data and note sheets are stored in a notebook in the Bioassay Laboratory. Copies are made for attachment in reports or other uses as required. Details with respect to entry of data onto data sheets are provided in the SOP Manual.

Parameter	Standard Unit
pH	pH units
Salinity	ppt or ‰
Temperature	° C
Dissolved Oxygen	mg/L
Total Ammonia	mg/L
Unionized Ammonia	mg/L

10.2 DATA REDUCTION AND REPORTING

Data reduction is the process of transforming raw data into reportable material. Mathematical manipulation and summary statistics are generated by means of computer programs and laboratory equipment that perform these functions.

Once all measurements have been recorded on data sheets, data is manually entered into spreadsheets or statistical programs. All phases of data transfer are double checked. When computer programs are used to derive calculations, individual calculations are performed by hand at random. Summaries and results are compared with raw data entries to assure accurate data entry. Any suspect data is reported.

Depending on the objectives of the study, toxicity data are statistically analyzed using a variety of tools, including ToxCalc 5.0, a software package designed specifically for whole effluent toxicity test data. The staff is trained in the proper use of ToxCalc to derive NOECs and LOECs using hypothesis testing and LC50/EC50 values using point estimation techniques. The software is designed to be in accordance with EPA recommend procedures for the analysis of whole effluent toxicity data. ToxCalc summary sheets are saved on the computer in Rm 116 and printed out and placed into a folder designated for the specific study for future reference. The "Statistical Analysis of Data" SOP provides a detailed description of data analysis procedures for the staff to follow.

11.0 REPLICATION AND TEST SENSITIVITY

The sensitivity of toxicity tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant the sensitivity of the test will increase as the number of replicates is increased. Minimum numbers of replicates are dictated by the individual protocol. The actual numbers will depend on the objectives of the test and the statistical method used for analysis of the data. For example, 20 fish per concentration are generally considered optimal for Probit analysis. This typically equates to 4 replicates of 5 fish each. The Bioassay Laboratory meets minimum requirements for replication at all times. The actual number of replicates used is typically a function of costs and project goals, and may be discussed with the project manager/sponsor/client.

12.0 CORRECTIVE ACTION

12.1 DETERMINING THE PROBLEM

Since each situation is unique regarding resolution of erroneous data, the following guidelines are used in a manner that best suits the existing circumstance. The analyst and laboratory director generally work on this together. Documentation of the troubleshooting techniques used is required to allow follow up on the situation.

1. Compare results from the reference toxicant test to control chart values to assess quality of test results. In general, if LC50 values fall within 2 standard deviations of the mean, they are acceptable.

2. Compare the control (e.g. negative, solvent, salt, brine control) response to test acceptability requirements as dictated by the SOP for the method.

3. Double-check all calculations (e.g. those for making reference toxicant stock solutions and sub-stock solutions, dilutions and test organism counts).

4. Verify quality of the reference toxicant solutions or samples (e.g. were they correctly made, did they violate holding time, are water quality parameters within range tolerated by test organisms?).

5. Check all water quality parameters (pH, salinity, dissolved oxygen, ammonia, temperature).

6. If there are any other tests being run via an analogous procedure, then compare results. This may allow the analyst to exclude certain factors.

7. Assure properly treated/cleaned glassware was used in all phases of the test set up and testing.

8. Re-read all notes taken during testing to determine if there are any conditions in the laboratory that may conflict with proper testing procedures (light sources, temperature, debris in glassware, incorrect calibration of equipment, etc.).

9. Review scientific journals of relevance that may offer possible cause or resolution.

10. If the erroneous data is being observed during data analysis, verify all entries and confirm all calculations by hand.

12.2 RESOLUTION

If the source of error is determined, appropriate action to resolve issues (e.g. repeat test, note and remove erroneous data, re-calibrate equipment, etc.) is taken. Detailed accounts of the corrective action are made for future reference. Although some data may be considered "unacceptable" by not meeting DQOs, the data may still be potentially "useful" to the study. Data should be evaluated for its usefulness on a case-by-case basis.

If the source of error is not determined, follow up testing or other measures may be required. The approach may be discussed with the principal investigator or project sponsor.

13.0 AUDITS AND QUALITY ASSURANCE REPORTS

13.1 INTERNAL AUDITS

The laboratory director and analyst meet regularly to discuss status, required changes, and proper execution of the Bioassay Lab's QA/QC program. Any deficiencies in the program are documented and corrective action is taken towards remediation of the problem(s). The following topics are typically covered in the review of the QA program to ensure that:

- QA Manual and SOPs are up to date
- Equipment and Facilities are functioning properly
- Equipment is being calibrated correctly
- Reagents/standards/solvents are available and not expired
- Samples are being handled properly
- QC measures are being applied correctly
- QC records (e.g. control charts) indicate lab is in control
- Data is being properly managed and reported
- Staff is receiving appropriate training

13.2 EXTERNAL AUDITS

To date, the Bioassay Lab has not been subjected to external audits. The laboratory, however, agrees to comply and assist with future external audits that may be required.

13.3 QUALITY ASSURANCE REPORTS

The laboratory director and analyst meet on a regular basis to discuss results of internal audits and evaluate the status of the quality assurance program. A report documenting issues associated with any of the below topics is compiled as needed, but on a quarterly basis at a minimum, depending on the level of testing taking place in the lab. Reports are available to the Bioassay Lab staff as well as relevant principal investigators, Environmental Sciences and Applied Systems (71750) Branch Head, and Advanced Systems and Applied Sciences (717) Division Head. Areas covered in the quality assurance report may include:

- Audit findings
- Certification status
- Problematic data and effectiveness of corrective action taken
- Modification/addition of SOPs
- Personnel and instrumentation changes
- Training courses held/attended
- Status of new methods evaluated/new lab capabilities

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I. WATER SAMPLES

General Methodology

Water samples for copper analysis were collected in 30-mL acid-cleaned low-density polyethylene bottles, which were acidified to pH ≤2 with quartz still-grade nitric acid (Q-HNO_3) in a High Efficiency Particle Air (HEPA) class-100 all polypropylene working area. Copper concentrations were measured with a Perkin-Elmer SCIEX ELAN DRC II inductively coupled plasma with detection by mass spectrometry (ICP-MS; USEPA, 1994). If deemed necessary, samples were diluted with 0.1 N Q-HNO_3 made up in high-purity (18 MΩ cm^{-1}) water in order to minimize matrix related interferences inherent to seawater. The samples were injected directly into the ICP-MS via a Perkin-Elmer Autosampler 100. Analytical standards were made with Perkin-Elmer multi-element standard solution (PEMES-3) diluted in 1N Q-HNO_3, which was matrix matched to the salinity of the test samples. Standards were analyzed at the beginning and end of the run. The analysis also included measurement of the Standard Reference Material (SRM) 1643e from the National Institute of Standards & Technology (NIST), and analytical blanks made up of 1N Q-HNO_3 after every five samples. A coefficient of variation (CV) of ≤5% for replicate measurements will be observed, as well as a recovery within 15% of SRM 1643e.

II. SEDIMENT SAMPLES

Sediment Digestion

Adapted from:

David Strom, Stuart L. Simpson, Graeme E. Batley, and Diane F. Jolley. 2011. The influence of sediment particle size and organic carbon on toxicity of copper to benthic invertebrates in oxic/sub-oxic surface sediments. *Environmental Toxicology and Chemistry* **30**(7): 1599-1610.

- Weigh a pre-labeled, pre-dried 125 mL LDPE bottle with cap and record the **BOTTLE tare mass (g).**
- Include at least six (6) blanks of a sample bottle with no sediment that will go thru all the treatments of a regular sample, and the three (3) Standard Reference Materials (SRMs), PACS-1, BCSS-1 and NIST 2709, each in triplicate (3X).
- Pour 0.20 ± 0.05 g dry sediment sample in the bottle. In case of using wet sediment, then pour 2.0 ± 0.05 g wet sediment.
- Weigh about 0.25 ± 0.05 g of each SRMs in each of three (3) separate bottles.
- Set bottle with sediment in an oven at 60°C for at least 24 hours. Take the cap out from the bottle and set it down-side up by the bottle. NOTE: make sure the sediment is completely dry before weighing and proceeding to add any acid.
- Set bottle with dry sediment in a desiccator to cool down to room temperature.

- Weigh and record the **BOTTLE plus DRY SEDIMENT mass (g)**
- Add 1.0 mL of concentrated trace metal grade (TMG) hydrochloric acid (HCl).
- Add 0.5 mL of concentrated TMG nitric acid (HNO_3).
- Allow the sample to digest at room temperature for 24 hours with loose cap.
- Warm up on hot plate in clean bench for 1 hour with loose cap. Hot plates are set to warm up to a temperature that does not melt the bottle
- Alternatively: Microwave 2 times for 20 minutes at 100 W with loose cap
- Add 1N HNO_3 TMG to neck of bottle, about 130 g, and weigh and record **BOTTLE plus DIGESTATE final mass (g)**
- Allow particles to settle down and pipette volume required of digestate from the overlying water
- Alternatively: Filter thru 0.45 µm pore-size and pipette from filtered solution

Make up the appropriate dilution (e.g., 25, 50 or 100 µL of digestate to 15 mL or so) in an 15 mL ICP-MS test tube with 1N quartz-still grade HNO_3 (Q-HNO_3) for analysis using methodology described above.

III. CLEAN ROOM TECHNIQUES

The clean room is located in Building 111 Room 242

To turn on Epure System

1. Turn on the red valve located to the left of the Barnstead filter unit. The valve is on when it is parallel with the pipe.
2. Turn the switch located on the Barnstead unit on.
3. Allow digital read-out to reach 18.0 mega-ohm before using milli-Q (MQ) water.
4. Retrieve a lab coat and booties from adjacent room if there aren't any in the clean room already.
5. Place a bootie over one shoe at a time while stepping onto sticky pad at the entrance of room. Never let shoes touch the sticky pad without booties. This pad serves to remove any dust particles that have gotten on the booties.
6. Once inside the room, close the door, put on lab coat and affix all buttons.
7. Put on nylon gloves.
8. Put on one pair of plastic gloves without touching anything above the wrist area of the glove.
9. Put on a second pair of plastic gloves without touching anything above the wrist area on the glove. Consider the first pair of plastic gloves contaminated and do not touch the second pair anywhere above the wrist.
10. Gloves can be tightened over fingers by overlapping fingers. Hand should be kept in this position when not in use to avoid contamination. See figure 1.

Figure 1.

11. Do not touch the walls or the inside of the hood glass with hands.
12. Using the spicket, rinse gloves with MQ water, and then rinse the spicket itself.
13. Rinse grating inside hood to remove any settled dust particles.

Bringing an item into the Clean room or Hood

1. When bringing new items into the clean room, place in container on the floor until use.
2. When bringing item into hood, rinse the outside entirely three to four times with MQ water from the spicket.
3. Next, rinse the inside entirely three to four times with MQ water from the spicket.
4. Water will be exiting into a bucket, be sure not to overflow.
5. Keep item near grating until rinsed thoroughly.

After use of clean room and hood

1. Rinse area down with spicket.
2. Use kimwipe to absorb water; do not repeat exposure of kimwipe surface after touching another.

Filtering Samples

1. Loosen all caps from samples
2. Bring in Container and label it (acid washed scint vial) to place filtered sample in.
3. You should have the following items; 5% nitric acid in small container, 2 MQ water containers, disposal container, cup with syringes, and filters.
4. Fill syringe with 5% nitric acid and place back into cup.
5. Empty acid into waste container from syringe
6. Semirinse with MQ water
7. Swirl sample and take 5 ml through plastic tip on syringe

8. Remove plastic tip from syringe within sample allowing it to drain the tip into the original sample bottle. replace tip with a clean filter
9. Dispose of the first 5-10 drops
10. Place remaining sample (about 5ml) in the labeled clean scint vial in hood.
11. If there is any sample remaining, place into the disposal container
12. Use additional MQ container to rinse syringe 2-3 times
13. Clean with nitric acid by allowing it to sit in syringe.
14. Rinse once with MQ water.

Begin with the lowest concentration and filter in order to the highest conc.