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Final Report: Interlaboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Vol. 2: Appendix

LIST OF APPENDICES

STUDY PLAN FOR DETERMINING INTERLABORATORY VARIABILITY OF THE EPA SHORT-TERM CHRONIC AND ACUTE WHOLE EFFLUENT TOXICITY TEST METHODS

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SECTION 1: INTRODUCTION AND BACKGROUND

The Clean Water Act (CWA) requires the U.S. Environmental Protection Agency (EPA) to promulgate guidelines establishing test procedures for data gathering and compliance monitoring under the National Pollutant Discharge Elimination System (NPDES). Test procedures are specified at 40 CFR Part 136. On October 16, 1995, EPA promulgated a final rule approving the use of seventeen whole effluent toxicity (WET) test methods to protect aquatic life in NPDES compliance monitoring (60 FR 53529). Whole effluent toxicity is defined as the aggregate toxic effect of an effluent or receiving water measured directly as an organism response in a toxicity test. The Agency-approved WET test methods are listed in 40 CFR §136.3, Table IA. These WET test procedures employ a suite of standardized freshwater, marine and estuarine plants, invertebrates, and vertebrates to measure acute and short-term chronic toxicity. The EPA-approved WET methods resulted from many years of development and testing by EPA, States, municipalities, academia, and the regulated community. As part of a settlement agreement to resolve a judicial challenge to the WET methods rule, EPA will conduct the WET Interlaboratory Variability Study (hereinafter referred to as the "WET Study").

Twelve of the seventeen promulgated WET methods will be evaluated in the WET study. These include five acute and seven short-term chronic WET methods. The study will be implemented in three rounds. Freshwater tests will be conducted in Round 1, and marine tests will be conducted in Rounds 2 and 3. The WET methods and the round in which they will be performed in the WET Study are listed in Table 1. Table 2 identifies the test duration and test endpoints for the five acute and seven short-term chronic methods included in the WET Study.

The WET Study was designed to quantify the interlaboratory variability of the 12 WET test methods. This will be accomplished through (at a minimum) the determination of the coefficient of variation (CV) for the LC_{50} and IC_{25} endpoints and the range of values for the NOEC endpoints for each method in the study. Other measurements of method variability such as ASTM's h and k statistics also may be used to quantify interlaboratory variability. The study was designed to provide data on the rate at which participating laboratories successfully complete tests initiated (test completion rate) and the rate at which the tests indicate the presence of toxicity when measuring non-toxic samples (false positive rate).

The general design of the WET Study is as follows:

- A total of 12 WET methods (5 freshwater methods and 7 marine methods) will be conducted (See Tables 1 and 2).

- A minimum of 9 and a maximum of 20 participant laboratories (that meet prequalification requirements) will be selected to perform each WET test method. This will constitute the "base" study design. Additional laboratories (above 20) may participate on a more limited basis as part of an "extended" study design (see Section 4.1.3).

- Referee laboratories will conduct WET tests for each method during preliminary testing and simultaneously with participant laboratories during interlaboratory testing. Preliminary testing will document sample characteristics and consistency, and referee laboratory results during interlaboratory testing will provide further information on sample consistency and may be pooled with participant laboratory data in the evaluation of interlaboratory method variability.

- For each method, laboratories participating in the base study design will conduct WET tests with four blind test samples. A "test sample" is a single bulk sample preparation (i.e, matrix, recipe) that is divided and distributed by the referee laboratory to participant laboratories for the conduct of a given test. Aliquots of the bulk sample will be shipped to the participant laboratories as

whole volume (volume necessary to conduct the test) or ampules (to mix and dilute to required volume) for test initiation and test renewals (if necessary).

- Laboratories that are participating in the extended study design will conduct WET tests with two or three blind test samples received as ampules.

- Test samples received by participant laboratories will include some combination of the following test sample types: reference toxicants, industrial and/or municipal wastewater effluents, ambient receiving water, and method "blanks"(i.e., moderately hard reagent water prepared as explained in the test method manuals).

- Replicate (i.e., duplicate) test samples will be included among the four blind test samples distributed to participant laboratories for each test method.

Table 1. WET Methods Included in the WET Interlaboratory Variability Study

Round 1 - Freshwater Tests
(1) Fathead Minnow, *Pimephales promelas*, Acute Test[1]
(2) Method 1000.0: Fathead Minnow, *Pimephales promelas,* Larval Survival and Growth Test[2]
(3) Cladoceran, *Ceriodaphnia dubia*, Acute Test[1]
(4) Method 1002.0: Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test[2]
(5) Method 1003.0: Green Alga, *Selenastrum capricornutum*, Growth Test[2]

Round 2 - Marine Tests
(1) Inland Silverside, *Menidia beryllina*, Acute Test[1]
(2) Method 1006.0: Inland Silverside, *Menidia beryllina,* Larval Survival and Growth Test[3]
(3) Mysid, *Holmesimysis costata*, Acute Test[1]
(4) Method 1009.0: Red Macroalga, *Champia parvula*, Reproduction Test[3]

Round 3 - Marine Tests
(1) Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test[1]
(2) Method 1004.0: Sheepshead Minnow, *Cyprinodon variegatus,* Larval Survival and Growth Test[3]
(3) Method 1007.0: Mysid, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test[3]

[1]USEPA, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, Fourth Edition, EPA-600-4-90-027F, August 1993
[2]USEPA, *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms*, Third Edition, EPA-600-4-91-002, July 1994
[3]USEPA, *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Marine and Estuarine Organisms*, Second Edition, EPA-600-4-91-003, July 1994
NOTE: EPA will conduct the WET Interlaboratory Variability Study using the specific test protocols promulgated at 40 CFR Part 136, including, as appropriate, reference to EPA guidance entitled "Clarifications Regarding Flexibility in 40 CFR Part 136 Whole Effluent Toxicity (WET) Test Methods" dated April 10, 1996 from Tudor T. Davies, EPA Office of Science and Technology to EPA Water Management Division Directors and EPA Environmental Services Division Directors. Additional corrections to the method manuals are included in the following document: USEPA, *Errata for Effluent and Receiving Water Toxicity Test Manuals: Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms; Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms; and Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms*, EPA-600/R-98/182, January 1999.

Table 2. Twelve Acute and Short-Term Chronic WET Methods.

Round	EPA Methods for the WET Interlaboratory Variability Study	Acute Tests		Short-Term Chronic Tests			
		Survival LC_{50}	Test Duration (Hours)	Survival LC_{50} NOEC	Growth IC_{25} NOEC	Reprod IC_{25} NOEC	Test Duration (Days)
1	Fathead Minnow, *Pimephales promelas*, Acute Test	X	96				
1	Method 1000.0: Fathead Minnow, *Pimephales promelas*, Larval Survival & Growth Test			X	X		7
1	Cladoceran, *Ceriodaphnia dubia*, Acute Test	X	48				
1	Method 1002.0: Cladoceran, *Ceriodaphnia dubia*, Survival & Reproduction Test			X		X	8[1]
1	Method 1003.0: Green Alga, *Selenastrum capricornutum*, Growth Test				X		4
2	Inland Silverside, *Menidia beryillina*, Acute Test	X	96				
2	Method 1006.0: Inland Silverside, *Menidia beryillina*, Larval Survival and Growth Test			X	X		7
2	Mysid, *Holmesimysis costata*, Acute Test[2]	X	96				
2	Method 1009.0: Red Macroalga, *Champia parvula*, Reproduction (cystocarp production) Test					X	7 - 9[3]
3	Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test	X	96				
3	Method 1004.0: Sheepshead Minnow, *Cyprinodon variegatus* - Larval Survival & Growth Test			X	X		7
3	Method 1007.0: Mysid, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test			X	X	X	7

[1] The *C. dubia* test acceptability criteria states that the test is complete when 60% of controls have 3 broods (approximately 7 days); for purposes of this study, all tests will continue for 8 days and each laboratory must carefully distinguish and carefully record the number of broods (see Section 4.4.3 and 4.4.4 of this study plan).

[2] The EPA-approved acute test with *Holmesimysis costata* will be performed using the acute test procedures for *Mysidopsis bahia* and test conditions optimized for *H. costata*.

[3] *C. parvula* are exposed to test substance for two days, followed by a 5-7 day recovery period in control water.

The remainder of this study plan describes the design of the WET Study. In the performance of each WET method, participating laboratories shall follow the specific instructions that EPA (or EPA's authorized representative) provides to perform the testing in accordance with their routine laboratory practices using the applicable test methods from the WET final rule. Additionally, EPA will provide all laboratories interested in the referee or participant laboratory role with detailed statements of work (SOWs) that articulate the specific tasks, instructions, deliverables, and turnaround requirements associated with each task. EPA may modify this study plan, the SOWs, or any specific instructions prior to or during the performance of the WET Study.

SECTION 2: OBJECTIVES

The primary objectives of the WET Study are to (1) generate data to characterize the interlaboratory variability of the 12 WET methods targeted in the study, (2) obtain data on the rate at which participating laboratories successfully completed WET tests initiated, and (3) generate data on the rate at which WET tests indicate "toxicity" is present when measuring non-toxic samples.

The WET Study will be conducted in four phases designed to accomplish the overall study objectives. These phases, and the specific objectives associated with each phase, are shown in Table 3.

Table 3. Four Phases of the WET Interlaboratory Variability Study.

Phase	Objectives
1 - Laboratory Procurement	• Identify potential referee and participant laboratories to support the study • Prequalify and select referee laboratories for Phases 2, 3, and 4 • Prequalify and select participant laboratories for Phase 4 of the study
2 - Preliminary Testing	• Determine the suitability of selected real-world sample matrices for use in the study through characterization of physical, chemical, and toxicological properties of the test sample • Determine the appropriate spiking concentrations for reference toxicant samples to achieve the desired range of toxicity • Determine the persistence of toxicity in real-world test samples • Assess whether the desired range of sample toxicity will be maintained in test samples following shipping and handling
3 - Sample Preparation and Distribution	• Prepare real-world and synthetic test samples for use by referee and participant laboratories in Phase 4 • Minimize variability between samples prepared for and distributed to each of the Phase 4 laboratories • Distribute blind test samples to all qualified laboratories for initial use within 36 hours of individual sample shipment from the referee laboratories
4 - Interlaboratory Testing	• Obtain interlaboratory test data for each WET method using four real-world and synthetic test samples to evaluate precision of the test methods, the rate at which laboratories successfully completed tests initiated, and the rate at which the tests indicate "toxicity" is present when measuring non-toxic samples

Six data quality objectives (DQOs) have been identified as necessary to ensure that data produced will meet the study objectives described above. These are:

(1) All data produced in the study must be generated in accordance with the analytical and quality assurance/quality control (QA/QC) procedures defined in this study plan and the following documents:

- *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Marine and Estuarine Organisms*, Second Edition, EPA-600-4-91-003, July 1994; (hereinafter referred to as the "Marine Chronic Methods Manual").
- *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms*, Third Edition, EPA-600-4-91-002, July 1994, (hereinafter referred to as the "Freshwater Chronic Methods Manual").
- *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms,* Fourth Edition, EPA-600-4-90-027F, August 1993; (hereinafter referred to as the "Acute Methods Manual").

- "Clarifications Regarding Flexibility in 40 CFR Part 136 Whole Effluent Toxicity (WET) Test Methods", memorandum from Tudor Davies, Office of Science and Technology, USEPA dated April 10, 1996.
- *Errata for Effluent and Receiving Water Toxicity Test Manuals: Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms; Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms; and Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms*, EPA-600/R-98/182, January 1999.

The first three documents are referred to collectively as the "methods manuals" throughout this document. The test requirements in Sections 4.4.3 and 4.4.4 of this study plan and the specific instructions provided by EPA will define the allowable flexibility in the WET methods included in this study. This study plan and the specific instructions will address items agreed to by EPA in the settlement that are currently not specified in the methods manuals.

(2) All test results from controls must meet the required test acceptability criteria (i.e., survival, minimum growth, minimum offspring/reproduction, average dry weight) specified in the methods manuals and Section 4.4.4 of this study plan to be considered valid. The *Ceriodaphnia dubia* Survival and Reproduction Test (Method 1002.0) will be conducted according to the method manuals as a three brood test, with careful notation of times of broods. In addition, the test will be conducted for eight days, with survival and reproduction measurements continuing past the third brood (see Section 4.4.3 and 4.4.4 for further clarification).

(3) Test parameters must meet the range of chemical and physical test conditions (such as temperature, hardness, alkalinity, ammonia, conductivity, pH, salinity, etc.) outlined in the appropriate methods manual and as detailed in Section 4.4.3 and 4.4.4 of this study plan.

(4) All calculations and data produced in this study must be capable of being verified through an independent review of the final data package by an analyst familiar with WET testing.

(5) Interlaboratory CVs must be calculated from a minimum of six complete and useable data sets for each WET test method evaluated in the study. Therefore, EPA's objective is to increase the number of laboratories participating in the study sufficiently beyond six to assure that at least six sets of complete and useable data are available after outliers and non-usable data are removed. To meet this DQO, EPA will directly support a minimum of nine participant laboratories. In addition, non-EPA-sponsored laboratories will be included in the study (up to 20 laboratories in the base study design and additional laboratories in the extended design).

(6) Participant laboratories must represent a cross-section of the laboratory community qualified to conduct WET tests using the proper test procedures and QA/QC provisions detailed in the method manuals.

To meet these DQOs, each participating laboratory will be required to have a comprehensive QA program in place and operating throughout this study.

SECTION 3: STUDY MANAGEMENT

The WET Study will be directed by EPA with contractual support by DynCorp Information & Engineering Technology, Inc. under the Sample Control Center (SCC) contract (EPA Contract No. 68-C-98-139). Overall management and technical oversight of this study will be provided by EPA Office of Water Engineering and Analysis Division's Analytical Methods Staff (AMS) and EPA's Office of Research and Development (ORD) staff. Laboratory procurement, day-to-day management, coordination of study activities, data review, and preparation of the final study report will be performed by SCC under AMS and ORD guidance. Referee laboratories will also be contracted to support the study through the preparation and distribution of blind test samples to participant laboratories conducting WET tests in the WET Study. The general responsibilities of each party contributing to the WET Study are detailed in Table 4.

Table 4. General Responsibilities of Parties Contributing to the WET Interlaboratory Variability Study.

Organization	Responsibilities
EPA	• Assemble a WET technical workgroup from OW, AMS, ORD, and Office of General Council (OGC) staff. This workgroup will be responsible for developing and finalizing the study plan and providing technical oversight during the study. • Provide overall management for the study (AMS). • Secure funding for the study. • Manage and approve the production of study reports.
SCC	• Support WET technical workgroup in development of study plan. • Draft statements of work (SOW) and standard operating procedures (SOP) for referee and participant laboratories. • Procure referee and participant laboratories (Phase 1 of the study). • Coordinate and provide day-to-day management of referee and participant laboratories during study Phases 2, 3, and 4. • Track sample shipment/receipt during study Phase 4. • Review, validate, and analyze study data. • Provide draft interim and final study reports to EPA.
Referee Laboratory	• Collect real-world samples. • Conduct preliminary testing on real-world and synthetic test samples (Phase 2). • Prepare, package, and distribute test samples (Phase 3) to laboratories participating in the base and extended study design. • Conduct WET tests concurrently with interlaboratory testing (Phase 4).
Participant Laboratory	• Conduct WET tests during interlaboratory testing and report results to SCC (Phase 4).

SECTION 4: TECHNICAL APPROACH

4.1 Phase 1 - Laboratory Procurement

The purpose of Phase 1 is to contract referee and participant laboratory support for the WET Study. EPA will attempt to maximize the number of qualified laboratories participating in the study and select laboratories that are representative of laboratories throughout the United States that routinely conduct WET tests for permittees. At the same time, EPA will only select laboratories that possess the capacity and capabilities, experience and proficiency, and quality assurance and quality control necessary to meet the needs of the study. To achieve these goals, EPA will identify and solicit a large number of laboratories, but select participant laboratories only from those that meet prequalification requirements.

A smaller more select list of laboratories that possess exceptional qualifications (based on EPA technical staff recommendations) will be solicited for the referee laboratory positions, since the responsibilities of the referee laboratory are demanding and critical to successful implementation of the WET Study.

4.1.1 Identification of Potential Laboratories

Laboratories participating in the WET Study may include EPA, state, academic, municipal, industrial and/or private laboratories. A list of potential participant laboratories will be identified from a variety of sources, including EPA and State environmental agencies, the Society of Environmental Toxicology and Chemistry (SETAC), reviews of the public literature, the *Directory of Environmental Laboratories*[1], and EPA's Discharge Monitoring Report Quality Assurance (DMRQA) list of laboratories conducting testing for the DMRQA program. A list of laboratories interested in participating without EPA sponsorship was also provided by the petitioners. All laboratories included in the compiled potential laboratory list will be solicited as participant laboratories. A subset of potential referee laboratories will be selected from the laboratory list based on EPA technical staff recommendations.

4.1.2 Selection of Referee Laboratories

At least one referee laboratory for Round 1 and at least one referee laboratory for both Round 2 and 3 will be required to conduct preliminary testing, collect and prepare blind test samples, distribute test samples to participant laboratories, and conduct WET tests concurrently with participant laboratories during Phase 4. Potential referee laboratories will be forwarded a bid solicitation package that includes the following documents: (1) referee laboratory prequalification document, (2) SOW, including a preliminary study schedule, and (3) referee laboratory bid sheet. Referee laboratories must meet all of the prequalification requirements given in Section 4.1.4 for participant laboratories. In addition to the requirements for participant laboratories, the referee laboratory must submit three client references and provide background information on potential real-world effluent and receiving water sample sources. Referee laboratory prequalification materials will be evaluated based on the rejection criteria listed in Section 4.1.4 and the additional reference and sample source requirements. The capacity and capabilities of potential referee laboratories will be highly scrutinized to ensure that the laboratory can meet the sample collection, preparation, distribution, and testing requirements of the study. Potential referee laboratories will be initially screened based on the prequalification requirements. For each WET test method, the referee laboratory that meets the prequalification requirements and has the lowest bid will be selected.

4.1.3 Selection of Participant Laboratories

All laboratories identified as described in Section 4.1.1 will receive a solicitation package from SCC that includes the following documents: (1) a detailed cover letter describing the solicitation, (2) participant laboratory prequalification document, (3) SOW, including a preliminary study schedule, and (4) participant laboratory bid sheet.

All laboratories seeking to participate in the WET Study **must** prequalify for each WET test method they would like to conduct according to the requirements in Section 4.1.4. From the pool of prequalified laboratories submitting bids, the nine lowest cost laboratories will be selected for EPA-sponsorship to support each WET test method. An additional maximum of 11 laboratories (for each WET test method) will be randomly selected from the pool of prequalified laboratories to participate in the base study design at their own cost or an external sponsor's cost (non-EPA sponsorship). The 9 EPA-sponsored laboratories and the 11 randomly chosen non-EPA-sponsored laboratories will constitute the 20

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[1] *Directory of Environmental Laboratories*, DynCorp, 1996.

laboratories included in the base study design for each WET test method. All remaining prequalified laboratories not selected for the base study design yet willing to participate without EPA sponsorship may participate in the extended study design.

Laboratories participating in the base design will each test four blind test samples received as whole volume samples or ampules. Laboratories participating in the extended design will each test two or three blind test samples received as test ampules. SCC will formally notify all laboratories of their selection and level of participation.

4.1.4 Prequalification Requirements

The prequalification process consists of submitting information that documents WET testing experience, proficiency, capacity, and quality control. Laboratories may choose to prequalify to perform one or more of the twelve WET test methods listed in Table 1. The **entire** prequalification process must be completed for **each** WET method potential participant laboratories are interested in performing. Laboratories **may not** qualify to fill both the referee and participant laboratory role for the same test species in the study.

Laboratories **must** be willing and able to abide by the statement of work and preliminary study schedule for the conduct and timing of each WET test method for which prequalification materials are submitted. Participant laboratories must have the capacity and capability to accommodate the testing schedule. It may be necessary for participant laboratories to limit the number of test methods for which they submit prequalification materials if laboratory facilities cannot meet the demands of the full testing schedule. Laboratories should recognize that selection for participation is more likely for those methods that are less common, however, laboratories must be prepared to perform all methods for which they submit prequalification information.

To prepare a complete prequalification package, laboratories must address all prequalification requirements, attach all required documentation, provide an explanation for the omission of any requisite information, and submit the material in accordance with the turnaround requirements in Section 4.1.5 of this document. Laboratories also must complete the attached laboratory bid sheet based on the performance of the tasks outlined in the participant laboratory SOW.

Prequalification materials must document that the potential participant laboratory has the capacity and capabilities to perform the necessary tasks in this study, experience and proficiency in conducting the WET test methods, and established quality assurance and quality control practices. To demonstrate these aspects, each potential participant laboratory **must** provide the following:

General information

(1) Information (on a cover page) including the laboratory name, address, telephone number, fax number, e-mail address, contact person, and additional contacts for day-to-day sample tracking and technical issues if different from primary contact.

(2) A statement on the number of tests that the laboratory can conduct at one time with the proposed staff, including the number of tests using a single test method and the number of tests using multiple test methods (e.g., three *C. dubia* survival and reproduction tests, three fathead minnow survival and growth tests, and two of each simultaneously). This information will not affect prequalification, but may be used for evaluating alternate study schedules if the preliminary study schedule must be further compressed.

Capacity and capabilities

(3) A statement that the combination of facilities, equipment, staff and laboratory capabilities are sufficient to meet study needs. In determining the sufficiency of laboratory capabilities, attention must be payed to the preliminary testing schedule. Participant laboratories must have the equipment, organisms, and personnel to accommodate this testing schedule. It may be necessary for participant laboratories to limit the number of test methods for which they submit prequalification materials if laboratory facilities cannot meet the demands of the full testing schedule.

(4) Detailed information on the type and size of laboratory and test equipment used for conducting each test method. Include information on temperature control, sample storage, water purification devices (i.e., Millipore Milli-Q® filtration and ion exchange), and dilution water sources. Laboratories must provide summaries of routine water quality monitoring data on dilution water and water used for culturing or maintaining each species (e.g., 3-4 months of pH, alkalinity, hardness, and salinity measurements on dilution and culture waters).

(5) A statement that the laboratory can receive next day deliveries (including Saturday deliveries) via overnight carriers (i.e., Fed Ex, UPS, etc.) and initiate a test on the same day as receipt.

(6) A list of laboratory staff able to participate in the study, including resumes and titles.

(7) Information on the source of organisms. This information must include whether organisms are cultured in-house or obtained externally. If cultured in-house, provide standard operating procedures for organism culturing (as required in number 9 below), provide a summary of how culture performance is assessed, and provide data on culture performance. For example, provide *Ceriodaphnia dubia* brood board monitoring data for the past six month or records of *Pimephales promelas* egg production. If obtained from an external source, include source, number of organisms that can be obtained from this source on a given day, age of obtained organisms, and organism holding and maintenance conditions.

Experience and proficiency

(8) Copies of internal Standard Operating Procedures (SOPs) for conducting each of the test methods for which prequalification is sought. Internal laboratory SOPs for each test method must be in place with dates of SOP origination.

(9) Copies of supporting internal laboratory SOPs for organism culturing, food preparation, and dilution water preparation for each species and each method.

(10) A statement on the number of effluent tests conducted in the last year using each of the WET test methods for which prequalification is sought. Include the frequency with which test acceptability criteria were met in these tests and the average control response measured in these tests.

(11) A statement regarding State or regional certifications. Does the given State or region certify toxicity testing laboratories? If so, is the laboratory currently certified? Provide documentation of current certifications.

Quality assurance/quality control

(12) Evidence that the laboratory maintains control (cusum) charts for reference toxicant tests for each method. The laboratory must submit the most current control chart for each test method, covering at least 12-24 data points and showing control limits. The raw data (actual data sheets and summarized data) for each data point also must be provided. Data charts with NOEC and/or IC_{25} for the same test values should be provided or describe why one is used rather than the other. Explanations must be included if methods used to develop control charts using reference toxicants deviate from promulgated methods or from the previous edition of a relevant test protocol.

(13) Evidence that reference toxicant tests are conducted at the appropriate frequency (e.g., monthly for tests that are routinely run for NPDES permits). Along with control chart information described above, provide a statement on the frequency of reference toxicant testing. If control charts (particularly for less common test methods) are composed of fewer than 12-24 data points, include an explanation.

(14) Copies of internal laboratory SOPs for conducting reference toxicant tests and constructing control charts. This information must include a narrative explanation of the width of the control limits for the laboratory and a statement of corrective action for any toxicity test endpoint value that falls outside the control limits.

(15) Results of the most recent DMRQA study, if the lab participated. The laboratory must also readily provide data point(s) for each method performed for the previous year's DMRQA study. If the laboratory did not participate, a narrative statement to that effect must be included.

(16) A signed statement of accuracy and completeness. The following statement should be included with the prequalification information and signed and dated by an authorized representative of the laboratory: "I certify that the information provided in this prequalification package is complete and accurate to the best of my knowledge."

Rejection of laboratories would be based on the following:

(1) Combination of facilities, equipment, staff and lab capacity and capabilities were insufficient to meet study needs.

(2) Organism source information was not provided, culture and or collection information was severely lacking, or source information was inadequate to assess the health of the organisms routinely used.

(3) Internal laboratory SOPs for each method were vague and could not be discerned and/or were generally insufficient to support performance of the methods in accordance with specific instructions provided by EPA.

(4) Statements regarding the number of effluent tests conducted per year, test acceptability rates, average control response, and/or State certifications were not provided, did not adequately demonstrate proficiency in the test method, or did not adequately demonstrate that the laboratory is representative of laboratories throughout the United States that routinely conduct WET testing for permittees.

(5) Control charts were not adequately maintained for reference toxicant tests, or data were not provided (cusum chart for each endpoint and raw data for each data point). Control charts should cover 12-24 data points for each species and test method, or an acceptable explanation given.

(6) Reference toxicant tests were not conducted at the appropriate frequency (monthly for tests that are routinely run for permits) and a satisfactory explanation was not provided.

(7) No acceptable explanation or evidence of corrective action was provided for any control chart value falling outside the control limits.

(8) Laboratory did not provide the most recent DMRQA study results, or an acceptable explanation for non-passing results was not provided. If the laboratory did not participate in the DMRQA study, the laboratory did not include an acceptable explanation as to why they did not participate.

(9) No signed statement of accuracy and completeness was included.

4.1.5 Prequalification Information Turnaround Requirements

All required prequalification information must be received by SCC in accordance with the turnaround requirements listed below to be considered valid.

- Prequalification information should address each item listed in Section 4.1.4 and the order and format of submitted information should follow the list in Section 4.1.4.

- The laboratory must submit two copies of all prequalification information and a completed participant laboratory bid response sheet (if seeking EPA sponsorship) to SCC at the address provided below within 15 business days (three calendar weeks) of receipt of the bid solicitation package.

Participant laboratory procurement for this study will be conducted by SCC. Laboratories should submit prequalification information to the following address:

DynCorp I&ET, Sample Control Center Contract
6101 Stevenson Avenue
Alexandria, VA 22304
Attn: Robert Brent

Laboratories will be required to assume responsibility for ensuring that prequalification materials are received within the 15 business day deadline.

4.2 Phase 2 - Preliminary Testing

The referee laboratories that are contracted to support each Round of the study will be responsible for conducting preliminary testing for each WET test method. This preliminary testing will be completed two weeks prior to commencement of each testing round. A four part preliminary testing scheme will be instituted to accomplish the following goals of preliminary testing:

(1) Determine the suitability of selected real-world sample matrices (i.e., effluent, receiving water) for use in the study through characterization of physical, chemical, and toxicological properties of the test sample

(2) Determine the appropriate spiking concentrations for reference toxicant samples to achieve the desired range of toxicity

(3) Determine the persistence of toxicity in real-world test samples
(4) Assess whether test samples will provide the desired range of sample toxicity following shipping and handling.

4.2.1 Part 1- Characterization of Physical, Chemical, and Toxicological Properties of Real-World Matrix Types

Part 1 of preliminary testing will verify that selected real-world sample matrices are acceptable for study use by assessing the physical, chemical, and toxicological characteristics of the samples. Selection of potential real-world effluent and receiving water sample sources will begin with the list submitted by referee laboratories as part of prequalification materials and a review of historical information from the source (if available). Through consultation with SCC and EPA, a preliminary selection of the real-world sample sources will be made for each test species. Following this determination, the referee laboratory will initiate Part 1 of preliminary testing.

Following sample collection, physical and chemical analysis of both the effluent sample and the receiving water sample including alkalinity, hardness, pH, temperature, total residual chlorine, total ammonia, dissolved oxygen, total dissolved solids, total suspended solids, total organic carbon, biological oxygen demand, and chemical oxygen demand will be conducted. For samples that are to be used in marine tests (Round 2 and 3), salinity also will be measured.

Following chemical and physical characterization of the sample, a single background definitive test using each of the test species will be conducted on a sample from each real-world source. For acute and chronic methods using the same species, the conduct of the acute background definitive test may be omitted (acute results may be obtained from measurements nested within the chronic test). If historical information (chemistry analysis or toxicological analysis) on the real-world matrix source is available, this information will be submitted along with results of the background testing. Following completion of analysis and historical data gathering, all historical and current information will be provided to SCC and EPA to accept or reject the sample source for use as the real-world sample matrix.

4.2.2 Part 2 - Determination of the Toxicity of Spiked Reference Toxicants in the Sample Matrix

The goal of Part 2 of preliminary testing is to determine the spiking concentration of reference toxicants to achieve the desired range of toxicity for reference toxicant samples. It may also be necessary to spike real-world matrix samples to achieve the desired range of toxicity. In Part 2 preliminary testing, a range-finding test using each WET test method will be conducted on each sample that is to be spiked. The range-finding test will use a range of spiking concentrations, and results will be used to isolate the appropriate spiking level to achieve the desired range of toxicity.

4.2.3 Part 3 - Holding Time Testing

Part 3 of preliminary testing will determine the persistence of toxicity in the real-world samples. Excess volume of the real-world samples will be retained from Part 1(if real-world sample is to be unspiked) or Part 2 (if real-world sample is to be spiked) of preliminary testing and stored in the dark at 4°C. Following storage for 7 days, a second test (using each WET test method for which the given sample is to be used) will be conducted and results compared to that of the initial test. The results of holding time testing will provide valuable information on the persistence of sample toxicity that will allow determinations of appropriate holding times for real-world samples. This information will be useful in the timing and scheduling of sample preparation for interlaboratory testing. This information may also be useful in the event that participant laboratories do not receive samples or are not able to conduct testing on the day

specified in the final study schedule. If Part 3 testing reveals that significant changes to toxicity occur during sample holding, the real-world sample sources may be reconsidered at this time.

4.2.4 Part 4 - Definitive Testing

Part 4 of preliminary testing will validate that the samples and spiking concentrations (if applicable) are appropriate for use in the study. Each sample type that will be used in interlaboratory testing will be prepared or collected, packaged, and shipped exactly as described for interlaboratory testing (Phase 4). The samples will be shipped by the referee laboratory round-trip back to the referee laboratory. Upon receipt, the referee laboratory will then conduct the definitive WET tests as described for interlaboratory testing (Phase 4). If samples produce the desired and expected range of toxicity in Part 4 preliminary testing, then the sample selection and preparation will be validated and preliminary testing is complete. If WET test values are not within the target range, SCC and EPA will be consulted and additional testing may be conducted to determine more appropriate spiking concentrations or sample sources.

4.3 Phase 3 - Sample Preparation, Packaging, and Distribution

4.3.1 Description of Test Samples

As mentioned in Section 1, a "test sample" is a single bulk sample preparation (i.e, matrix, recipe) that is provided to a participant laboratory. Aliquots of the single bulk sample will be used for test initiation and renewal(s) for the WET test method under study.

Four types of test samples will be tested using each WET test method. The four test sample types include: reference toxicants, industrial and/or municipal wastewater effluents, ambient receiving water, and method "blanks"(i.e., moderately hard reagent water prepared as explained in the test method manuals). Within each test sample type, EPA will select specific test samples that reflect the precision of the tests and not the variability of the toxicant or sample. Test samples also will be selected to exhibit a range of toxicity across test sample types. Preliminary testing (Phase 2) will validate the selection of real-world samples and spiking concentrations for reference toxicants.

EPA will randomly distribute "blind" test samples to each laboratory for evaluation. Each participant laboratory will receive some combination of the four test sample types. The combination of blind test samples received at any given laboratory may include duplicates of one or more test sample types and may exclude one or more test sample types. Neither EPA, EPA's authorized representatives, nor selected referee laboratories shall disclose the nature, number, or composition of any of the various samples distributed to laboratories participating in the studies.

4.3.2 Collection of Real-World Samples

The referee laboratories will collect real-world samples for the industrial and/or municipal wastewater effluent and ambient receiving water test sample types. Sample collection will be conducted to supply sufficient test sample volume for preliminary testing (Phase 2) and interlaboratory testing (Phase 4). Samples will be collected in accordance with the procedures detailed in specific instructions provided to the referee laboratories, the referee laboratory SOW, and Section 8 of the methods manuals. All real-world samples will be collected as grab samples. Grab samples of effluent will be collected from the designated NPDES sampling locations using a peristaltic pump. Between sampling events the sampling hose will be cleaned and rinsed thoroughly. Prior to the collection of a sample during each sampling period, three hose volumes of the sample will be pumped, purged, and disposed.

Real-world samples will be collected in pre-rinsed polyethylene containers of the appropriate size to accommodate the necessary volume of sample. Alternatively, multiple smaller polyethylene containers may be used to ease in the collection and transport of samples, provided that the individual containers are combined and homogenized in a bulk container prior to sample preparation. Immediately following sample collection, samples will be refrigerated and placed in the dark or in darkened containers.

The referee laboratory will use an SCC-assigned episode number to track each sampling event. All samples will be identified with a five-digit EPA sample number and documented on EPA traffic reports. Sample numbers, sample labels, and EPA traffic reports will be provided to the referee laboratory by SCC along with detailed instructions for sample documentation.

The referee laboratory SOW and specific instructions provided to the referee laboratories will give detailed instructions about the volume of each real-world test samples that should be collected for the WET methods included in this study.

4.3.3 Preparation of Test Samples

The referee laboratories will prepare test samples for use in Phase 2 preliminary testing and Phase 4 interlaboratory testing. For Phase 4, the referee laboratory will prepare four bulk test samples that will be divided and distributed to the participant laboratories for test initiation and test renewals (if necessary). A portion of each bulk test sample will also remain in the referee laboratory for WET testing to be conducted by the referee laboratory. An additional portion (20%) will remain in the referee laboratory until all shipped samples (including renewals) have been documented as arriving in good condition at the participant laboratories. This is to ensure that extra sample is available in the case of loss or damage during shipment.

Test samples will be prepared in large, thoroughly cleaned, and rinsed, polyethylene containers or tanks. Containers may be reused for preparation of separate bulk samples provided that they are properly cleaned before reuse. Containers will be cleaned according to recommendations for cleaning of laboratory apparatus stated in the WET methods manuals. Containers may be reused for preparation of an identical sample following only a rinse with deionized water. Similar type containers will be used to prepare samples for preliminary testing and for interlaboratory testing.

Test samples will be prepared in bulk in large containers or tanks that satisfy the volume requirements of the test sample needed for interlaboratory testing (Phase 4). Ideally, each of the bulk samples (for all laboratories and all renewals) should be prepared in a single batch container. For several tests, however, the minimum prepared volumes may be too large to be prepared in a single batch container. Under these circumstances the samples for test initiation and each renewal may be prepared individually.

Bulk samples will be mixed thoroughly using a paddle or impeller to ensure homogenization prior to division of test sample aliquots for shipment. For spiked test samples, the bulk volume will be homogenized prior to spiking, following spiking, and prior to division of test sample aliquots for shipment. The bulk samples will be prepared and mixed at least 12 hours, but not more than 36 hours prior to division of sample aliquots and shipment to participating laboratories. The holding time requirements may be relaxed if Part 3 preliminary testing indicates that the toxicity of samples is persistent during sample holding. During bulk sample mixing and holding, samples will remain refrigerated at 4^{o}C in the dark.

4.3.4 Packaging and Distribution of Test Samples

After bulk test samples have been prepared according to Section 4.3.3, each bulk test sample will be divided into individual test sample aliquots for shipment to participant laboratories. Test sample aliquots will be divided into containers appropriate for the individual test sample volumes. Sample containers will be pre-rinsed with the sample, filled, and then sealed with zero head-space. All samples for a given test method will be shipped in the same container style and size. Samples will be cooled to 4°C ± 2°C prior to shipment and then packed in coolers (e.g., 28, 48, 54-qt) containing ice packs (i.e., blue ice). Depending on the test method being performed by an individual participant laboratory, multiple test samples may be shipped in one cooler. The maximum volume of sample that can be shipped in one cooler (about 54 qt) is approximately 21-L. Test sample volumes above 21-L will exceed the maximum weight limit for overnight shipping. Test sample volumes above 21-L will be sent in separate coolers. Ideally, duplicate test sample aliquots will be shipped in the same cooler; if test sample volume prohibits shipping duplicates in the same cooler, they will be shipped under the same airbill to ensure they are shipped together. An EPA traffic report form and any additional information for participant laboratories regarding test sample preparation or testing (such as reconstitution instructions for ampule samples) will be included with each sample shipment. Referee laboratories will follow guidelines and recommendations for sample shipment given in Section 8 of the method manuals and the referee laboratory SOW that will be provided by SCC.

SCC will provide the referee laboratory with a list of participant laboratories for each method. The list of participant laboratories will include addresses and contacts, as well as specifications for the test samples each participant laboratory is to receive. The referee laboratory will ship aliquots of test samples to each participating laboratory that is conducting the given test. Samples for testing at the referee laboratory will be prepared and shipped round-trip back to the referee laboratory. Testing will be scheduled to occur simultaneously at each participant laboratory, so samples will be shipped overnight to arrive at each participant laboratory on the day of scheduled testing.

4.3.5 Sample Tracking

Sample Labeling: Each WET test method will receive an EPA episode number to designate samples prepared for that test method. Each sample aliquot that is prepared and shipped will be assigned a unique sample number. Duplicate samples will receive different sample numbers to retain the blind sample aspect of the study design. For tests that require additional shipments for sample renewal, the sample number will be the same for each initiation and renewal shipment with the addition of a letter (A, B, and C) after the sample number to designate the sample for use as initiation (A), renewal 1 (B), or renewal 2 (C). The sample number will appear clearly and permanently on each container and on each EPA traffic report form included with the shipment.

Referee Laboratory Tracking: SCC will provide referee laboratories with EPA traffic report forms that must accompany each sample shipped. The referee laboratory will clearly indicate on the traffic report form the episode number, sample number, name and address of the referee laboratory, name and address of the participant laboratory, date shipped, airbill number, tests requested, and pre-shipment sample information (sample preparation date and initial water chemistry). A traffic report form specific to each sample will be placed in a waterproof enclosure (i.e., Ziploc bag) and packed in the cooler with the respective sample.

For each shipment event, the referee laboratory will also complete a sample shipment documentation form. The form will be faxed along with a copy of the airbill to SCC immediately after sample pickup by

the overnight carrier. This form will document the identity of each sample that is shipped. Information reported on this form will include:

- sample number - the unique identifying number for each sample aliquot
- sample description - identifies the sample as either blank, spiked effluent, spiked effluent duplicate, spiked receiving water, reference toxicant, or reference toxicant duplicate
- participant laboratory name - the name of the laboratory that the sample is shipped to
- airbill number - the overnight shipping service's number that identifies each individual shipment
- size of test containers - the size of the test container in which the sample is shipped
- cooler number - a unique identifying number for the cooler in which the sample is shipped. Each cooler used in the study should be permanently numbered and labeled (with the referee laboratory name and address) to assist in locating lost coolers and to assist in retrieving coolers from participant laboratories.
- comments - any miscellaneous comment related to sample shipment.

Participant Laboratory Tracking: Upon receipt of each sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. Laboratories will be required to fax the completed traffic report form to SCC immediately upon sample receipt and retain a copy for inclusion in the data report. If individual test samples are unusable or not received, the participant laboratories must contact SCC on the day of expected shipment arrival for problem resolution.

4.4 Phase 4 - Interlaboratory Testing

The general conduct of interlaboratory testing will proceed as described in Section 1 of this study plan. Round 1 will include the acute and short-term chronic *Ceriodaphnia dubia* and *Pimephales promelas* tests and the short-term chronic *Selenastrum capricornutum* test. Round 2 includes the acute and short-term chronic *Menidia beryllina* tests, the acute *Holmesimysis costata* test, and the short-term chronic *Champia parvula* test. Round 3 includes the acute and short-term chronic *Cyprinodon variegatus* tests and the short-term chronic *Mysidopsis bahia* test. Participant and referee laboratories will conduct interlaboratory testing simultaneously according to the final study schedule.

4.4.1 Study Initiation

Following prequalification, EPA will notify participant laboratories that have been selected to take part in the WET Study. This notification will be accompanied by a final study schedule. EPA will provide adequate time for laboratories to prepare for study initiation.

4.4.2 Preliminary Study Schedule

The interlaboratory testing phase of the WET Study will be conducted from approximately August 1999 to February 2000, with final data reports from each participant laboratory due 30 days following termination of the round. A preliminary schedule for the timing of each round is provided in Table 5. *Note: This is a preliminary schedules for planning purposes only; a final study schedule will be provided to participant laboratories with bid acceptance notification. The structure of the schedule will remain the same, but dates may be slightly altered*. Testing will be scheduled to occur simultaneously at each participant laboratory, so adherence to the finalized schedule is mandatory for all participant laboratories. Samples will arrive at each participant laboratory on the day scheduled for test commencement.

In order to meet project deadlines, it is necessary to overlap Rounds 1 and 2 of the study causing some marine methods to be conducted concurrently with freshwater methods. Within each round, the study schedule was designed to allow the conduct of only one WET test method at a time, however, one test method may begin on the day that another test method ends. During the study, samples will be distributed in pairs and numbered 1-4 for each test method. Testing of samples #1 and 2 will be conducted concurrently, and testing of samples #3 and 4 will be conducted concurrently.

Table 5. Preliminary Schedule for WET Interlaboratory Study

Approximate Date	Activity
6/11/99 - 7/5/99	**Participant laboratory prequalification**
6/11/99	DynCorp SCC solicits participant labs
7/5/99	Prequalification materials due
8/9/99	DynCorp SCC to award participant labs
8/24/99 - 10/25/99	**Round 1 Testing**
8/24/99 - 8/26/99	Conduct Cladoceran, *Ceriodaphnia dubia*, Acute Test with samples #1&2
8/26/99 - 8/28/99	Conduct Cladoceran, *Ceriodaphnia dubia*, Acute Test with samples #3&4
8/31/99 - 9/4/99	Conduct Green Alga, *Selenastrum capricornutum*, Growth Test with samples #1&2
9/9/99 - 9/13/99	Conduct Green Alga, *Selenastrum capricornutum*, Growth Test with samples #3&4
9/14/99 - 9/21/99	Conduct Fathead Minnow, *Pimephales promelas,* Larval Survival and Growth Test with samples #1&2
9/21/99 - 9/28/99	Conduct Fathead Minnow, *Pimephales promelas,* Larval Survival and Growth Test with samples #3&4
9/28/99 - 10/6/99	Conduct Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test with samples #1&2
10/7/99 - 10/11/99	Conduct Fathead Minnow, *Pimephales promelas*, Acute Test with samples #1&2
10/12/99 - 10/20/99	Conduct Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test with samples #3&4
10/21/99 - 10/25/99	Conduct Fathead Minnow, *Pimephales promelas*, Acute Test with samples #3&4
11/24/99	**Round 1 data due**
8/24/99 - 10/30/99	**Round 2 Testing**
8/24/99 - 9/2/99	Conduct Red Macroalga, *Champia parvula*, Reproduction Test with samples #1&2
9/9/99 - 9/18/99	Conduct Red Macroalga, *Champia parvula*, Reproduction Test with samples #3&4
9/21/99 - 9/25/99	Conduct Mysid, *Holmesimysis costata*, Acute Test with samples #1&2
9/28/99 - 10/2/99	Conduct Mysid, *Holmesimysis costata*, Acute Test with samples #3&4
10/5/99 - 10/12/99	Conduct Inland Silverside, *Menidia beryllina,* Larval Survival and Growth Test with samples #1&2
10/12/99 - 10/19/99	Conduct Inland Silverside, *Menidia beryllina,* Larval Survival and Growth Test with samples #3&4
10/19/99 - 10/23/99	Conduct Inland Silverside, *Menidia beryllina*, Acute Test with samples #1&2
10/26/99 - 10/30/99	Conduct Inland Silverside, *Menidia beryllina*, Acute Test with samples #3&4
11/29/99	**Round 2 data due**
1/11/00 - 2/19/00	**Round 3 Testing**
1/11/00 - 1/18/00	Conduct Sheepshead Minnow, *Cyprinodon variegatus,* Larval Survival and Growth Test with samples #1&2
1/18/00 - 1/25/00	Conduct Mysid, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test with samples #1&2
1/25/00 - 2/1/00	Conduct Sheepshead Minnow, *Cyprinodon variegatus,* Larval Survival and Growth Test with samples #3&4
2/1/00 - 2/8/00	Conduct Mysid, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test with samples #3&4
2/8/00 - 2/12/00	Conduct Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test with samples #1&2
2/15/00 - 2/19/00	Conduct Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test with samples #3&4
3/20/00	**Round 3 data due**

4.4.3 General Testing Requirements

Each laboratory selected to participate in the base study design will receive four blind test samples (as whole volume samples or ampules) for each method they are prequalified to perform. Additionally, sample aliquots of each test sample type will be analyzed in the referee laboratories. Each laboratory participating in the extended study design will receive two or three blind test samples (as ampules) for each method they are prequalified to perform. Instructions will be included for reconstituting the ampule samples. Whole volume samples and reconstituted ampule samples should be treated as if they are effluent samples being tested for compliance monitoring purposes. Except where indicated in Sections 4.4.3 and 4.4.4 of this study plan and specific instructions provided to participant laboratories, each test will be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. The finalized study schedule will be distributed to participating laboratories prior to commencement of each study round and in ample time to prepare for testing. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this study plan, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) will be provided to the testing laboratories with the sample. Test samples received at participant laboratories must be refrigerated (at 4 °C ± 2 °C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, conductivity or salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals.

(5) The specified dilution and control waters (listed in Tables 6 - 17 for each test method) must be used and prepared according to instructions in Section 7 of the methods manuals. Marine waters must also be prepared to meet the salinity ranges for each test (given in Tables 11 - 17).

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 4.4.4.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals. In addition, block randomization and use of known parentage will be required for the *Ceriodaphnia dubia* survival and reproduction test as described in Method 1002.0. The Agency plans to amend Method 1002.0 (*Ceriodaphnia dubia* Survival and Reproduction test) to require that test organisms be allocated among test replicates so that offspring of each female are evenly distributed among test replicates ("blocking-by-parentage").

(10) The *Ceriodaphnia dubia* Survival and Reproduction Test (Method 1002.0), which would otherwise be terminated after 3 broods according to Section 13.12.1 of that method, must be conducted for 8 days, with endpoints including survival, number of young per day, and number of broods recorded each day. These readings are to be made at the end of the 6th, 7th and 8th day (specifically, at 144 hours, at 168 hours, and at 192 hours, respectively, from test initiation). This will be done to assess the effect of that test acceptance criterion on test results. No test shall be terminated prior to the eighth day for any reason, including a failure to meet test acceptance criteria. The additional measurements on days 6, 7, and 8 should be included as raw data in the final data report, but should not affect the data analysis of test results. The analysis of data from the *C. dubia* chronic test shall be conducted as specified in the method manual using the three brood approach.

(11) The Green Algae, *Selenastrum capricornutum*, Growth Test shall be conducted simultaneously with and without EDTA for each sample. For laboratories participating in the base study design, four samples will be tested with and without EDTA for a total of eight analyses. For laboratories participating in the extended study design, two or three samples will be tested with and without EDTA for a total of four or six analyses.

(12) Daily observation of mortality and removal of dead organisms for each test is required, except for the *Selenastrum capricornutum* and *Champia parvula* tests. Daily young counts are required for the *Ceriodaphnia dubia* survival and reproduction test, along with determining the number of broods at each count.

(13) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible

causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(14) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(15) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(16) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(17) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(18) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of this study plan. <u>All</u> bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet, or equivalent) that will be provided by SCC prior to test initiation.

(19) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(20) An LC_{50} must be reported for each acute test. An NOEC and LC_{50} for survival, and an NOEC and IC_{25} for growth/reproduction must be reported as appropriate for each short-term chronic test as described in the method manuals and Table 2 of this study plan. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

4.4.4 Method-Specific Requirements

The summary of test conditions for the twelve WET methods to be evaluated in the WET Study are provided in Tables 6 - 17. These tables are extracted from the summary test condition tables in the methods manuals and modified to fit the scope of this study. Items that are bold italic in these tables represent conditions standardized for the purposes of this study where method manuals provide a range. Final SOPs for sample preparation (i.e., reconstitution of ampules) and test conduct will be provided to each participant laboratory prior to study initiation.

Table 6. Fathead Minnow, *Pimephales promelas,* Acute Test. Summary of test conditions and test acceptability criteria for fathead minnow, *Pimephales promelas*, acute toxicity tests with effluents and receiving waters

1. Test type:	***Static-renewal***
2. Test duration:	***96 h***
3. Temperature:	***25 ± 1 °C***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***250 mL***
8. Test solution volume:	***200 mL***
9. Renewal of test solutions:	***At 48 h***
10. Age of test organisms:	1-14 days; 24-h range in age
11. No. organisms per test chamber:	***10***
12. No. replicate chambers per concentration:	***2***
13. No. organisms per concentration:	***20***
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	***Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor	≥0.5
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	***2 L for effluents***
23. Test acceptability criterion:	90% or greater survival in controls

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 7. Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test. Summary of test conditions and test acceptability criteria for fathead minnow, *Pimephales promelas*, larval survival and growth toxicity tests with effluents and receiving waters

1. Test type:	Static renewal
2. Temperature:	25 ± 1°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h darkness
6. Test chamber size:	***500 mL***
7. Test solution volume:	***250 mL***
8. Renewal of test solutions:	Daily
9. Age of test organisms:	Newly hatched larvae less than 24h old. If shipped, not more than 48h old, 24h range in age
10. No. larvae per test chamber:	***10***
11. No. replicate chambers per concentration:	***4***
12. No. larvae per concentration:	***40***
13. Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old)
14. Feeding regime:	Feed 0.1 g newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 g twice daily, 6 h between feedings (at the beginning of the work day prior to renewal, and at the end of the work day following renewal). Sufficient nauplii are added to provide an excess. Larvae fish are not fed during the final 12 h of the test
15. Cleaning:	Siphon daily, immediately before test solution renewal
16. Aeration:	None, unless DO concentration falls below 4.0 mg/L. Rate should not exceed 100 bubbles/min
17. Dilution water:	***Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor	≥0.5
20. Test duration:	7 days
21. Endpoints:	***Survival and growth (weight as mean per original)***
22. Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers equals or exceeds 0.25 mg/surviving
23. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
24. Sample volume required:	2.5 L/day

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 8. Cladoceran, *Ceriodaphnia dubia,* Acute Test. Summary of test conditions and test acceptability criteria for daphnid, *Ceriodaphnia dubia* acute toxicity tests with effluents and receiving waters

1. Test type:	***Static non-renewal***
2. Test duration:	***48 h***
3. Temperature:	***25 ± 1 ℃***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***30 mL***
8. Test solution volume:	***15 mL***
9. Renewal of test solutions:	***None***
10. Age of test organisms:	Less than 24-h old
11. No. organisms per test chamber:	***5***
12. No. replicate chambers per concentration:	***4***
13. No. organisms per concentration:	***20***
14. Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test.
15. Test chamber cleaning:	Cleaning not required
16. Test chamber aeration:	None
17. Dilution water:	***Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor	≥0.5
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	1 L
23. Test acceptability criterion:	90% or greater survival in controls

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 9. Cladoceran, *Ceriodaphnia dubia,* Survival and Reproduction Test. Summary of test conditions and test acceptability criteria for daphnid, *Ceriodaphnia dubia*, survival and reproduction toxicity tests with effluents and receiving waters

1. Test type:	Static renewal
2. Temperature (°C):	25 ± 1°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 μE/m^2/s, or 50-100 ft-c (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h dark
6. Test chamber size:	***30 mL***
7. Test solution volume:	***15 mL***
8. Renewal of test solutions:	Daily
9. Age of test organisms:	Less than 24 h; and all released within a 8-h period
10. No. neonates per test chamber:[1]	1
11. No. replicate test chambers per concentration:	10
12. No. neonates per test concentration:	10
13. Feeding regime:	Feed 0.1 mL each of YCT and algal suspension per test chamber daily.
14. Cleaning:	Use freshly cleaned glass beakers or new plastic cups daily
15. Aeration:	None
16. Dilution water:	***Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)***
17. Test concentrations:	***Five concentrations and a control***
18. Dilution factor:	≥0.5
19. Test duration:[2]	***8 days***
20. Endpoints:	***Survival and reproduction***
21. Test acceptability criteria:	80% or greater survival and an average of 15 or more young per surviving female in the control solutions. 60% of surviving control organisms must produce three broods.
22. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
23. Sample volume required:	1 L/day

[1] Test vessels shall be randomized in accordance with the method manuals. In addition, block randomization and use of known parentage will be required for the *Ceriodaphnia* survival and reproduction test as described in the manual and guidance will be reiterated in the specific instructions provided to the laboratories.

[2] The *Ceriodaphnia dubia* test which would otherwise be terminated after 3 broods according to methods manual Section 13.12.1 of that Method must be conducted for 8 days, with endpoints (survival and number of young per day and number of broods at each recording interval) recorded at the end of the 6th, 7th and 8th day (specifically, at 144, 168, and 192 hours, respectively, from test initiation). No test shall be terminated prior to the eighth day for any reason, including a failure to meet test acceptance criteria.

Table 10. Green Alga, *Selenastrum capricornutum*, Growth Test. Summary of test conditions and test acceptability criteria for green alga, *Selenastrum capricornutum*, growth toxicity tests with effluents and receiving waters. Test will be conducted with EDTA and without EDTA.

1. Test type:	Static non-renewal
2. Temperature:	25 ± 1°C
3. Light quality:	"Cool white" fluorescent lighting
4. Light intensity:	86 ± 8.6 µE/m^2/s (400 ± 40 ft-c or 4306 lux)
5. Photoperiod:	Continuous illumination
6. Test chamber size:	***250 mL***
7. Test solution volume:	***100 mL***
8. Renewal of test solutions:	None
9. Age of test organisms:	4 to 7 days
10. Initial cell density in test chambers:	10,000 cells/mL
11. No. replicate chambers per concentration:	***4***
12. Shaking rate:	***100 cpm continuous***
13. Aeration:	None
14. Dilution water:	***Algal stock culture medium, moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals(see Methods Manual Section 7, Dilution Water)***
15. Test concentrations:	***Five concentrations and a control***
16. Test dilution factor:	≥0.5
17. Test duration:	96 h
18. Endpoint:	***Growth (cell counts)***
19. Test acceptability criteria:	1 X 10^6 cells/mL with EDTA or 2 X 10^5 cells/mL without EDTA in the controls; Variability of controls should not exceed 20%
20. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
21. Sample volume required:	***2 L***

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 11. Inland Silverside, *Menidia beryllina* , Acute Test. Summary of test conditions and test acceptability criteria for inland silverside, *Menidia beryllina,* acute toxicity test with effluents and receiving waters

1. Test type:	***Static-renewal***
2. Test duration:	***96 h***
3. Temperature:	***25 °C ± 1 °C***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***250 mL***
8. Test solution volume:	***200 mL***
9. Renewal of test solutions:	***At 48 h***
10. Age of test organisms:	9-14 days; 24-h range in age
11. No. organisms per test chamber:	***10***
12. No. replicate chambers per concentration:	***2***
13. No. organisms per concentration:	***20***
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	***25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor:	≥0.5
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	***1 L for effluents***
23. Test acceptability criterion:	90% or greater survival in controls
24. Salinity:	***25‰ (± 2‰)***

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 12. Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test. Summary of test conditions and test acceptability criteria for the inland silverside, *Menidia beryllina*, larval survival and growth test with effluents and receiving waters

1. Test type:	Static renewal
2. Salinity:	***25‰ (±2‰)***
3. Temperature:	25 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	l0-20 μE/m^2/s (50-100 ft-c) (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***1 L containers***
8. Test solution volume:	***750 mL/replicate (loading and DO restrictions must be met)***
9. Renewal of test solutions:	Daily
10. Age of test organisms:	7-11 days post hatch; 24-h range in age
11. No. larvae per test chamber:	***10***
12. No. replicate chambers per concentration:	***4***
13. No. larvae per concentration:	***40***
14. Source of food:	Newly hatched *Artemia* nauplii (survival of 7-9 days old *Menidia beryllina* larvae improved by feeding 24 h old *Artemia*)
15. Feeding regime:	Feed 0.10 g wet weight *Artemia* nauplii per replicate on days 0-2; Feed 0.15 g wet weight *Artemia* nauplii per replicate on days 3-6
16. Cleaning:	Siphon daily, immediately before test solution renewal and feeding
17. Aeration:	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min.
18. Dilution water:	***25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
19. Test concentrations:	***Five concentrations and a control***
20. Dilution factor:	≥0.5
21. Test duration:	7 days
22. Endpoints:	Survival and growth (weight)
23. Test acceptability criteria:	80% or greater survival in controls, 0.50 mg average dry weight of control larvae when larvae dried immediately after test termination, or 0.43 mg or greater average dry weight of control larvae, preserved not more than 7 days in 4% formalin or 70% ethanol
24. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
25. Sample volume required:	6 L/day

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 13. Mysid, *Holmesimysis costata*, Acute Test. Summary of test conditions and test acceptability criteria for mysid, *Holmesimysis costata*, acute toxicity tests with effluents and receiving waters. The acute test procedure described in the Acute Methods Manual for *Mysidopsis bahia* will be used for this test with a salinity of 32‰ (± 2‰) and a temperature of 12 °C ± 1 °C.

1. Test type:	***Static-renewal***
2. Test duration:	***96 h***
3. Temperature:	***12 °C ± 1 °C***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***250 mL***
8. Test solution volume:	***200 mL***
9. Renewal of test solutions:	***At 48 h***
10. Age of test organisms:	1-5 days; 24-h range in age
11. No. organisms per test chamber:	***10***
12. No. replicate chambers per concentration:	***4***
13. No. organisms per concentration:	***40***
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of *Artemia* nauplii ≤ 24-h old, daily (approximately 100 nauplii per mysid)
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	***32‰ salinity natural seawater***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor:	≥0.5
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	***1 L for effluents***
23. Test acceptability criterion:	90% or greater survival in controls
24. Salinity:	***32‰ (±2‰)***

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 14. Red Macroalga, *Champia parvula*, Reproduction Test. Summary of test conditions and test acceptability criteria for the red macroalga, *Champia parvula*, sexual reproduction test

1. Test type:	Static non-renewal
2. Salinity:	30‰ (± 2‰)
3. Temperature:	23 ± 1°C
4. Photoperiod:	16 h light, 8 h darkness
5. Light intensity:	75 µE/m^2/s (500 ft-c)
6. Light source:	Cool-white fluorescent lights
7. Test chamber size:	200 mL polystyrene cups, or 250 mL Erlenmeyer flasks
8. Test solution volume:	***100 mL***
9. No. organisms per test chamber:	5 female branch tips and 1 male plant
10. No. replicate chambers per concentration:	***4***
11. No. organisms per concentrations:	***24***
12. Dilution water:	***30‰ salinity natural seawater***
13. Test concentrations:	***Five concentrations and a control***
14. Test dilution factor:	≥0.5
15. Test duration:	2 day exposure to effluent, followed by 5 to 7-day recovery period in control medium for cyctocarp development
16. Endpoints:	Reduction in cystocarp production compared to controls
17. Test acceptability criteria:	80% or greater survival, and an average of 10 cystocarps per plant in controls
18. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
19. Sample volume required:	2 L per test

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 15. Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test. Summary of test conditions and test acceptability criteria for sheepshead minnow, *Cyprinodon variegatus*, acute toxicity tests with effluents and receiving waters

1. Test type:	***Static renewal***
2. Test duration:	***96 h***
3. Temperature:	***25 °C ± 1 °C***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s or (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***250 mL***
8. Test solution volume:	***200 mL***
9. Renewal of test solutions:	***At 48 h***
10. Age of test organisms:	1-14 days; 24-h range in age
11. No. organisms per test chamber:	***10***
12. No. replicate chambers per concentration:	***2***
13. No. organisms per concentration:	***20***
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	***25 ‰ ± 2‰ Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor	≥0.5
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	***1 L for effluents***
23. Test acceptability criterion:	90% or greater survival in controls
24. Salinity:	***25‰ (±2‰)***

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 16. Sheepshead Minnow, *Cyprinodon variegatus,* Larval Survival And Growth Test. Summary of test conditions and test acceptability criteria for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test with effluents and receiving waters

1. Test type:	Static renewal
2. Salinity:	***25‰ (±2‰)***
3. Temperature:	25 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***600 mL beaker***
8. Test solution volume:	***500 mL/replicate (loading and DO restrictions must be met)***
9. Renewal of test solutions:	Daily
10. Age of test organisms:	Newly hatched larvae (less than 24 h old; 24-h range in age)
11. No. larvae per test chamber:	***10***
12. No. replicate chambers per concentration:	***4***
13. No. larvae per concentration:	***40***
14. Source of food:	Newly hatched *Artemia* nauplii, (less than 24-h old)
15. Feeding regime:	Feed once a day 0.10 g wet weight *Artemia* nauplii per replicate on Days 0-2; Feed 0.15 g wet weight *Artemia* nauplii per replicate on Days 3-6
16. Cleaning:	Siphon daily, immediately before test solution renewal and feeding
17. Aeration:	None, unless DO falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min
18. Dilution water:	***25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
19. Test concentrations:	***Five concentrations and a control***
20. Dilution factor:	≥0.5
21. Test duration:	7 days
22. Endpoints:	Survival and growth (weight)
23. Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers should be 0.60 mg or greater, if unpreserved, or 0.50 mg or greater after no more than 7 days in 4% formalin or 70% ethanol
24. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
25. Sample volume required:	6 L per day

NOTE: Test vessels shall be randomized in accordance with the method manuals.

Table 17. Mysid Shrimp, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test. Summary of test conditions and test acceptability criteria for the mysid, *Mysidopsis bahia*, seven day survival, growth, and fecundity test with effluents and receiving waters

1. Test type:	Static renewal
2. Salinity:	***25‰ (±2‰)***
3. Temperature:	26 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c.) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness, with phase in/out period
7. Test chamber:	8 oz plastic disposable cups, or 400 mL glass beakers
8. Test solution volume:	150 mL per replicate
9. Renewal of test solutions:	Daily
10. Age of test organisms:	7 days
11. No. organisms per test chamber:	***5***
12. No. replicate chambers per concentration:	***8***
13. No. larvae per concentration:	***40***
14. Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old)
15. Feeding regime:	Feed 150 24 h old nauplii per mysid daily, half after test solution renewal and half after 8-12 h.
16. Cleaning:	Pipette excess food from cups daily immediately before test solution renewal and feeding.
17. Aeration:	None unless DO falls below 4.0 mg/L, then gently aerate in all cups
18. Dilution water:	***25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
19. Test concentrations:	***Five concentrations and a control***
20. Dilution factor:	≥0.5
21. Test duration:	7 days
22. Endpoints:	Survival, growth, and egg development
23. Test acceptability criteria:	80% or greater survival, average dry weight 0.20 mg or greater in controls; fecundity may be used if 50% or more of females in controls produce eggs
24. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
25. Sample volume required:	3 L per day

NOTE: Test vessels shall be randomized in accordance with the method manuals.

SECTION 5: DATA REPORTING AND EVALUATION

Each referee and participant laboratory will be required to submit data reports in a hard copy format that is consistent with the applicable methods manual. Submission of data reports will be required within 30 calendar days of the completion of each testing round. At a minimum, this report should follow the data reporting format outlined in Table 18 and include all laboratory bench sheets. Laboratories also will be required to submit selected data in an electronic format (Microsoft Excel® spreadsheet, or equivalent) that will allow SCC to create a database of study results. This database will facilitate automated review and statistical analysis of study results. Specific instructions regarding the electronic format will be provided to referee and participant laboratories prior to study initiation. Raw data will be made available in the public record.

Upon receipt of each laboratory data package, SCC will review the results to ensure that they were generated in accordance with the required procedures. Data generated by all qualified participating laboratories will be considered in the evaluation of the test methods and will be compiled in a study database and statistically analyzed to determine the interlaboratory variability of the acute and short-term chronic methods under study. Statistical methods appropriate to the data received will be used in the analysis process. This may include outlier analysis if warranted by the data. Data also will be assessed to determine the success rate for test initiation and test completion for each method and the rate at which the tests indicate "toxicity" is present when measuring non-toxic samples. Overall, EPA will evaluate the study results to draw conclusions about the performance of standardized WET tests. Participant laboratories that fail to initiate tests in Phase 4 or fail to complete tests due to reasons unrelated to the test methods themselves (i.e., laboratory error, sample receipt problems) will not be included in the success rate calculations nor statistical analyses. SCC will assemble background information and study data into a final study report for review by EPA staff.

EPA will evaluate results from the WET Study in accordance with the criteria for evaluating the adequacy of biological methods described in "Availability, Adequacy, and Comparability for the Analysis of Pollutants Established Under Section 304(h) of the Federal Water Pollution Control Act," EPA/600/9-87/030 (September 1988), and, to the extent applicable, the "Data Quality Objectives" guidance (from EPA's Permit Writers' Guide dated November 1990 and Guidance for Planning for Data Collection, EPA/QA/G-4).

Note: Laboratories may not independently publish the results of analyses they are paid by EPA to perform under this study plan.

Table 18. Data Reporting Format.

Section 1 - Summary Page

1.1 Laboratory name
1.2 Laboratory address and phone number
1.3 Name and signature of laboratory QA Officer, certifying that data have been internally reviewed and that personnel meticulously followed the methods, and the procedures are deemed to be compliant with the methods and acceptable for reporting purposes.
1.4 Laboratory contact responsible for study
1.5 Analyst(s) who performed WET tests (full name)
1.6 Toxicity tests performed
1.7 Detailed explanations of any difficulties encountered and any approved modifications to the techniques specified in the SOW, specific instructions, or the methods manuals.
1.8 Number of successful tests completed

Section 2 - Sample Information

2.1 Number of samples received and EPA sample number assigned to each sample
2.2 Dates of sample receipt
2.3 Sample temperature when received at laboratory
2.4 Physical and chemical data of sample contents (as required in appropriate method)
2.5 Dilution water
  2.5.1 Source and time frame water is used or how maintained
  2.5.2 Collection or preparation date(s), where applicable
  2.5.3 Pretreatment information
  2.5.4 Physical and chemical characteristics (pH, hardness, conductivity, salinity, etc.)
2.6 Sample storage information
2.7 Sample preparation for testing information

Section 3 - Test Conditions

3.1 Toxicity test method used (title, number, source)
3.2 Endpoint(s) of test(s)
3.3 Deviations from reference method(s), if any, and reason(s)
3.4 Date and time test(s) started, date and time samples were prepared and solutions transferred for renewals
3.5 Date and time test(s) terminated
3.6 Type and volume of test chambers
3.7 Volume of solution used per chamber
3.8 Number of organisms per test chamber
3.9 Number of replicate test chambers per treatment
3.10 Feeding frequency and amount and type of food (be specific with sources, concentrations of foods (i.e, algae concentration, YCT solids level, preparation dates)
3.11 Acclimation of test organisms (temperature mean and range and, where applicable, salinity mean and range)
3.12 Test temperature (mean and range)
3.13 Test salinity, where applicable (mean and range)
3.14 Specify if aeration was needed
3.15 Specify if organisms were dried immediately for weighing or preserved prior to drying
3.16 Specify how food was prepared and sources of food. Include test results that validate the quality of batch food preparations (i.e., *Ceriodaphnia dubia* tests on YCT preparation)
3.17 Describe how routine chemistries on new solutions were made (in actual test chamber or in beakers after dispensing)
3.18 Describe how randomization was conducted, especially blocking and known parentage. Report how brood distinctions were made and male (if any) identification was made

Section 4 - Test Organisms

4.1 Scientific name of test species, verification of species documented
4.2 Age (life stage) of test species (be specific for all species). Age at time of test initiation (for example, for *C. dubia* be sure to clarify the window of age of the neonates as well as the overall age of the animals)
4.3 Mean length and weight (where applicable)
4.4 Source and QA/QC test conditions
4.5 Holding Conditions
4.6 Diseases and treatment (where applicable)
4.7 Taxonomic key used for species identification

Section 5 - Quality Assurance

5.1 Reference toxicant used routinely; source; date received; lot number
5.2 Date and time of most recent reference toxicant test; test results and current control (cusum) chart including 20 most recent data points
5.3 Dilution water used in reference toxicant tests (with characteristics provided)
5.4 Physical and chemical methods used
5.5 Reference toxicant results (NOEC, IC_{25}, or LC_{50} where applicable, LOEC or EC_{50})

Section 6 - Results

6.1 Copies of all bench sheets. Be sure to count and notate broods for reproduction test with *Ceriodaphnia*

6.2 Raw toxicity data in tabular form, including daily records of affected organisms in each replicate at each concentration (including controls) and plots of toxicity data

6.3 Table of endpoints (LC_{50}, IC_{25}, NOEC for each endpoint) and confidence limits (where applicable)

6.4 Statistical methods and software used to calculate endpoints

6.5 Summary table of physical and chemical data

Appendix B:

Participant Laboratory Standard Operating Procedures

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the *Ceriodaphnia dubia* Acute Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: October 25, 1999

SUBJECT: Final Guidance and SOP for the *Ceriodaphnia dubia* Acute Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the *Ceriodaphnia dubia* acute test method in EPA's WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. The SOP details the sample distribution and testing schedule and provides important information for completing participant laboratory tasks, such as instructions for the reconstitution of ampule samples. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

Also enclosed is the electronic data reporting format disk for the *Ceriodaphnia dubia* acute method. A description and instructions for use of the electronic data report form are provided in the SOP.

Please ensure that the enclosed SOP and disk pertaining to the *Ceriodaphnia dubia* acute test method are distributed to laboratory staff that will be performing the test method in the study.

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Ceriodaphnia dubia Acute Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Ceriodaphnia dubia acute test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Ceriodaphnia dubia* acute method will occur between November 9 and 13, 1999 with final reports due 30 days following termination of all tests (December 13, 1999). The schedule for the testing is provided below. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Laboratories participating in the base study design will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 11/9/99 for test initiation on that day, and two samples will arrive on 11/11/99 for test initiation on that day. Laboratories participating in the extended study design will each receive three ampule samples. Some laboratories will receive two samples on 11/9/99 and one on 11/11/99, while other laboratories in the extended design will receive one on 11/9/99 and two on 11/11/99. Laboratories participating in the extended design will be notified by fax prior to study initiation to confirm the number of samples (one or two) that should be expected for each testing period.

Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For whole volume samples, one aliquot will be received on test Day 0. This aliquot shall be used for test initiation on Day 0. For ampule samples, one aliquot will be received on test Day 0. This aliquot shall be reconstituted on Day 0, and the reconstituted sample shall be used for test initiation.

Table 1. Schedule for *Ceriodaphnia dubia* Acute Testing.

Date (start date - finish date)	Activity
11/9/99 - 11/11/99	Conduct Cladoceran, *Ceriodaphnia dubia*, Acute Test with samples #1&2
11/11/99 - 11/13/99	Conduct Cladoceran, *Ceriodaphnia dubia*, Acute Test with samples #3&4
12/13/99	Cladoceran, *Ceriodaphnia dubia*, Acute Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and conductivity or salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. No sample preparation or adjustment steps (e.g. pH adjustment) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using moderately hard synthetic freshwater as the dilution water (prepared according to Section 7 of the method manuals).

3.1.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in 125ml plastic containers. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using moderately hard synthetic freshwater as the dilution water (prepared according to Section 7 of the method manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the *Ceriodaphnia dubia* acute test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at 4 °C ± 2 °C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, conductivity or salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) Daily observation of mortality and removal of dead organisms for each test is required.

(11) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(12) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. All bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An LC_{50} must be reported for each acute test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all *Ceriodaphnia dubia* acute tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in this table represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Cladoceran, *Ceriodaphnia dubia,* Acute Test. Summary of test conditions and test acceptability criteria for daphnid, *Ceriodaphnia dubia* acute toxicity tests with effluents and receiving waters

1. Test type:	***Static non-renewal***
2. Test duration:	***48 h***
3. Temperature:	***25 ± 1 ℃***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***30 mL***
8. Test solution volume:	***15 mL***
9. Renewal of test solutions:	***None***
10. Age of test organisms:	Less than 24-h old
11. No. organisms per test chamber:	***5***
12. No. replicate chambers per concentration:	***4***
13. No. organisms per concentration:	***20***
14. Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test.
15. Test chamber cleaning:	Cleaning not required
16. Test chamber aeration:	None
17. Dilution water:	***Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor	***0.5***
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	1 L
23. Test acceptability criterion:	90% or greater survival in controls

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the *Ceriodaphnia dubia* acute test method, the 48 hour LC_{50} shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Additional Information on Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. Deliverables #1 and 2 shall be submitted according to the requirements specified in the SOW. This section provides additional instructions for the submission of the electronic results synopsis.

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the *Ceriodaphnia dubia* acute test method. The disk contains a Microsoft Excel 97 spreadsheet file named CDA___.xls. The CDA indicates that this template is for the *Ceriodaphnia dubia* acute test method, and the number following is a unique identifying number for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template.

Notice the following characteristics of the electronic template file:

1. The file contains four worksheet pages labeled “Sample #1", “Sample #2", “Sample #3", and “Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
2. Each worksheet page contains six information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, and summarized test results) in which data should be entered. The seventh information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested. If participating in the extended study design, the last worksheet page (Sample #4) may be left empty.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Cladoceran, *Ceriodaphnia, dubia*, Acute Test

For each sample, a single liquid ampule will be received. The container shall be reconstituted and used to initiate the test. Follow the directions below for the reconstitution of the sample ampule.

1. Volumetrically add 100 mL of the liquid ampule sample to approximately 600mL of moderately-hard synthetic freshwater (MHSF) prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 1L (measured using volumetric glassware) with MHSF dilution water.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

6. This 1L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation. Store sample at 4°C.

8. Perform the Cladoceran, *Ceriodaphnia dubia*, Acute Test as described in the method manuals, the SOW for this study, and this SOP.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the *Ceriodaphnia dubia* Chronic Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: October 8, 1999

SUBJECT: Additional Question from the WET Participant Laboratory Meeting

On October 6, 1999, the meeting notes from the WET Participant Laboratory Meeting were distributed along with the final guidance SOP and electronic data reporting disk for the *Ceriodaphnia dubia* chronic test method. Unfortunately, one question and answer from the meeting pertaining to the *Ceriodaphnia dubia* chronic test method was inadvertently omitted from the meeting notes. The question and answer are provided below:

Q: Is the addition of selenium to synthetic freshwater required in the preparation of dilution water for *Ceriodaphnia dubia* tests?

A: The addition of selenium is not specifically required in this study, but is recommended. Laboratories should follow their standard procedures and the method manuals for preparation of synthetic freshwater.

Thank you for your attention to this addition, and I apologize for the omission of this question in the meeting notes.

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Ceriodaphnia dubia Survival and Reproduction Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Ceriodaphnia dubia survival and reproduction test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Ceriodaphnia dubia* survival and reproduction method will occur between October 12 and November 3, 1999 with final reports due 30 days following termination of all tests (December 3, 1999). The schedule for the testing is provided below. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Laboratories participating in the base study design will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 10/12/99 for test initiation on that day, and two samples will arrive on 10/26/99 for test initiation on that day. Laboratories participating in the extended study design will each receive three ampule samples. Some laboratories will receive two samples on 10/12/99 and one on 10/26/99, while other laboratories in the extended design will receive one on 10/12/99 and two on 10/26/99. Laboratories participating in the extended design will be notified by fax prior to study initiation to confirm the number of samples (one or two) that should be expected for each testing period.

Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For tests that require additional shipments for sample renewal, the sample number shall be the same for each initiation and renewal shipment with the addition of a letter (A, B, or C) after the sample number to designate the sample for use as initiation (A), renewal 1 (B), or renewal 2 (C).

For whole volume samples, separate aliquots will be received on test Day 0, Day 2, and Day 4. The first aliquot (identified with the sample number and the letter "A") shall be used for test initiation on Day 0 and renewal on Day 1. The second aliquot (identified with the sample number and the letter "B") shall be used for test renewals on Day 2 and Day 3. The final aliquot (identified with the sample number and the letter "C") shall be used for test renewal on Day 4, Day 5, Day 6, and Day 7. This sample shipment schedule mimics the typical schedule for chronic monitoring of effluent for compliance.

For ampule samples, three separate ampules containers (marked with the sample number followed by A, B, or C) will be received in a single shipment on test Day 0. The container marked "A" shall be reconstituted on test Day 0 and used for test initiation and renewal on Day 1. The other aliquots of the sample shall be refrigerated and stored until use on Day 2 and Day 4, respectively. The container marked "B" shall be reconstituted on test Day 2 and used for renewal on Day 2 and Day 3. The container marked "C" shall be reconstituted on Day 4 and used for renewal on Day 4, Day 5, Day 6, and Day 7. The sample reconstitution schedule for ampules attempts to mimic the typical sample shipment schedule for chronic monitoring of effluents for compliance.

Table 1. Schedule for *Ceriodaphnia dubia* Survival and Reproduction Testing.

Date (start date - finish date)	Activity
10/12/99 - 10/20/99	Conduct Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test with samples #1&2
10/26/99 - 11/3/99	Conduct Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test with samples #3&4
12/3/99	Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and conductivity or salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00 AM (local laboratory

time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00 AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary. If renewal shipments do not arrive on the expected day, DynCorp will provide guidance for test renewal on a case-by-case basis. Depending on the volume of sample remaining from previous shipments, laboratories may be instructed to conduct full renewals with the remaining sample, conduct partial renewals with the remaining sample, or omit the sample renewal for that day but carefully record dissolved oxygen throughout the day and remove excess food and dead organisms from the test containers.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. No sample preparation or adjustment steps (e.g. pH adjustment) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using moderately hard synthetic freshwater as the dilution water (prepared according to Section 7 of the method manuals).

3.1.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in 125ml plastic containers. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using moderately hard synthetic freshwater as the dilution water (prepared according to Section 7 of the method manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the *Ceriodaphnia dubia* survival and reproduction test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any

adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at 4 °C ± 2 °C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, conductivity or salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals. In addition, block randomization and use of known parentage will be required for the *Ceriodaphnia dubia* survival and reproduction test as described in Method 1002.0.

(10) The *Ceriodaphnia dubia* Survival and Reproduction Test (Method 1002.0), which would otherwise be terminated after 3 broods according to Section 13.12.1 of that method, must be conducted for 8 days, with endpoints including survival, number of young per day, and number of broods recorded each day. These readings are to be made at the end of the 6th, 7th and 8th day (specifically, at 144 hours, at 168 hours, and at 192 hours, respectively, from test initiation). This will be done to assess the effect of that test acceptance criterion on test results. No test shall be terminated prior to the eighth day for any reason, including a failure to meet test acceptance criteria. The additional measurements on days 6, 7, and 8 should be included as raw data in the final data report, but should not affect the data analysis of test results. The analysis of data from the *C. dubia* chronic test shall be conducted as specified in the method manual using the three brood approach.

(11) Daily observation of mortality and removal of dead organisms for each test is required. Daily young counts are required for the *Ceriodaphnia dubia* survival and reproduction test, along with determining the number of broods at each count.

(12) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(13) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(14) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(15) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(16) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(17) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. All bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(18) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(19) An NOEC and LC_{50} for survival, and an NOEC and IC_{25} for growth/reproduction must be reported as appropriate for each short-term chronic test as described in the method manuals. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all *Ceriodaphnia dubia* survival and reproduction tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in this table represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Cladoceran, *Ceriodaphnia dubia,* Survival and Reproduction Test. Summary of test conditions and test acceptability criteria for daphnid, *Ceriodaphnia dubia*, survival and reproduction toxicity tests with effluents and receiving waters

1. Test type:	Static renewal
2. Temperature (°C):	25 ± 1°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 μE/m^2/s, or 50-100 ft-c (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h dark
6. Test chamber size:	***30 mL***
7. Test solution volume:	***15 mL***
8. Renewal of test solutions:	Daily
9. Age of test organisms:	Less than 24 h; and all released within a 8-h period
10. No. neonates per test chamber:[1]	1
11. No. replicate test chambers per concentration:	10
12. No. neonates per test concentration:	10
13. Feeding regime:	Feed 0.1 mL each of YCT and algal suspension per test chamber daily.
14. Cleaning:	Use freshly cleaned glass beakers or new plastic cups daily
15. Aeration:	None
16. Dilution water:	***Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)***
17. Test concentrations:	***Five concentrations and a control***
18. Dilution factor:	***0.5***
19. Test duration:[2]	***8 days***
20. Endpoints:	***Survival and reproduction***
21. Test acceptability criteria:	80% or greater survival and an average of 15 or more young per surviving female in the control solutions. 60% of surviving control organisms must produce three broods.
22. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
23. Sample volume required:	1 L/day

[1] Test vessels shall be randomized in accordance with the method manuals. In addition, block randomization and use of known parentage will be required for the *Ceriodaphnia* survival and reproduction test as described in the manual and guidance will be reiterated in the specific instructions provided to the laboratories.

[2] The *Ceriodaphnia dubia* test which would otherwise be terminated after 3 broods according to methods manual Section 13.12.1 of that Method must be conducted for 8 days, with endpoints (survival and number of young per day and number of broods at each recording interval) recorded at the end of the 6th, 7th and 8th day (specifically, at 144, 168, and 192 hours, respectively, from test initiation). No test shall be terminated prior to the eighth day for any reason, including a failure to meet test acceptance criteria.

3.2.3 Data Analysis

The *Ceriodaphnia dubia* survival and reproduction test shall not be terminated until day 8, however, survival and reproduction endpoints should be calculated according to the method manuals using the three brood approach. Raw data beyond the three brood time period should be recorded and reported, yet this data should not be included in endpoint determination. EPA will perform additional analysis using this data.

For survival endpoints, a NOEC and an LC_{50} should be calculated at the time of normal test termination (after 60% of controls have had three broods). For reproduction endpoints, a NOEC and an IC_{25} should be calculated at the time of normal test termination (after 60% of controls have had three broods). Data analysis and statistical procedures should be conducted according to the methods manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample should be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Additional Information on Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. Deliverables #1 and 2 shall be submitted according to the requirements specified in the SOW. This section provides additional instructions for the submission of the electronic results synopsis.

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the *Ceriodaphnia dubia* survival and reproduction test method. The disk contains a Microsoft Excel 97 spreadsheet file named CDC___.xls. The CDC indicates that this template is for the Ceriodaphnia dubia chronic test method, and the number following is a unique identifying number for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template.

Notice the following characteristics of the electronic template file:

1. The file contains four worksheet pages labeled “Sample #1", “Sample #2", “Sample #3", and “Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
2. Each worksheet page contains seven information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, summarized biological data, and summarized test results) in which data should be entered. The eighth information

box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested. If participating in the extended study design, the last worksheet page (Sample #4) may be left empty.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test

For each sample, three liquid ampules will be received (marked with the sample number followed by an "A", "B", or "C"). The three containers shall be reconstituted as described below to mimic the sample shipment schedule for effluent samples. The container marked "A" shall be reconstituted on the day of test initiation (Day 0) and used for renewal on Day 1. The container marked "B" shall be reconstituted on Day 2 and used for renewals on Day 2 and Day 3. The container marked "C" shall be reconstituted on Day 4 and used for renewals on Day 4, Day 5, Day 6, and Day 7. Follow the directions below for the reconstitution of each sample ampule.

1. Volumetrically add 100 mL of the liquid ampule sample to approximately 1L of moderately-hard synthetic freshwater (MHSF) prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 3L (measured using volumetric glassware) with MHSF dilution water.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

6. This 3L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation and test renewal on Day 1. Store sample at 4°C.

8. Perform the Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test as described in the SOW for this study and the methods manuals.

9. Follow Steps 1 through 6 with each sample container to prepare the reconstituted sample on Day 2 and Day 4 for subsequent daily renewals.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the Fathead Minnow Acute Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: October 13, 1999

SUBJECT: Final Guidance and SOP for the Fathead Minnow Acute Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the fathead minnow acute test method in EPA's WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. The SOP details the sample distribution and testing schedule and provides important information for completing participant laboratory tasks, such as instructions for the reconstitution of ampule samples. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

Also enclosed is the electronic data reporting format disk for the fathead minnow acute method. A description and instructions for use of the electronic data report form are provided in the SOP.

Please ensure that the enclosed SOP and disk pertaining to the fathead minnow acute test method are distributed to laboratory staff that will be performing the test method in the study.

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Pimephales promelas Acute Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Pimephales promelas acute test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Pimephales promelas* acute method will occur between October 21 and November 8, 1999 with final reports due 30 days following termination of all tests (December 8, 1999). The schedule for the testing is provided below. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Laboratories participating in the base study design will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 10/21/99 for test initiation on that day, and two samples will arrive on 11/4/99 for test initiation on that day. Laboratories participating in the extended study design will each receive three ampule samples. Some laboratories will receive two samples on 10/21/99 and one on 11/4/99, while other laboratories in the extended design will receive one on 10/21/99 and two on 11/4/99. Laboratories participating in the extended design will be notified by fax prior to study initiation to confirm the number of samples (one or two) that should be expected for each testing period.

Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For whole volume samples, one aliquot will be received on test Day 0. This aliquot shall be used for test initiation on Day 0 and test renewal at 48 hours. For ampule samples, one aliquot will be received on test Day 0. This aliquot shall be reconstituted on Day 0, and the reconstituted sample shall be used for test initiation on Day 0 and test renewal at 48 hours.

Table 1. Schedule for *Pimephales promelas* Acute Testing.

Date (start date - finish date)	Activity
10/21/99 - 10/25/99	Conduct Fathead minnow, *Pimephales promelas,* Acute Test with samples #1&2
11/4/99 - 11/8/99	Conduct Fathead minnow, *Pimephales promelas,* Acute Test with samples #3&4
12/8/99	Fathead minnow, *Pimephales promelas,* Acute Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and conductivity or salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00 AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00 AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track

lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. No sample preparation or adjustment steps (e.g. pH adjustment) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using moderately hard synthetic freshwater as the dilution water (prepared according to Section 7 of the method manuals).

3.1.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in 125ml plastic containers. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using moderately hard synthetic freshwater as the dilution water (prepared according to Section 7 of the method manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the Fathead minnow, *Pimephales promelas*, Acute test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms “must” and “shall”) and recommendations and guidance (indicated by discretionary terms “should” and “may”). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at 4 °C ± 2 °C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, conductivity or salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) Daily observation of mortality and removal of dead organisms for each test is required.

(11) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(12) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. All bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An IC_{25} must be reported for each chronic test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all fathead minnow acute tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in this table represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Fathead Minnow, *Pimephales promelas,* Acute Test. Summary of test conditions and test acceptability criteria for fathead minnow, *Pimephales promelas*, acute toxicity tests with effluents and receiving waters

1. Test type:	***Static-renewal***
2. Test duration:	***96 h***
3. Temperature:	***25 ± 1 °C***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***250 mL***
8. Test solution volume:	***200 mL***
9. Renewal of test solutions:	***At 48 h***
10. Age of test organisms:	1-14 days; 24-h range in age
11. No. organisms per test chamber:	***10***
12. No. replicate chambers per concentration:	***2***
13. No. organisms per concentration:	***20***
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	***Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor	***0.5***
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	***2 L for effluents***
23. Test acceptability criterion:	90% or greater survival in controls

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the Fathead minnow acute test method, the 96 hour LC_{50} and NOEC shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Additional Information on Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. Deliverables #1 and 2 shall be submitted according to the requirements specified in the SOW. This section provides additional instructions for the submission of the electronic results synopsis.

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the Fathead minnow acute test method. The disk contains a Microsoft Excel 97 spreadsheet file named FHA___.xls. The FHA indicates that this template is for the Fathead minnow acute test method, and the number following is a unique identifying number for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template.

Notice the following characteristics of the electronic template file:

1. The file contains four worksheet pages labeled “Sample #1", “Sample #2", “Sample #3", and “Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
2. Each worksheet page contains six information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, and summarized test results) in which data should be entered. The seventh information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested. If participating in the extended study design, the last worksheet page (Sample #4) may be left empty.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Fathead Minnow, *Pimephales promelas*, Acute Test

For each sample, a single liquid ampule will be received. The container shall be reconstituted and used to initiate the test. The same reconstituted sample shall be used for test renewal at 48 hours. Follow the directions below for the reconstitution of the sample ampule.

1. Volumetrically add 100 mL of the liquid ampule sample to approximately 1L of moderately-hard synthetic freshwater (MHSF) prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 4L (measured using volumetric glassware) with MHSF dilution water.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

6. This 4L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation and test renewal at 48 hr. Store sample at 4°C.

8. Perform the Fathead Minnow, *Pimephales promelas*, Acute Test as described in the SOW for this study and the methods manuals.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the Fathead Minnow Chronic Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: September 23, 1999

SUBJECT: Final Guidance and SOP for the Fathead Minnow Chronic Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the fathead minnow chronic test method of the WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

At the Participant Laboratory Meeting held on September 16, 1999, EPA and DynCorp staff presented the general study design, discussed participant laboratory tasks, and answered questions from participant laboratories. Notes from the meeting, including handouts, slide copies, and a list of questions and answers, will be provided to participant laboratories by next week. Unfortunately, the fathead chronic test method is scheduled to begin before these meeting notes are finalized and distributed, so items from the meeting that specifically apply to the fathead chronic test method are addressed in this memo. Participant laboratory tasks that were discussed in the meeting presentations are covered in the SOW and SOP provided. Specific questions and answers from the meeting that pertain to the fathead chronic test are listed below:

(1) **Q:** Is residual chlorine measurement required?
A: The requirements of the method manuals should be followed for each test method. Residual chlorine measurement is not specifically required for the fathead chronic test, however, if your laboratory routinely tests residual chlorine on each sample, this information should be included in the data report deliverables.

(2) **Q:** Our laboratory's moderately hard synthetic water typically has a pH of 8.0-8.2, above the range given in Section 7 of the method manual. Should we adjust the pH of the dilution water to within the given range in Table 3 (Section 7 of the method manuals)?
A: No. The table in Section 7 gives expected approximate ranges, not required ranges. No adjustment should be made, however, the laboratory should confirm that the water is prepared properly and prepared using the proper chemicals (correct hydrate forms of the chemicals).

(3) **Q:** Is distilled water acceptable for use as the base for moderately hard synthetic dilution water?
A: No. The method manuals state that the synthetic dilution water must be prepared from deionized water obtained from a Millipore Milli-Q or equivalent system. An equivalent system should be

interpreted to imply a deionizing system that produces water of equivalent quality (i.e., conductivity).

(4) **Q:** Can plastic beakers be used as test containers for the fathead chronic test?
A: Yes. Glass or plastic may be used.

(5) **Q:** Can a 500mL plastic disposable beaker be used for the fathead chronic test?
A: In the original solicitation, the test chamber size for the fathead chronic test was stated as 500mL. This was an error that was corrected in the table of test conditions provided in the final SOP. The intended test chamber was a 600mL glass beaker that is graduated up to 500mL (and is often referred to as a 500mL beaker). Because of the error in the original solicitation document, a test chamber size of 500 - 600mL will be acceptable for the study, provided that the chamber has similar dimensions to the glass 600mL beaker. Glass or plastic is acceptable within these volume constraints.

(6) **Q:** How is mean dry weight measured for the growth endpoint in the fathead chronic test?
A: According to the method manual, the mean dry weight is measured for each replicate as the total dry weight of larvae (total weight minus tare weight) divided by the number of original larvae in that replicate. This endpoint is in essence a combined survival and growth endpoint. For the determination of test acceptability, the mean dry weight per surviving larvae should be calculated for control replicates.

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Pimephales promelas Larval Survival and Growth Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Pimephales promelas larval survival and growth test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Pimephales promelas* larval survival and growth method will occur between September 28 and October 12, 1999 with final reports due 30 days following termination of all tests (November12, 1999). The schedule for the testing is provided below. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Laboratories participating in the base study design will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 9/28/99 for test initiation on that day, and two samples will arrive on 10/5/99 for test initiation on that day. Laboratories participating in the extended study design will each receive three ampule samples. Some laboratories will receive two samples on 9/28/99 and one on 10/5/99, while other laboratories in the extended design will receive one on 9/28/99 and two on 10/5/99. Laboratories participating in the extended design will be notified by fax prior to study initiation to confirm the number of samples (one or two) that should be expected for each testing period.

Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For tests that require additional shipments for sample renewal, the sample number shall be the same for each initiation and renewal shipment with the addition of a letter (A, B, or C) after the sample number to designate the sample for use as initiation (A), renewal 1 (B), or renewal 2 (C).

For whole volume samples, separate aliquots will be received on test Day 0, Day 2, and Day 4. The first aliquot (identified with the sample number and the letter "A") shall be used for test initiation on Day 0 and renewal on Day 1. The second aliquot (identified with the sample number and the letter "B") shall be used for test renewals on Day 2 and Day 3. The final aliquot (identified with the sample number and the letter "C") shall be used for test renewal on Day 4, Day 5, and Day 6. This sample shipment schedule mimics the typical schedule for chronic monitoring of effluent for compliance.

For ampule samples , three separate ampules containers (marked with the sample number followed by A, B, or C) will be received in a single shipment on test Day 0. The container marked "A" shall be reconstituted on test Day 0 and used for test initiation and renewal on Day 1. The other aliquots of the sample shall be refrigerated and stored until use on Day 2 and Day 4, respectively. The container marked "B" shall be reconstituted on test Day 2 and used for renewal on Day 2 and 3. The container marked "C" shall be reconstituted shall be reconstituted on Day 4 and used for renewal on Day 4, Day 5 and Day 6. The sample reconstitution schedule for ampules attempts to mimic the typical sample shipment schedule for chronic monitoring of effluents for compliance.

Table 1. Schedule for *Pimephales promelas* Larval Survival and Growth Testing.

Date (start date - finish date)	Activity
9/28/99 - 10/5/99	Conduct Fathead minnow, *Pimephales promelas,* Larval Survival and Growth Test with samples #1&2
10/5/99 - 10/12/99	Conduct Fathead minnow, *Pimephales promelas,* Larval Survival and Growth Test with samples #3&4
11/12/99	Fathead minnow, *Pimephales promelas,* Larval Survival and Growth Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and conductivity or salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall stay with the ampules and rechecked when renewals are prepared. The temperature check shall be discarded after the final temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00 AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or at FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00 AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary. If renewal shipments do not arrive on the expected day, DynCorp will provide guidance for test renewal on a case-by-case basis. Depending on the volume of sample remaining from previous shipments, laboratories may be instructed to conduct full renewals with the remaining sample, conduct partial renewals with the remaining sample, or omit the sample renewal for that day but carefully record dissolved oxygen throughout the day and remove excess food and dead organisms from the test containers.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. No sample preparation or adjustment steps (e.g. pH adjustment) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using moderately hard synthetic freshwater as the dilution water (prepared according to Section 7 of the methods manuals).

3.1.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in 125ml plastic containers. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using moderately hard synthetic freshwater as the dilution water (prepared according to Section 7 of the methods manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the Fathead minnow, *Pimephales promelas*, Chronic test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at $4\,^{\circ}C \pm 2\,^{\circ}C$) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, conductivity or salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) Daily observation of mortality and removal of dead organisms for each test is required.

(11) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(12) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. All bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An IC_{25} must be reported for each chronic test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all fathead minnow chronic tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that

are bold italic in these tables represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test. Summary of test conditions and test acceptability criteria for fathead minnow, *Pimephales promelas*, larval survival and growth toxicity tests with effluents and receiving waters

1. Test type:	Static renewal
2. Temperature:	25 ± 1°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h darkness
6. Test chamber size:	***600 mL***
7. Test solution volume:	***250 mL***
8. Renewal of test solutions:	Daily
9. Age of test organisms:	Newly hatched larvae less than 24h old. If shipped, not more than 48h old, 24h range in age
10. No. larvae per test chamber:	***10***
11. No. replicate chambers per concentration:	***4***
12. No. larvae per concentration:	***40***
13. Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old)
14. Feeding regime:	Feed 0.1 g newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 g twice daily, 6 h between feedings (at the beginning of the work day prior to renewal, and at the end of the work day following renewal). Sufficient nauplii are added to provide an excess. Larvae fish are not fed during the final 12 h of the test
15. Cleaning:	Siphon daily, immediately before test solution renewal
16. Aeration:	None, unless DO concentration falls below 4.0 mg/L. Rate should not exceed 100 bubbles/min
17. Dilution water:	***Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor ***0.5***	
20. Test duration: 7 days	
21. Endpoints:	***Survival and growth (weight as mean per original)***
22. Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers equals or exceeds 0.25 mg/surviving
23. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
24. Sample volume required:	2.5 L/day

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the Fathead minnow chronic test method, the 7 day survival LC_{50}, 7 day survival NOEC, growth IC_{25}, and growth NOEC shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Additional Information on Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. Deliverables #1 and 2 shall be submitted according to the requirements specified in the SOW. This section provides additional instructions for the submission of the electronic results synopsis.

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the Fathead minnow chronic test method. The disk contains a Microsoft Excel 97 spreadsheet file named FHC___.xls. The FHC indicates that this template is for the Fathead minnow chronic test method, and the number following is a unique identifying number for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template.

Notice the following characteristics of the electronic template file:

1. The file contains four worksheet pages labeled “Sample #1", “Sample #2", “Sample #3", and “Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
2. Each worksheet page contains seven information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, weight data, and summarized test results) in which data should be entered. The eighth information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested. If participating in the extended study design, the last worksheet page (Sample #4) may be left empty.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test

For each sample, three liquid ampules will be received (marked with the sample number followed by an "A", "B", or "C"). The three containers shall be reconstituted as described below to mimic the sample shipment schedule for effluent samples. The container marked "A" shall be reconstituted on the day of test initiation (Day 0) and used for renewal on Day 1. The container marked "B" shall be reconstituted on Day 2 and used for renewals on Day 2 and Day 3. The container marked "C" shall be reconstituted on Day 4 and used for renewals on Day 4, Day 5, and Day 6. Follow the directions below for the reconstitution of each sample ampule.

1. Volumetrically add 100 mL of the liquid ampule sample to approximately 1L of moderately-hard synthetic freshwater (MHSF) prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 8L (measured using volumetric glassware) with MHSF dilution water.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. Cubitainer) is recommended.

6. This 8L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation and test renewal on Day 1. Store sample at 4°C.

8. Perform the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test as described in the SOW for this study and the methods manuals.

9. Follow Steps 1 through 6 with each sample container to prepare the reconstituted sample on Day 2 and Day 4 for subsequent daily renewals.

EPA

United States
Environmental Protection Agency
Washington, DC 20460

EPISODE NO:

SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report
USEPA ENGINEERING AND ANALYSIS DIVISION
SAMPLE CONTROL CENTER

Fax completed form immediately upon completion, and include hardcopy in final data report to:

DynCorp - Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
phone: (703) 461-2100
fax: (703) 461-8056

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	**Pre-Shipment**	**Post-Shipment**
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the *Selenastrum capricornutum* Growth Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: February 25, 2000

SUBJECT: Final Guidance and SOP for the *Selenastrum capricornutum* Growth Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the *Selenastrum capricornutum* growth test method in EPA's WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. The SOP details the sample distribution and testing schedule and provides important information for completing participant laboratory tasks, such as instructions for the reconstitution of ampule samples. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

Also enclosed is the electronic data reporting format disk for the *Selenastrum capricornutum* growth test method. A description and instructions for use of the electronic data report form are provided in the SOP.

Please ensure that the enclosed SOP and disk pertaining to the *Selenastrum capricornutum* growth test method are distributed to laboratory staff that will be performing the test method in the study. Also, please see that laboratory staff read over the SOP carefully to ensure the proper data is collected and reported. **Please note that the ampule reconstitution instructions for this method require the addition of 200mL of the ampule sample instead of 100mL that has been used previously for other methods. For this reason, ampule samples will be provided in 500mL bottles for this method.**

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Green Alga, *Selenastrum capricornutum* Growth Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Selenastrum capricornutum growth test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the green alga, *Selenastrum capricornutum,* growth method will occur between March 9 and April 3, 2000 with final reports due 30 days following termination of all tests (May 3, 2000). The testing schedule is provided below in Table 1. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Participant laboratories in the study will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. One sample will arrive on 3/9/00 for test initiation on that day, the second, third, and fourth samples will arrive on 3/16/00, 3/23/00, and 3/30/00, respectively. **On each testing date, two side-by-side tests will be conducted on the sample received. One test will analyze the sample with the addition of EDTA, and one test will analyze the sample without the addition of EDTA**.

Each sample that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For whole volume samples that are received, the laboratory shall split the sample as described in Section 3.2.1 for analysis with and without EDTA. For ampule samples, the laboratory shall reconstitute the sample according to instructions in Appendix A and then split the sample for analysis with and without EDTA.

Table 1. Schedule for *Selenastrum capricornutum* Growth Testing.

Date (start date - finish date)	Activity
3/9/00 - 3/13/00	Conduct Green Alga, *Selenastrum capricornutum*, Growth Test with sample #1 (with and without EDTA)
3/16/00 - 3/20/00	Conduct Green Alga, *Selenastrum capricornutum*, Growth Test with sample #2 (with and without EDTA)
3/23/00 - 3/27/00	Conduct Green Alga, *Selenastrum capricornutum*, Growth Test with sample #3 (with and without EDTA)
3/30/00 - 4/3/00	Conduct Green Alga, *Selenastrum capricornutum*, Growth Test with sample #4 (with and without EDTA)
5/3/00	Green Alga, *Selenastrum capricornutum*, Growth Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and conductivity or salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary.

3.0 WET Test Analysis

3.1 Dilution Water Preparation

Two separate dilution waters must be prepared for the *Selenastrum* growth test. One must be prepared as described in the methods manual with the addition of EDTA, and one must be prepared without the addition of EDTA. Acceptable dilution waters for this test include the algal culture media or moderately hard synthetic water with the correct addition of nutrients.

3.2 Sample Preparation

3.2.1 Whole Volume Samples

Whole volume samples will be received in 4L cubitainers with sufficient volume for the conduct of two tests and required water chemistry analysis. Prior to preparing test solutions, the whole volume sample shall be split into two portions of 2L each. One portion should be labeled "with EDTA", and 2mL (1mL per liter) of each nutrient stock including EDTA shall be added according to the method manual. The second portion should be labeled "without EDTA", and 2mL (1mL per liter) of each nutrient stock excluding EDTA shall be added according to the method manual. Each sample portion shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. The *Selenastrum capricornutum* growth test shall be conducted on each sample portion (with and without EDTA). Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from each portion using the appropriate dilution water (Section 3.1)

3.2.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in 500ml plastic bottles. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. According to the instructions, the reconstituted sample will then be split into two portions and appropriate nutrients added to prepare a "with EDTA" and "without EDTA" portion. These portions shall then be treated as a typical 100% effluent sample received for

NPDES compliance monitoring. The *Selenastrum capricornutum* growth test shall be conducted on each sample portion (with and without EDTA). Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from each portion using the appropriate dilution water (Section 3.1)

3.3 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the *Selenastrum capricornutum* growth test method. Except where indicated in Sections 3.3.1 and 3.3.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.3.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at 4 °C ± 2 °C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals. NOTE: Refer to the electronic benchsheet for required and recommended information.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(11) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(12) **The Green Algae, *Selenastrum capricornutum*, Growth Test shall be conducted simultaneously with and without EDTA for each sample. For participant laboratories, four samples will be tested with and without EDTA for a total of eight analyses.**

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. All bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An IC_{25} must be reported for each chronic test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all *Selenastrum capricornutum* growth tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in this table represent conditions standardized for the purposes of this study where method manuals provide a range.

For the WET interlaboratory study, the algal growth endpoint must be measured as cell counts using an approved counting method (see method manual Section 14.10.6.2). Automatic particle counters or manual microscopic counting methods are acceptable. If laboratories use multiple counting methods, submission of data from each counting method is encouraged (but not required) and would improve the evaluation of the method and allow comparison of counting techniques.

Table 2. Green Alga, *Selenastrum capricornutum*, Growth Test. Summary of test conditions and test acceptability criteria for green alga, *Selenastrum capricornutum*, growth toxicity tests with effluents and receiving waters. Test will be conducted with EDTA and without EDTA.

1. Test type:	Static non-renewal
2. Temperature:	25 ± 1°C
3. Light quality:	"Cool white" fluorescent lighting
4. Light intensity:	86 ± 8.6 μE/m^2/s (400 ± 40 ft-c or 4306 lux)
5. Photoperiod:	Continuous illumination
6. Test chamber size:	***250 mL***
7. Test solution volume:	***100 mL***
8. Renewal of test solutions:	None
9. Age of test organisms:	4 to 7 days
10. Initial cell density in test chambers:	10,000 cells/mL
11. No. replicate chambers per concentration:	***4***
12. Shaking rate:	***100 cpm continuous***
13. Aeration:	None
14. Dilution water:	***Algal stock culture medium, moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals(see Methods Manual Section 7, Dilution Water)***
15. Test concentrations:	***Five concentrations and a control***
16. Test dilution factor:	***0.5***
17. Test duration:	96 h
18. Endpoint:	***Growth (cell counts)***
19. Test acceptability criteria:	1 X 10^6 cells/mL with EDTA or 2 X 10^5 cells/mL without EDTA in the controls; Variability of controls should not exceed 20%
20. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
21. Sample volume required:	***2 L***

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the *Selenastrum capricornutum* growth test method, the 96 hour growth IC_{25} and NOEC shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Additional Information on Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. This section provides instructions for the submission of each of these deliverables.

4.1 Narrative Summary of Testing

This narrative summary shall clearly identify the laboratory, test method, samples tested, summarized test results, and any problems associated with the samples or conduct of the tests. This summary must list any tests that were initiated but not completed and fully explain the reason for not completing the test. This summary must also include a detailed written description of any approved modification to the procedures provided in this SOW, specific instructions, or the method manuals. This will include any telephone log and written correspondence received from the referee laboratory and/or DynCorp during the course of testing. Lastly, this summary should also provide comments on the performance of the method.

4.2 Hardcopy Results Synopsis and Full Report

At a minimum, this report must consist of the items outlined below in section 5.0, all raw data (biological and chemical), and laboratory bench sheets. This report must include all pertinent sample information including copies of all completed traffic report forms, all pertinent test condition and test organism information, all pertinent quality assurance information including results of the monthly QA/QC reference toxicant tests, and all summarized and raw results.

4.3 Electronic Results Synopsis

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the *Selenastrum capricornutum* growth test method. The disk contains a Microsoft Excel 97 spreadsheet file named SCG___.xls. The SCG indicates that this template is for the *Selenastrum capricornutum* growth test method, and the number following is a unique identifying number for your

laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template. **It is recommended to view the electronic benchsheet prior to initiating the test**, so the analyst can verify all the information collected on the laboratory benchsheet will be sufficient to complete the electronic results.

Notice the following characteristics of the electronic template file:

1. The file contains eight worksheet pages labeled "Sample #1w/ EDTA", "Sample #1w/o EDTA","Sample #2 w/EDTA","Sample #2 w/o EDTA", "Sample #3 w/EDTA", "Sample #3 w/o EDTA", "Sample #4 w/ EDTA", and "Sample #4 w/o EDTA". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file. NOTE: Each sample has two sheets, one for each sample tested with EDTA and one for each sample tested without EDTA.
2. Each worksheet page contains six information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, and summarized test results) in which data should be entered. The seventh information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Also keep a copy of the file for laboratory records (a backup in case the diskette crashes when redelivered to DynCorp). Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Data Report Format

Final hardcopy data reports should be submitted in the following format:
Note: Adapted from Section 10 of the methods manuals.

Section 1 - Summary Page

1.1 Laboratory name
1.2 Laboratory address and phone number
1.3 Name and signature of laboratory QA Officer, certifying that data have been internally reviewed and that personnel meticulously followed the methods, and the procedures are deemed to be compliant with the methods and acceptable for reporting purposes.
1.4 Laboratory contact responsible for study
1.5 Analyst(s) who performed WET tests (full names)
1.6 Toxicity tests performed
1.7 Detailed explanations of any difficulties encountered and any approved modifications to the techniques specified in this SOW, specific instructions, or the methods manuals.
1.8 Number of successful tests completed

Section 2 - Sample Information

2.1 Number of samples received and EPA sample number assigned to each sample. Copies of all completed traffic report forms should be included.
2.2 Dates of sample receipt
2.3 Sample temperature when received at laboratory
2.4 Physical and chemical data of sample contents (as required in appropriate method)
2.5 Dilution water
  2.5.1 Source and time frame water is used or how maintained
  2.5.2 Collection or preparation date(s), where applicable
  2.5.3 Pretreatment information
  2.5.4 Physical and chemical characteristics (pH, hardness, conductivity, salinity, etc.)
2.6 Sample storage information
2.7 Sample preparation for testing information

Section 3 - Test Conditions

3.1 Toxicity test method used (title, number, source)
3.2 Endpoint(s) of test(s)
3.3 Deviations from reference method(s), if any, and reason(s)
3.4 Date and time test(s) started, date and time samples were prepared and solutions transferred for renewals.
3.5 Date and time test(s) terminated
3.6 Type and volume of test chambers
3.7 Volume of solution used per chamber
3.8 Number of organisms per test chamber
3.9 Number of replicate test chambers per treatment
3.10 Feeding frequency and amount and type of food (be specific with sources, concentrations of foods (i.e, algae concentration, YCT solids level, preparation dates))
3.11 Acclimation of test organisms (temperature mean and range and, where applicable, salinity mean and range)
3.12 Test temperature (mean and range)
3.13 Test salinity, where applicable (mean and range)
3.14 Specify if aeration was needed
3.15 Specify if organisms were dried immediately for weighing or preserved prior to drying
3.16 Specify how food was prepared and sources of food. Include test results that validate the quality of batch food preparations (i.e., *Ceriodaphnia dubia* tests on YCT preparation).

3.17 Describe how routine chemistries on new solutions were made (in actual test chamber or in beakers after dispensing)
3.18 Describe how randomization was conducted, especially blocking and known parentage. Report how brood distinctions were made and male (if any) identification was made.

Section 4 - Test Organisms

4.1 Scientific name of test species, verification of species documented
4.2 Age (life stage) of test species (be specific for all species). Age at time of test initiation (for example, for *C. dubia* be sure to clarify the window of age of the neonates as well as the overall age of the animals.)
4.3 Mean length and weight (where applicable)
4.4 Source and QA/QC test conditions
4.5 Holding conditions
4.6 Diseases and treatment (where applicable)
4.7 Taxonomic key used for species identification

Section 5 - Quality Assurance

5.1 Reference toxicant used routinely; source; date received; lot number
5.2 Date and time of most recent reference toxicant test; test results and current control (cusum) chart including 20 most recent data points
5.3 Dilution water used in reference toxicant tests (with characteristics provided)
5.4 Physical and chemical methods used
5.5 Reference toxicant results (NOEC, IC_{25}, or LC_{50} where applicable, LOEC or EC_{50})

Section 6 - Results

6.1 Copies of all bench sheets. Be sure to count and notate broods for reproduction test with *Ceriodaphnia*
6.2 Raw toxicity data in tabular form, including daily records of affected organisms in each replicate at each concentration (including controls) and plots of toxicity data
6.3 Table of endpoints (LC_{50}, IC_{25}, NOEC for each endpoint) and confidence limits (where applicable)
6.4 Statistical methods and software used to calculate endpoints
6.5 Summary table of physical and chemical data

6.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Green Alga, *Selenastrum capricornutum* Growth Test

For each sample, a single liquid ampule will be received. The container shall be reconstituted, split into a "with EDTA" and "without EDTA" portion, and used to initiate two tests. Follow the directions below for the reconstitution of the sample ampule.

1. Volumetrically add **200 mL** of the liquid ampule sample to approximately 1L of moderately-hard synthetic freshwater (MHSF) prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 4L (measured using volumetric glassware) with MHSF dilution water.

4. Mix again by swirling and gently shaking.

5. Split the 4L reconstituted sample into two portions of 2L each, labeling one portion "with EDTA" and one portion "without EDTA."

6. Place the reconstituted sample portions in separate plastic containers of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

7. To the portion labeled "with EDTA," add 2mL (1mL per liter of sample) of nutrient stock including EDTA. Nutrient stock should be prepared according to the methods manual. This sample portion shall be used to conduct the *Selenastrum capricornutum* Growth Test with EDTA.

8. To the portion labeled "without EDTA," add 2mL (1mL per liter of sample) of nutrient stock excluding EDTA. Nutrient stock should be prepared according to the methods manual. This sample portion shall be used to conduct the *Selenastrum capricornutum* Growth Test without EDTA.

9. The two sample portions should be used directly for the 100% effluent test concentrations and diluted using the respective dilution water (with or without EDTA) to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

10. Perform the Green Alga, *Selenastrum capricornutum* Growth Test with and without EDTA using the respective sample portions. Perform the tests as described in the SOW for this study and the methods manuals.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the *Mysidopsis bahia* Chronic Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: February 10, 2000

SUBJECT: Final Guidance and SOP for the *Mysidopsis bahia* Chronic Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the *Mysidopsis bahia* chronic test method in EPA's WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. The SOP details the sample distribution and testing schedule and provides important information for completing participant laboratory tasks, such as instructions for the reconstitution of ampule samples. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

Also enclosed is the electronic data reporting format disk for the *Mysidopsis bahia* chronic method. A description and instructions for use of the electronic data report form are provided in the SOP.

Please ensure that the enclosed SOP and disk pertaining to the *Mysidopsis bahia* chronic test method are distributed to laboratory staff that will be performing the test method in the study. Also, please see that laboratory staff read over the SOP carefully to ensure the proper data is collected and reported.

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Mysidopsis bahia Survival, Growth, and Fecundity Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Mysidopsis bahia survival, growth, and fecundity test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Mysidopsis bahia* survival, growth, and fecundity (chronic) method will occur between February 22 and March 7, 2000 with final reports due 30 days following termination of all tests (April 6, 2000). The testing schedule is provided below in Table 1. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Participant laboratories will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 2/22/00 for test initiation on that day, and two samples will arrive on 2/29/00 for test initiation on that day.

Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For tests that require additional shipments for sample renewal, the sample number shall be the same for each initiation and renewal shipment with the addition of a letter (A, B, or C) after the sample number to designate the sample for use as initiation (A), renewal 1 (B), or renewal 2 (C).

For whole volume samples, separate aliquots will be received on test Day 0, Day 2, and Day 4. The first aliquot (identified with the sample number and the letter "A") shall be used for test initiation on Day 0 and renewal on Day 1. The second aliquot (identified with the sample number and the letter "B") shall be used for test renewals on Day 2 and Day 3. The final aliquot (identified with the sample number and the letter "C") shall be used for test renewal on Day 4, Day 5, and Day 6. This sample shipment schedule mimics the typical schedule for chronic monitoring of effluent for compliance.

For ampule samples, three separate ampule containers (marked with the sample number followed by A, B, or C) will be received in a single shipment on test Day 0. The container marked "A" shall be reconstituted on test Day 0 and used for test initiation and renewal on Day 1. The other aliquots of the sample shall be refrigerated and stored until use on Day 2 and Day 4, respectively. The container marked

"B" shall be reconstituted on test Day 2 and used for renewal on Day 2 and 3. The container marked "C" shall be reconstituted on Day 4 and used for renewal on Day 4, Day 5 and Day 6. The sample reconstitution schedule for ampules attempts to mimic the typical sample shipment schedule for chronic monitoring of effluents for compliance.

Table 1. Schedule for *Mysidopsis bahia* Survival, Growth, and Fecundity Testing.

Date (start date - finish date)	Activity
2/22/00 - 2/29/00	Conduct Mysid, *Mysidopsis bahia,* Survival, Growth, and Fecundity Test with samples #1&2
2/29/00 - 3/7/00	Conduct Mysid, *Mysidopsis bahia,* Survival, Growth, and Fecundity Test with samples #3&4
4/6/00	Mysid, *Mysidopsis bahia,* Survival, Growth, and Fecundity Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary. If renewal shipments do not arrive on the expected day, DynCorp will provide guidance for test renewal on a case-by-case basis. Depending on the volume of sample remaining from previous shipments, laboratories may be instructed to conduct full renewals with the remaining sample, conduct partial renewals with the remaining sample, or omit the sample renewal for that day but carefully record dissolved oxygen throughout the day and remove excess food and dead organisms from the test containers.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. No sample preparation or adjustment steps (e.g. pH adjustment or salinity adjustments) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using synthetic seawater as the dilution water (prepared according to Section 7 of the methods manuals).

3.1.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in 125ml plastic containers. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%,

25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using synthetic seawater as the dilution water (prepared according to Section 7 of the methods manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the Mysid, *Mysidopsis bahia,* chronic test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at 4 °C ± 2 °C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals. NOTE: Refer to the electronic benchsheet for required and recommended information.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) Daily observation of mortality and removal of dead organisms for each test is required.

(11) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(12) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. <u>All</u> bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An LC_{50} and IC_{25} must be reported for each chronic test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

(1) Following termination of the mysid chronic test, surviving organisms must be examined by a skilled analyst to determine sex and the presence of eggs. Fecundity endpoints shall be calculated for tests if 50% or more of females in the controls produce eggs.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all mysid chronic tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in these tables represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Mysid Shrimp, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test. Summary of test conditions and test acceptability criteria for the mysid, *Mysidopsis bahia*, seven day survival, growth, and fecundity test with effluents and receiving waters

1. Test type:	Static renewal
2. Salinity:	***25‰ (±2‰)***
3. Temperature:	26 ± 1 °C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c.) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness, with phase in/out period
7. Test chamber:	8 oz plastic disposable cups, or 400 mL glass beakers
8. Test solution volume:	150 mL per replicate
9. Renewal of test solutions:	Daily
10. Age of test organisms:	7 days
11. No. organisms per test chamber:	***5***
12. No. replicate chambers per concentration:	***8***
13. No. larvae per concentration:	***40***
14. Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old)
15. Feeding regime:	Feed 150 24 h old nauplii per mysid daily, half after test solution renewal and half after 8-12 h.
16. Cleaning:	Pipette excess food from cups daily immediately before test solution renewal and feeding.
17. Aeration:	None unless DO falls below 4.0 mg/L, then gently aerate in all cups
18. Dilution water:	***25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
19. Test concentrations:	***Five concentrations and a control***
20. Dilution factor:	***0.5***
21. Test duration:	7 days
22. Endpoints:	Survival, growth, and egg development
23. Test acceptability criteria:	80% or greater survival, average dry weight 0.20 mg or greater in controls; fecundity may be used if 50% or more of females in controls produce eggs
24. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
25. Sample volume required:	3 L per day

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the mysid chronic test method, the 7 day survival LC_{50}, 7 day survival NOEC, growth IC_{25}, growth NOEC, 7 day fecundity IC_{25}, and 7 day fecundity NOEC shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals. **Note: Fecundity should be calculated if 50% or more of females in controls produce eggs.**

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. This section provides instructions for the submission of each of these deliverables.

4.1 Narrative Summary of Testing

This narrative summary shall clearly identify the laboratory, test method, samples tested, summarized test results, and any problems associated with the samples or conduct of the tests. This summary must list any tests that were initiated but not completed and fully explain the reason for not completing the test. This summary must also include a detailed written description of any approved modification to the procedures provided in this SOW, specific instructions, or the method manuals. This will include any telephone log and written correspondence received from the referee laboratory and/or DynCorp during the course of testing. Lastly, this summary should also provide comments on the performance of the method.

4.2 Hardcopy Results Synopsis and Full Report

At a minimum, this report must consist of the items outlined below in section 5.0, all raw data (biological and chemical), and laboratory bench sheets. This report must include all pertinent sample information including copies of all completed traffic report forms, all pertinent test condition and test organism information, all pertinent quality assurance information including results of the monthly QA/QC reference toxicant tests, and all summarized and raw results.

4.3 Electronic Results Synopsis

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the Mysid chronic test method. The disk contains a Microsoft Excel 97 spreadsheet file named MBC___.xls. The MBC indicates that this template is for the *Mysidopsis bahia* chronic test

method, and the number following is a unique identifying number for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template. **It is recommended to view the electronic benchsheet prior to initiating the test**, so the analyst can verify all the information collected on the laboratory benchsheet will be sufficient to complete the electronic results.

Notice the following characteristics of the electronic template file:

(1) The file contains four worksheet pages labeled "Sample #1", "Sample #2", "Sample #3", and "Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
(2) Each worksheet page contains seven information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, weight data, and summarized test results) in which data should be entered. The eighth information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
(3) Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
(4) The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

(1) Record information into a separate worksheet page for each sample tested.
(2) Record requested information or data in all required cells (pale yellow).
(3) Record requested information or data in optional cells (blue) if data is available.
(4) Check entered data against hardcopy bench sheets to ensure accuracy.
(5) Save the file onto the diskette provided. Also keep a copy of the file for laboratory records (a backup in case the diskette crashes when redelivered to DynCorp). Do not change the file name.
(6) Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Data Report Format

Final hardcopy data reports should be submitted in the following format:
Note: Adapted from Section 10 of the methods manuals.

Section 1 - Summary Page
1.1 Laboratory name

1.2 Laboratory address and phone number
1.3 Name and signature of laboratory QA Officer, certifying that data have been internally reviewed and that personnel meticulously followed the methods, and the procedures are deemed to be compliant with the methods and acceptable for reporting purposes.
1.4 Laboratory contact responsible for study
1.5 Analyst(s) who performed WET tests (full names)
1.6 Toxicity tests performed
1.7 Detailed explanations of any difficulties encountered and any approved modifications to the techniques specified in this SOW, specific instructions, or the methods manuals.
1.8 Number of successful tests completed

Section 2 - Sample Information

2.1 Number of samples received and EPA sample number assigned to each sample. Copies of all completed traffic report forms should be included.
2.2 Dates of sample receipt
2.3 Sample temperature when received at laboratory
2.4 Physical and chemical data of sample contents (as required in appropriate method)
2.5 Dilution water
  2.5.1 Source and time frame water is used or how maintained
  2.5.2 Collection or preparation date(s), where applicable
  2.5.3 Pretreatment information
  2.5.4 Physical and chemical characteristics (pH, hardness, conductivity, salinity, etc.)
2.6 Sample storage information
2.7 Sample preparation for testing information

Section 3 - Test Conditions

3.1 Toxicity test method used (title, number, source)
3.2 Endpoint(s) of test(s)
3.3 Deviations from reference method(s), if any, and reason(s)
3.4 Date and time test(s) started, date and time samples were prepared and solutions transferred for renewals.
3.5 Date and time test(s) terminated
3.6 Type and volume of test chambers
3.7 Volume of solution used per chamber
3.8 Number of organisms per test chamber
3.9 Number of replicate test chambers per treatment
3.10 Feeding frequency and amount and type of food (be specific with sources, concentrations of foods (i.e, algae concentration, YCT solids level, preparation dates))
3.11 Acclimation of test organisms (temperature mean and range and, where applicable, salinity mean and range)
3.12 Test temperature (mean and range)
3.13 Test salinity, where applicable (mean and range)
3.14 Specify if aeration was needed
3.15 Specify if organisms were dried immediately for weighing or preserved prior to drying
3.16 Specify how food was prepared and sources of food. Include test results that validate the quality of batch food preparations (i.e., *Ceriodaphnia dubia* tests on YCT preparation).
3.17 Describe how routine chemistries on new solutions were made (in actual test chamber or in beakers after dispensing).

3.18 Describe how randomization was conducted, especially blocking and known parentage. Report how brood distinctions were made and male (if any) identification was made.

Section 4 - Test Organisms

4.1 Scientific name of test species, verification of species documented
4.2 Age (life stage) of test species (be specific for all species). Age at time of test initiation (for example, for *C. dubia* be sure to clarify the window of age of the neonates as well as the overall age of the animals.)
4.3 Mean length and weight (where applicable)
4.4 Source and QA/QC test conditions
4.5 Holding conditions
4.6 Diseases and treatment (where applicable)
4.7 Taxonomic key used for species identification

Section 5 - Quality Assurance

5.1 Reference toxicant used routinely; source; date received; lot number
5.2 Date and time of most recent reference toxicant test; test results and current control (cusum) chart including 20 most recent data points
5.3 Dilution water used in reference toxicant tests (with characteristics provided)
5.4 Physical and chemical methods used
5.5 Reference toxicant results (NOEC, IC_{25}, or LC_{50} where applicable, LOEC or EC_{50})

Section 6 - Results

6.1 Copies of all bench sheets. Be sure to count and notate broods for reproduction test with *Ceriodaphnia*
6.2 Raw toxicity data in tabular form, including daily records of affected organisms in each replicate at each concentration (including controls) and plots of toxicity data
6.3 Table of endpoints (LC_{50}, IC_{25}, NOEC for each endpoint) and confidence limits (where applicable)
6.4 Statistical methods and software used to calculate endpoints
6.5 Summary table of physical and chemical data

6.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Mysid Shrimp, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test

For each sample, three containers of liquid reagent will be received (marked with the sample number followed by an "A", "B", or "C"). The three containers shall be reconstituted as described below to mimic the sample shipment schedule for effluent samples. The container marked "A" shall be reconstituted on the day of test initiation (Day 0) and used for renewal on Day 1. The container marked "B" shall be reconstituted on Day 2 and used for renewals on Day 2 and Day 3. The container marked "C" shall be reconstituted on Day 4 and used for renewals on Day 4, Day 5, and Day 6. Follow the directions below for the reconstitution of each sample ampule.

1. Volumetrically add 100 mL of the liquid ampule sample to approximately 1L of synthetic seawater. The synthetic seawater should be prepared to a salinity of 25‰ (±2‰) using Bioassay Grade Forty Fathoms artificial sea salts and MILLIPORE MILLI-Q® or equivalent deionized water according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 9L (measured using volumetric glassware) with synthetic seawater dilution water.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

6. This 9L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation and Day 1 test renewal. Store sample at 4°C.

8. Perform the Mysid Shrimp, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test as described in the SOW for this study and the methods manuals.

9. Follow Steps 1 through 6 with each sample container to prepare the reconstituted sample on Day 2 and Day 4 for subsequent daily renewals.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the Sheepshead Minnow Acute Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: February 25, 2000

SUBJECT: Final Guidance and SOP for the Sheepshead Minnow Acute Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the Sheepshead Minnow acute test method in EPA's WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. The SOP details the sample distribution and testing schedule and provides important information for completing participant laboratory tasks, such as instructions for the reconstitution of ampule samples. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

Also enclosed is the electronic data reporting format disk for the sheepshead minnow acute method. A description and instructions for use of the electronic data report form are provided in the SOP.

Please ensure that the enclosed SOP and disk pertaining to the sheepshead minnow acute test method are distributed to laboratory staff that will be performing the test method in the study. Also, please see that laboratory staff read over the SOP carefully to ensure the proper data is collected and reported. **Please note that the ampule reconstitution instructions for this method require the addition of 500mL of the ampule sample instead of 100mL that has been used previously for other methods. For this reason, ampule samples will be provided in 500mL bottles for this method.**

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Cyprinodon variegatus Acute Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Cyprinodon variegatus acute test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Cyprinodon variegatus* acute method will occur between March 7 and March 18, 2000 with final reports due 30 days following termination of all tests (April 17, 2000). The testing schedule is provided below in Table 1. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Participant laboratories will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 3/7/00 for test initiation on that day, and two samples will arrive on 3/14/00 for test initiation on that day. Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample.

For whole volume samples, one aliquot will be received on test Day 0. This aliquot shall be used for test initiation on Day 0 and test renewal at 48 hours. For ampule samples, one aliquot will be received on test Day 0. This aliquot shall be reconstituted on Day 0 and shall be used for test initiation on Day 0 and test renewal at 48 hours.

Table 1. Schedule for *Cyprinodon variegatus* Acute Testing.

Date (start date - finish date)	Activity
3/7/00 - 3/11/00	Conduct Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test with samples #1&2
3/14/00 - 3/18/00	Conduct Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test with samples #3&4
4/17/00	Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and conductivity or salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. No sample preparation or adjustment steps (e.g. pH or salinity adjustment) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using bioassay grade Forty Fathoms synthetic seawater as the dilution water (prepared according to Section 7 of the method manuals).

3.1.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in a 500 ml plastic bottle. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using bioassay grade Forty Fathoms synthetic seawater as the dilution water (prepared according to Section 7 of the method manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the Sheepshead minnow, *Cyprinodon variegatus*, acute test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms “must” and “shall”) and recommendations and guidance (indicated by discretionary terms “should” and “may”). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or

other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples) are provided herein. Test samples received at participant laboratories must be refrigerated (at 4°C ± 2°C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals. NOTE: Refer to the electronic benchsheet for required and recommended information.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) Daily observation of mortality and removal of dead organisms for each test is required.

(11) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(12) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. All bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An IC_{25} must be reported for each chronic test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a method's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all sheepshead minnow acute tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in these tables represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test. Summary of test conditions and test acceptability criteria for sheepshead minnow, *Cyprinodon variegatus*, acute toxicity tests with effluents and receiving waters

1. Test type:	***Static renewal***
2. Test duration:	***96 h***
3. Temperature:	***25 °C ± 1 °C***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s or (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***250 mL***
8. Test solution volume:	***200 mL***
9. Renewal of test solutions:	***At 48 h***
10. Age of test organisms:	1-14 days; 24-h range in age
11. No. organisms per test chamber:	***10***
12. No. replicate chambers per concentration:	***2***
13. No. organisms per concentration:	***20***
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	***25 ‰ ± 2‰ Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor	***0.5***
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	***1 L for effluents***
23. Test acceptability criterion:	90% or greater survival in controls
24. Salinity:	***25‰ (±2‰)***

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the Sheepshead Minnow acute test method, the 96 hour LC_{50} shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. This section provides instructions for the submission of each of these deliverables.

4.1 Narrative Summary of Testing

This narrative summary shall clearly identify the laboratory, test method, samples tested, summarized test results, and any problems associated with the samples or conduct of the tests. This summary must list any tests that were initiated but not completed and fully explain the reason for not completing the test. This summary must also include a detailed written description of any approved modification to the procedures provided in this SOW, specific instructions, or the method manuals. This will include any telephone log and written correspondence received from the referee laboratory and/or DynCorp during the course of testing. Lastly, this summary should also provide comments on the performance of the method.

4.2 Hardcopy Results Synopsis and Full Report

At a minimum, this report must consist of the items outlined below in section 5.0, all raw data (biological and chemical), and laboratory bench sheets. This report must include all pertinent sample information including copies of all completed traffic report forms, all pertinent test condition and test organism information, all pertinent quality assurance information including results of the monthly QA/QC reference toxicant tests, and all summarized and raw results.

4.3 Electronic Results Synopsis.

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the Sheepshead minnow acute test method. The disk contains a Microsoft Excel 97 spreadsheet file named SHMA___.xls. The SHMA indicates that this template is for the Sheepshead minnow acute test method, and the number following is a unique identifying number for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file,

please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template. **It is recommended to view the electronic benchsheet prior to initiating the test**, so the analyst can verify all the information collected on the laboratory benchsheet will be sufficient to complete the electronic results.

Notice the following characteristics of the electronic template file:

1. The file contains four worksheet pages labeled "Sample #1", "Sample #2", "Sample #3", and "Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
2. Each worksheet page contains six information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, and summarized test results) in which data should be entered. The seventh information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Also keep a copy of the file for laboratory records (a backup in case the diskette crashes when redelivered to DynCorp). Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Data Report Format

Final hardcopy data reports should be submitted in the following format:
Note: Adapted from Section 10 of the methods manuals.

Section 1 - Summary Page

1.1 Laboratory name
1.2 Laboratory address and phone number

1.3 Name and signature of laboratory QA Officer, certifying that data have been internally reviewed and that personnel meticulously followed the methods, and the procedures are deemed to be compliant with the methods and acceptable for reporting purposes.
1.4 Laboratory contact responsible for study
1.5 Analyst(s) who performed WET tests (full names)
1.6 Toxicity tests performed
1.7 Detailed explanations of any difficulties encountered and any approved modifications to the techniques specified in this SOW, specific instructions, or the methods manuals.
1.8 Number of successful tests completed

Section 2 - Sample Information

2.1 Number of samples received and EPA sample number assigned to each sample. Copies of all completed traffic report forms should be included.
2.2 Dates of sample receipt
2.3 Sample temperature when received at laboratory
2.4 Physical and chemical data of sample contents (as required in appropriate method)
2.5 Dilution water
  2.5.1 Source and time frame water is used or how maintained
  2.5.2 Collection or preparation date(s), where applicable
  2.5.3 Pretreatment information
  2.5.4 Physical and chemical characteristics (pH, hardness, conductivity, salinity, etc.)
2.6 Sample storage information
2.7 Sample preparation for testing information

Section 3 - Test Conditions

3.1 Toxicity test method used (title, number, source)
3.2 Endpoint(s) of test(s)
3.3 Deviations from reference method(s), if any, and reason(s)
3.4 Date and time test(s) started, date and time samples were prepared and solutions transferred for renewals.
3.5 Date and time test(s) terminated
3.6 Type and volume of test chambers
3.7 Volume of solution used per chamber
3.8 Number of organisms per test chamber
3.9 Number of replicate test chambers per treatment
3.10 Feeding frequency and amount and type of food (be specific with sources, concentrations of foods (i.e, algae concentration, YCT solids level, preparation dates))
3.11 Acclimation of test organisms (temperature mean and range and, where applicable, salinity mean and range)
3.12 Test temperature (mean and range)
3.13 Test salinity, where applicable (mean and range)
3.14 Specify if aeration was needed
3.15 Specify if organisms were dried immediately for weighing or preserved prior to drying
3.16 Specify how food was prepared and sources of food. Include test results that validate the quality of batch food preparations (i.e., *Ceriodaphnia dubia* tests on YCT preparation).
3.17 Describe how routine chemistries on new solutions were made (in actual test chamber or in beakers after dispensing)

3.18 Describe how randomization was conducted, especially blocking and known parentage. Report how brood distinctions were made and male (if any) identification was made.

Section 4 - Test Organisms

4.1 Scientific name of test species, verification of species documented
4.2 Age (life stage) of test species (be specific for all species). Age at time of test initiation (for example, for *C. dubia* be sure to clarify the window of age of the neonates as well as the overall age of the animals.)
4.3 Mean length and weight (where applicable)
4.4 Source and QA/QC test conditions
4.5 Holding conditions
4.6 Diseases and treatment (where applicable)
4.7 Taxonomic key used for species identification

Section 5 - Quality Assurance

5.1 Reference toxicant used routinely; source; date received; lot number
5.2 Date and time of most recent reference toxicant test; test results and current control (cusum) chart including 20 most recent data points
5.3 Dilution water used in reference toxicant tests (with characteristics provided)
5.4 Physical and chemical methods used
5.5 Reference toxicant results (NOEC, IC_{25}, or LC_{50} where applicable, LOEC or EC_{50})

Section 6 - Results

6.1 Copies of all bench sheets. Be sure to count and notate broods for reproduction test with *Ceriodaphnia*
6.2 Raw toxicity data in tabular form, including daily records of affected organisms in each replicate at each concentration (including controls) and plots of toxicity data
6.3 Table of endpoints (LC_{50}, IC_{25}, NOEC for each endpoint) and confidence limits (where applicable)
6.4 Statistical methods and software used to calculate endpoints
6.5 Summary table of physical and chemical data

6.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test

For each sample, a single liquid ampule will be received. The container shall be reconstituted and used to initiate the test. The same reconstituted sample shall be used for test renewal at 48 hours. Follow the directions below for the reconstitution of the sample ampule.

1. Volumetrically add **500 mL** of the liquid ampule sample to approximately 1L of synthetic seawater. The synthetic seawater should be prepared to a salinity of 25‰ (±2‰) using Bioassay Grade Forty Fathoms artificial sea salts and MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 4L (measured using volumetric glassware) with synthetic seawater.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

6. This 4L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation and test renewal at 48 hr. Store sample at 4°C.

8. Perform the Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test as described in the SOW for this study and the methods manuals.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the Sheepshead Minnow Chronic Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: March 9, 2000

SUBJECT: Final Guidance and SOP for the Sheepshead Minnow Chronic Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the Sheepshead Minnow chronic test method in EPA's WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. The SOP details the sample distribution and testing schedule and provides important information for completing participant laboratory tasks, such as instructions for the reconstitution of ampule samples. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

Also enclosed is the electronic data reporting format disk for the sheepshead minnow chronic method. A description and instructions for use of the electronic data report form are provided in the SOP.

Please ensure that the enclosed SOP and disk pertaining to the sheepshead minnow chronic test method are distributed to laboratory staff that will be performing the test method in the study. Also, please see that laboratory staff read over the SOP carefully to ensure the proper data is collected and reported. **Please note that the ampule reconstitution instructions for this method require the addition of 500mL of the ampule sample instead of 100mL that has been used previously for other methods. For this reason, ampule samples will be provided in 500mL bottles for this method.**

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Cyprinodon variegatus Larval Survival and Growth Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Cyprinodon variegatus larval survival and growth test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Cyprinodon variegatus* survival and growth method will occur between March 21 and April 4, 2000 with final reports due 30 days following termination of all tests (May 4, 2000). The schedule for the testing is provided below. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Laboratories participating in the base study design will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 3/21/00 for test initiation on that day, and two samples will arrive on 3/28/00 for test initiation on that day.

Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For tests that require additional shipments for sample renewal, the sample number shall be the same for each initiation and renewal shipment with the addition of a letter (A, B, or C) after the sample number to designate the sample for use as initiation (A), renewal 1 (B), or renewal 2 (C).

For whole volume samples, separate aliquots will be received on test Day 0, Day 2, and Day 4. The first aliquot (identified with the sample number and the letter "A") shall be used for test initiation on Day 0 and renewal on Day 1. The second aliquot (identified with the sample number and the letter "B") shall be used for test renewals on Day 2 and Day 3. The final aliquot (identified with the sample number and the letter "C") shall be used for test renewal on Day 4, Day 5, and Day 6. This sample shipment schedule mimics the typical schedule for chronic monitoring of effluent for compliance.

For ampule samples , three separate ampule containers (marked with the sample number followed by A, B, or C) will be received in a single shipment on test Day 0. The container marked "A" shall be reconstituted on test Day 0 and used for test initiation on Day 0 and renewal on Day 1. The other aliquots of the sample shall be refrigerated and stored until use on Day 2 and Day 4, respectively. The container

marked “B” shall be reconstituted on test Day 2 and used for renewal on Day 2 and 3. The container marked “C” shall be reconstituted on Day 4 and used for renewal on Day 4, Day 5 and Day 6. The sample reconstitution schedule for ampules attempts to mimic the typical sample shipment schedule for chronic monitoring of effluents for compliance.

Table 1. Schedule for *Cyprinodon variegatus* Survival and Growth Testing.

Date (start date - finish date)	Activity
3/21/00 - 3/28/00	Conduct Sheepshead minnow, *Cyprinodon variegatus,* Larval Survival and Growth Test with samples #1&2
3/28/00 - 4/4/00	Conduct Sheepshead minnow, *Cyprinodon variegatus,* Larval Survival and Growth Test with samples #3&4
5/4/00	Sheepshead minnow, *Cyprinodon variegatus,* Larval Survival and Growth Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the “For Participant Lab Use Only” box and in the “Post-Shipment” column of the “Sample Collection/Receipt Information” box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled “temperature check” will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary. If renewal shipments do not arrive on the expected day, DynCorp will provide guidance for test renewal on a case-by-case basis. Depending on the volume of sample remaining from previous shipments, laboratories may be instructed to conduct full renewals with the remaining sample, conduct partial renewals with the remaining sample, or omit the sample renewal for that day but carefully record dissolved oxygen throughout the day and remove excess food and dead organisms from the test containers.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. Samples will be received at the proper salinity range, so no sample preparation or adjustment steps (e.g. pH adjustment or salinity adjustment) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using synthetic seawater as the dilution water (prepared according to Section 7 of the methods manuals).

3.1.2 Ampule Samples

Ampule samples will be received as liquid samples in 500ml plastic bottles. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent

sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using synthetic seawater as the dilution water (prepared according to Section 7 of the methods manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the Sheepshead minnow*, Cyprinodon variegatus,* larval survival and growth test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at $4\,^{\circ}C \pm 2\,^{\circ}C$) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals. **NOTE: Refer to the electronic benchsheet for required and recommended information.**

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) Daily observation of mortality and removal of dead organisms for each test is required.

(11) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(12) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. <u>All</u> bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An IC_{25} must be reported for each chronic test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all Sheepshead minnow larval survival and growth tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in these tables represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Sheepshead Minnow, *Cyprinodon variegatus,* Larval Survival And Growth Test. Summary of test conditions and test acceptability criteria for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test with effluents and receiving waters

1. Test type:	Static renewal
2. Salinity:	***25‰ (±2‰)***
3. Temperature:	25 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***600 mL beaker***
8. Test solution volume:	***500 mL/replicate (loading and DO restrictions must be met)***
9. Renewal of test solutions:	Daily
10. Age of test organisms:	Newly hatched larvae (less than 24 h old; 24-h range in age)
11. No. larvae per test chamber:	***10***
12. No. replicate chambers per concentration:	***4***
13. No. larvae per concentration:	***40***
14. Source of food:	Newly hatched *Artemia* nauplii, (less than 24-h old)
15. Feeding regime:	Feed once a day 0.10 g wet weight *Artemia* nauplii per replicate on Days 0-2; Feed 0.15 g wet weight *Artemia* nauplii per replicate on Days 3-6
16. Cleaning:	Siphon daily, immediately before test solution renewal and feeding
17. Aeration:	None, unless DO falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min
18. Dilution water:	***25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
19. Test concentrations:	***Five concentrations and a control***
20. Dilution factor:	***0.5***
21. Test duration:	7 days
22. Endpoints:	Survival and growth (weight)
23. Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers should be 0.60 mg or greater, if unpreserved, or 0.50 mg or greater after no more than 7 days in 4% formalin or 70% ethanol
24. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
25. Sample volume required:	6 L per day

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the Sheepshead minnow larval survival and growth test method, the 7 day survival LC_{50}, 7 day survival NOEC, growth IC_{25}, and growth NOEC shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Additional Information on Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. This section provides instructions for the submission of each of these deliverables.

4.1 Narrative Summary of Testing

This narrative summary shall clearly identify the laboratory, test method, samples tested, summarized test results, and any problems associated with the samples or conduct of the tests. This summary must list any tests that were initiated but not completed and fully explain the reason for not completing the test. This summary must also include a detailed written description of any approved modification to the procedures provided in this SOW, specific instructions, or the method manuals. This will include any telephone log and written correspondence received from the referee laboratory and/or DynCorp during the course of testing. Lastly, this summary should also provide comments on the performance of the method.

4.2 Hardcopy Results Synopsis and Full Report

At a minimum, this report must consist of the items outlined below in section 5.0, all raw data (biological and chemical), and laboratory bench sheets. This report must include all pertinent sample information including copies of all completed traffic report forms, all pertinent test condition and test organism information, all pertinent quality assurance information including results of the monthly QA/QC reference toxicant tests, and all summarized and raw results.

4.3 Electronic Results Synopsis

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the Sheepshead minnow larval survival and growth test method. The disk contains a Microsoft Excel 97 spreadsheet file named SHMC___.xls. The SHMC indicates that this template is for the Sheepshead minnow chronic test method, and the number following is a unique identifying number

for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template. **It is recommended to view the electronic benchsheet prior to initiating the test**, so the analyst can verify all the information collected on the laboratory benchsheet will be sufficient to complete the electronic results.

Notice the following characteristics of the electronic template file:

1. The file contains four worksheet pages labeled "Sample #1", "Sample #2", "Sample #3", and "Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
2. Each worksheet page contains seven information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, weight data, and summarized test results) in which data should be entered. The eighth information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Also keep a copy of the file for laboratory records (a backup in case the diskette crashes when redelivered to DynCorp). Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Data Report Format

Final hardcopy data reports should be submitted in the following format:
Note: Adapted from Section 10 of the methods manuals.

Section 1 - Summary Page

1.1 Laboratory name
1.2 Laboratory address and phone number

1.3 Name and signature of laboratory QA Officer, certifying that data have been internally reviewed and that personnel meticulously followed the methods, and the procedures are deemed to be compliant with the methods and acceptable for reporting purposes.
1.4 Laboratory contact responsible for study
1.5 Analyst(s) who performed WET tests (full names)
1.6 Toxicity tests performed
1.7 Detailed explanations of any difficulties encountered and any approved modifications to the techniques specified in this SOW, specific instructions, or the methods manuals.
1.8 Number of successful tests completed

Section 2 - Sample Information

2.1 Number of samples received and EPA sample number assigned to each sample. Copies of all completed traffic report forms should be included.
2.2 Dates of sample receipt
2.3 Sample temperature when received at laboratory
2.4 Physical and chemical data of sample contents (as required in appropriate method)
2.5 Dilution water
  2.5.1 Source and time frame water is used or how maintained
  2.5.2 Collection or preparation date(s), where applicable
  2.5.3 Pretreatment information
  2.5.4 Physical and chemical characteristics (pH, hardness, conductivity, salinity, etc.)
2.6 Sample storage information
2.7 Sample preparation for testing information

Section 3 - Test Conditions

3.1 Toxicity test method used (title, number, source)
3.2 Endpoint(s) of test(s)
3.3 Deviations from reference method(s), if any, and reason(s)
3.4 Date and time test(s) started, date and time samples were prepared and solutions transferred for renewals.
3.5 Date and time test(s) terminated
3.6 Type and volume of test chambers
3.7 Volume of solution used per chamber
3.8 Number of organisms per test chamber
3.9 Number of replicate test chambers per treatment
3.10 Feeding frequency and amount and type of food (be specific with sources, concentrations of foods (i.e, algae concentration, YCT solids level, preparation dates))
3.11 Acclimation of test organisms (temperature mean and range and, where applicable, salinity mean and range)
3.12 Test temperature (mean and range)
3.13 Test salinity, where applicable (mean and range)
3.14 Specify if aeration was needed
3.15 Specify if organisms were dried immediately for weighing or preserved prior to drying
3.16 Specify how food was prepared and sources of food. Include test results that validate the quality of batch food preparations (i.e., *Ceriodaphnia dubia* tests on YCT preparation).
3.17 Describe how routine chemistries on new solutions were made (in actual test chamber or in beakers after dispensing).

3.18 Describe how randomization was conducted, especially blocking and known parentage. Report how brood distinctions were made and male (if any) identification was made.

Section 4 - Test Organisms

4.1 Scientific name of test species, verification of species documented
4.2 Age (life stage) of test species (be specific for all species). Age at time of test initiation (for example, for *C. dubia* be sure to clarify the window of age of the neonates as well as the overall age of the animals.)
4.3 Mean length and weight (where applicable)
4.4 Source and QA/QC test conditions
4.5 Holding conditions
4.6 Diseases and treatment (where applicable)
4.7 Taxonomic key used for species identification

Section 5 - Quality Assurance

5.1 Reference toxicant used routinely; source; date received; lot number
5.2 Date and time of most recent reference toxicant test; test results and current control (cusum) chart including 20 most recent data points
5.3 Dilution water used in reference toxicant tests (with characteristics provided)
5.4 Physical and chemical methods used
5.5 Reference toxicant results (NOEC, IC_{25}, or LC_{50} where applicable, LOEC or EC_{50})

Section 6 - Results

6.1 Copies of all bench sheets. Be sure to count and notate broods for reproduction test with *Ceriodaphnia*
6.2 Raw toxicity data in tabular form, including daily records of affected organisms in each replicate at each concentration (including controls) and plots of toxicity data
6.3 Table of endpoints (LC_{50}, IC_{25}, NOEC for each endpoint) and confidence limits (where applicable)
6.4 Statistical methods and software used to calculate endpoints
6.5 Summary table of physical and chemical data

6.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test

For each sample, three ampules containing liquid will be received (marked with the sample number followed by an "A", "B", or "C"). The three containers shall be reconstituted as described below to mimic the sample shipment schedule for effluent samples. The container marked "A" shall be reconstituted on the day of test initiation (Day 0) and used for renewal on Day 1. The container marked "B" shall be reconstituted on Day 2 and used for renewals on Day 2 and Day 3. The container marked "C" shall be reconstituted on Day 4 and used for renewals on Day 4, Day 5, and Day 6. Follow the directions below for the reconstitution of each sample ampule.

1. Volumetrically add **500 mL** of the liquid ampule sample to approximately 1L of synthetic seawater. The synthetic seawater should be prepared to a salinity of 25‰ (±2‰) using Bioassay Grade Forty Fathoms artificial sea salts and MILLIPORE MILLI-Q® or equivalent deionized water according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 21L (measured using volumetric glassware) with synthetic seawater dilution water.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

6. This 21L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation and Day 1 test renewal. Store sample at 4°C.

8. Perform the Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test as described in the SOW for this study and the methods manuals.

9. Follow Steps 1 through 6 with each sample container to prepare the reconstituted sample on Day 2 and Day 4 for subsequent daily renewals.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the *Menidia beryllina* Acute Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: October 25, 1999

SUBJECT: Final Guidance and SOP for the *Menidia beryllina* Acute Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the *Menidia beryllina* acute test method in EPA's WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. The SOP details the sample distribution and testing schedule and provides important information for completing participant laboratory tasks, such as instructions for the reconstitution of ampule samples. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

Also enclosed is the electronic data reporting format disk for the *Menidia beryllina* acute method. A description and instructions for use of the electronic data report form are provided in the SOP.

Please ensure that the enclosed SOP and disk pertaining to the *Menidia beryllina* acute test method are distributed to laboratory staff that will be performing the test method in the study.

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Menidia beryllina Acute Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Menidia beryllina acute test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Menidia beryllina* acute method will occur between November 2 and November 13, 19990 with final reports due 30 days following termination of all tests (December 13, 1999). The schedule for the testing is provided below. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Laboratories participating in the base study design will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 11/2/99 for test initiation on that day, and two samples will arrive on 11/9/99 for test initiation on that day.

Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For whole volume samples, one aliquot will be received on test Day 0. This aliquot shall be used for test initiation on Day 0 and test renewal at 48 hours. For ampule samples, one aliquot will be received on test Day 0. This aliquot shall be reconstituted on Day 0, and the reconstituted sample shall be used for test initiation on Day 0 and test renewal at 48 hours.

Table 1. Schedule for *Menidia beryllina* Acute Testing.

Date (start date - finish date)	Activity
11/2/99 - 11/6/99	Conduct Inland Silverside, *Menidia beryllina*, Acute Test with samples #1&2
11/9/99 - 11/13/99	Conduct Inland Silverside, *Menidia beryllina*, Acute Test with samples #3&4
12/13/99	Inland Silverside, *Menidia beryllina*, Acute Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and conductivity or salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. No sample preparation or adjustment steps (e.g. pH adjustment) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using synthetic seawater as the dilution water (prepared according to Section 7 of the method manuals).

3.1.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in 125ml plastic containers. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using synthetic seawater as the dilution water (prepared according to Section 7 of the method manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the Inland Silverside, *Menidia beryllina*, acute test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms “must” and “shall”) and recommendations and guidance (indicated by discretionary terms “should” and “may”). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or

other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

(1) Personnel that conduct tests must be the same personnel that routinely conduct the WET tests at that laboratory facility and who were identified in the prequalification materials. If these individuals cannot be available during any part of the study, the laboratory must contact SCC. Personnel conducting the tests must be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at 4 °C ± 2 °C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, conductivity or salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) Daily observation of mortality and removal of dead organisms for each test is required.

(11) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible

causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(12) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. All bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An IC_{25} must be reported for each chronic test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all Inland Silverside acute tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in this table represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Inland Silverside, *Menidia beryllina*, Acute Test. Summary of test conditions and test acceptability criteria for inland silverside, *Menidia beryllina,* acute toxicity test with effluents and receiving waters

1. Test type:	***Static-renewal***
2. Test duration:	***96 h***
3. Temperature:	***25 °C ± 1 °C***
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***250 mL***
8. Test solution volume:	***200 mL***
9. Renewal of test solutions:	***At 48 h***
10. Age of test organisms:	9-14 days; 24-h range in age
11. No. organisms per test chamber:	***10***
12. No. replicate chambers per concentration:	***2***
13. No. organisms per concentration:	***20***
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	***25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
18. Test concentrations:	***Five concentrations and a control***
19. Dilution factor:	***0.5***
20. Endpoint:	***Mortality (LC50)***
21. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
22. Sample volume required:	***1 L for effluents***
23. Test acceptability criterion:	90% or greater survival in controls
24. Salinity:	***25‰ (± 2‰)***

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the Inland Silverside acute test method, the 96 hour LC_{50} and NOEC shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Additional Information on Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. Deliverables #1 and 2 shall be submitted according to the requirements specified in the SOW. This section provides additional instructions for the submission of the electronic results synopsis.

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the Inland Silverside minnow acute test method. The disk contains a Microsoft Excel 97 spreadsheet file named ISA___.xls. The ISA indicates that this template is for the Inland Silverside minnow acute test method, and the number following is a unique identifying number for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template.

Notice the following characteristics of the electronic template file:

1. The file contains four worksheet pages labeled “Sample #1", “Sample #2", “Sample #3", and “Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
2. Each worksheet page contains six information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, and summarized test results) in which data should be entered. The seventh information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Inland Silverside, *Menidia beryllina*, Acute Test

For each sample, a single liquid ampule will be received. The container shall be reconstituted and used to initiate the test. The same reconstituted sample shall be used for test renewal at 48 hours. Follow the directions below for the reconstitution of the sample ampule.

1. Volumetrically add 100 mL of the liquid ampule sample to approximately 1L of of synthetic seawater. The synthetic seawater should be prepared to a salinity of 25‰ (±2‰) using Bioassay Grade Forty Fathoms artificial sea salts and MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 4L (measured using volumetric glassware) with synthetic seawater.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

6. This 4L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation and test renewal at 48 hr. Store sample at 4°C.

8. Perform the Inland Silverside, *Menidia beryllina*, Acute Test as described in the SOW for this study and the methods manuals.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

DynCorp
Information & Enterprise Technology

TO: Participant Laboratories for the *Menidia beryllina* Chronic Test Method

FROM: Robert Brent, WET Study Coordinator

DATE: October 13, 1999

SUBJECT: Final Guidance and SOP for the *Menidia beryllina* Chronic Test

Enclosed is the final standard operating procedure (SOP) for laboratories participating in the *Menidia beryllina* chronic test method in EPA's WET Interlaboratory Variability Study. This SOP is a supplement to the statement of work (SOW) that was distributed on July 9, 1999 with the solicitation for this study. The SOP details the sample distribution and testing schedule and provides important information for completing participant laboratory tasks, such as instructions for the reconstitution of ampule samples. Participant laboratories should follow the guidance in the enclosed SOP, the SOW, and the method manuals.

Also enclosed is the electronic data reporting format disk for the *Menidia beryllina* chronic method. A description and instructions for use of the electronic data report form are provided in the SOP.

Please ensure that the enclosed SOP and disk pertaining to the *Menidia beryllina* chronic test method are distributed to laboratory staff that will be performing the test method in the study.

STANDARD OPERATING PROCEDURE
Participant Laboratory Support for EPA's WET Interlaboratory Study

Menidia beryllina Larval Survival and Growth Method

***Preamble**:*
This standard operating procedure document (SOP) is a supplement to the participant laboratory Statement of Work (SOW). This SOP details specific information in the SOW regarding the conduct of the Menidia beryllina larval survival and growth test method in the WET Interlaboratory Variability Study. All modifications to the SOP must be approved by DynCorp prior to implementation.

All data deliverables submitted for this interlaboratory study become the property of EPA and may be incorporated into the public record. Laboratories may not independently publish the results of analyses for which they are paid to perform under this SOP.

1.0 Sample Distribution and Testing Schedule

Participant laboratory testing for the *Menidia beryllina* survival and growth method will occur between October 19 and November 2, 1999 with final reports due 30 days following termination of all tests (December 2, 1999). The schedule for the testing is provided below. The date ranges on the schedule indicate the test start dates and test completion dates. Samples will be shipped FedEx Priority Overnight to arrive at the participant laboratory on the test start date by 10:00AM. All tests must be initiated on the day of sample arrival. Testing is scheduled to occur simultaneously at each participant laboratory, so adherence to the testing schedule is mandatory for all participant laboratories.

Each participant laboratory will receive 4 blind test samples (reflected in the schedule) that may be whole volume or ampule samples. Two samples will arrive on 10/19/99 for test initiation on that day, and two samples will arrive on 10/26/99 for test initiation on that day.

Each sample aliquot that is prepared and shipped will be assigned a unique sample number. The sample number will appear clearly and permanently on each container and on an EPA traffic report form that will accompany each sample. For tests that require additional shipments for sample renewal, the sample number shall be the same for each initiation and renewal shipment with the addition of a letter (A, B, or C) after the sample number to designate the sample for use as initiation (A), renewal 1 (B), or renewal 2 (C).

For whole volume samples, separate aliquots will be received on test Day 0, Day 2, and Day 4. The first aliquot (identified with the sample number and the letter "A") shall be used for test initiation on Day 0 and test renewal on Day 1. The second aliquot (identified with the sample number and the letter "B") shall be used for test renewals on Day 2 and Day 3. The final aliquot (identified with the sample number and the letter "C") shall be used for test renewal on Day 4, Day 5, and Day 6. This sample shipment schedule mimics the typical schedule for chronic monitoring of effluent for compliance.

For ampule samples, three separate ampule containers (marked with the sample number followed by A, B, or C) will be received in a single shipment on test Day 0. The container marked "A" shall be reconstituted on test Day 0 and used for test initiation and renewal on Day 1. The other aliquots of the sample shall be refrigerated and stored until use on Day 2 and Day 4, respectively. The container marked

"B" shall be reconstituted on test Day 2 and used for renewal on Day 2 and 3. The container marked "C" shall be reconstituted shall be reconstituted on Day 4 and used for renewal on Day 4, Day 5 and Day 6. The sample reconstitution schedule for ampules also attempts to mimic the typical sample shipment schedule for chronic monitoring of effluents for compliance.

Table 1. Schedule for *Menidia beryllina* Survival and Growth Testing.

Date (start date - finish date)	Activity
10/19/99 - 10/26/99	Conduct Inland Silverside, *Menidia beryllina,* Larval Survival and Growth Test with samples #1&2
10/26/99 - 11/2/99	Conduct Inland Silverside, *Menidia beryllina,* Larval Survival and Growth Test with samples #3&4
12/2/99	Inland Silverside, *Menidia beryllina,* Larval Survival and Growth Test data due

2.0 Sample Traffic Reporting Tasks

2.1 Sample Receipt Confirmation

An EPA traffic report form (Appendix B) will be received with each sample. The traffic report form will document important sample collection, shipment, and receipt information. Portions of the form will be completed by the referee laboratory prior to shipment and will indicate the episode number, sample number, referee laboratory information, participant laboratory shipping information, sample pre-shipment information, and the requested analysis. Immediately upon receipt of the sample, participant laboratories will be responsible for determining that the sample arrived in satisfactory condition and for documenting receipt of the sample, post-shipment sample water quality (temperature, pH, dissolved oxygen, and conductivity or salinity), and any problems on the EPA traffic report form. The participant laboratory shall complete the traffic report form by recording the required information in the "For Participant Lab Use Only" box and in the "Post-Shipment" column of the "Sample Collection/Receipt Information" box.

For ampule samples, the pre-shipment and post-shipment measurement of pH, dissolved oxygen, and conductivity or salinity shall be omitted. This will avoid possible contamination of the ampule sample prior to reconstitution. Pre-shipment and post-shipment temperature shall be measured in a temperature check sample. An additional sample container labeled "temperature check" will be included with each cooler shipment of ampules. The temperature shall be measured in this container and recorded on the EPA traffic report form as a surrogate measure of temperature for all ampule samples within that cooler. The temperature check shall be discarded after temperature measurement, and must not be used in any way for WET testing.

Immediately upon completion of the EPA traffic report form, the participant laboratory shall fax the completed form to DynCorp and retain a copy for inclusion in the final data report. Receipt of the faxed traffic report form by DynCorp will be confirmation of successful sample arrival, so it is important that the form be completed and faxed immediately. Forms should be faxed by 11:00AM (local laboratory time) to facilitate sample tracking and potential problem resolution. The EPA traffic report form shall be faxed to:

Brian Rusignuolo
Sample Coordinator
DynCorp SCC
fax: (703)461-8056
phone: (703)461-2401

2.2 Problem Resolution

Prior to test initiation, notify Brian Rusignuolo of any special shipment receiving instructions (i.e., hold shipment at airport or FedEx shipment center for pickup). Notification must also be made if any specific instructions are required for Saturday deliveries.

Participant laboratories should be expecting the arrival of samples based on the provided schedule. If samples do not arrive as expected, if samples are damaged in shipment, if samples arrive without accompanying traffic report forms, if sample numbers are not clearly identified on samples, or if any other sample shipment or receipt problem is encountered immediately notify Brian Rusignuolo by phone at (703)461-2401.

Brian must be notified by 11:00AM (local laboratory time) if samples fail to arrive by the morning FedEx shipment or if other shipment or sample receipt problems are encountered. DynCorp will track lost shipments and instruct referee laboratories to resend test samples for arrival the following day if necessary. If renewal shipments do not arrive on the expected day, DynCorp will provide guidance for test renewal on a case-by-case basis. Depending on the volume of sample remaining from previous shipments, laboratories may be instructed to conduct full renewals with the remaining sample, conduct partial renewals with the remaining sample, or omit the sample renewal for that day but carefully record dissolved oxygen throughout the day and remove excess food and dead organisms from the test containers.

3.0 WET Test Analysis

3.1 Sample Preparation

3.1.1 Whole Volume Samples

Whole volume samples will be received in cubitainers with sufficient volume for test conduct and required water chemistry analysis. No sample preparation or adjustment steps (e.g. pH adjustment or salinity adjustment) should be conducted prior to test initiation. The whole volume sample shall be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%, 25%, 12.5%, and 6.25% sample shall be prepared from the whole volume sample for use in the test. These test concentrations and a dilution water control shall be prepared using synthetic seawater as the dilution water (prepared according to Section 7 of the methods manuals).

3.1.2 Ampule Samples

Ampule samples will be received as small volume liquid samples in 125ml plastic containers. Prior to test initiation the ampule samples must be reconstituted according to the instructions in Appendix A to provide the necessary volume for testing. The reconstituted sample shall then be treated as a typical 100% effluent sample received for NPDES compliance monitoring. Test concentrations of 100%, 50%,

25%, 12.5%, and 6.25% sample shall be prepared from this reconstituted sample for use in the test. These test concentrations and a dilution water control shall be prepared using synthetic seawater as the dilution water (prepared according to Section 7 of the methods manuals).

3.2 Test Conduct

This section of the SOP reiterates testing requirements from Section 3 of the Participant Laboratory Statement of Work that must be followed for the conduct of the Inland Silverside minnow, *Menidia beryllina,* larval survival and growth test method. Except where indicated in Sections 3.2.1 and 3.2.2 of this SOP, each test shall be conducted in accordance with the general guidance and method specific requirements for effluent testing included in the methods manuals.

3.2.1 General Testing Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion and when standardization is necessary to assure the predictability of the methods to provide reliable results. Additionally, the method manuals allow variations of the methods which are typically fixed in the permit; therefore, for the purposes of this study, a set of variables will be defined by EPA (for example, dilution water, salinity, and acute test duration). Any deviation from defined test procedures and/or conditions, such as the necessity to change reagents, equipment, test conditions, or other specified test parameters must be reported to SCC, recorded at the time of modification, noted in telephone logs of communications, documented in a memorandum, and approved by EPA.

Additional WET test requirements and general requirements listed in the methods manuals of special note are provided below:

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(2) To coordinate testing at participant laboratories, testing of each sample with each method must be initiated on the precise day specified in the finalized study schedule. Samples should be tested within 36 hours from the time of sample preparation (determined in this study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Deviation from this schedule must be reported to SCC immediately for approval.

(3) Physical and chemical properties of the test samples must be in the ranges specified in this SOP, the SOW, specific instructions, and the methods manuals. Method specific instructions for any adjustments to the test samples prior to sample use (such as reconstitution of ampule samples or salinity adjustments) are provided herein. Test samples received at participant laboratories must be refrigerated (at 4 °C ± 2 °C) immediately upon receipt and throughout the period of testing. The temperature of the refrigeration unit should be routinely or continuously monitored to ensure that these sample holding requirements are met.

(4) Measurement of test conditions (pH, conductivity or salinity, total alkalinity, total hardness, and dissolved oxygen) shall be performed for each method by the participant laboratories following guidance in method manuals.

(5) The specified dilution and control waters must be used and prepared according to instructions in Section 7 of the methods manuals.

(6) All WET tests are to be definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) All tests must be conducted using the number of replicates and number of test containers per concentration as specified in Section 3.2.2.

(8) Test chambers used within a test must be of the same type, size, shape, and material. The material must be allowed by the method manuals for the method used.

(9) Test vessels shall be randomized in accordance with the method manuals.

(10) Daily observation of mortality and removal of dead organisms for each test is required.

(11) If test results indicate too great of toxicity (i.e., control mortalities, or complete mortality in all concentrations), the laboratories must contact SCC immediately and then investigate possible causes, first by checking for transcription and calculation mistakes, and then by investigating possible contamination in dilution waters, organism cultures, equipment, or other procedural steps.

(12) If any test that has been initiated fails to be completed for any reason, the laboratory must contact SCC immediately for problem resolution and scheduling of additional testing. The incomplete test data and the reason for not completing the test must be fully documented in the final report.

(13) Each laboratory shall be required to report all data obtained during the course of testing, including the response of control samples.

(14) Laboratories must perform all QA/QC tests listed in Section 4 of the method manuals. Laboratories that purchase organisms must supply QA/QC from the test organism supplier and follow method manuals for the appropriate QA/QC for purchasing organisms.

(15) A reference toxicant QC test must be performed for each test method in the month that testing for this study occurs. Results of this test must be submitted with the final data package.

(16) Data and statistical analyses must be submitted in hard copy in the standardized format specified in Section 5 of the SOW. All bench sheets and raw data, including sample tracking and chemistry analysis data must be submitted. Data must also be submitted electronically according to an electronic template (Microsoft Excel® spreadsheet) that is provided with this SOP.

(17) Data analysis must be performed in accordance with the statistical programs specified in the methods manuals. Statistical methods and programs used must be reported along with sample calculations.

(18) An IC_{25} must be reported for each chronic test. The laboratories must report individual toxicity endpoints; laboratories are not allowed to average or perform other data manipulations unless required by a methods's instructions.

3.2.2 Method-Specific Requirements

Table 2 provides a summary of test conditions that shall be followed for the conduct of all Inland Silverside minnow larval survival and growth tests performed in the WET Interlaboratory Study. This table is extracted from the summary test condition table in the method manual and modified to fit the scope of this study. Items that are bold italic in this table represent conditions standardized for the purposes of this study where method manuals provide a range.

Table 2. Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test. Summary of test conditions and test acceptability criteria for the inland silverside, *Menidia beryllina*, larval survival and growth test with effluents and receiving waters

1. Test type:	Static renewal
2. Salinity:	***25‰ (±2‰)***
3. Temperature:	25 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	l0-20 μE/m^2/s (50-100 ft-c) (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***1 L containers***
8. Test solution volume:	***750 mL/replicate (loading and DO restrictions must be met)***
9. Renewal of test solutions:	Daily
10. Age of test organisms:	7-11 days post hatch; 24-h range in age
11. No. larvae per test chamber:	***10***
12. No. replicate chambers per concentration:	***4***
13. No. larvae per concentration:	***40***
14. Source of food:	Newly hatched *Artemia* nauplii (survival of 7-9 days old *Menidia beryllina* larvae improved by feeding 24 h old *Artemia*)
15. Feeding regime:	Feed 0.10 g wet weight *Artemia* nauplii per replicate on days 0-2; Feed 0.15 g wet weight *Artemia* nauplii per replicate on days 3-6
16. Cleaning:	Siphon daily, immediately before test solution renewal and feeding
17. Aeration:	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min.
18. Dilution water:	***25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)***
19. Test concentrations:	***Five concentrations and a control***
20. Dilution factor:	***0.5***
21. Test duration:	7 days
22. Endpoints:	Survival and growth (weight)
23. Test acceptability criteria:	80% or greater survival in controls, 0.50 mg average dry weight of control larvae when larvae dried immediately after test termination, or 0.43 mg or greater average dry weight of control larvae, preserved not more than 7 days in 4% formalin or 70% ethanol
24. Sample handling and holding requirements:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule***
25. Sample volume required:	6 L/day

NOTE: Test vessels shall be randomized in accordance with the method manuals.

3.2.3 Data Analysis

For the Inland Silverside larval survival and growth test method, the 7 day survival LC_{50}, 7 day survival NOEC, growth IC_{25}, and growth NOEC shall be calculated and reported. Data analysis and statistical procedures should be conducted according to the method manuals.

3.2.4 Problem Resolution

In the event that problems are encountered during the WET test analysis contact Brian Rusignuolo at (703)461-2401 for guidance. Brian will direct your call as appropriate for the resolution of technical issues.

3.2.5 Sample Disposal

Following the termination of the test, any excess sample shall be disposed of according to standard laboratory procedures for disposal of effluent samples.

4.0 Additional Information on Data Reporting Deliverables

According to the Participant Laboratory Statement of Work, the submission of three deliverables are required: 1). Narrative summary of testing, 2). Hardcopy results synopsis and full report, and 3). Electronic results synopsis. Deliverables #1 and 2 shall be submitted according to the requirements specified in the SOW. This section provides additional instructions for the submission of the electronic results synopsis.

Enclosed with this package is a computer diskette that contains the template for the electronic submission of results from the Inland Silverside minnow larval survival and growth test method. The disk contains a Microsoft Excel 97 spreadsheet file named ISC___.xls. The ISC indicates that this template is for the Inland Silverside minnow chronic test method, and the number following is a unique identifying number for your laboratory. If your laboratory does not have the hardware or software capabilities to view and enter data into this file, please contact Brian Rusignuolo at (703)461-2401 to arrange distribution of an alternative version of the electronic template.

Notice the following characteristics of the electronic template file:

1. The file contains four worksheet pages labeled “Sample #1", “Sample #2", “Sample #3", and “Sample #4". The results from each of the samples that your laboratory tested in this study should be entered on a separate worksheet page within the same file.
2. Each worksheet page contains seven information boxes (general information, sample collection/receipt information, test information, biological data, water quality data, weight data, and summarized test results) in which data should be entered. The eighth information box (data quality flags) is for SCC use only and will aid in the automated review and quality control check of data.
3. Cells that are highlighted in pale yellow indicate required information. Cells highlighted in blue indicate optional information that may be entered if available. Cells highlighted in red are for SCC use only.
4. The file has been protected such that data can only be entered in yellow or blue cells. No other cells can be changed.

To complete the electronic results synopsis data deliverable:

1. Record information into a separate worksheet page for each sample tested.
2. Record requested information or data in all required cells (pale yellow).
3. Record requested information or data in optional cells (blue) if data is available.
4. Check entered data against hardcopy bench sheets to ensure accuracy.
5. Save the file onto the diskette provided. Do not change the file name.
6. Submit the diskette with the other data reporting deliverables by the due date to:

DynCorp I&ET, Sample Control Center
6101 Stevenson Avenue
Alexandria, VA 22304
Phone: (703) 461-2064
Fax: (703) 461-8056
Attention: Robert Brent

5.0 Coolers

All coolers will include a prepaid return FedEx label for returning the cooler to the referee laboratory. Fill out the sender portion of the FedEx label and place it on the cooler. Coolers will be returned by 2 Day shipment, as already marked on the prepared label. Coolers must be returned to the referee laboratory within 7 days of sample receipt. Please empty and wipe out the cooler prior to returning it.

Appendix A: Instructions for Reconstitution of Liquid Ampule Sample for Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test

For each sample, three liquid ampules will be received (marked with the sample number followed by an "A", "B", or "C"). The three containers shall be reconstituted as described below to mimic the sample shipment schedule for effluent samples. The container marked "A" shall be reconstituted on the day of test initiation (Day 0) and used for renewal on Day 1. The container marked "B" shall be reconstituted on Day 2 and used for renewals on Day 2 and Day 3. The container marked "C" shall be reconstituted on Day 4 and used for renewals on Day 4, Day 5, and Day 6. Follow the directions below for the reconstitution of each sample ampule.

1. Volumetrically add 100 mL of the liquid ampule sample to approximately 1L of synthetic seawater. The synthetic seawater should be prepared to a salinity of 25‰ (±2‰) using Bioassay Grade Forty Fathoms artificial sea salts and MILLIPORE MILLI-Q® or equivalent deionized water according to Section 7 of the methods manuals.

2. Mix by swirling or gently shaking.

3. Bring final volume to 21L (measured using volumetric glassware) with synthetic seawater dilution water.

4. Mix again by swirling and gently shaking.

5. Place reconstituted sample in a plastic container of appropriate volume. A container that minimizes head space (i.e. cubitainer) is recommended.

6. This 21L sample is the whole volume reconstituted sample and should be treated as a typical effluent sample. It should be used directly for the 100% effluent test concentration and diluted appropriately to prepare other test concentrations (50%, 25%, 12.5%, and 6.25%).

7. Use the reconstituted sample for test initiation and Day 1 test renewal. Store sample at 4°C.

8. Perform the Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test as described in the SOW for this study and the methods manuals.

9. Follow Steps 1 through 6 with each sample container to prepare the reconstituted sample on Day 2 and Day 4 for subsequent daily renewals.

EPA	United States **Environmental Protection Agency** Washington, DC 20460	EPISODE NO: SAMPLE NO:

WET Interlaboratory Variability Study Traffic Report USEPA ENGINEERING AND ANALYSIS DIVISION SAMPLE CONTROL CENTER	**Fax** completed form immediately upon completion, and include hardcopy in final data report to:	DynCorp - Sample Control Center 6101 Stevenson Avenue Alexandria, VA 22304 phone: (703) 461-2100 **fax: (703) 461-8056**

Referee Laboratory Information	**Participant Lab Shipping Information**	FOR PARTICIPANT LAB USE ONLY
Name:	Lab Name:	Received by:
Address:	Address:	Sample condition on receipt:
City:	City:	
State:	State:	
	Airbill no:	
Sampler name:	Date shipped:	

SAMPLE COLLECTION / RECEIPT INFORMATION

	Requested Information	Pre-Shipment	Post-Shipment
1.	Sample use description	☐Initiation ☐renewal 1 ☐renewal 2	
2.	Sample collection/receipt date		
3.	Sample collection/receipt time		
4.	Sampler / recipient signature		
5.	pH		
6.	Temperature		
7.	Conductivity (freshwater methods) / Salinity (marine methods)		
8.	Dissolved Oxygen concentration		

REQUESTED ANALYSES

9.	☐ Pimephales promelas Acute	☐ Menidia beryllina Acute	☐ Cyprinodon variegatus Acute
	☐ Pimephales promelas Chronic	☐ Menidia beryllina Chronic	☐ Cyprinodon variegatus Chronic
	☐ Ceriodaphnia dubia Acute	☐ Holmesmysis costata Acute	☐ Mysidopsis bahia Chronic
	☐ Ceriodaphnia dubia Chronic	☐ Champia parvula Chronic	
	☐ Selenastrum capricornutum Chronic		

Appendix C:

List of Referee and Participant Laboratories

Referee Laboratories Involved in the WET Variability Study

EA Engineering, Science, and Technology, Inc.
Ogden Environmental and Energy Services, Inc.
MEC Analytical, Inc.
EnviroSystems, Inc.

Participant Laboratories Involved in the WET Variability Study

Analytical Environmental Testing, Inc.
Analytical Services, Inc.
Aqua Survey, Inc.
AQUA-Science
Aquatech Environmental Services, Inc.
Aquatic Bioassay Consulting Laboratories, Inc.
Aquatic Consulting & Testing, Inc.
Beckmar Environmental Laboratory
Bio-Aquatic Testing, Inc.
Biological Monitoring, Inc.
Block Environmental Services, Inc.
Burlington Research, Inc.
C-K Associates, Inc.
Central Virginia Laboratories & Consultants, Inc.
CH2M Hill
Chadwick & Associates, Inc.
City & County of Honolulu, Water Quality Laboratory
City of Phoenix Water Services Department Laboratory
City of San Diego, Marine Biology Laboratory
City of San Jose, Environmental Services Department Laboratory
Coastal Bioanalysts, Inc.
Cosper Environmental Services, Inc.
County Sanitation Districts of Los Angeles, San Jose Creek Water Quality Laboratory
Eastman Chemical Company
EnviroData Group, LLC
Environmental Science & Engineering, Inc.
EnviroScience, Inc.
EnviroSystems, Inc.
ETT Environmental, Inc.
EVS Environment Consultants
Global Environmental Consulting, LLC
Hampton Roads Sanitation District
Hydrosphere Research
King County Environmental Lab
Law Engineering & Environmental Services, Inc.

Participant Laboratories Involved in the WET Variability Study (continued)

MEC Analytical Systems, Inc.- Carlsbad, CA
MEC Analytical Systems, Inc.- Tiburon, CA
Metro Wastewater Reclamation District - Denver
Metropolitan Water Reclamation District of Greater Chicago
NEORSD Analytical Services
New England Bioassay, Inc.
Northshore Sanitation District
Ogden Environmental & Energy Services Company, Inc. - Fife, WA
Ogden Environmental & Energy Services Company, Inc. - San Diego, CA
Pacific EcoRisk
Pima County Wastewater Management Department - Ina Road Water Pollution Control Facility
QC Laboratories, Inc
Research, Environmental & Industrial (REI) Consultants, Inc.
Shealy Environmental Services, Inc.
Tetra Tech, Inc.
The ADVENT Group, Inc.
The SeaCrest Group
Toxikon Corporation
ToxScan, Inc.
Whole Effluent Toxicity Testing Laboratories, Inc.

Appendix D:

Preliminary Testing Results

D.1 Preliminary Testing for *Ceriodaphnia* Acute and Chronic Test Methods

D.1.1 Reference Toxicant Sample Type

The reference toxicant sample type was composed of moderately hard synthetic freshwater prepared according to Section 7 of the WET method manuals (USEPA, 1994a) and spiked with KCl. The spiking level for this sample type was targeted to produce a test result (LC50 for the *Ceriodaphnia* acute test and IC25 for the *Ceriodaphnia* chronic test) of 50% sample. Spiking levels for Part 2 preliminary testing were selected based on historical testing at the referee laboratory for the *Ceriodaphnia* acute test method and from an initial Part 2 range-finding test for the *Ceriodaphnia* chronic test method. Tables D1 and D2 show the results from preliminary testing for *Ceriodaphnia* acute and chronic test methods, respectively. For the *Ceriodaphnia* acute test method, Part 2 testing resulted in an LC50 of 424 mg KCl/L. Based on this result, the spiking level for Part 4 testing was increased to 850 mg KCl/L (approximately 424 / the target result of 50% sample). Part 4 *Ceriodaphnia* acute testing produced an LC50 of 574 mg KCl/L or 67.6% sample. The spiking level for the interlaboratory testing phase (1000 mg KCl/L) was based on an average of the LC50 results obtained in Part 2 and Part 4 testing (approximately the average result of 500 / the target result of 50% sample). Referee laboratory testing of this interlaboratory sample yielded LC50s of 40.6% and 34.4% sample.

Table D1. Results from *Ceriodaphnia* acute preliminary testing.

Sample type	Part[a]	Concentrations tested (mg KCl/L)	NOAEC (mg KCl/L)	LC50 (mg KCl/L)	LC50 (% sample)
Reference toxicant	2	37.5, 75, 150, 300, 600	300	424	70.7
	4	53, 106, 212.5, 425, 850	425	574	67.6
	IL	62.5, 125, 250, 500, 1000	250	406	40.6 & 34.4[b]
Effluent	2	131, 262.5, 525, 1050, 2100	525	651	31.0
	3	131, 262.5, 525, 1050, 2100	525	689	32.8
	4	167.5, 335, 670, 1340, 2680	670	948	35.4
	IL	167.5, 335, 670, 1340, 2680	335	670	25.0
Receiving water	2	106, 212, 424, 848, 1696	424	526	31.0
	3	106, 212, 424, 848, 1696	424	487	28.7
	4	125, 250, 500, 1000, 2000	250	365	18.3
	IL	112.5, 225, 450, 900, 1800	450	554	30.8

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL). Part 1 preliminary testing was conducted using only the chronic method, and Part 3 was conducted using only the acute method.
[b] The referee laboratory tested two reference toxicant samples due to a sample distribution error (see Section 6.4 in the main body of this report).

Part 2 preliminary testing for the *Ceriodaphnia* chronic test method produced IC25 values of 323 mg KCl/L and 138 mg KCl/L in two separate tests. The referee laboratory selected a Part 4 spiking level of 650 mg KCl/L so that the IC25 would potentially fall between 50% (if the true IC25 was closer to 323 mg KCl/L) and 25% sample (if the true IC25 was closer to 138 mg KCl/L). Results of this test revealed an IC25 of 132 mg KCl/L. The referee laboratory then repeated the Part 4 test with a slightly lower spiking level. This test produced an IC25 of 134 mg KCl/L. Since three consecutive tests produced IC25 values between 132 and 138 mg KCl/L, the referee laboratory selected a final spiking level of 270 mg KCl/L (the average result of 135 / the target result of 50% sample) to achieve the target result of 50% sample in interlaboratory testing. Unfortunately, this spiking level did not produce the desired effect during interlaboratory testing. The resulting sample was marginally toxic and produced toxic results in only some laboratories (see Section 5.3 in the main body of this report).

Table D2. Results from *Ceriodaphnia* chronic preliminary testing.

Sample type	Part[a]	Concentrations tested (mg KCl/L)	Survival NOEC (mg KCl/L)	Reproduction NOEC (mg KCl/L)	IC25 (mg KCl/L)	IC25 (% sample)
Reference toxicant	2[b]	56, 100, 180, 320, 560	320	320	323	57.7
	2	43.75, 87.5, 175, 350, 700	175	87.5	138	19.8
	4	40.5, 81, 162.5, 325, 650	325	81	132	20.3
	4[c]	31.25, 62.5, 125, 250, 500	125	125	134	26.9
	IL	17, 34, 67.5, 135, 270	270	270	>270	>100
Effluent	1[d]	6.25, 12.5, 25, 50, 100	100	100	-	>100
	2	87.5, 175, 350, 700, 1400	350	350	424	30.3
	4	106.3, 212.5, 425, 850, 1700	425	425	538	31.6
	IL	131, 263, 525, 1050, 2100	263	263	389	18.5
Receiving water	1[d]	6.25, 12.5, 25, 50, 100	100	100	-	>100
	2	75, 150, 300, 600, 1200	300	300	368	30.7
	4	92.5, 185, 370, 740, 1480	185	92.5	114	7.7
	4[c]	62.5, 125, 250, 500, 1000	250	250	342	34.2
	IL	75, 150, 300, 600, 1200	300	300	372	31.3

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL). Part 1 preliminary testing was conducted using only the chronic method, and Part 3 was conducted using only the acute method.
[b] An initial Part 2 range-finding test was conducted due to a lack of historical referee laboratory data for this method and toxicant.
[c] Part 4 testing was repeated to confirm spiking levels.
[d] Part 1 testing was conducted on unspiked samples. The units for test concentrations in Part 1 were percent sample.

D.1.2 Effluent Sample Type

The effluent sample type was composed of a municipal wastewater treatment plant effluent spiked with KCl. The referee laboratory (EA Engineering, Science and Technology, Inc.) collected the effluent from a municipal wastewater treatment plant that is designed to treat 180 mgd, is able to handle peak flows of 400 mgd, and currently treats 140 to 150 mgd. The facility employs tertiary treatment for biological nutrient removal including single-stage nitrification/denitrification, sand filtration, chlorination/dechlorination, and anaerobic digestion. The effluent source was selected based on historical consistency in chemical and toxicological testing conducted by the referee laboratory. This same effluent source was used for all freshwater methods, the *Mysidopsis* chronic test method, and the sheepshead acute and chronic test methods. Water chemistry of the effluent on each sample collection date is shown in Table D3. More detailed chemical analyses (including total dissolved solids, total suspended solids, total organic carbon, biological oxygen demand, and chemical oxygen demand) were performed at the beginning and end of preliminary testing to better characterize the sample source. Historically, the effluent has demonstrated low to no acute or chronic toxicity to freshwater organisms. Part 1 preliminary testing confirmed this consistency (Table D2).

For Part 2 of preliminary testing, the effluent sample was spiked with KCl to provide a consistently toxic sample. The spiking level for this sample type was targeted to produce a test result (LC50 for the *Ceriodaphnia* acute test and IC25 for the *Ceriodaphnia* chronic test) of 25% sample. Part 2 preliminary testing for the *Ceriodaphnia* acute test method produced an LC50 of 651 mg KCl /L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an LC50 of 689 mg KCl/L, which represents only a 5.8% change from the original Part 2 test result. The spiking level for Part 4 preliminary testing (2680 mg KCl/L) was based on an average of the LC50 results obtained in Part 2 and Part 3 testing (the average result of 670 / the target result of 25% sample). Part 4 testing produced an LC50 of 948 mg KCl/L or 35.4% sample. The same spiking level was used for the interlaboratory testing phase. Referee laboratory testing of the interlaboratory sample produced an LC50 of 25.0% sample.

For the *Ceriodaphnia* chronic test method, Part 1 testing of the unspiked effluent resulted in no toxicity (IC25 > 100% sample). Spiking of the effluent sample in Part 2 testing produced an IC25 of 424 mg KCl/L. Based on this result, the spiking level was increased to 1700 mg KCl/L (approximately 424 / the target result of 25% sample) for Part 4 testing. Part 4 testing produced an IC25 of 538 mg KCl/L or 31.6% sample. Based on this result, the spiking level used for interlaboratory testing was further increased to 2100 mg KCl/L (approximately 538 / the target result of 25% sample). The interlaboratory sample yielded an IC25 of 18.5% sample in referee laboratory testing.

Table D3. Water chemistry of effluent sample source for freshwater test methods.

Parameters	Sampling date[a]										
	08/11/99	**08/19/99**	**08/24/99**	**09/07/99**	**09/20/99**	**11/29/99**	**12/07/99**	**12/10/99**	**01/06/00**	**01/24/00**	**02/08/00**
Alkalinity (mg/L as $CaCO_3$)	76	62	68	78	68	54	38	70	-	76	82
Hardness (mg/L as $CaCO_3$)	148	160	160	172	156	152	148	128	-	156	172
Conductivity (μS/cm)	828	933	894	909	728	695	675	766	798	959	933
pH	7.4	7.1	7.2	7.2	7.5	6.8	6.6	7.3	7.1	7.7	7.4
Temperature (°C)	28.1	26	25.7	23.9	23.3	12.9	17.6	17.0	15.6	11.9	10.9
Total residual chlorine (mg/L)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	-	-	<0.01	-	<0.01
Dissolved oxygen (mg/L)	7.5	7.2	7.8	7.6	8.1	8.9	7.7	8.9	9.1	9.5	10.3
Total ammonia (mg/L)	1.17	0.284	0.122	0.191	-	0.0371	0.024	0.079	-	-	1.88
Total dissolved solids (mg/L)	420	-	-	-	-	-	-	-	-	-	626
Total suspended solids (mg/L)	7.0	-	-	-	-	-	-	-	-	-	<2.5
Total organic carbon (mg/L)	21.4	-	-	-	-	-	-	-	-	-	26.6
Biological oxygen demand (mg/L)	3.8	-	-	-	-	-	-	-	-	-	4.8
Chemical oxygen demand (mg/L)	59.9	-	-	-	-	-	-	-	-	-	77.2

[a] "-" indicates that the parameter was not tested on the given sampling date.

D.1.3 Receiving Water Sample Type

The receiving water sample type was composed of a natural surface freshwater spiked with KCl. The referee laboratory (EA Engineering, Science and Technology, Inc.) collected the receiving water from the Gunpowder River, in Baltimore County, Maryland. Initial samples were collected from a location near Bunker Hill Road. Results of preliminary testing indicated that these unspiked samples occasionally showed toxicity to fathead minnows and *Selenastrum capricornutum*. To avoid the potential problems associated with intermittent ambient toxicity, subsequent freshwater samples were collected from a new location (near Falls Road), upstream from potential sources of non-point source runoff. The results from toxicity tests conducted with the Falls Road samples indicated no acute or chronic toxicity to *Ceriodaphnia dubia* (Table D2) or other freshwater species. Water chemistry of the receiving water is shown in Table D4 for each sample collection date. More detailed chemical analyses (including total dissolved solids, total suspended solids, total organic carbon, biological oxygen demand, and chemical oxygen demand) were performed at the beginning and end of preliminary testing to better characterize the sample source.

The receiving water sample was spiked with KCl to provide a consistently toxic sample. The spiking level for this sample type was targeted to provide a test result (LC50 for the *Ceriodaphnia* acute test and IC25 for the *Ceriodaphnia* chronic test) of 25% sample. Part 2 preliminary testing for the *Ceriodaphnia* acute test method produced an LC50 of 526 mg KCl/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an LC50 of 487 mg KCl/L, which represents only a 7.4% change from the original Part 2 test result. For Part 4 preliminary testing, the referee laboratory increased the spiking level to 2000 mg KCl/L to better achieve the target result of 25% sample. Part 4 testing produced an LC50 of 365 mg KCl/L or 18.3% sample. The spiking level used for interlaboratory testing (1800 mg KCl/L) was based on an average of the LC50 results obtained in Part 2, 3, and 4 testing (approximately the average result of 460 / the target result of 25% sample). Referee laboratory testing of this sample produced an LC50 of 30.8% sample during the interlaboratory testing phase.

For the *Ceriodaphnia* chronic test method, Part 1 testing of the unspiked receiving water resulted in no toxicity (IC25 > 100% sample). Spiking of the receiving water sample in Part 2 testing produced an IC25 of 368 mg KCl/L. Based on this result, the spiking level was increased to 1480 mg KCl/L for Part 4 testing (approximately 368 / the target result of 25% sample). Part 4 testing produced an IC25 of 114 mg KCl/L or 7.7% sample. Due to the discrepancy between the results of Part 2 and Part 4 testing, Part 4 testing was repeated. Repeated Part 4 testing produced an IC25 of 342 mg KCl/L, which was consistent with Part 2 testing results. Final spiking levels were based on these tests and set at 1200 mg KCl/L for the interlaboratory testing phase. Referee laboratory testing of the interlaboratory sample produced an IC25 of 31.3% sample.

Table D4. Water chemistry of receiving water sample source for freshwater test methods.

Parameters	Sampling date[a]								
	06/24/99	**07/07/99**	**07/14/99**	**07/28/99**	**08/05/99**	**08/20/99**	**09/21/99**	**12/21/99**	**01/31/00**
Alkalinity (mg/L as $CaCO_3$)	30	28	30	24	28	34	34	26	28
Hardness (mg/L as $CaCO_3$)	80	60	60	60	48	44	60	36	56
Conductivity (μS/cm)	183	154	148	159	161	155	157	139	124
pH	7.7	7.9	6.8	7.9	7.9	7.3	7.5	8.3	7.8
Temperature (°C)	14.1	15.0	16.3	18.3	16.6	16.5	14.6	7.8	2.1
Total residual chlorine (mg/L)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	-	-
Dissolved oxygen (mg/L)	10.4	9.6	10.3	9.8	10.7	10.9	10.0	12.6	14.6
Total ammonia (mg/L)	0.056	0.096	-	-	0.092	0.307	-	-	-
Total dissolved solids (mg/L)	94.5	-	-	-	-	-	-	-	-
Total suspended solids (mg/L)	5.5	-	-	-	-	-	-	-	-
Total organic carbon (mg/L)	1.3	-	-	-	-	-	-	-	-
Biological oxygen demand (mg/L)	<1.0	-	-	-	-	-	-	-	-
Chemical oxygen demand (mg/L)	12.8	-	-	-	-	-	-	-	-

[a] "-" indicates that the parameter was not tested on the given sampling date.

D.2 Preliminary Testing for Fathead Acute and Chronic Test Methods

D.2.1 Reference Toxicant Sample Type

The reference toxicant sample type was composed of moderately hard synthetic freshwater prepared according to Section 7 of the WET method manuals (USEPA, 1994a) and spiked with KCl. The spiking level for this sample type was targeted to produce a test result (LC50 for the fathead acute test and IC25 for the fathead chronic test) of 50% sample. Tables D5 and D6 show the results from preliminary testing using fathead acute and chronic test methods, respectively. Spiking levels selected for Part 2 preliminary testing resulted in an LC50 of 915 mg KCl/L for the fathead acute test method. Based on this result, the spiking level for Part 4 testing was increased to 1830 mg KCl/L (915 / the target result of 50% sample). Part 4 fathead acute testing produced an LC50 of 1167 mg KCl/L (63.8% sample). Spiking levels were further increased to 2200 mg KCl/L for the interlaboratory testing phase to better achieve the target result of 50% sample. Referee laboratory testing of the interlaboratory sample produced an LC50 of 42.4% sample.

Part 2 preliminary testing using the fathead chronic test method produced an IC25 of 545 mg KCl/L. Based on this result, the spiking level for Part 4 testing was decreased to 1090 mg KCl /L (545 / the target result of 50% sample). Part 4 testing yielded an IC25 of 610 mg KCl/L. Based on Part 4 testing results, the spiking level for the interlaboratory sample was further increased to 1220 mg KCl/L (610 / the target result of 50% sample). Referee laboratory testing of the interlaboratory sample yielded an IC25 of 63.3% sample.

Table D5. Results from fathead acute preliminary testing.

Sample type	Part[a]	Concentrations tested (mg KCl/L)	NOAEC (mg KCl/L)	LC50 (mg KCl/L)	LC50 (% sample)
Reference toxicant	2	104.9, 209.8, 419.5, 839, 1678	419.5	915	54.5
	4	114.5, 229, 457.5, 915, 1830	915	1167	63.8
	IL	138, 275, 550, 1100, 2200	550	924	42.4
Effluent	2	300, 600, 1200, 2400, 4800	600	1308	27.3
	3	300, 600, 1200, 2400, 4800	600	1356	28.3
	4	350, 700, 1400, 2800, 5600	700	990	17.7
	IL	333, 666, 1332, 2664, 5328	666	1028	19.3
Receiving water	2	250, 500, 1000, 2000, 4000	1000	1270	31.7
	3	250, 500, 1000, 2000, 4000	500	1168	29.2
	4	300, 600, 1200, 2400, 4800	600	1256	26.2
	IL	313, 625, 1250, 2500, 5000	625	985	19.7

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL). Part 1 preliminary testing was conducted using only the chronic method, and Part 3 was conducted using only the acute method.

Table D6. Results from fathead chronic preliminary testing.

Sample type	Part[a]	Concentrations tested (mg/L KCl)	Survival NOEC (mg/L KCl)	Reproduction NOEC (mg/L KCl)	IC25 (mg/L KCl)	IC25 (% sample)
Reference toxicant	2	88.4, 176.8, 353.5, 707, 1414	353.5	353.5	545	38.5
	4	68, 136, 272.5, 545, 1090	545	545	610	56.0
	IL	76, 153, 305, 610, 1220	610	610	772	63.3
Effluent	1[b]	6.25, 12.5, 25, 50, 100	100	100	-	>100
	2	144, 287.5, 575, 1150, 2300	575	575	721	31.4
	4	181.3, 362.5, 725, 1450, 2900	725	725	901	31.1
	IL	225, 450, 900, 1800, 3600	900	900	968	26.9
Receiving water	1[b, c]	6.25, 12.5, 25, 50, 100	100	50	-	5.6
	1[b, d]	6.25, 12.5, 25, 50, 100	100	100	-	>100
	2	136, 272.5, 545, 1090, 2180	545	545	566	26.0
	4	140, 280, 560, 1120, 2240	560	560	606	27.0
	IL	150, 300, 600, 1200, 2400	600	600	708	29.5

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL). Part 1 preliminary testing was conducted using only the chronic method, and Part 3 was conducted using only the acute method.
[b] Part 1 testing was conducted on unspiked samples. The units for test concentrations in Part 1 were percent sample.
[c] Receiving water sample was collected from Bunker Hill Road site.
[d] Receiving water sample was collected from Falls Road site.

D.2.2 Effluent Sample Type

The effluent sample source used for the fathead acute and chronic test methods was the same as described in Section D.1.2. This effluent sample was spiked with KCl to provide a consistently toxic sample. The spiking level for this sample type was targeted to produce a test result (LC50 for the fathead acute test and IC25 for the fathead chronic test) of 25% sample. Part 2 preliminary testing for the fathead acute test method produced an LC50 of 1308 mg KCl/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an LC50 of 1356 mg KCl/L, which represents only a 3.7% change from the original Part 2 test result. For Part 4 preliminary testing, the referee laboratory increased the spiking level to 5600 mg KCl/L to better achieve the target effect level of 25% sample. This test produced an LC50 of 990 mg KCl/L or 17.7% sample; however, less than 90% (65%) survival was experienced in the control. Since Part 4 testing was unreliable, the spiking level for interlaboratory testing was based on an average of Part 2 and Part 3 testing results and set at 5328 mg KCl/L (the average result of 1332 / the target result of 25% sample). This interlaboratory sample yielded an LC50 of 19.3% sample in referee laboratory testing.

For the fathead chronic test method, Part 1 testing of the unspiked effluent resulted in no toxicity (IC25 > 100% sample). Spiking of the effluent sample in Part 2 testing produced an IC25 of 721 mg KCl/L. Based on this result, the spiking level was increased to 2900 mg KCl/L for Part 4 testing (approximately 721 / the target result of 25% sample). Part 4 testing produced an IC25 of 901 mg KCl/L or 31.1% sample. Based on Part 4 testing, the spiking level for interlaboratory testing was further increased to 3600 mg KCl/L (approximately 901 / the target result of 25% sample). This interlaboratory sample yielded an IC25 of 26.9% sample in referee laboratory testing.

D.2.3 Receiving Water Sample Type

The receiving water sample source used for the fathead acute and chronic test methods was the same as described in Section D.1.3. The receiving water sample was spiked with KCl to provide a consistently toxic sample. The spiking level for this sample type was targeted to provide a test result (LC50 for the fathead acute test and IC25 for the fathead chronic test) of 25% sample. Part 2 preliminary testing for the fathead acute test method produced an LC50 of 1270 mg KCl/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an LC50 of 1168 mg KCl/L, which represented only a 8.7% change from the original Part 2 test result. The spiking level for Part 4 preliminary testing (4800 mg KCl/L) was based on an average of the LC50 results obtained in Part 2 and Part 3 testing (approximately the average result of 1219 / the target result of 25% sample). Part 4 testing produced an LC50 of 1256 mg KCl/L or 26.2% sample. Based on this result, the spiking level was further increased to 5000 mg KCl/L (approximately 1256 / the target result of 25% sample) for the interlaboratory testing phase. Referee laboratory testing of the interlaboratory sample yielded an LC50 of 19.7% sample.

For the fathead chronic test method, Part 1 testing of the unspiked receiving water collected from the Bunker Hill Road site indicated toxicity (IC25 of 5.6% sample). Following this test, the referee laboratory moved the receiving water collection site farther upstream to the Falls Road site. Part 1 testing of receiving water from the new location revealed no toxicity (IC25 >100% sample). Part 2 testing of the spiked receiving water produced an IC25 of 566 mg KCl/L. Based on this result, the spiking level was increased to 2240 mg KCl/L for Part 4 testing (approximately 566 / the target result of 25% sample). Part 4 testing produced an IC25 of 606 mg KCl/L or 27.0% sample. Based on this result, the final spiking level for interlaboratory testing was increased to 2400 mg KCl/L (approximately 606 / the target result of 25% sample). This interlaboratory sample yielded an IC25 of 29.5% sample in referee laboratory testing.

D.3 Preliminary Testing for the *Selenastrum* Chronic Test Method

D.3.1 Reference Toxicant Sample Type

The reference toxicant sample type for the *Selenastrum* chronic test method was composed of deionized water spiked with KCl. The spiking level for this sample type was targeted to produce an IC50 of 38% sample (see Section D.9). Table D7 shows the results from preliminary testing for the *Selenastrum* chronic test method. Spiking levels for Part 2 preliminary testing were based on the results of an initial Part 2 range-finding test. The range-finding test resulted in an IC50 of 925 mg KCl/L. Spiking levels selected for Part 2 testing resulted in an IC50 of 2925 mg KCl/L for the *Selenastrum* chronic test method. Based on this result, the spiking level for Part 4 testing was increased to 7888 mg KCl/L. Part 4 testing with EDTA produced an IC50 of 1713 mg KCl/L (or 57.1% sample) and Part 4 testing without EDTA produced an IC50 of 2943 mg KCl/L (or 98.1% sample). Due to the discrepancy between Part 4 results with EDTA and Part 2 results with EDTA, Part 4 testing was repeated. In additional Part 4 testing, IC50s of 1808 mg KCl/L (or 22.9% sample) and 462 mg KCl/L (or 5.9% sample) were produced with EDTA and without EDTA, respectively. The spiking level for interlaboratory testing was set at 5655 mg KCl/L (average result of 2149 / the target result of 38% sample) based on an average of Part 2 and Part 4 testing with EDTA. Referee laboratory testing of the interlaboratory sample yielded IC50s of 35.5% sample and 37.6% sample with and without EDTA, respectively.

D.3.2 Effluent Sample Type

The effluent sample source used for the *Selenastrum* chronic test method was the same as described in Section D.1.2. Part 1 testing of the unspiked effluent resulted in no toxicity (IC50 > 100% sample). The effluent sample was then spiked with KCl to provide a consistently toxic sample. The spiking level for this sample was targeted to produce a test result (IC50) of 38% sample. Part 2 preliminary testing for the *Selenastrum* chronic test method produced an IC50 of 4383 mg KCl/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an IC50 of 5143 mg KCl/L, which represents a 17% difference from the initial Part 2 test result. Based on Part 2 testing results, the spiking level for Part 4 testing was set at 11540 mg KCl/L (approximately 4383 / the target result of 38% sample). Part 4 testing resulted in an IC50 of 4609 mg KCl/L or 39.9% sample with EDTA and an IC50 of 4821 mg KCl/L or 41.8% sample without EDTA. Since Part 4 testing results were very close to the 38% sample target, the same spiking level was maintained for interlaboratory testing. Referee laboratory results during interlaboratory testing were 29.9% sample with EDTA and 19.8% sample without EDTA.

Table D7. Results from *Selenastrum* chronic preliminary testing.

Sample type	Part[a]	Concentrations tested (mg KCl/L)	NOEC (mg KCl/L)	IC50 (mg KCl/L)	IC50 (% sample)
Reference toxicant	2[b]	100, 1000, 10000, 30000, 60000	100	925	1.5
	2	375, 750, 1500, 3000, 6000	375	2925	48.7
	4 w/EDTA[c]	188, 375, 750, 1500, 3000	375	1713	57.1
	4 w/EDTA	493, 986, 1972, 3944, 7888	986	1808	22.9
	4 w/o EDTA	188, 375, 750, 1500, 3000	187.5	2943	98.1
	4 w/o EDTA[c]	493, 986, 1972, 3944, 7888	<493	462	5.9
	IL w/ EDTA	353, 707, 1414, 2828, 5655	1414	2007	35.5
	IL w/o EDTA	353, 707, 1414, 2828, 5655	1414	2126	37.6
Effluent	1[d]	6.25, 12.5, 25, 50, 100	100	-	>100
	2 w/ EDTA	938, 1875, 3750, 7500, 15000	937.5	4383	29.2
	3 w/ EDTA	938, 1875, 3750, 7500, 15000	937.5	5143	34.3
	4 w/ EDTA	721, 1443, 2885, 5770, 11540	2885	4609	39.9
	4 w/o EDTA	721, 1443, 2885, 5770, 11540	2885	4821	41.8
	IL w/ EDTA	721, 1443, 2885, 5770, 11540	2885	3502	29.9
	IL w/o EDTA	721, 1443, 2885, 5770, 11540	1443	2319	19.8
Receiving water	1[d, e]	6.25, 12.5, 25, 50, 100	100	-	97
	1[d, f]	50, 100	100	-	>100
	2 w/ EDTA	225, 450, 900, 1800, 3600	3600	>3600	>100
	2 w/ EDTA[g]	1000, 2000, 4000, 8000, 16000	1000	4885	30.5
	2 w/ EDTA[g]	500, 1000, 2000, 4000, 8000	<500	4399	55.0
	3 w/ EDTA	500, 1000, 2000, 4000, 8000	1000	4928	61.6
	4 w/ EDTA	807, 1614, 3228, 6456, 12912	3228	4069	31.5
	4 w/o EDTA	807, 1614, 3228, 6456, 12912	1624	2098	16.2
	IL w/ EDTA	732, 1464, 2928, 5857, 11713	2928	3093	26.8
	IL w/o EDTA	732, 1464, 2928, 5857, 11713	2928	4201	36.4

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL).
[b] An initial Part 2 rangefinding test was conducted due to a lack of historical referee laboratory data for this method and toxicant.
[c] Part 4 testing was repeated to confirm spiking levels.
[d] Part 1 testing was conducted on unspiked samples. The units for test concentrations in Part 1 were percent sample.
[e] Receiving water sample was collected from Bunker Hill Road site.
[f] Receiving water sample was collected from Falls Road site.
[g] Part 2 testing was repeated to confirm spiking levels.

D.3.3 Receiving Water Sample Type

The receiving water sample source used for the *Selenastrum* chronic test method was the same as described in Section D.1.3. Part 1 testing on the unspiked receiving water collected from the Bunker Hill Road site indicated toxicity (IC50 of 97% sample). Following this test, the referee laboratory moved the receiving water collection site farther upstream to the Falls Road site. Part 1 testing of receiving water from this site revealed no toxicity (IC50 >100% sample). The receiving water sample was then spiked with KCl to provide a consistently toxic sample. The spiking level for this sample was targeted to produce a test result (IC50) of 38% sample. Part 2 preliminary testing for the *Selenastrum* chronic test method produced an IC50 of >3600 mg KCl/L. Since the test result was outside of the concentration range tested, Part 2 testing was repeated. Two additional Part 2 tests produced IC50s of 4885 and 4399 mg KCl/L. The sample from the latter Part 2 test was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an IC50 of 4928 mg KCl/L, which represents only a 12% difference from the original Part 2 test result. Based on an average of results from the second Part 2 test and Part 3 testing, the spiking level for Part 4 testing was set at 12912 mg KCl/L (average result of 4906 / the target result of 38% sample). Part 4 testing produced IC50s of 4069 mg KCl/L (or 31.5% sample) and 2098 mg KCl/L (or 16.2% sample) with and without EDTA, respectively. The spiking level for interlaboratory testing was based on an average of results from Part 2 and Part 4 testing and set at 11713 mg KCl/L (average result of 4451 / the target result of 38% sample). Referee laboratory results during interlaboratory testing yielded IC50s of 26.8% and 36.4% sample for tests conducted with and without EDTA, respectively.

D.4 Preliminary Testing for the *Mysidopsis* Chronic Test Method

D.4.1 Reference Toxicant Sample Type

The reference toxicant sample type was composed of synthetic seawater (prepared using bioassay grade Forty Fathoms® artificial sea salts added to deionized water) spiked with KCl. The spiking level for this sample was targeted to produce a test result (IC25) of 50% sample. Spiking levels for Part 2 preliminary testing were selected based on historical testing at the referee laboratory for the *Mysidopsis* chronic test method. Table D8 shows the results from preliminary testing for the *Mysidopsis* chronic test method. Part 2 preliminary testing resulted in an IC25 of 426 mg KCl/L. Based on this result, the spiking level for Part 4 testing was increased to 900 mg KCl/L (approximately 426 / the target result of 50% sample). Part 4 testing resulted in an IC25 of 530 mg KCl/L (58.9% sample). Based on this result, the spiking level for interlaboratory testing was further increased to 1200 mg KCl/L. Referee laboratory testing of the interlaboratory sample resulted in an IC25 of 36.4% sample.

D.4.2 Effluent Sample Type

The effluent sample source used for the *Mysidopsis* chronic test method was the same as described in Section D.1.2. The salinity of the effluent was adjusted to 25 ppt prior to the conduct of marine tests. Part 1 testing on the unspiked effluent resulted in an IC25 of 64.4% sample. The effluent sample was then spiked with KCl to provide a consistently toxic sample. The spiking level for this sample type was targeted to produce a test result (IC25) of 25% sample. Part 2 preliminary testing for the *Mysidopsis* chronic test method produced an IC25 of 486 mg KCl/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an IC25 of 420 mg KCl/L, which represented a 14% difference from the original Part 2 test result. Based on the result of Part 2 testing, the spiking level for Part 4 testing was increased to 1960 mg KCl/L (approximately 486 / the target result of 25% sample). Part 4 testing produced an IC25 of 521 mg KCl/L (26.6% sample). Based on an average of results from Part 2 and Part 4 testing, the spiking level for interlaboratory testing was increased to 2000 mg KCl/L (approximately the average result of 504 / the target result of 25% sample). Referee laboratory testing of the interlaboratory sample resulted in an IC25 of 29.9% sample.

Table D8. Results from *Mysidopsis* chronic preliminary testing.

Sample type	Part[a]	Concentrations tested (mg KCl/L)	Survival NOEC (mg KCl/L)	Growth NOEC (mg KCl/L)	IC25 (mg KCl/L)	IC25 (% sample)
Reference toxicant	2	45.25, 90.5, 181, 362, 724	362	362	426	58.8
	4	56.3, 113, 225, 450, 900	450	450	530	58.9
	IL	75, 150, 300, 600, 1200	300	300	437	36.4
Effluent	1[b]	6.25, 12.5, 25, 50, 100	100	50	-	64.4
	2	90, 180, 360, 720, 1440	360	360	486	33.7
	3	90, 180, 360, 720, 1440	360	360	420	29.2
	4	122.5, 245, 490, 980, 1960	490	490	521	26.6
	IL	125, 250, 500, 1000, 2000	500	500	598	29.9
Receiving water	1[b]	6.25, 12.5, 25, 50, 100	100	50	-	88
	2	90, 180, 360, 720, 1440	360	360	486	33.8
	3	90, 180, 360, 720, 1440	720	360	634	44.0
	4	122.5, 245, 490, 980, 1960	490	490	608	31.0
	IL	150, 300, 600, 1200, 2400	300	300	564	23.5

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL).
[b] Part 1 testing was conducted on unspiked samples. The units for test concentrations in Part 1 were percent sample.

D.4.3 Receiving Water Sample Type

The receiving water sample type was composed of natural seawater spiked with KCl. The referee laboratory (EA Engineering, Science and Technology, Inc.) collected receiving water from Manasquan Inlet, in Manasquan, Monmouth County, New Jersey. Seawater from this location has historically been non-toxic to the test species, and is currently used by EPA's Division of Environmental Science and Assessment as dilution water for toxicity testing. Water chemistry of this receiving water on each sample collection date is shown in Table D9.

Table D9. Water chemistry of receiving water sample source for *Mysidopsis* chronic and sheepshead acute and chronic test methods.

Parameters	Sampling date[a]			
	11/03/99	**11/23/99**	**12/29/99**	**01/13/00**
Alkalinity (mg/L as $CaCO_3$)	98	110	-	-
pH	8.0	8.2	8.3	7.5
Temperature (°C)	3.5	3.9	2.5	10.6
Total residual chlorine (mg/L)	<0.01	-	-	-
Dissolved oxygen (mg/L)	-	10.9	9.2	10.1
Salinity (ppt)	28.3	31.7	35	31.9
Copper (μg/L)	<10	-	-	-
Total ammonia (mg/L)	0.980	-	-	-
Total dissolved solids (mg/L)	33,300	-	-	-
Total suspended solids (mg/L)	10.5	-	-	-
Total organic carbon (mg/L)	<1.0	-	-	-
Biological oxygen demand (mg/L)	2.1	-	-	-
Chemical oxygen demand (mg/L)	854	-	-	-

[a] "-" indicates that the parameter was not tested on the given sampling date.

The receiving water sample was filtered and adjusted to a salinity of 25 ppt prior to toxicity testing. Part 1 testing on the unspiked receiving water sample indicated moderate toxicity (IC25 of 88% sample). The receiving water sample was then spiked with KCl to provide a consistently toxic sample. The spiking level for this sample type was targeted to produce a test result (IC25) of 25% sample. Part 2 preliminary testing for the *Mysidopsis* chronic test method produced an IC25 of 486 mg KCl/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an IC25 of 634 mg KCl/L, which represented a 30% change from the initial Part 2 test result. For Part 4 preliminary testing, the spiking level was increased to 1960 mg KCl/L (approximately 486 / the target result of 25% sample) based on Part 2 testing results. Part 4 testing resulted in an IC25 of 608 mg KCl/L. Based on this result, the spiking level was further increased to 2400 mg KCl/L (approximately 608 / the

target result of 25% sample) for the interlaboratory testing phase. Referee laboratory testing of the interlaboratory sample resulted in an IC25 of 23.5% sample.

D.5 Preliminary Testing for Sheepshead Acute and Chronic Test Methods

D.5.1 Reference Toxicant Sample Type

The reference toxicant sample type for the sheepshead acute and chronic test methods was composed of synthetic seawater (prepared using bioassay grade Forty Fathoms® artificial sea salts added to deionized water) spiked with KCl. The spiking level for this sample type was targeted to produce a test result (LC50 for the sheepshead acute test and IC25 for the sheepshead chronic test) of 50% sample. Tables D10 and D11 show the preliminary testing results for sheepshead acute and chronic test methods, respectively. Spiking levels selected for Part 2 testing resulted in an LC50 of 1580 mg KCl/L for the sheepshead acute test method. Based on this result, the spiking level for Part 4 testing was increased to 3160 mg KCl/L (1580 / the target result of 50% sample). Part 4 sheepshead acute testing produced an LC50 of 1157 mg KCl/L or 36.6% sample. The same spiking level was used for the interlaboratory testing phase. Referee laboratory testing of the interlaboratory sample yielded an LC50 of 40.6% sample.

Part 2 preliminary testing for the sheepshead chronic method produced an IC25 of 1257 mg KCl/L. Based on this result, the spiking level for Part 4 testing was increased to 2600 mg KCl/L (approximately 1257 / the target result of 50% sample). Results of this test revealed an IC25 of 1528 mg KCl/L. Based on Part 4 testing results, the spiking level for interlaboratory testing was further increased to 3000 mg KCl/L (approximately 1528 / the target result of 50% sample). Referee laboratory testing of the interlaboratory sample yielded an IC25 of 54.3% sample.

D.5.2 Effluent Sample Type

The effluent sample source used for the sheepshead acute and chronic test methods was the same as described in Section D.1.2. This effluent sample was adjusted to a salinity of 25 ppt and spiked with KCl to provide a consistently toxic sample. The spiking level for this sample type was targeted to produce a test result (LC50 for the sheepshead acute test and IC25 for the sheepshead chronic test) of 25% sample. Part 2 preliminary testing for the sheepshead acute test method produced an LC50 of 1329 mg KCl/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an LC50 of 1694 mg KCl/L, which represents a 27% change from the original Part 2 test result. For Part 4 preliminary testing, the referee laboratory increased the spiking level to 5200 mg KCl/L. This test produced an LC50 of 1172 mg KCl/L or 22.5% sample. The same spiking level was used for the interlaboratory testing phase. Referee laboratory testing of the interlaboratory sample yielded an LC50 of 35.4% sample.

Table D10. Results from sheepshead acute preliminary testing.

Sample type	Part[a]	Concentrations tested (mg KCl/L)	NOAEC (mg KCl/L)	LC50 (mg KCl/L)	LC50 (% survival)
Reference toxicant	2	155, 310, 620, 1240, 2480	1240	1580	63.7
	4	198, 395, 790, 1580, 3160	790	1157	36.6
	IL	198, 395, 790, 1580, 3160	790	1283	40.6
Effluent	2	310, 620, 1240, 2480, 4960	620	1329	26.8
	3	310, 620, 1240, 2480, 4960	1240	1694	34.2
	4	325, 650, 1300, 2600, 5200	650	1172	22.5
	IL	325, 650, 1300, 2600, 5200	1300	1840	35.4
Receiving water	2	310, 620, 1240, 2480, 4960	1240	1580	31.9
	3	310, 620, 1240, 2480, 4960	620	1488	30.0
	4	398, 795, 1590, 3180, 6360	795	1247	19.6
	IL	350, 700, 1400, 2800, 5600	700	1450	25.9

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL). Part 1 preliminary testing was conducted using only the chronic method, and Part 3 was conducted using only the acute method.

Table D11. Results from sheepshead chronic preliminary testing.

Sample type	Part[a]	Concentrations tested (mg KCl/L)	Survival NOEC (mg KCl/L)	Growth NOEC (mg KCl/L)	IC25 (mg KCl/L)	IC25 (% sample)
Reference toxicant	2	98, 195, 390, 780, 1560	780	780	1257	80.6
	4	163, 325, 650, 1300, 2600	1300	1300	1528	58.8
	IL	188, 375, 750, 1500, 3000	1500	1500	1629	54.3
Effluent	1[b]	6.25, 12.5, 25, 50, 100	100	100	-	>100
	2	250, 500, 1000, 2000, 4000	1000	500	1158	28.9
	4	275, 550, 1100, 2200, 4400	1100	1100	1276	29.0
	IL	300, 600, 1200, 2400, 4800	1200	1200	1406	29.3
Receiving water	1[b]	6.25, 12.5, 25, 50, 100	100	100	-	>100
	2	195, 390, 780, 1560, 3120	780	780	1028	33.0
	4	253, 505, 1010, 2020, 4040	1010	505	1172	29.0
	IL	275, 550, 1100, 2200, 4400	1100	1100	1210	27.5

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL). Part 1 preliminary testing was conducted using only the chronic method, and Part 3 was conducted using only the acute method.
[b] Part 1 testing was conducted on unspiked samples. The units for test concentrations in Part 1 were percent sample.

For the sheepshead chronic test method, Part 1 testing on the unspiked effluent resulted in no toxicity (IC25 > 100% sample). Spiking of the effluent sample in Part 2 testing produced an IC25 of 1158 mg KCl/L. Based on this result, the spiking level was increased to 4400 mg KCl/L for Part 4 preliminary testing (approximately 1158 / the target result of 25% sample). Part 4 testing produced an IC25 of 1276 mg KCl/L or 29.0% sample. Based on the average of results from Part 2 and Part 4 testing, the spiking level for interlaboratory testing was further increased to 4800 mg/L (approximately the average result of 1217 / the target result of 25% sample). Referee laboratory testing of the interlaboratory sample yielded an IC25 of 29.3% sample.

D.5.3 Receiving Water Sample Type

The receiving water sample source used for the sheepshead acute and chronic test methods was the same as described in Section D.4.3. The receiving water sample was filtered, adjusted to a salinity of 25 ppt, and spiked with KCl to provide a consistently toxic sample. The spiking level for this sample type was targeted to provide a test result (LC50 for the sheepshead acute test and IC25 for the sheepshead chronic test) of 25% sample. Part 2 preliminary testing for the sheepshead acute test method produced an LC50 of 1580 mg KCl/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an LC50 of 1488 mg KCl/L, which represented only a 5.8% change from the original Part 2 test result. Based on the results of Part 2 testing, the spiking level for Part 4 testing was set at 6360 mg KCl/L (approximately 1580 / the target result of 25% sample). Part 4 testing resulted in an LC50 of 1247 mg KCl/L or 19.6% sample. Based on an average of Part 2 and Part 4 preliminary testing results, the spiking level was reduced to 5600 mg KCl/L (approximately the average result of 1414 / the target result of 25% sample) for the interlaboratory testing phase. Referee laboratory testing of the interlaboratory sample yielded an LC50 of 25.9% sample.

For the sheepshead chronic test method, Part 1 testing of the unspiked receiving water revealed no toxicity (IC25 >100% sample). Part 2 testing of the spiked receiving water produced an IC25 of 1028 mg KCl/L. Based on this result, the spiking level was increased to 4040 mg KCl/L for Part 4 testing (approximately 1028 / the target result of 25% sample). Part 4 testing produced an IC25 of 1172 mg KCl/L or 29.0% sample. Based on an average of test results from Part 2 and Part 4 testing, the spiking level was set at 4400 mg KCl/L (the average result of 1100 / the target result of 25% sample) for the interlaboratory testing phase. Referee laboratory testing of the interlaboratory sample yielded an IC25 of 27.5% sample.

D.6 Preliminary Testing for Silverside Acute and Chronic Test Methods

D.6.1 Reference Toxicant Sample Type

The reference toxicant sample type for the silverside acute and chronic test methods was composed of synthetic seawater (prepared using bioassay grade Forty Fathoms® artificial sea salts added to deionized water) spiked with copper sulfate ($CuSO_4$). The spiking level for this sample type was targeted to produce a test result (LC50 for the silverside acute test and IC25 for the silverside chronic test) of 50% sample. Tables D12 and D13 show the results of preliminary testing for silverside acute and chronic test methods, respectively. Spiking levels selected for Part 2 testing resulted in an LC50 of >0.25 mg Cu/L for the silverside acute test method. Based on this result, the spiking level for Part 4 testing was increased to 1 mg Cu/L. Part 4 sheepshead acute testing produced an LC50 of 0.29 mg Cu/L or 29% sample. The same spiking level was used during the interlaboratory testing phase; however, test results on the interlaboratory sample were >100% sample due to an error in the preparation of this sample type (see Section 5.3 in the main body of this report).

Part 2 preliminary testing for the silverside chronic method produced an IC25 of >0.3 mg Cu/L. Based on this result, the spiking level for Part 4 testing was increased to 1 mg Cu/L. Part 4 testing produced an IC25 of 0.189 mg Cu/L or 18.9% sample. The same spiking level was used during the interlaboratory testing phase, and referee laboratory testing of this sample yielded an IC25 of 15.2% sample.

Table D12. Results from silverside acute preliminary testing.

Sample type	Part[a]	Concentrations tested (mg Cu/L)	LC50 (mg Cu/L)	LC50 (% sample)
Reference toxicant	2	0.016, 0.031, 0.063, 0.125, 0.25	>0.25	>100
	4	0.063, 0.125, 0.25, 0.5, 1.000	0.29	29.0
	IL	0.063, 0.125, 0.25, 0.5, 1.000	>1	>100
Effluent	2	0.031, 0.063, 0.125, 0.25, 0.5	0.234	46.9
	3	0.031, 0.063, 0.125, 0.25, 0.5	0.347	69.5
	4	0.058, 0.115, 0.231, 0.461, 0.922	0.171	18.5
	IL	0.058, 0.115, 0.231, 0.461, 0.922	0.296	32.1
Receiving water	2	0.031, 0.063, 0.125, 0.25, 0.5	0.141	28.2
	3	0.031, 0.063, 0.125, 0.25, 0.5	0.146	29.2
	4	0.035, 0.071, 0.141, 0.283, 0.565	0.154	27.3
	IL	0.035, 0.071, 0.141, 0.283, 0.565	0.276	48.9

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL). Part 1 preliminary testing was conducted using only the chronic method, and Part 3 was conducted using only the acute method.

Table D13. Results from silverside chronic preliminary testing.

Sample type	Part[a]	Concentrations tested (mg Cu/L)	Survival NOEC (mg Cu/L)	Growth NOEC (mg Cu/L)	IC25 (mg Cu/L)	IC25 (% sample)
Reference toxicant	2	0.019, 0.038, 0.075, 0.15, 0.3	0.15	0.15	>0.3	>100
	4	0.063, 0.125, 0.25, 0.5, 1.0	0.125	0.125	0.189	18.9
	IL	0.063, 0.125, 0.25, 0.5, 1.0	0.125	0.125	0.152	15.2
Effluent	1[b]	6.25, 12.5, 25, 50, 100	50	25	-	43.9
	2	0.038, 0.075, 0.15, 0.3, 0.6	0.15	0.15	0.227	37.8
	4	0.075, 0.15, 0.3, 0.6, 1.2	0.15	0.15	0.171	14.3
	IL	0.05, 0.1, 0.2, 0.4, 0.8	0.1	0.1	0.23	28.8
Receiving water	1[b]	6.25, 12.5, 25, 50, 100	100	100	-	>100
	2	0.038, 0.075, 0.15, 0.3, 0.6	0.75	0.75	0.155	25.9
	4	0.031, 0.062, 0.124, 0.247, 0.494	0.124	0.124	0.149	30.2
	IL	0.031, 0.062, 0.124, 0.247, 0.494	0.062	0.062	0.103	20.9

[a] Preliminary testing Parts 1-4 and referee laboratory testing during the interlaboratory testing phase (IL). Part 1 preliminary testing was conducted using only the chronic method, and Part 3 was conducted using only the acute method.
[b] Part 1 testing was conducted on unspiked samples. The units for test concentrations in Part 1 were percent sample.

D.6.2 Effluent Sample Type

The effluent sample type used for the silverside acute and chronic test methods was composed of an industrial wastewater effluent spiked with $CuSO_4$. The referee laboratory (Ogden Environmental and Energy Services, Inc.) collected the effluent from an industrial wastewater treatment facility designed to treat oil refinery waste. This effluent source was selected based on historical consistency in chemical and toxicological testing conducted by the referee laboratory. Water chemistry from the effluent source is listed in Table D14.

The effluent sample was adjusted to a salinity of 25 ppt and then spiked with $CuSO_4$ to provide a consistently toxic sample that was appropriate for the silverside acute and chronic test method. The spiking level for this sample type was targeted to produce a test result (LC50 for the silverside acute test and IC25 for the silverside chronic test) of 25% sample. Part 2 preliminary testing for the silverside acute test method produced an LC50 of 0.234 mg Cu/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an LC50 of 0.347 mg Cu/L, which represents a 48% change from the original Part 2 test result. Based on Part 2 results, the referee laboratory increased the spiking level in Part 4 testing to 0.922 mg Cu/L (approximately 0.234 / the target result of 25% sample). Part 4 testing resulted in an LC50 of 0.171 mg Cu/L or 18.5% sample. The same

spiking level was used during the interlaboratory testing phase, and referee laboratory testing of this sample yielded an LC50 of 32.1% sample.

For the silverside chronic test method, Part 1 testing of the unspiked effluent resulted in an IC25 of 43.9% sample. Spiking of the effluent sample in Part 2 testing produced an IC25 of 0.227 mg Cu/L. Based on this result, the spiking level was increased to 1.2 mg Cu/L for Part 4 testing. Part 4 testing produced an IC25 of 0.171 mg Cu/L or 14.3% sample. Based on an average of Part 2 and Part 4 testing results, the spiking level for interlaboratory testing was set at 0.8 mg Cu/L (approximately the average result of 0.199 / the target result of 25% sample). Referee laboratory testing of the interlaboratory sample produced an IC25 of 28.8% sample.

Table D14. Water chemistry of effluent sample source for silverside acute and chronic test methods.

Parameters	Sampling date[a]				
	08/02/99	**08/23/99**	**08/26/99**	**08/28/99**	**09/17/99**
Alkalinity (mg/L as $CaCO_3$)	217	202	214	208	182
Hardness (mg/L as $CaCO_3$)	287	289	282	288	292
Conductivity (μS/cm)	1457	1424	1476	1486	1320
pH	7.37	7.08	7.31	7.35	7.16
Temperature (°C)	16.7	23.5	18.5	22.0	19.2
Total residual chlorine (mg/L)	0.02	0.03	0.02	0.02	<0.01
Dissolved oxygen (mg/L)	7.9	4.2	3.9	8.9	8.7
Salinity (ppt)	0	0	0	0	0
Copper (μg/L)	22	-	-	-	-
Total ammonia (mg/L)	14.0	-	-	-	-
Total dissolved solids (mg/L)	975	-	-	-	-
Total suspended solids (mg/L)	4	-	-	-	-
Total organic carbon (mg/L)	13.2	-	-	-	-
Biological oxygen demand (mg/L)	15	-	-	-	-
Chemical oxygen demand (mg/L)	41	-	-	-	-

[a] "-" indicates that the parameter was not tested on the given sampling date.

D.6.3 Receiving Water Sample Type

The receiving water sample type used for the silverside acute and chronic test methods was composed of a natural seawater spiked with $CuSO_4$. The referee laboratory (Ogden Environmental and Energy Services, Inc.) collected natural seawater from the Scripps Institution of Oceanography (Scripps) seawater system in La Jolla, CA. Scripps pumps seawater from a fixed collection site 320 m offshore of La Jolla, CA,

filters the seawater through a sand filter, and incorporates the seawater into a flow-through system for use in supplying aquariums housed at Scripps. The referee laboratory routinely uses natural seawater from Scripps' seawater system for in-house organism culturing and dilution water. The referee laboratory transported water from Scripps to the laboratory where it was incorporated into the laboratory's flow-through natural seawater system that includes two 2,200-gallon storage tanks, an in-line 20-µm filter, and an in-line heater/chiller unit. Table D15 shows the water chemistry of the receiving water sample collected for preliminary testing. Prior to testing, receiving water was filtered through a 0.2-µm filter and adjusted to a salinity of 25 ppt with the addition of deionized water.

Table D15. Water chemistry of the receiving water sample source for silverside acute and chronic test methods.

Parameters	Sampling date
	08/02/99
Alkalinity (mg/L as $CaCO_3$)	75
Hardness (mg/L as $CaCO_3$)	>2000
Conductivity (μS/cm)	53,100
pH	8.08
Temperature (°C)	9.9
Total residual Chlorine (mg/L)	<0.01
Dissolved oxygen (mg/L)	7.2
Salinity (ppt)	34
Copper (μg/L)	5.4
Total ammonia (mg/L)	<0.1
Total dissolved solids (mg/L)	28,000
Total suspended solids (mg/L)	<1.0
Total organic carbon (mg/L)	<0.5
Biological oxygen demand (mg/L)	2
Chemical oxygen demand (mg/L)	26

The receiving water sample was then spiked with $CuSO_4$ to provide a consistently toxic sample. The spiking level for this sample type was targeted to produce a test result (LC50 for the silverside acute test and IC25 for the silverside chronic) of 25% sample. Part 2 preliminary testing for the silverside acute test method produced an LC50 of 0.141 mg Cu/L. This sample was held for 7 days at 4°C and then retested

for Part 3 preliminary testing. After holding, the sample produced an LC50 of 0.146 mg Cu/L, which represented only a 3.5% change from the initial Part 2 test result. Based on Part 2 testing results, the spiking level for Part 4 preliminary testing was increased to 0.565 mg Cu/L(approximately 0.141 / the target result of 25% sample). Part 4 testing produced an LC50 of 0.154 mg Cu/L or 27.3% sample. The same spiking level was used during the interlaboratory testing phase, and referee laboratory testing of this sample yielded an LC50 of 48.9% sample.

For the silverside chronic test method, Part 1 testing of the unspiked effluent resulted in no toxicity (IC25 > 100% sample). Spiking of the receiving water sample in Part 2 testing produced an IC25 of 0.155 mg Cu/L. Based on this result, the spiking level was decreased slightly to 0.494 mg Cu/L for Part 4 testing. Part 4 testing produced an IC25 of 0.149 mg Cu/L or 30.2% sample. The same spiking level was used during the interlaboratory testing phase, and referee laboratory testing of this sample yielded an IC25 of 20.9% sample.

D.7 Preliminary Testing for the *Champia* Chronic Test Method

The referee laboratory supporting the *Champia* chronic test method (EnviroSystems, Inc.) originally was instructed to conduct preliminary testing as described in Section 4 (in the main body of this report). On January 28, 2000, interlaboratory testing for the *Champia* chronic test method was canceled due to a lack of participant laboratory support (see Section 2.1 in the main body of this report). With this cancellation, the objectives of any uncompleted preliminary tests were adjusted to better direct the use of preliminary test data toward single-laboratory testing rather than preparation for interlaboratory testing. As a result, preliminary testing occurred during two time periods; testing from July to September 1999 was conducted in preparation for interlaboratory testing, and testing from March to May 2000 was conducted to provide additional single-laboratory data for the *Champia* chronic test method.

D.7.1 Reference Toxicant Sample Type

The reference toxicant sample type was composed of natural seawater spiked with $CuSO_4$. Since natural seawater is recommended for the *Champia* chronic test method, the same natural seawater source was used as the matrix for the reference toxicant sample, dilution water in all tests, and the receiving water sample matrix. This natural seawater source is described in more detail in Section D.7.3. For use as the reference toxicant sample matrix and dilution water, the natural seawater was filtered through a 0.45-µm membrane filter and steam sterilized at 150°C for 30 minutes. Seawater used for the receiving water sample matrix was unfiltered and unsterilized.

Table D16 shows the results from preliminary testing for the *Champia* chronic test method. Spiking levels selected for Part 2 testing of the reference toxicant sample type resulted in an IC25 of 0.155 μg Cu/L. Part 4 preliminary testing using the same spiking levels produced an IC25 of 0.265 μg Cu/L. When the reference toxicant sample was retested in the spring of 2000, this sample type resulted in an

IC25 of 0.263 μg Cu/L. For the three tests conducted on the reference toxicant sample type, a mean IC25 of 0.228 μg Cu/L and a CV of 27.6% was calculated.

Table D16. Results from *Champia* chronic preliminary testing.

Sample type	Part[a]	Test date	Sample description	Units	Concentrations tested	NOEC (units)	IC25 (units)
Reference toxicant	2	7/27/99	filtered, sterilized natural seawater spiked with Cu	μg Cu/L	0.5, 1.0, 5, 10, 15	<0.5	0.155
	4	8/17/99		μg Cu/L	0.5, 1, 5, 10, 15	<0.5	0.265
	A	5/16/00		μg Cu/L	0.15, 0.5, 1, 5, 10	0.15	0.263
Effluent	1	7/28/99	unspiked municipal effluent adjusted to salinity of 30ppt	percent	0.2, 0.7, 2.0, 7.0, 10.0	<0.2	0.172
	1	8/3/99		percent	0.156, 0.312, 0.625, 1.25, 2.5, 5, 10	0.156	0.240
	1	8/10/99		percent	0.156, 0.312, 0.625, 1.25, 2.5, 5, 10	<0.156	0.119
	3	8/4/99		percent	0.2, 0.7, 2.0, 7.0, 10.0	<0.2	0.162
	4	9/14/99		percent	0.05, 0.1, 0.2, 0.7, 2, 7, 10	0.050	0.064
	A	5/9/00		percent	0.05, 0.1, 0.2, 0.7, 2, 7, 10	2.0	0.407
	A	5/9/00		percent	0.05, 0.1, 0.2, 0.7, 2, 7, 10	0.70	0.852
Receiving water	1	7/28/99	unspiked natural seawater (unfiltered and unsterilized)	percent	100[b]	NA	NA
	2	7/27/99	spiked natural seawater (unfiltered and unsterilized)	μg Cu/L	0.625, 1.25, 2.5, 5, 10, 20	0.625	0.699
	3	8/3/99		μg Cu/L	0.625, 1.25, 2.5, 5, 10, 20	<0.625	0.438
	4	8/18/99		μg Cu/L	0.625, 1.25, 2.5, 5, 10, 20, 40	<0.625	0.866
	A	5/31/00		μg Cu/L	0.15, 0.5, 1, 5, 10	1.0	1.45
	A	5/23/00	unspiked natural seawater (unfiltered and unsterilized)	percent	6.25, 12.5, 25, 50, 100	6.25	7.53[c]
	A	5/23/00		percent	6.25, 12.5, 25, 50, 100	100	90.4

[a] Preliminary testing Parts 1-4 and additional testing (A) requested following cancellation of interlaboratory testing.

[b] Tested as a single concentration (100%) receiving water. No toxicity was indicated.

[c] Based on test review and guidance on evaluating concentration-response relationships (USEPA, 2000a), this test result was determined to be inconclusive.

D.7.2 Effluent Sample Type

The effluent sample type used for the *Champia* chronic test method was composed of a municipal wastewater treatment plant effluent. This effluent source was selected based on historical testing by the referee laboratory that demonstrated relatively consistent levels of toxicity. No spiking of this effluent was necessary; the unspiked effluent sample produced IC25 values in the range of 0.064% to 0.852% sample. All tests were performed on unspiked effluent adjusted to a salinity of 30 ppt. Water chemistry of the effluent on each sample collection date is shown in Table D17.

Table D17. Water chemistry of the effluent sample source for the *Champia* chronic test method.

Parameters	Sampling date[a]					
	07/28/99	08/03/99	08/04/99[b]	08/10/99	09/14/99	05/09/00
Salinity (ppt)	3	1	1	2	5	1
Conductivity (μS/cm)	2250	2740	2300	2550	2980	2156
pH	7.56	6.93	7.67	7.24	6.99	6.94
Total residual chlorine (mg/L)	0.15	0.22	<0.05	0.17	0.38	0.84
Total ammonia (mg/L)	22.6	26.0	-	15.8	14.4	-

[a] "-" indicates that the parameter was not tested on the given sampling date.

[b] Sample collected 07/28/99 and held for 7 days at 4°C prior to testing.

Part 1 preliminary testing confirmed that the effluent was relatively consistent in toxicity to *Champia parvula*. Results of three separate effluent samples collected on three separate days ranged from 0.119% sample to 0.240% sample with a mean of 0.177% sample and a CV of 34.3%. The effluent sample collected on 7/28/99 was held for 7 days at 4°C and tested on 8/4/99 for Part 3 preliminary testing. This test resulted in an IC25 of 0.162% sample, which represents a 5.8% change from the initial test conducted on that sample. The effluent was tested again on 9/14/99 for Part 4 testing, and resulted in an IC25 of 0.064% sample. In the spring of 2000, the referee laboratory conducted duplicate testing of the effluent. The resulting IC25s were 0.407% and 0.852% effluent sample, yielding a CV of 50.0% for the duplicate samples.

D.7.3 Receiving Water Sample Type

The receiving water sample type used for the *Champia* chronic test method was composed of a natural seawater spiked with $CuSO_4$. The receiving water was collected from Rye Harbor, Rye, New Hampshire. The harbor provides anchorage for small pleasure craft and a limited number of small commercial fishing vessels. The harbor receives no direct discharges of treated or untreated wastewater. The water in the harbor is classified as SA-1 and has been used by the referee laboratory since 1991 to maintain *Champia parvula* cultures. Receiving water was collected from a boat offshore and away from other boat traffic or potential contamination. The physical and chemical characteristics of the receiving water are listed in Table D18 for each sample collection date. The same water source was used for the reference toxicant sample matrix and for dilution water in all tests; however, water was filtered and sterilized for these uses. The receiving water sample type was tested without filtration or sterilization.

Table D18. Water chemistry of the receiving water sample source for the *Champia* chronic test method.

Parameters	Sampling date[a]								
	07/28/99	**08/03/99**	**08/04/99**	**08/10/99**	**09/14/99**	**05/16/00**	**05/23/00**	**05/23/00**	**05/31/00**
Salinity (ppt)	33	33	33	33	32	32	33	33	33
Conductivity (μS/cm)	48100	48600	49100	49300	45400	42500	42700	43000	43010
pH	7.92	8.29	8.54	8.04	7.70	7.89	8.00	8.07	8.01
Total residual chlorine (mg/L)	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Total ammonia (mg/L)	<0.10	<0.10	<0.10	<0.10	0.22	-	-	-	-

[a] "-" indicates that the parameter was not tested on the given sampling date.

Part 1 preliminary testing initially was conducted using a single concentration (100%) test. Results from this test showed no toxicity in the receiving water. In the spring of 2000, the referee laboratory conducted duplicate testing of the unspiked receiving water. IC25 results from a split sample collected on 5/23/00 were 7.53% and 90.4% sample. This represents high variability between duplicate samples; however, data review revealed that results from the first test may not be reliable. This test produced an interrupted concentration-response curve, with statistically significant effects at the 12.5% and 100% test concentrations but not at the 25% and 50% test concentrations. Based on EPA guidance for evaluating concentration-response relationships (USEPA, 2000a), this test should be considered inconclusive if the PMSD is above recommended bounds (USEPA, 2000d). Unfortunately, upper PMSD bounds have not yet been recommended for the *Champia* chronic method. In this test, the PMSD was 47%, which is higher than the recommended PMSD upper bound for other chronic methods (23 - 37%) and higher than the average PMSD (31%) for other *Champia* chronic tests conducted during preliminary testing. Also, the average control response in this test (10.8 cystocarps per plant) was barely above the test acceptability criteria (10 cystocarps per plant) for the method and was well below the average control response (17.5 cystocarps per plant) in other *Champia* chronic tests conducted during preliminary testing. Based on test review and guidance on evaluating concentration-response relationships (USEPA, 2000a), this test was determined to be inconclusive.

In Part 2 preliminary testing, the receiving water sample was spiked with $CuSO_4$ to provide a consistently toxic sample. Part 2 testing for the *Champia* chronic method produced an IC25 of 0.699 μg $CuSO_4$/L. This sample was held for 7 days at 4°C and then retested for Part 3 preliminary testing. After holding, the sample produced an IC25 of 0.438 μg $CuSO_4$/L, which represents a 37% change from the original Part 2 test result. Part 4 testing resulted in an IC25 of 0.866 μg $CuSO_4$/L. An additional test of the spiked receiving water in the Spring of 2000 produced an IC25 of 1.45 μg $CuSO_4$/L. For the four tests

conducted on the spiked receiving water sample type, a mean IC25 of 0.863 μg Cu/L and a CV of 49.7% was calculated.

D.8 Preliminary Testing for the *Holmesimysis* Acute Test Method

The referee laboratory supporting the *Holmesimysis* acute test method (MEC Analytical, Inc.) was originally instructed to conduct preliminary testing according to Section 4 (in the main body of this report). Due to difficulties encountered in obtaining test organisms, a limited number of preliminary tests were conducted by the referee laboratory from July to September 1999. On January 28, 2000, interlaboratory testing for the *Holmesimysis* acute test method was canceled due to a lack of participant laboratory support (see Section 2.1 in the main body of this report). With this cancellation, the objectives of any uncompleted preliminary tests were adjusted to better direct the use of preliminary test data toward single-laboratory testing rather than preparation for interlaboratory testing. The referee laboratory attempted to complete preliminary testing from April through June 2000; however, the laboratory was unable to obtain test organisms during this period. On June 29, 2000, any further preliminary testing at the referee laboratory was canceled due to the unavailability of test organisms.

In all, only five preliminary *Holmesimysis* acute tests were performed (Table D19); one test was conducted on receiving water, and two tests were conducted on each of two seawater effluent sources. Of the five tests conducted, only two met test acceptability criteria for survival, and these two tests were not conducted according to the WET method manual test requirement for test organism age. Neonates for these tests were collected directly from the field rather than hatched in the laboratory from field-collected adults. For this reason, exact ages of neonates used for testing could not be determined (see Section D.9).

Table D19. Results from *Holmesimysis* acute preliminary testing.

Sample type	Test date	Control survival (%)	NOAEC (% sample)	LC50 (% sample)
Effluent 1	7/21/99	80[a]	12.5	25.3
Effluent 1	7/27/99	80[a]	12.5	15.3
Effluent 2	8/31/99[b]	92.5	25	35.1
Effluent 2	9/7/99[b]	90	12.5	16.6
Receiving water	7/21/99	80[a]	100	>100

[a] Failed to meet test acceptability criteria of ≥90% control survival.

[b] Tests were conducted on field collected neonates.

D.8.1 Effluent Sample Type

Two seawater effluent sources were tested and considered for use as the effluent sample source. Both effluent sources were from municipal wastewater treatment facilities, and water chemistry for both sources is listed in Table D20. Two *Holmesimysis* acute tests were conducted on effluent 1; these tests produced LC50s of 23.9% and 12.4% sample (Table D19). Control survival in each of these tests was only 80%, which fails to meet the test acceptability criteria for the method. Synthetic seawater (prepared using bioassay grade Forty Fathoms® artificial sea salts added to deionized water) was used for dilution in these tests rather than natural seawater as stated in the SOP. The dilution water may have been a factor in the poor control survival. Due to difficulties in obtaining test organisms, tests conducted on effluent 2 used neonates directly collected from the field, rather than neonates hatched in the laboratory from field collected adults. These two tests resulted in LC50s of 28.2% and 12.6% sample.

Table D20. Water chemistry of the effluent sample sources for the *Holmesimysis* acute test method.

Parameters	Effluent 1 Sampling date[a]		Effluent 2 Sampling date[a]	
	07/21/99	07/26/99	08/30/99	09/07/99
Alkalinity (mg/L as $CaCO_3$)	-	250	-	-
Hardness (mg/L as $CaCO_3$)	-	180	-	-
pH	7.28	7.26	7.09	7.88
Temperature (°C)	19.2	18.0	23.3	24.0
Total residual chlorine (mg/L)	0.07	0.07	-	0.09
Dissolved oxygen (mg/L)	3.3	6.0	6.2	6.9
Salinity (ppt)	0.6	0.5	0	0
Copper (μg/L)	-	10	-	-
Total ammonia (mg/L)	22.3	18.9	18.7	14.7
Total dissolved solids (mg/L)	-	562	-	-
Total suspended solids (mg/L)	-	13.5	-	-
Biological oxygen demand (mg/L)	-	32.2	-	-
Chemical oxygen demand (mg/L)	-	98	-	-

[a] "-" indicates that the parameter was not tested on the given sampling date.

D.8.2 Receiving Water Sample Type

The receiving water sample type used for the *Holmesimysis* acute test method consisted of natural seawater collected from San Francisco Bay off of Point Chauncey on the Tiburon Peninsula. Samples were collected away from direct discharges of treated or untreated wastewater. Water from this source has been used by the referee laboratory as dilution water for several years without exhibiting toxicity. The physical and chemical characteristics of the receiving water collected from the bay are listed in Table D21. A single Part 1 preliminary test was conducted on the receiving water resulting in an LC50 of >100% sample. This test failed to meet test acceptability criteria due to control survival of 80%. As mentioned above, use of synthetic seawater for dilution may have contributed to low control survival.

Table D21. Water chemistry of the receiving water sample source for the *Holmesimysis* acute test method.

Parameters	Sampling date
	07/20/99
Alkalinity (mg/L as $CaCO_3$)	122[a]
Hardness (mg/L as $CaCO_3$)	4558[a]
pH	7.85
Temperature (°C)	17.9
Total residual chlorine (mg/L)	0.03
Dissolved oxygen (mg/L)	7.6
Salinity (ppt)	24
Copper (μg/L)	ND[a]
Total ammonia (mg/L)	<0.10
Total dissolved solids (mg/L)	27,200[a]
Total suspended solids (mg/L)	1.25[a]
Biological oxygen demand (mg/L)	ND[a]
Chemical oxygen demand (mg/L)	1930[a]

[a] Analyses were conducted on the sample collected 7/27/99, but this sample was not used for *Holmesimysis* testing due to insufficient test organisms. ND = not detected.

D.9 Problems Encountered in Preliminary Testing

For the *Selenastrum* chronic test method, spiking levels were originally targeted to produce an IC25 of 50% for the reference toxicant sample type and 25% for the effluent and receiving water sample type. In Part 2 preliminary testing, IC25 values in repeated tests were variable and the referee laboratory had difficulty isolating the targeted spiking level. The referee laboratory observed that IC50 values were less variable than IC25 values in repeated tests. For this reason, the target spiking levels were based on IC50 values rather than IC25 values. Target spiking levels were set to produce an IC50 of 38% for reference toxicant, effluent, and receiving water sample types. The 38% level was selected in an attempt to allow IC25 and IC50 results from interlaboratory testing to fall within the test concentration range.

For the *Mysidopsis* chronic and sheepshead acute and chronic test methods, $CuSO_4$ was originally selected as the spiking agent. During preliminary testing for these methods, results on spiked samples were highly variable. Upon further investigation, the referee laboratory determined that this variability was due to precipitation of copper in the spiked seawater samples. A combination of factors including spiking concentrations, salinity of the sample, pH, other dissolved ions in the sample matrix, and storage of the spiked sample at <4°C contributed to the precipitation of copper in spiked samples. Due to this precipitation, the spiking agent for these marine methods was changed to KCl. The referee laboratory had experience in the use of KCl as the spiking agent for freshwater methods in the WET Variability Study and had experience in the use of KCl as a reference toxicant for these marine methods. The same problem was encountered for the silverside acute reference toxicant sample (see Section 5.3 in the main body of this report), but the problem was not identified in time to change the spiking agent. Precipitation of copper did not appear to affect the other sample types for the silverside acute and chronic test methods, possibly due to the lower spiking concentrations used for these methods.

For the *Holmesimysis* acute test method, problems were encountered in obtaining test organisms. Organisms for this test method are generally field collected from kelp beds off the coast of California, but they are not present in sufficient numbers during the winter months. From April through June 2000, the referee laboratory attempted to collect organisms to complete preliminary testing, but *Holmesimysis costata* populations were still not at sufficient densities at potential sites in San Diego or Santa Cruz, CA. Even when field-collected adult *Holmesimysis costata* were available (July through September 1999), obtaining sufficient neonates within the required age range was difficult. Field-collected gravid females were held in the laboratory and culled daily to obtain neonates within a 24-hour age range. This required maintaining a large number of gravid females to produce the necessary neonates. In addition, survival of newly hatched neonates was poor, which added to difficulties in obtaining a sufficient number of test organisms. To avoid these difficulties, the referee laboratory collected neonates directly from the field for use in two preliminary tests. The smallest of collected organisms were used in these tests. This technique of obtaining test organisms did not allow an exact determination of the age of test organisms, so the age of test organisms could have been outside of the required range.

A second problem encountered in *Holmesimysis* acute preliminary testing was poor survival of neonates in test controls. Three of the five preliminary tests conducted failed to meet the test acceptability criteria of 90% survival. It is believed that poor control survival in these tests was due to the use of a synthetic seawater rather than a natural seawater for organism holding and test dilution.

Appendix E:

Analysis of Percent Minimum Significant Differences

The percent minimum significant difference (PMSD) is a measure of within-test variability and test sensitivity. The PMSD for a given WET test can be defined as the smallest percentage difference between the control and a treatment (an effluent dilution) that could be declared as statistically significant. As test variability increases, the ability of a test to detect small toxic effects diminishes and the test becomes a less sensitive measure of toxicity. Appendix C of the WET method manuals (USEPA, 1994a; USEPA, 1994b) describes the calculation of the minimum significant difference (MSD) as:

$$MSD = d \times S_w \sqrt{(1/n_1) + (1/n)}$$

where, d = critical value for the Dunnett's procedure
S_w = the square root of the within mean square
n = the number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations
n_1 = number of replicates in the control

The PMSD is the MSD expressed as a percentage of the control response (i.e., PMSD = MSD/control mean * 100).

In June 2000, EPA published guidance on WET test variability that recommended placing upper and lower bounds on the PMSD to control variability and ensure a specified range of test sensitivity (the WET Variability Guidance Document; USEPA, 2000d). Based on this guidance, tests for which the PMSD exceeds an upper bound would not be acceptable, if the test leads to a decision that there is no significant toxicity at the concentration identified in the permit as a limit ("Instream Waste Concentration" (IWC) or "Receiving Water Concentration"). This guidance also applies lower PMSD bounds for the purpose of determining the no observed effect concentration (NOEC). The purpose of the lower PMSD bound is to avoid declaring as "significant" toxic effects that are smaller than can generally and routinely be detected by the method as currently conducted by qualified laboratories.

To derive recommended PMSD bounds for the WET Variability Guidance Document, EPA compiled and analyzed a database of more than 1800 reference toxicant tests conducted for 23 different methods between 1988 and 1999 in 75 laboratories. EPA derived the lower and upper bounds as the 10th and 90th percentiles, respectively, of PMSDs from this reference toxicant test database.

This appendix reports PMSD values calculated for short-term chronic tests in the WET Variability Study, and compares the distribution of those values to the PMSD distributions and bounds derived in the WET Variability Guidance Document (USEPA, 2000d). While the WET Variability Study results contain fewer tests than the database analyzed for the guidance document, the number of laboratories contained in each database is comparable. The WET Variability Study database contained only 25% to 67% (depending on the method) of the number of tests contained in the guidance document database but 63% to 142% (depending on the method) of the number of laboratories included in the guidance document database. For four of the six chronic methods, the WET Variability Study database contained a larger

number of laboratories. Also, while the guidance document database contains only data from reference toxicant tests, the WET Variability Study database contains results from the analysis of blank, reference toxicant, effluent, and receiving water samples.

The percentiles of PMSD values calculated in the WET Variability Study for chronic test methods are displayed in Table E1. This data represents the PMSDs calculated for valid tests (i.e., those that met the test acceptability criteria). This data is from 28 to 100 tests per method and 7 to 32 laboratories per method. In the WET Variability Study, median PMSD values ranged from 12% to 23% for the various methods. The median PMSD values from the WET Variability Study were also very similar to the median PMSD values in the WET Variability Guidance Document. Median PMSD values from both databases were within 2% of each other for the *Ceriodaphnia* chronic (23% in both databases), *Mysidopsis* chronic (18% in the WET Variability Study database and 20% in EPA's reference toxicant database), sheepshead chronic (12% in the WET Variability Study database and 13% in EPA's reference toxicant database), and silverside chronic (19% in the WET Variability Study database and 18% in EPA's reference toxicant database) test methods. The median PMSD for the fathead chronic test was 4% lower in the WET Variability Study (16%) than the reference toxicant database (20%); and the median PMSD for the *Selenastrum* chronic test was 3% higher in the WET Variability Study (17%) than the reference toxicant database (14%). The PMSDs for the fathead chronic test may have been lower in the WET Variability Study because all tests used four replicates, while some tests in the reference toxicant database used only three replicates. The PMSDs for the *Selenastrum* chronic test may have been higher in the WET Variability Study due to the inclusion of tests conducted both with and without EDTA. PMSD values for this method are more similar when only tests conducted with EDTA in the WET Variability Study (median of 15%) are compared to the reference toxicant database (median of 14%).

While median PMSD values were very similar between the two databases, more variability was exhibited in the tails of the distributions. This is not unexpected, given the differences in the size of each database. Table E2 compares the 10^{th} and 90^{th} percentile PMSDs between the WET Variability Study and the WET Variability Guidance Document. PMSD 10^{th} percentiles from the two databases differed by less than 3% for all test methods, and PMSD 90^{th} percentiles differed by 5 to 10%. PMSD 90^{th} percentiles were higher in the WET Variability Study than the guidance document for the *Ceriodaphnia* chronic, *Selenastrum* chronic, and *Mysidopsis* chronic test methods; but were lower for the fathead chronic, sheepshead chronic, and silverside chronic test methods.

Table E3 shows the number of tests in the WET Variability Study that had PMSD values outside of the lower or upper PMSD bounds recommended in the WET Variability Guidance Document (USEPA, 2000d). From 2 to 15% of tests had PMSDs below the recommended lower bound, and from 0 to 31% of tests had PMSDs above the upper bound. These upper and lower PMSD bounds were not used to exclude or modify test results in the WET Variability Study. Based on the guidance (USEPA, 2000d), decisions regarding the validity of test results exceeding the upper PMSD bounds are dependent upon the permit

IWC concentration. Because IWC concentrations were not established or applicable to an interlaboratory variability study, determinations of test validity were not made based on PMSD bounds.

Table E1. Percentiles of PMSD for sublethal endpoints of chronic WET methods evaluated in the WET Variability Study.

Parameter		Test Method							
		***Ceriodaphnia* chronic**	**Fathead chronic**	***Selenastrum* chronic[a]**	***Selenastrum* chronic (EDTA)**	***Selenastrum* chronic (w/o EDTA)**	***Mysidopsis* chronic**	**Sheepshead chronic**	**Silverside chronic**
No. of tests[b]		100	99	57	28	29	43	28	40
No. of labs		32	27	11	8	9	11	7	10
Endpoint		reproduction	growth	growth	growth	growth	growth	growth	growth
PMSD Percentile	5%	10	10	8.2	8.0	9.1	11	7.0	11
	10%	13	12	9.5	9.1	10	11	7.3	11
	15%	14	13	10	10	10	12	8.1	12
	20%	16	13	11	11	11	15	9.1	13
	25%	17	14	12	11	12	15	9.4	14
	50%	23	16	17	15	18	18	12	19
	75%	34	21	23	21	26	26	15	23
	80%	35	23	27	22	30	29	16	25
	85%	39	25	30	27	33	31	16	26
	90%	47	30	32	29	38	37	17	28
	95%	53	34	41	31	52	61	19	31

[a] Results of tests conducted with EDTA and without EDTA are combined.
[b] Number of valid tests. Tests failing to meet test acceptability criteria were excluded from analysis.

Table E2. Comparison of PMSD percentiles observed in the WET Variability Study and those reported in the WET Variability Guidance Document (USEPA, 2000d).

Test method	WET Variability Guidance Document		WET Variability Study	
	PMSD 10th percentile	PMSD 90th percentile	PMSD 10th percentile	PMSD 90th percentile
Ceriodaphnia chronic	11	37	13	47
Fathead chronic	9.4	35	12	30
Selenastrum chronic	9.3	23	9.5	32
Mysidopsis chronic	12	32	11	37
Sheepshead chronic	6.3	23	7.3	17
Silverside chronic	12	35	11	28

Table E3. Percentage of tests in the WET Variability Study with calculated PMSDs outside of recommended bounds (USEPA, 2000d).

Test Method	Total no. of tests[a]	Below lower PMSD bound		Above upper PMSD bound	
		No. of tests	% of tests	No. of tests	% of tests
Ceriodaphnia chronic	100	6	6.0	18	18
Fathead chronic	99	2	2.0	4	4.0
Selenastrum chronic	57	5	8.8	14	25
Selenastrum chronic (EDTA)	28	3	11	5	18
Selenastrum chronic (w/o EDTA)	29	2	6.9	9	31
Mysidopsis chronic	43	7	16	6	14
Sheepshead chronic	28	1	3.6	0	0.0
Silverside chronic	40	6	15	2	5.0

[a] Number of valid tests. Tests failing to meet test acceptability criteria were excluded from analysis.

Appendix F:

Method Performance Including Referee Laboratory Data

The WET Variability Study evaluated the successful test completion rate, false positive rate, and precision of WET test methods. In the analysis of these test performance measures, data from the referee laboratories were not included. Referee laboratories conducted testing of each sample type simultaneously with participant laboratories; however, the identity and expected result of test samples was not blinded to referee laboratories as they were to participant laboratories. For this reason, referee laboratory results were excluded from the calculation of test performance measures. This appendix presents summarized results of the study including referee laboratory data.

Table F1 shows the successful test completion rates achieved in the WET Variability Study when referee laboratory data is included. Including referee laboratory data had very little effect on successful test completion rates. Successful test completion rates remained unchanged for three test methods, increased for five test methods, and decreased for two test methods. Successful test completion rates increased by only 0.1 to 1%, and decreased by only 1.8 to 1.9%.

Table F2 shows the false positive rates reported in the WET Variability Study when referee laboratory data is included. Inclusion of referee laboratory data only affected the false positive rates for the *Ceriodaphnia* chronic and fathead chronic test methods. For these two methods, false positive rates decreased by 0.13% and 0.18%, respectively.

Table F3 shows the precision of WET methods achieved in the WET Variability Study when referee laboratory data is included. For most test methods, the inclusion of referee laboratory data had very little effect on measured test precision. Interlaboratory CVs (based on total variance) of LC50s for acute tests and IC25s for chronic tests remained unchanged for two methods, increased for two methods, and decreased for six methods. For the six methods that decreased in variability, interlaboratory CVs decreased by 0.2 to 2.1%. For the sheepshead chronic method, the CV increased by 0.5%, and for the *Selenastrum* chronic method the CV increased by 8.6% (when conducted with EDTA) and 8.4% (when conducted without EDTA). Referee laboratory results for the *Selenastrum* chronic test method were consistently more sensitive (i.e., lower IC25) than results from most participant laboratories.

Table F1. Successful test completion rates for test methods evaluated in the WET Variability Study (including referee laboratory data).

Test method	N	No. of invalid tests	Successful test completion rate (%)
Ceriodaphnia acute	108	5	95.4
Ceriodaphnia chronic	126	22	82.5
Fathead acute	111	2	98.2
Fathead chronic	105	2	98.1
Selenastrum chronic (with EDTA)	48	17	64.6
Selenastrum chronic (without EDTA)	48	16	66.7
Mysidopsis chronic	48	2	95.8
Sheepshead acute	32	0	100
Sheepshead chronic	32	0	100
Silverside acute	40	2	95.0
Silverside chronic	44	0	100

Table F2. False positive rates for test methods evaluated in the WET Variability Study (including referee laboratory data).

Test method	N	False positive rate (%)					
		Survival endpoint		Growth endpoint		Reproduction endpoint	
		LC50	NOEC	IC25	NOEC	IC25	NOEC
Ceriodaphnia acute	33	0.00	-	-	-	-	-
Ceriodaphnia chronic	28	0.00	0.00	-	-	3.57	3.57
Fathead acute	28	0.00	-	-	-	-	-
Fathead chronic	25	0.00	0.00	4.00	4.17[a]	-	-
Selenastrum chronic (with EDTA)	5	-	-	0.00	0.00	-	-
Selenastrum chronic (without EDTA)	6	-	-	33.3	20.0[b]	-	-
Mysidopsis chronic	7	0.00	0.00	0.00	0.00	0.00[c]	0.00[c]
Sheepshead acute	8	0.00	-	-	-	-	-
Sheepshead chronic	8	0.00	0.00	0.00	0.00	-	-
Silverside acute	7	0.00	-	-	-	-	-
Silverside chronic	8	0.00	0.00	0.00	0.00	-	-

[a] N for the growth NOEC endpoint was 24.
[b] N for the growth NOEC endpoint was 5.
[c] N for the fecundity endpoints was 4.

Table F 3. Within-laboratory, between-laboratory, and total variability observed for test methods evaluated in the WET Variability Study (including referee laboratory data).

Test method	CV (%)[a]					
	Survival endpoint[b]			Sublethal endpoint[c]		
	Within-laboratory	Between-laboratory	Total[d]	Within-laboratory	Between-laboratory	Total[d]
Ceriodaphnia acute	12.0	23.5	28.4	-	-	-
Ceriodaphnia chronic	7.03	21.5	21.2	17.3	26.6	33.5
Fathead acute	8.96	19.4	19.8	-	-	-
Fathead chronic	7.84	11.2	13.2	14.5	15.0	20.7
Selenastrum chronic (with EDTA)	-	-	-	25.6	28.0	42.9
Selenastrum chronic (without EDTA)	-	-	-	25.5	78.4	66.9
Mysidopsis chronic[e]	6.44	26.6	30.1	6.89	36.9	39.2
Sheepshead acute[f]	-	-	24.1	-	-	-
Sheepshead chronic[f]	-	-	8.17	-	-	11.0
Silverside acute	10.1	49.2	38.5	-	-	-
Silverside chronic	11.0	40.3	41.3	14.7	41.7	43.8

[a] Within-laboratory, between-laboratory, and total CVs presented are averaged across sample types.
[b] CVs for the survival endpoint are based on LC50 values.
[c] CVs for the sublethal endpoint are based on IC25 values.
[d] CVs based on total variance may not necessarily be greater than CVs based on within and between-laboratory variance because the CVs presented are averaged across sample types. No within-laboratory replication was provided for the receiving water sample type, so CVs based on within and between-laboratory variance are averaged across only the reference toxicant and effluent sample types; CVs based on total variance are averaged across the reference toxicant, effluent, and receiving water sample types.
[e] For the *Mysidopsis* chronic test method, sublethal enpdoint CVs are for the growth endpoint.
[f] Within and between-laboratory components of variability were not estimated for the sheepshead acute and chronic test methods because no within-laboratory replication was provided for these methods.