

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

TABLE OF CONTENTS

SECTION 1: INTRODUCTION AND BACKGROUND	1
SECTION 2: OBJECTIVES	4
SECTION 3: STUDY MANAGEMENT	6
SECTION 4: TECHNICAL APPROACH	6
4.1 Phase 1 - Laboratory Procurement	6
4.1.1 Identification of Potential Laboratories	7
4.1.2 Selection of Referee Laboratories	7
4.1.3 Selection of Participant Laboratories	7
4.1.4 Prequalification Requirements	8
4.1.5 Prequalification Information Turnaround Requirements	11
4.2 Phase 2 - Preliminary Testing	11
4.2.1 Part 1- Characterization of Physical, Chemical, and Toxicological Properties of Real-World Matrix Types	12
4.2.2 Part 2 - Determination of the Toxicity of Spiked Reference Toxicants in the Sample Matrix	12
4.2.3 Part 3 - Holding Time Testing	12
4.2.4 Part 4 - Definitive Testing	13
4.3 Phase 3 - Sample Preparation, Packaging, and Distribution	13
4.3.1 Description of Test Samples	13
4.3.2 Collection of Real-World Samples	13
4.3.3 Preparation of Test Samples	14
4.3.4 Packaging and Distribution of Test Samples	15
4.3.5 Sample Tracking	15
4.4 Phase 4 - Interlaboratory Testing	16
4.4.1 Study Initiation	16
4.4.2 Preliminary Study Schedule	16
4.4.3 General Testing Requirements	19
4.4.4 Method-Specific Requirements	21
SECTION 5: DATA REPORTING AND EVALUATION	34

LIST OF TABLES

<u>TABLE</u>	<u>TITLE</u>	<u>PAGE</u>
1.	WET Methods Included in the WET Interlaboratory Variability Study	2
2.	Twelve Acute and Short-Term Chronic WET Methods	3
3.	Four Phases of the WET Interlaboratory Variability Study.	4
4.	General Responsibilities of Parties Contributing to the WET Interlaboratory Variability Study	6
5.	Preliminary Schedule for WET Interlaboratory Study	18
6.	Fathead Minnow, <i>Pimephales promelas</i> , Acute Test	22
7.	Fathead Minnow, <i>Pimephales promelas</i> , Larval Survival and Growth Test	23
8.	Cladoceran, <i>Ceriodaphnia dubia</i> , Acute Test	24
9.	Cladoceran, <i>Ceriodaphnia dubia</i> , Survival and Reproduction Test	25
10.	Green Alga, <i>Selenastrum capricornutum</i> , Growth Test	26
11.	Inland Silverside, <i>Menidia beryllina</i> , Acute Test	27
12.	Inland Silverside, <i>Menidia beryllina</i> , Larval Survival and Growth Test	28
13.	Mysid, <i>Holmesimysis costata</i> , Acute Test	29
14.	Red Macroalga, <i>Champia parvula</i> , Reproduction Test	30
15.	Sheepshead Minnow, <i>Cyprinodon variegatus</i> , Acute Test	31
16.	Sheepshead Minnow, <i>Cyprinodon variegatus</i> , Larval Survival And Growth Test	32
17.	Mysid Shrimp, <i>Mysidopsis bahia</i> , Survival, Growth, and Fecundity Test	33
18.	Data Reporting Format	34

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 LC₅₀ and IC₂₅ 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 Test¹
- (2) Method 1000.0: Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test²
- (3) Cladoceran, *Ceriodaphnia dubia*, Acute Test¹
- (4) Method 1002.0: Cladoceran, *Ceriodaphnia dubia*, Survival and Reproduction Test²
- (5) Method 1003.0: Green Alga, *Selenastrum capricornutum*, Growth Test²

Round 2 - Marine Tests

- (1) Inland Silverside, *Menidia beryllina*, Acute Test¹
- (2) Method 1006.0: Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test³
- (3) Mysid, *Holmesimysis costata*, Acute Test¹
- (4) Method 1009.0: Red Macroalga, *Champia parvula*, Reproduction Test³

Round 3 - Marine Tests

- (1) Sheepshead Minnow, *Cyprinodon variegatus*, Acute Test¹
- (2) Method 1004.0: Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test³
- (3) Method 1007.0: Mysid, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test³

¹USEPA, *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

²USEPA, *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

³USEPA, *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, <i>Pimephales promelas</i> , Acute Test	X	96				
1	Method 1000.0: Fathead Minnow, <i>Pimephales promelas</i> , Larval Survival & Growth Test			X	X		7
1	Cladoceran, <i>Ceriodaphnia dubia</i> , Acute Test	X	48				
1	Method 1002.0: Cladoceran, <i>Ceriodaphnia dubia</i> , Survival & Reproduction Test			X		X	8 ¹
1	Method 1003.0: Green Alga, <i>Selenastrum capricornutum</i> , Growth Test				X		4
2	Inland Silverside, <i>Menidia beryllina</i> , Acute Test	X	96				
2	Method 1006.0: Inland Silverside, <i>Menidia beryllina</i> , Larval Survival and Growth Test			X	X		7
2	Mysid, <i>Holmesimysis costata</i> , Acute Test ²	X	96				
2	Method 1009.0: Red Macroalga, <i>Champia parvula</i> , Reproduction (cystocarp production) Test					X	7 - 9 ³
3	Sheepshead Minnow, <i>Cyprinodon variegatus</i> , Acute Test	X	96				
3	Method 1004.0: Sheepshead Minnow, <i>Cyprinodon variegatus</i> - Larval Survival & Growth Test			X	X		7
3	Method 1007.0: Mysid, <i>Mysidopsis bahia</i> , 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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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-useable 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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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)

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

will be randomly selected from the pool of prequalified laboratories to participate in the base study design at their own cost or an external sponsors cost (non-EPA sponsorship). The 9 EPA-sponsored laboratories and the 11 randomly chosen non-EPA-sponsored laboratories will constitute the 20 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 paid 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 IC₂₅ 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°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, <i>Ceriodaphnia dubia</i> , Acute Test with samples #1&2
8/26/99 - 8/28/99	Conduct Cladoceran, <i>Ceriodaphnia dubia</i> , Acute Test with samples #3&4
8/31/99 - 9/4/99	Conduct Green Alga, <i>Selenastrum capricornutum</i> , Growth Test with samples #1&2
9/9/99 - 9/13/99	Conduct Green Alga, <i>Selenastrum capricornutum</i> , Growth Test with samples #3&4
9/14/99 - 9/21/99	Conduct Fathead Minnow, <i>Pimephales promelas</i> , Larval Survival and Growth Test with samples #1&2
9/21/99 - 9/28/99	Conduct Fathead Minnow, <i>Pimephales promelas</i> , Larval Survival and Growth Test with samples #3&4
9/28/99 - 10/6/99	Conduct Cladoceran, <i>Ceriodaphnia dubia</i> , Survival and Reproduction Test with samples #1&2
10/7/99 - 10/11/99	Conduct Fathead Minnow, <i>Pimephales promelas</i> , Acute Test with samples #1&2
10/12/99 - 10/20/99	Conduct Cladoceran, <i>Ceriodaphnia dubia</i> , Survival and Reproduction Test with samples #3&4
10/21/99 - 10/25/99	Conduct Fathead Minnow, <i>Pimephales promelas</i> , 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, <i>Champia parvula</i> , Reproduction Test with samples #1&2
9/9/99 - 9/18/99	Conduct Red Macroalga, <i>Champia parvula</i> , Reproduction Test with samples #3&4
9/21/99 - 9/25/99	Conduct Mysid, <i>Holmesimysis costata</i> , Acute Test with samples #1&2
9/28/99 - 10/2/99	Conduct Mysid, <i>Holmesimysis costata</i> , Acute Test with samples #3&4
10/5/99 - 10/12/99	Conduct Inland Silverside, <i>Menidia beryllina</i> , Larval Survival and Growth Test with samples #1&2
10/12/99 - 10/19/99	Conduct Inland Silverside, <i>Menidia beryllina</i> , Larval Survival and Growth Test with samples #3&4
10/19/99 - 10/23/99	Conduct Inland Silverside, <i>Menidia beryllina</i> , Acute Test with samples #1&2
10/26/99 - 10/30/99	Conduct Inland Silverside, <i>Menidia beryllina</i> , 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, <i>Cyprinodon variegatus</i> , Larval Survival and Growth Test with samples #1&2
1/18/00 - 1/25/00	Conduct Mysid, <i>Mysidopsis bahia</i> , Survival, Growth, and Fecundity Test with samples #1&2
1/25/00 - 2/1/00	Conduct Sheepshead Minnow, <i>Cyprinodon variegatus</i> , Larval Survival and Growth Test with samples #3&4
2/1/00 - 2/8/00	Conduct Mysid, <i>Mysidopsis bahia</i> , Survival, Growth, and Fecundity Test with samples #3&4
2/8/00 - 2/12/00	Conduct Sheepshead Minnow, <i>Cyprinodon variegatus</i> , Acute Test with samples #1&2
2/15/00 - 2/19/00	Conduct Sheepshead Minnow, <i>Cyprinodon variegatus</i> , 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 $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$) 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. 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, 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 LC₅₀ must be reported for each acute test. An NOEC and LC₅₀ for survival, and an NOEC and IC₂₅ 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:	<i>Static-renewal</i>
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:	<i>Artemia</i> nauplii are made available while holding prior to the test; add 0.2 mL <i>Artemia</i> 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:	<i>Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)</i>
18. Test concentrations:	<i>Five concentrations and a control</i>
19. Dilution factor	≥0.5
20. Endpoint:	<i>Mortality (LC50)</i>
21. Sample handling and holding requirements:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
22. Sample volume required:	<i>2 L for effluents</i>
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 <i>Artemia</i> 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:	<i>Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)</i>
18. Test concentrations:	<i>Five concentrations and a control</i>
19. Dilution factor	≥0.5
20. Test duration:	7 days
21. Endpoints:	<i>Survival and growth (weight as mean per original)</i>
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:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
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:	<i>Static non-renewal</i>
2. Test duration:	48 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:	30 mL
8. Test solution volume:	15 mL
9. Renewal of test solutions:	<i>None</i>
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 <i>Selenastrum</i> 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:	<i>Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)</i>
18. Test concentrations:	<i>Five concentrations and a control</i>
19. Dilution factor	≥0.5
20. Endpoint:	<i>Mortality (LC50)</i>
21. Sample handling and holding requirements:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
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:	<i>Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals (see Methods Manual Section 7, Dilution Water)</i>
17. Test concentrations:	<i>Five concentrations and a control</i>
18. Dilution factor:	≥0.5
19. Test duration: ²	8 days
20. Endpoints:	<i>Survival and reproduction</i>
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:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
23. Sample volume required:	1 L/day

¹Test 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.

²The *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:	<i>Static-renewal</i>
2. Test duration:	<i>96 h</i>
3. Temperature:	<i>25°C ± 1°C</i>
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu\text{E}/\text{m}^2/\text{s}$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	<i>250 mL</i>
8. Test solution volume:	<i>200 mL</i>
9. Renewal of test solutions:	<i>At 48 h</i>
10. Age of test organisms:	9-14 days; 24-h range in age
11. No. organisms per test chamber:	<i>10</i>
12. No. replicate chambers per concentration:	<i>2</i>
13. No. organisms per concentration:	<i>20</i>
14. Feeding regime:	<i>Artemia</i> nauplii are made available while holding prior to the test; add 0.2 mL <i>Artemia</i> 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:	<i>25‰ (±2‰) Bioassay Grade Forty Fathoms® artificial seawater prepared with MILLIPORE MILLI-Q® or equivalent deionized water (see Methods Manual Section 7, Dilution Water)</i>
18. Test concentrations:	<i>Five concentrations and a control</i>
19. Dilution factor:	≥0.5
20. Endpoint:	<i>Mortality (LC50)</i>
21. Sample handling and holding requirements:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
22. Sample volume required:	<i>1 L for effluents</i>
23. Test acceptability criterion:	90% or greater survival in controls
24. Salinity:	<i>25‰ (± 2‰)</i>

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:	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:	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 <i>Artemia</i> nauplii (survival of 7-9 days old <i>Menidia beryllina</i> larvae improved by feeding 24 h old <i>Artemia</i>)
15. Feeding regime:	Feed 0.10 g wet weight <i>Artemia</i> nauplii per replicate on days 0-2; Feed 0.15 g wet weight <i>Artemia</i> 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‰ ($\pm 2\%$) and a temperature of 12 °C ± 1 °C.

1. Test type:	<i>Static-renewal</i>
2. Test duration:	96 h
3. Temperature:	12 °C ± 1 °C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu\text{E}/\text{m}^2/\text{s}$ (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:	<i>Artemia</i> nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of <i>Artemia</i> 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:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
22. Sample volume required:	1 L for effluents
23. Test acceptability criterion:	90% or greater survival in controls
24. Salinity:	32‰ ($\pm 2\%$)

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 cystocarp 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:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
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:	<i>Static renewal</i>
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:	<i>Artemia</i> nauplii are made available while holding prior to the test; add 0.2 mL <i>Artemia</i> 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:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
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 <i>Artemia</i> nauplii, (less than 24-h old)
15. Feeding regime:	Feed once a day 0.10 g wet weight <i>Artemia</i> nauplii per replicate on Days 0-2; Feed 0.15 g wet weight <i>Artemia</i> 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, <u>or</u> 0.50 mg or greater after no more than 7 days in 4% formalin or 70% ethanol
24. Sample handling and holding requirements:	<i>Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the finalized interlaboratory study testing schedule</i>
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 <i>Artemia</i> 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, IC₂₅, or LC₅₀ where applicable, LOEC or EC₅₀)

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 (LC₅₀, IC₂₅, 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