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Final Report: Interlaboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Vol. 1

NOTE: COASTAL BIOANALYSTS' LAB ID NO. IS 73
(TEST RESULTS IN YELLOW HIGHLIGHT)

Disclaimer

This final report has been reviewed by the Analytical Methods Staff in the Engineering and Analysis Division within the USEPA Office of Water and EPA's WET Interlaboratory Variability Workgroup. Mention of company names, trade names, or commercial products in this report does not constitute endorsement or recommendation for use.

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Acknowledgement

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Executive Summary

In 1995, the U.S. Environmental Protection Agency (EPA) promulgated 17 whole effluent toxicity (WET) test methods for use in monitoring toxicity under the National Pollutant Discharge Elimination System (NPDES) [60 FR 53529; October 16, 1995]. As part of a settlement agreement (Edison Electric Institute *et al.* v. USEPA, Settlement Agreement, July 24, 1998) to resolve a judicial challenge to this rulemaking, EPA conducted an interlaboratory variability study of 12 EPA short-term chronic and acute whole effluent toxicity test methods (the WET Variability Study). This report presents the results of the WET Variability Study.

The purpose of the WET Variability Study was to characterize (1) interlaboratory variability, (2) the rate of successful test completion, and (3) the rate of false positive incidence for the following 12 approved WET test methods:

- Cladoceran, *Ceriodaphnia dubia*, acute test (*Ceriodaphnia* acute)
- Cladoceran, *Ceriodaphnia dubia*, survival and reproduction test (*Ceriodaphnia* chronic)
- Fathead minnow, *Pimephales promelas*, acute test (fathead acute)
- Fathead minnow, *Pimephales promelas*, larval survival and growth test (fathead chronic)
- Green alga, *Selenastrum capricornutum*, growth test (*Selenastrum* chronic)
- Mysid, *Mysidopsis bahia*, survival, growth, and fecundity test (*Mysidopsis* chronic)
- Sheepshead minnow, *Cyprinodon variegatus*, acute test (sheepshead acute)
- Sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test (sheepshead chronic)
- Inland silverside, *Menidia beryllina*, acute test (silverside acute)
- Inland silverside, *Menidia beryllina*, larval survival and growth test (silverside chronic)
- Red macroalga, *Champia parvula*, reproduction test (*Champia* chronic)
- Mysid, *Holmesimysis costata*, acute test (*Holmesimysis* acute)

For two of these methods (the *Champia* chronic and *Holmesimysis* acute test methods), EPA was unable to obtain interlaboratory data due to laboratory unavailability (see Section 2.1). Intralaboratory data were obtained for the *Champia* chronic method, but no valid intralaboratory or interlaboratory data were obtained for the *Holmesimysis* acute method. For each of the remaining 10 methods, EPA selected a minimum of 7 and a maximum of 20 participant laboratories to constitute a "base" study design. Additional volunteer or externally sponsored laboratories (above 20) participated on a more limited basis as part of an "extended" study design (see Section 3). In total, 55 participant laboratories were involved in the study, with 7 to 35 participant laboratories testing each method. Each participating laboratory was required to prequalify for the study by documenting that their capacity and capabilities, experience and proficiency, and quality assurance and quality control systems met the needs of the study (see Section 3).

Four referee laboratories also were involved in the WET Variability Study (see Section 3). For each test method, a referee laboratory was responsible for conducting preliminary testing (see Section 4), collecting and preparing homogeneous bulk test samples (see Section 5), and distributing "blind" test sample

aliquots to participant laboratories (see Section 6). Referee laboratories prepared the following four test sample types for each test method: blank sample; reference toxicant sample; municipal or industrial effluent sample; and receiving water sample. Referee laboratories distributed some combination of these test sample types to participant laboratories for testing. Laboratories participating in the base study design received 4 blind test samples as whole volume (volume necessary to conduct the test) or ampule (to mix and dilute to a required volume) samples; laboratories participating in the extended study design received 3 blind test samples as ampule samples.

Participant laboratories were required to analyze each blind test sample according to the promulgated WET test method manuals and specific instructions in participant laboratory standard operating procedures (SOPs) developed for the study (see Section 7 and Appendix B). Interlaboratory testing was conducted from September 1999 through April 2000. In total, the WET Variability Study generated interlaboratory precision data from testing more than 700 blind samples among 55 participant laboratories.

Following testing, participant laboratories submitted all test data on analyzed samples to the Sample Control Center (SCC) operated by DynCorp Information & Enterprise Technology, Inc., for independent review and calculation (see Section 8). SCC biologists reviewed test data to verify that all pertinent information was provided, tests were conducted in accordance with the WET method manuals and the WET Variability Study SOP, and test results were accurately calculated. Following test review, results were compiled and method performance characteristics (interlaboratory variability, successful test completion rate, and false positive rate) were calculated for each WET test method (see Section 9).

Table 1 displays summarized results from the WET Variability Study. Successful test completion rates were greater than 90% for all WET test methods except the *Ceriodaphnia* chronic (82%) and *Selenastrum* chronic (63.6% with Ethylenediaminetetraacetic acid [EDTA] and 65.9% without EDTA) test methods. False positive rates were less than 5% for all WET test methods except the *Selenastrum* chronic test method conducted without EDTA (33.3%). Interlaboratory variability was described by the coefficient of variation (CV) calculated for point estimates. Interlaboratory CVs of LC50s (median lethal effect concentrations) ranged from 20.0% to 38.5% for acute test methods. Interlaboratory CVs of IC25s (25% inhibition concentrations) ranged from 10.5% to 58.5% for chronic test methods.

Table 1. Summarized test results from EPA's WET Variability Study.

Test method	Successful test completion rate (%)	False positive rate[a] (%)	Interlaboratory Precision (%CV)[b]
Ceriodaphnia acute	95.2	0.00	29.0
Ceriodaphnia chronic	82.0	3.70	35.0
Fathead acute	100	0.00	20.0
Fathead chronic	98.0	4.35	20.9
Selenastrum chronic (with EDTA)[c]	63.6	0.00	34.3
Selenastrum chronic (without EDTA)[c]	65.9	33.3	58.5
Mysidopsis chronic	97.7[d]	0.00	41.3
Sheepshead acute	100	0.00	26.0
Sheepshead chronic	100	0.00	10.5
Silverside acute	94.4	0.00	38.5
Silverside chronic	100	0.00	43.8
Champia chronic[e]	ND	ND	ND[f]
Holmesimysis acute[e]	ND	ND	ND

[a] False positive rates reported for each method represent the higher of false positive rates observed for hypothesis testing results or point estimates.

[b] Coefficients of variation (CVs) reported for each method represent the CV of LC50 values for acute test methods and IC25 values for chronic test methods. CVs reported are based on total variance and averaged across sample types.

[c] The *Selenastrum* chronic test method was conducted with and without ethylenediaminetetraacetic acid (EDTA) as a component of the nutrients added to test and control treatments.

[d] Successful test completion for the optional fecundity endpoint was 50%.

[e] ND = not determined. Due to insufficient laboratory support, interlaboratory data were not obtained for the *Champia* chronic and *Holmesimysis* acute test methods.

[f] While interlaboratory test data were not obtained for the *Champia* chronic method, intralaboratory data was obtained from the referee laboratory. Intralaboratory CVs were 27.6%, 49.7%, and 50.0% for reference toxicant, receiving water, and effluent sample types, respectively.

List of Acronyms and Abbreviations

ASTM	American Society for Testing and Materials
CFR	Code of Federal Regulations
$CuSO_4$	Copper sulfate
CV	Coefficient of variation
CWA	Clean Water Act
DMRQA	Discharge Monitoring Report Quality Assurance
DO	Dissolved oxygen
EAD	Engineering and Analysis Division
EC50	50% effect concentration
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
FR	Federal Register
HDPP	High-density polypropylene
IC25	25% inhibition concentration
IC50	50% inhibition concentration
ICp	Percentage inhibition concentration
KCl	Potassium chloride
LC50	Median lethal effect concentration
LOEC	Lowest observed effect concentration
MHSF	Moderately-hard synthetic freshwater
MSD	Minimum significant difference
NOEC	No observed effect concentration
NPDES	National Pollutant Discharge Elimination System
ORD	Office of Research and Development
PMSD	Percent minimum significant difference
QA	Quality assurance
QC	Quality control
SAS	Statistical Analysis Software
SCC	Sample Control Center
SETAC	Society of Environmental Toxicology and Chemistry
SOP	Standard operating procedure
SOW	Statement of work
TAC	Test acceptability criteria
WET	Whole effluent toxicity
YCT	Yeast-cerophyl-trout chow mixture

Glossary

Accuracy is used in this document only to describe the quality of being free from mistakes and errors. In other test method performance applications, accuracy of a test method is the closeness of agreement between measured values and an accepted reference (or known) value; however, accuracy in this sense cannot be determined for whole effluent toxicity test methods.

Acute Toxicity Test is a test to determine the concentration of effluent or ambient waters that causes an adverse effect (usually death) on a group of test organisms during a short-term exposure (e.g., 24, 48, or 96 hours).

Algal Suspension is a homogenized mixture of alga and liquid media.

Aliquot is a subsample of a larger homogenized sample.

Ambient Laboratory Illumination is the general lighting condition occurring daily in the laboratory.

Ampule Sample is a small volume (generally 100 mL) liquid sample that is reconstituted at participant laboratories to provide the necessary test sample volume.

Base Study Design used in the WET Variability Study consisted of participant laboratories receiving four blind test samples, which included some combination of the four test sample types (blank, reference toxicant, effluent, and receiving water). The base study design included a minimum of 7 and maximum of 20 participant laboratories, with up to 9 EPA-sponsored laboratories and up to 11 non-EPA-sponsored laboratories.

Between-Laboratory Variability is the variability of test results from different laboratories using the same test method and analyzing the same test material. Between-laboratory variability, as used in this document, does not include the within-laboratory component of variance. Between-laboratory coefficients of variation (CVs) are CVs calculated based on solely the between-laboratory component of variance.

Bioassay Grade is a rating on chemicals or chemical mixtures that have been tested and do not contain interferences to various bioassay tests.

Blank is a non-toxic sample prepared as the typical synthetic control dilution water for the method being tested.

Blind Sample is a sample that is of unknown composition to the testing laboratory.

Brood is a group of offspring produced from a female in a single reproductive event.

Bulk Sample is a large homogenized volume of a collected or prepared test sample. In this study, referee laboratories prepared bulk samples that were divided and distributed to participant laboratories for testing.

Chronic Toxicity Test is a short-term test in which sublethal effects (e.g., reduced growth or reproduction) are usually measured in addition to lethality.

Coefficient of Variation (CV) is a standard statistical measure of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean. It is also called the relative standard deviation (RSD). The CV can be used as a measure of precision within (within-laboratory) and between (between-laboratory) laboratories, or among replicates for each treatment concentration. In this study, within-laboratory, between-laboratory, and total CVs were calculated. The within-laboratory CV is used to express just the variability between duplicate samples tested in the same laboratory. The between-laboratory CV is used to express just the variability between laboratories testing duplicate samples. The total CV is used to express the total interlaboratory variability of the results, including both within-laboratory and between-laboratory components of variability.

Control Chart is a chronological graphical representation of the results from reference toxicant tests performed with the same reference toxicant, test species, test conditions, and endpoints repeated by the same laboratory.

Culture is an ongoing, reproducing population of organisms maintained in a laboratory to provide the laboratory with a supply of test organisms.

Data Qualifier Flag is an identifier for tests with deviations in test conditions, sample holding times, sample temperatures, test acceptability criteria, or test water quality.

Effect Concentration (EC) is a point estimate of the toxicant concentration that would cause an observable adverse effect (e.g., death, immobilization, or serious incapacitation) in a given percent of the test organisms, calculated from a continuous model (e.g., Probit Model). EC25 is a point estimate of the toxicant concentration that would cause an observable adverse effect in 25% of the test organisms.

Effluent is wastewater discharged from a facility.

Endpoint is the final measurement of a biological response (e.g., reproduction, growth, or survival).

Episode is a designation to group samples prepared for a specific test method**.**

Extended Study Design used in the WET Variability Study consisted of participant laboratories receiving three blind ampule samples, which included some combination of blank and reference toxicant samples. The extended study design included all non-EPA-sponsored laboratories not included in the base study design (those above the 20 laboratory maximum for the base study design).

False Positive is a test result that indicates toxicity in a non-toxic or blank sample.

False Positive Rate is the percentage of valid test results that indicate toxicity in blank samples.

Forty Fathoms® Artificial Sea Salts is a commercially available chemical mixture of dry reagents that is used to create synthetic seawater.

h Statistic is a calculated parameter defined by ASTM that represents the consistency of test results from laboratory to laboratory.

Holding Time is the elapsed time from the end of sample collection to the first use of the sample. Because bulk samples were prepared in the laboratory for this study, the holding time began when sample aliquots were divided for distribution to participant laboratories.

Hypothesis Testing is a statistical technique (e.g., Dunnett's test) for determining whether a tested concentration is statistically different from the control. Effect concentrations determined from hypothesis testing are NOEC and LOEC values. The two hypotheses commonly tested in WET testing are:

Null hypothesis (H_o): The effluent is not toxic.

Alternative hypothesis (H_a): The effluent is toxic.

Inhibition Concentration (IC) is a point estimate of the toxicant concentration that would cause a given percent reduction in a non-lethal biological measurement (e.g., reproduction or growth), calculated from a continuous model, (i.e., Interpolation Method). IC25 is a point estimate of the toxicant concentration that would cause a 25-percent reduction in a non-lethal biological measurement.

Interlaboratory Variability is the variability of test results from different laboratories using the same test method and analyzing the same test material. Interlaboratory variability, as used in this document, includes both within-laboratory and between-laboratory components of variance. Interlaboratory coefficients of variation (CVs) are CVs calculated based on the total variance of results for a given method, endpoint, and sample type.

k Statistic is a calculated parameter defined by ASTM that represents the consistency of within-laboratory precision from laboratory to laboratory.

Lowest Observed Effect Concentration (LOEC) is the lowest concentration of an effluent or toxicant that results in adverse effects on the test organisms (i.e., where the values for the observed endpoints are statistically different from the control).

Minimum Significant Difference (MSD) is the magnitude of difference from control where the null hypothesis is rejected in a statistical test comparing a treatment with a control (i.e., the smallest difference between control and treatment responses that can be determined as statistically significant). MSD is based on the number of replicates, control performance, and power of the test.

Moderately Hard Synthetic Freshwater (MHSF) is water prepared from deionized water and reagent grade chemicals as described in the WET method manuals to produce a hardness of 80 to100 mg $CaCO_3$/L.

No Observed Effect Concentration (NOEC) is the highest tested concentration of an effluent or toxicant that causes no observable adverse effect on the test organisms (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically different from the controls).

National Pollutant Discharge Elimination System (NPDES) program regulates discharges to the nation's waters. Discharge permits issued under the NPDES program are required by EPA regulation to contain, where necessary, effluent limits based on water quality criteria for the protection of aquatic life and human health.

Outlier is an extreme observation that is divergent from other observations of the same parameter. In this study, ASTM h and k statistics were used to identify potential outliers.

Participant Laboratory is a laboratory selected to conduct a specific test method and report data for use in the study.

Percent Minimum Significant Difference (PMSD) is the magnitude of difference from control, expressed as a percentage of the control response, where the null hypothesis is rejected in a statistical test comparing a treatment with a control (i.e., the smallest difference between control and treatment responses, expressed as a percentage of the control response, that can be determined as statistically significant).

Photoperiod is the diurnal cycle of light and darkness to which test organisms are exposed.

Point Estimate is a statistical estimate of some amount of adverse effect derived from a mathematical model that assumes a continuous concentration-response relationship.

Precision is a measure of reproducibility within a data set. Precision can be measured both within a laboratory (within-laboratory) and between laboratories (between-laboratory) using the same test method and toxicant.

Prequalification is a the process of determining whether laboratories meet specific requirements for participation in the study.

Quality Assurance (QA) is a practice in toxicity testing that addresses all activities affecting the quality of the final effluent toxicity data. QA includes practices such as effluent sampling and handling, source and condition of test organisms, equipment condition, test conditions, instrument calibration, replication, use of reference toxicants, recordkeeping, and data evaluation.

Quality Control (QC) is the set of more focused, routine, day-to-day activities carried out as part of the overall QA program.

Range-finding is preliminary testing to determine the range of toxicant concentrations that produce a targeted range of effects.

Receiving Water is water into which wastewater flows.

Reconstitute is to mix and dilute an ampule sample to the required volume for use in a toxicity test.

Referee Laboratory is a laboratory selected to provide support for the testing of a specific test method in the study. The referee laboratory was responsible for conducting preliminary testing and for collecting, preparing, and distributing test samples to participant laboratory.

Reference Toxicant is a known toxic chemical that is routinely tested to evaluate the consistency and precision of toxicity tests. Reference toxicant testing is a component of the quality assurance/quality control program in WET testing.

Reference Toxicant Test is a check of the sensitivity of the test organisms and the suitability of the test methodology. Reference toxicant data are part of a routine QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Sample Code is a unique identifying number for each sample.

Spiking is the addition of a reference toxicant to a sample matrix in order to elicit a toxicological effect.

Static Non-renewal Test is a toxicity test that exposes test organisms to the same test solution (without renewal) for the duration of the test.

Static Renewal Test is a toxicity test that exposes test organisms to a fresh solution of the sample at regular prescribed intervals (typically every 24 or 48 hours).

Successful Test Completion Rate is the percentage of initiated and properly terminated tests that met the test acceptability criteria as specified in the WET method manuals.

Synthetic Seawater is artificial saltwater that is prepared by adding commercial sea salts or reagent grade chemicals to deionized water.

Test Acceptability Criteria (TAC) are specific criteria for determining whether toxicity test results are acceptable. The effluent and reference toxicant must meet specific criteria as defined in the test method (e.g., for the *Ceriodaphnia dubia* survival and reproduction test, the criteria are as follows: the test must achieve at least 80 percent survival and an average of 15 young per surviving female in the controls).

Traffic Report Form is a form to document the chain-of-custody for each test sample shipped. The traffic report form identifies 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).

Valid Test is a test that met the required test acceptability criteria for the method as stated in the WET method manuals.

Variance is a measure of the dispersion in a set of values, defined as the sum of the squared deviations divided by their total number.

Whole Effluent Toxicity (WET) is the total toxic effect of an effluent measured directly with a toxicity test.

Whole Volume Sample is a sample that is provided in the appropriate volume for direct use in a toxicity test.

Within-laboratory Variability is the variability of test results from the same laboratory using the same test method and analyzing the same test material. Within-laboratory coefficients of variation (CVs) are CVs calculated based on solely the within-laboratory component of variance.

1.0 INTRODUCTION AND BACKGROUND

Whole effluent toxicity (WET) is defined as "the aggregate toxic effect of an effluent measured directly by an aquatic toxicity test" [54 FR 23686; June 2, 1989]. WET tests expose aquatic organisms to a range of effluent concentrations under controlled laboratory conditions. Exposure durations generally range from 24 hours (acute tests) to 7 days or more (short-term chronic or chronic tests). At the end of the exposure period, biological endpoints such as survival, growth, reproduction, or fertilization are measured in each effluent concentration and a control treatment. Toxicity of the effluent is determined by statistically comparing (either by hypothesis testing or point estimation) measured responses between the control and various effluent concentrations. Test results are expressed as the concentration of effluent estimated to produce a given effect (i.e., effect concentration). Effect concentrations such as the NOEC (No Observed Effect Concentration), LOEC (Lowest Observed Effect Concentration), LC50 (median lethal effect concentration), EC50 (median effect concentration), or IC25 (25% inhibition concentration) are commonly used to report the results of WET tests.

1.1 Regulatory Background

The Clean Water Act (CWA) was enacted in 1972 with the objective of "restoring the chemical, physical, and biological integrity of the Nation's waters." Along with other goals, CWA section 101(a)(3) states that "it is the national policy that the discharge of toxic pollutants in toxic amounts be prohibited." The U.S. Environmental Protection Agency (EPA) has pursued this goal through implementation of the water quality standards program and the National Pollutant Discharge Elimination System (NPDES) permitting program. These programs have adopted an integrated strategy of water quality-based toxics control that includes three approaches: chemical-specific control, whole effluent toxicity (WET) control, and biological criteria/bioassessment (USEPA, 1991).

To implement this strategy, States are encouraged to define numeric or narrative water quality standards that include chemical-specific criteria, criteria for WET, and biological criteria. Some states have included numeric criteria for WET, while others have relied on narrative criteria. These water quality standards and criteria are maintained by controlling the discharge of pollutants through the NPDES permitting program. When a discharge causes or has a reasonable potential to cause or contribute to the excursion above of numeric or narrative water quality standards, a NPDES permit limit will be issued to control the discharge. Permit limits for WET are established if the discharge has a reasonable potential to cause or contribute to the excursion above of water quality standards for WET.

In 1995, EPA approved 17 WET test methods for use in NPDES permit monitoring [60 FR 53529; October 16, 1995]. The EPA-approved WET test methods resulted from many years of development and testing by EPA, States, municipalities, academia, and the regulated community. These WET test methods measure the acute and short-term chronic toxicity of effluents and receiving waters to aquatic plants, invertebrates, and vertebrates from freshwater and marine environments. WET test methods approved for use in NPDES monitoring are listed in 40 CFR §136.3, Table IA, and standardized test procedures for

conducting the approved WET tests are published in the following three test method manuals (the WET method manuals).

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

1.2 The WET Variability Study

Following the promulgation of WET test methods in 1995 [60 FR 53529; October 16, 1995], various parties filed suit to challenge the rulemaking. To resolve that litigation, EPA entered into settlement agreements with the various parties. In a 1998 settlement agreement (Edison Electric Institute *et al.* v. USEPA, Settlement Agreement, July 24, 1998), EPA agreed to conduct an interlaboratory variability study of 12 EPA short-term chronic and acute whole effluent toxicity test methods (the WET Variability Study). EPA conducted the WET Variability Study from September 1999 to April 2000 and published preliminary results from the study in October 2000 (USEPA, 2000b; USEPA, 2000c). In 2001, EPA submitted the preliminary results of the study for expert peer review. This document incorporates peer review comments (where appropriate) and presents the final results of the WET Variability Study. EPA intends to comment on the significance of these results in subsequent rulemaking and propose to ratify or withdraw each of the WET test methods evaluated in the WET Variability Study.

1.3 Other EPA Documents

The WET method manuals (USEPA, 1993; USEPA, 1994a; USEPA, 1994b) were published in 1993 and 1994 and incorporated by reference in the 1995 WET rule [60 FR 53529; October 16, 1995]. Following this rulemaking, EPA issued clarifications to the WET test methods on April 10, 1996, via a memorandum from Tudor Davies, Director of EPA's Office of Science and Technology. This memorandum, titled "Clarifications Regarding Flexibility in 40 CFR Part 136 Whole Effluent Toxicity (WET) Test Methods" (USEPA, 1996), provided clarification on the following WET test issues: pH and ammonia control, temperature, hardness, test dilution concentrations, and acceptance criteria for *Champia parvula.*

In February 1999, EPA published an errata to the WET method manuals (USEPA, 1999). This errata amended the approved WET test methods to correct typographical errors and omissions, provide technical clarification, and establish consistency among the 1995 WET rule language and the WET method manuals.

In June 2000, EPA published a guidance document titled, "Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program" (USEPA, 2000d). This guidance document quantified WET test variability, evaluated statistical methods for deriving WET permit conditions, and provided guidance on accounting for and minimizing WET test variability and its effects on the regulatory process.

In July 2000, EPA published a guidance document titled, "Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR Part 136)" (USEPA, 2000a). This document included guidance and recommendations on nominal error rate adjustments, confidence intervals, concentration-response relationships, dilution series selection, and selection of an acceptable dilution water for WET testing.

The documents mentioned above were used in the conduct of WET tests and review of WET test data reported in this study. The WET method manuals (USEPA, 1993; USEPA, 1994a; USEPA, 1994b), clarification memo (USEPA, 1996), and errata document (USEPA, 1999) were used by referee and participant laboratories in the conduct of WET tests during the WET Variability Study. The two guidance documents published in 2000 were not available to laboratories at the time of testing, but were used by EPA in the review and analysis of WET test results from the study.

2.0 STUDY DESIGN AND OBJECTIVES

2.1 Objectives

In conducting the WET Variability Study, EPA's primary objectives were to:

- characterize the interlaboratory variability of 12 WET test methods through the determination of CVs for LC50s and IC25s and ranges for NOEC values,
- characterize the rate at which participating laboratories successfully completed WET tests initiated (successful test completion rate), and
- characterize the rate at which WET tests indicate "toxicity" is present when measuring non-toxic samples (false positive rate).

EPA developed a preliminary study plan to meet these objectives, and made this study plan available for public and peer review comment on October 9, 1998. EPA then revised the study plan in response to comments received and published a final study plan on June 11, 1999 (see Appendix A). The WET Variability Study was conducted according to the final study plan with the following exceptions.

- The minimum number of participant laboratories was reduced from nine to seven for the sheepshead acute and chronic test methods. Only seven laboratories qualified to participate in these methods. A minimum of seven participant laboratories still satisfied the data quality objective requiring a minimum of six complete and useable data sets for each WET test method.
- No interlaboratory testing was conducted for the *Champia* chronic and *Holmesimysis* acute test methods. The interlaboratory testing phase was canceled for these test methods due to insufficient participant laboratory support. Only one participant laboratory could be procured for the *Champia* chronic test method, and only two participant laboratories could be procured for the *Holmesimysis* acute test method. This report presents only referee laboratory preliminary testing results for these two methods.

2.2 General Study Design

The WET Variability Study was conducted in five phases designed to accomplish the overall study objectives. These phases are described in Table 2.1 and discussed in more detail in Sections 3 - 9 of this report.

2.2.1 Study Management

The WET Variability Study was directed by EPA with contractor support provided by DynCorp Information & Enterprise Technology, Inc., under the Sample Control Center (SCC) contract. EPA Office of Water's Engineering and Analysis Division (EAD) and EPA's Office of Research and Development (ORD) provided overall management and technical oversight of the study. Laboratory procurement, day-to-day management, coordination of study activities, data review, and preparation of the preliminary and final study reports were performed by SCC under EAD and ORD guidance. SCC

contracted four referee laboratories to conduct preliminary testing and prepare and distribute blind test samples to 55 participant laboratories (see Appendix C for a list of referee and participant laboratories). Participant laboratories conducted WET tests and submitted data reports to SCC.

Table 2.1. Four phases and specific objectives of the WET Variability Study.

Phase	Objectives
1 - Laboratory Procurement (See Section 3)	• 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 (See Section 4)	• Determine the suitability of selected effluent and receiving water 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 effluent and receiving water 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 (See Sections 5 and 6)	• Collect effluent and receiving water samples • Prepare blank, reference toxicant, effluent, and receiving water samples for use by referee and participant laboratories in Phase 4 • Minimize variability between samples prepared for and distributed to each participant laboratory in Phase 4 • 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 (See Section 7)	• Obtain interlaboratory test data for each WET method using four test sample types
5 - Data Review and Analysis (See Sections 8 and 9)	• Review test data and calculate test results • 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

2.2.2 Methods Evaluated

EPA evaluated 12 of the 17 promulgated WET test methods in the WET Variability Study. These included two acute freshwater methods, three short-term chronic freshwater methods, three acute marine methods, and four short-term chronic marine methods. The test methods evaluated in the WET Variability Study are displayed in Table 2.2, and the endpoints and effect concentrations evaluated for each test method are displayed in Table 2.3. Each test method was conducted in accordance with the WET method manuals (USEPA, 1993; USEPA, 1994a; USEPA, 1994b), and as appropriate, specific guidance provided in the WET Variability Study participant laboratory standard operating procedure

(SOP) documents (Appendix B), the WET method manuals errata (USEPA, 1999), and clarifications provided in an April 10, 1996, memorandum from Tudor Davies, Director of EPA's Office of Science and Technology (USEPA, 1996).

Table 2.2. WET test methods included in the WET Variability Study.

Test method	Common test method name[a]	Test method number[b]	Test type
Cladoceran, *Ceriodaphnia dubia*, acute test[c]	*Ceriodaphnia* acute	-	freshwater acute
Cladoceran, *Ceriodaphnia dubia*, survival and reproduction test[d]	*Ceriodaphnia* chronic	1002.0	freshwater chronic
Fathead minnow, *Pimephales promelas*, acute test[c]	Fathead acute	-	freshwater acute
Fathead minnow, *Pimephales promelas,* larval survival and growth test[d]	Fathead chronic	1000.0	freshwater chronic
Green alga, *Selenastrum capricornutum*, growth test[d]	*Selenastrum* chronic	1003.0	freshwater chronic
Mysid, *Mysidopsis bahia*, survival, growth, and fecundity test[e]	*Mysidopsis* chronic	1007.0	marine chronic
Sheepshead minnow, *Cyprinodon variegatus*, acute test[c]	Sheepshead acute	-	marine acute
Sheepshead minnow, *Cyprinodon variegatus,* larval survival and growth test[e]	Sheepshead chronic	1004.0	marine chronic
Inland silverside, *Menidia beryllina*, acute test[c]	Silverside acute	-	marine acute
Inland silverside, *Menidia beryllina,* larval survival and growth test[e]	Silverside chronic	1006.0	marine chronic
Red macroalga, *Champia parvula*, reproduction test[e]	*Champia* chronic	1009.0	marine chronic
Mysid, *Holmesimysis costata*, acute test[c,f]	*Holmesimysis* acute	-	marine acute

[a] Common test method names were used in this report to refer to the test methods in the WET Variability Study.
[b] Test method numbers were not designated for acute test methods in USEPA, 1993.
[c] USEPA, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, Fourth Edition, EPA-600-4-90-027F, August 1993
[d] USEPA, *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms*, Third Edition, EPA-600-4-91-002, July 1994
[e] USEPA, *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Marine and Estuarine Organisms*, Second Edition, EPA-600-4-91-003, July 1994
[f] The EPA-approved acute test with *Holmesimysis costata* was performed using the acute test procedures for *Mysidopsis bahia* and test conditions optimized for *H. costata*.

Table 2.3. Endpoints and effect concentrations evaluated for each test method in the WET Variability Study.

Test method	Acute tests		Short-Term Chronic Tests			
	Survival LC50	Test duration (hours)	Survival LC50 NOEC	Growth IC25 NOEC	Reproduction IC25 NOEC	Test duration (days)
Ceriodaphnia acute	X	48	-	-	-	-
Ceriodaphnia chronic	-	-	X	-	X	8[a]
Fathead acute	X	96	-	-	-	-
Fathead chronic	-	-	X	X	-	7
Selenastrum chronic	-	-	-	X	-	4
Mysidopsis chronic	-	-	X	X	X	7
Sheepshead acute	X	96	-	-	-	-
Sheepshead chronic	-	-	X	X	-	7
Silverside acute	X	96	-	-	-	-
Silverside chronic	-	-	X	X	-	7
Champia chronic	-	-	-	-	X	7[b]
Holmesimysis acute	X	96	-	-	-	-

[a] The *C. dubia* test acceptability criteria state that the test is complete when 60% of controls have 3 broods (approximately 7 days); for purposes of this study, all tests continued for 8 days and each laboratory was requested to carefully distinguish and carefully record the number of broods.
[b] *C. parvula* were exposed to test samples for 2 days, followed by a 5-day recovery period in control water.

2.2.3 Laboratories

A referee laboratory was selected to provide support for each test method evaluated in the WET Variability Study. The referee laboratory was responsible for conducting preliminary testing on sample types to ensure that samples used in the WET Variability Study were stable and provided the desired level of effect. Referee laboratories also collected, prepared, and distributed test samples for interlaboratory testing, and conducted testing simultaneously with participant laboratory testing to provide further information on sample consistency.

Interlaboratory testing was conducted by participant laboratories selected for each test method. Each participating laboratory was required to prequalify for the study by documenting that their capacity and capabilities, experience and proficiency, and quality assurance and quality control systems met the needs of the study (see Section 3). The number of participant laboratories conducting each method was

dependent upon the number of laboratories responding to the solicitation, the number of laboratories qualified to conduct the given test method, and the number of laboratories that were sponsored by parties external to EPA. The WET Variability Study plan called for a minimum of 9 and a maximum of 20 participant laboratories to constitute a base study design for each method. Additional laboratories (greater than 20) participated on a more limited basis as part of an extended study design. In deviation from this design, only seven qualified laboratories could be procured to participate in the sheepshead acute and chronic test methods. In addition, interlaboratory testing for the *Champia* chronic and *Holmesimysis* acute test methods was canceled due to insufficient participant laboratory support. See Section 3 for a detailed description of referee and participant laboratory selection, and the base and extended study designs.

2.2.4 Samples

For each test method, four test sample types were prepared in bulk by the referee laboratory, divided, and distributed to participant laboratories for testing. The four sample types included: 1) blank sample, 2) reference toxicant sample, 3) effluent sample, and 4) receiving water sample. Blank and reference toxicant samples were distributed to participant laboratories as liquid ampule samples (to mix and dilute to the required volume at the participant laboratory), while effluent and receiving water samples were distributed as whole-volume samples (consisting of the full volume necessary to conduct the test). The blank sample was a non-toxic sample prepared as the typical synthetic control dilution water for each test method. Testing of the blank sample provided a means of determining the false positive rate for each test method. Interlaboratory precision was evaluated through testing of the reference toxicant, effluent, and receiving water sample types. These sample matrix types (with the exception of the effluent sample type for the *Champia* chronic and *Holmesimysis* acute test methods) were spiked with a toxicant to achieve a desired level of effect and facilitate the evaluation of precision.

Laboratories participating in the base study design received four blind test samples, which included some combination of the four test sample types (blank, reference toxicant, effluent, and receiving water). Laboratories that participated in the extended study design received three blind ampule samples, which included some combination of blank and reference toxicant samples. The combination of blind test samples received by individual laboratories included replicate (i.e., duplicate) test samples for each test method except for methods with fewer than nine participant laboratories. Replicate samples were always shipped together and tested simultaneously, with the exception of the *Selenastrum* chronic test, where only one sample was tested per week. See Sections 5 and 6 for a detailed description of sample preparation and distribution, respectively.

2.2.5 Schedule

Interlaboratory testing in the WET Variability Study was conducted from September 1999 through April 2000. The final participant laboratory schedule for the WET Variability Study is provided in Table 2.4. For each method (with the exception of the *Selenastrum* chronic method), testing was conducted in two

testing periods. Each participant laboratory simultaneously tested two samples during each testing period. For the *Selenastrum* chronic method, testing was conducted in four testing periods, with a single sample tested with and without EDTA during each testing period.

Table 2.4. Final participant laboratory schedule for interlaboratory testing during the WET Variability Study.

Activity	Testing dates[a] (start date - finish date)
Fathead chronic testing period #1	9/28/99 - 10/5/99
Fathead chronic testing period #2	10/5/99 - 10/12/99
Ceriodaphnia chronic testing period #1	10/12/99 - 10/20/99
Silverside chronic testing period #1	10/19/99 - 10/26/99
Fathead acute testing period #1	10/21/99 - 10/25/99
Silverside chronic testing period #2	10/26/99 - 11/2/99
Ceriodaphnia chronic testing period #2	10/26/99 - 11/3/99
Silverside acute testing period #1	11/2/99 - 11/6/99
Fathead acute testing period #2	11/4/99 - 11/8/99
Ceriodaphnia acute testing period #1	11/9/99 - 11/11/99
Silverside acute testing period #2	11/9/99 - 11/13/99
Ceriodaphnia acute testing period #2	11/11/99 - 11/13/99
Mysidopsis chronic testing period #1	2/22/00 - 2/29/00
Mysidopsis chronic testing period #2	2/29/00 - 3/7/00
Sheepshead acute testing period #1	3/7/00 - 3/11/00
Selenastrum chronic testing period #1	3/9/00 - 3/13/00
Sheepshead acute testing period #2	3/14/00 - 3/18/00
Selenastrum chronic testing period #2	3/16/00 - 3/20/00
Sheepshead chronic testing period #1	3/21/00 - 3/28/00
Selenastrum chronic testing period #3	3/23/00 - 3/27/00
Sheepshead chronic testing period #2	3/28/00 - 4/4/00
Selenastrum chronic testing period #4	3/30/00 - 4/3/00

[a] Samples were shipped to arrive at participant laboratories on the indicated start date. Tests were initiated on the same day as sample receipt.

Samples were shipped on ice overnight to arrive at each participant laboratory on the day of scheduled testing. Sample distribution was organized such that all effluent samples were tested on the same day in all participant laboratories, and all receiving water samples were tested on the same day (with the exception of delays due to sample or organism shipments). Since the synthetic matrix and ampule form

of blank and reference toxicant samples provided more sample stability, these samples were distributed for testing in either the first or second testing periods. As a result, tests conducted on reference toxicant and blank samples were initiated on one of two testing dates. Replicate samples were always distributed together for simultaneous testing.

3.0 LABORATORY PROCUREMENT

3.1 Identification and Solicitation of Potential Referee Laboratories

Since the responsibilities of the referee laboratory would be demanding and critical to successful implementation of the WET Variability Study, SCC solicited a select list of laboratories that possessed exceptional qualifications, based on EPA technical staff recommendations. Solicitation bid packages included the following documents: (1) a referee laboratory prequalification document; (2) a referee laboratory statement of work (SOW), including a preliminary study schedule; and (3) a referee laboratory bid sheet. Referee laboratories were first solicited in December 1998. Additional solicitations to an expanded number of laboratories were necessary to fill referee laboratory positions for all of the test methods (Table 3.1).

Table 3.1. Summary of referee laboratory solicitation.

Solicitation date	Response deadline	Methods solicited	Laboratories solicited	Number of qualified responses
12/29/98	1/13/99	All twelve methods	7	2
1/22/99	2/4/99	All seven marine methods	12	2
2/19/99	3/5/99	Silverside acute and chronic; *Holmesimysis* acute	19	3

3.2 Identification and Solicitation of Potential Participant Laboratories

EPA attempted to maximize the number of qualified laboratories participating in the WET Variability Study and select laboratories that were representative of laboratories throughout the United States that routinely conduct WET tests for permittees. SCC and EPA staff identified a list of potential participant laboratories 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* (DynCorp, 1996), and the list of laboratories conducting testing for EPA's Discharge Monitoring Report Quality Assurance (DMRQA) program. In addition, the petitioners provided a list of laboratories interested in participating without EPA sponsorship and a list of potential non-EPA sponsors.

On July 9, 1999, SCC solicited all 319 laboratories included in the compiled potential laboratory list. Laboratories solicited included state, academic, municipal, industrial and private laboratories. Solicitation bid packages included the following documents: (1) a detailed cover letter describing the solicitation; (2) a participant laboratory prequalification document; (3) a participant laboratory SOW, including a preliminary study schedule; and (4) a participant laboratory bid sheet.

3.3 Prequalification Requirements for Participant Laboratories

To ensure that laboratories participating in the WET Variability Study possessed the capacity and capabilities, experience and proficiency, and quality assurance and quality control systems necessary to meet the needs of the study, EPA required all laboratories to meet specific prequalification requirements.

3.3.1 Prequalification Documentation

To demonstrate its qualifications, each potential participant laboratory was required to provide the 16 prequalification items listed below. Prequalification was conducted independently for each test method, and laboratories could submit prequalification materials for any or all methods evaluated in the WET Variability Study.

General information

(1) A cover page with 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).

Capacity and capabilities

(3) A statement that the combination of facilities, equipment, staff, and laboratory capabilities are sufficient to meet study needs.

(4) Detailed information on the type and size of laboratory and test equipment used for conducting each test method, including information on temperature control, sample storage, water purification devices (i.e., Millipore Milli-Q® filtration and ion exchange), and dilution water sources. Laboratories were required to 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, including whether organisms are cultured in-house or obtained externally. If organisms are cultured in-house, the laboratory was required to provide

standard operating procedures for organism culturing (as required in number 9 below), a summary of how culture performance is assessed, and data on culture performance (e.g., *Ceriodaphnia dubia* brood board monitoring data for the past six months or records of *Pimephales promelas* egg production). If organisms are obtained from an external source, the laboratory was required to specify the 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. Internal laboratory SOPs for each test method were required to 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. The laboratory was required to specify 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 and documentation of current certifications (if applicable).

Quality assurance/quality control

(12) Evidence that the laboratory maintains control (cusum) charts for reference toxicant tests for each method. The laboratory was required to 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 were required. Data charts with NOEC and/or IC25 for the same test values were requested as well as an explanation of why one is used rather than the other. Explanations were required if methods used to develop reference toxicant control charts deviated from promulgated methods or were 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 conducted for NPDES permits). Along with control chart information described above, the laboratory was required to provide a statement on the frequency of reference toxicant testing. If control charts were composed of fewer than 12-24 data points, an explanation was required.

(14) Copies of internal laboratory SOPs for conducting reference toxicant tests and constructing control charts. This information had to include a narrative explanation of the width of the control

limits for the laboratory and a statement of corrective action for any toxicity test result that falls outside the control limits.

(15) Results of the most recent DMRQA study, if the laboratory participated. The laboratory was required to 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 was required.

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

3.3.2 Prequalification Determination

SCC evaluated prequalification materials and recommended laboratory rejection based on the following criteria:

(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 did not cover 12-24 data points for each species and test method, and an acceptable explanation was not provided.

(6) Reference toxicant tests were not conducted at the appropriate frequency (monthly for tests that are routinely conducted 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) The 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 of prequalification materials was included.

Laboratories that failed to meet the prequalification criteria due to an incomplete submission were notified via fax of their deficiency and allowed an opportunity to submit the missing information. Laboratories that did not respond to this notification or were unable to provide the missing information failed to prequalify for the WET Variability Study. Only eight laboratories failed to prequalify for the WET Variability Study. Two of these laboratories failed to prequalify for more than one test method.

3.4 Prequalification and Selection of Referee Laboratories

Referee laboratories were required to submit the same prequalification materials listed in Section 3.3.1 for participant laboratories. In addition, referee laboratories were required to submit three client references and provide background information on potential effluent and receiving water sample sources. Referee laboratory prequalification materials were evaluated based on the rejection criteria listed in Section 3.3.2 and the additional reference and sample source requirements. The capacity and capabilities of potential referee laboratories were highly scrutinized to ensure that the laboratory could meet the sample collection, preparation, distribution, and testing requirements of the study. All potential referee laboratories that met prequalification criteria and were determined to possess the capacity and capabilities to meet the needs of the study were considered for the referee laboratory position. From this pool of qualified laboratories, the lowest bidder was selected as the referee laboratory for each bid lot of test methods. The referee laboratories selected for each test method are listed in Table 3.2.

3.5 Selection of Participant Laboratories

Laboratories that met all prequalification criteria (see Section 3.3) were eligible for participation in the WET Variability Study. From the pool of prequalified laboratories, those laboratories with the nine lowest bids per method were selected for EPA sponsorship and participation in the base study design (see Figure 3.1). If a tie for the ninth lowest bid was encountered, selection among equal bid prices from prequalified laboratories was determined based on business classification, with preferences granted in the order of small company, minority-owned, woman-owned, or large company. From the remaining prequalified laboratories, those that had identified a willing sponsor outside of EPA (non-EPA sponsorship) were also selected for participation in the WET Variability Study. From this group of prequalified, non-EPA sponsored laboratories, a maximum of 11 laboratories (for each WET test method) were randomly selected for participation in the base study design. The 9 EPA-sponsored laboratories and

the 11 randomly chosen non-EPA-sponsored laboratories constituted the 20 laboratories included in the base study design for each WET test method. All remaining prequalified laboratories with non-EPA sponsorship were selected for participation in the extended study design. Figure 3.1 displays the complete process of participant laboratory selection. SCC formally notified all laboratories on September 8, 1999, of their selection, sponsor for each method (EPA or non-EPA), and level of participation (base or extended design).

Table 3.2. Referee laboratories selected for the WET Variability Study.

Referee laboratory	Method(s) supported	Date awarded
EA Engineering, Science and Technology, Inc.	*Ceriodaphnia* acute and chronic; Fathead acute and chronic; *Selenastrum* chronic	03/10/1999
EA Engineering, Science and Technology, Inc.	*Mysidopsis* chronic; Sheepshead acute and chronic	04/01/1999
Ogden Environmental and Energy Services, Inc.	Silverside acute and chronic	06/08/1999
MEC Analytical, Inc.	*Holmesimysis* acute	06/02/1999
EnviroSystems, Inc.	*Champia* chronic	04/14/1999

The results of laboratory prequalification and selection are displayed in Table 3.3 for each test method. Because only one and two laboratories were procured for the *Champia* chronic and *Holmesimysis* acute test methods respectively, interlaboratory testing was canceled for these two methods. For all other methods, 7 to 35 laboratories prequalified and were selected for participation. A total of 55 laboratories were selected to participate in the WET Variability Study, with many laboratories participating in multiple methods. See Appendix C for an alphabetical list of the participant laboratories.

3.6 Participant Laboratory Meeting

EPA invited all laboratories that submitted prequalification materials to attend a participant laboratory meeting held in Chicago, Illinois, on September 16, 1999. At the participant laboratory meeting, EPA and SCC staff presented the study plan for the WET Variability Study and highlighted participant laboratory tasks and requirements. Participant laboratories had the opportunity to meet EPA and SCC staff and to ask questions regarding the WET Variability Study and their responsibilities. At the meeting, EPA and SCC staff did not release any information regarding sample contents, sample descriptions, or sample distribution schemes that would jeopardize the blind aspect of the study. Following the meeting, SCC distributed meeting notes to all participant laboratories that were unable to attend.

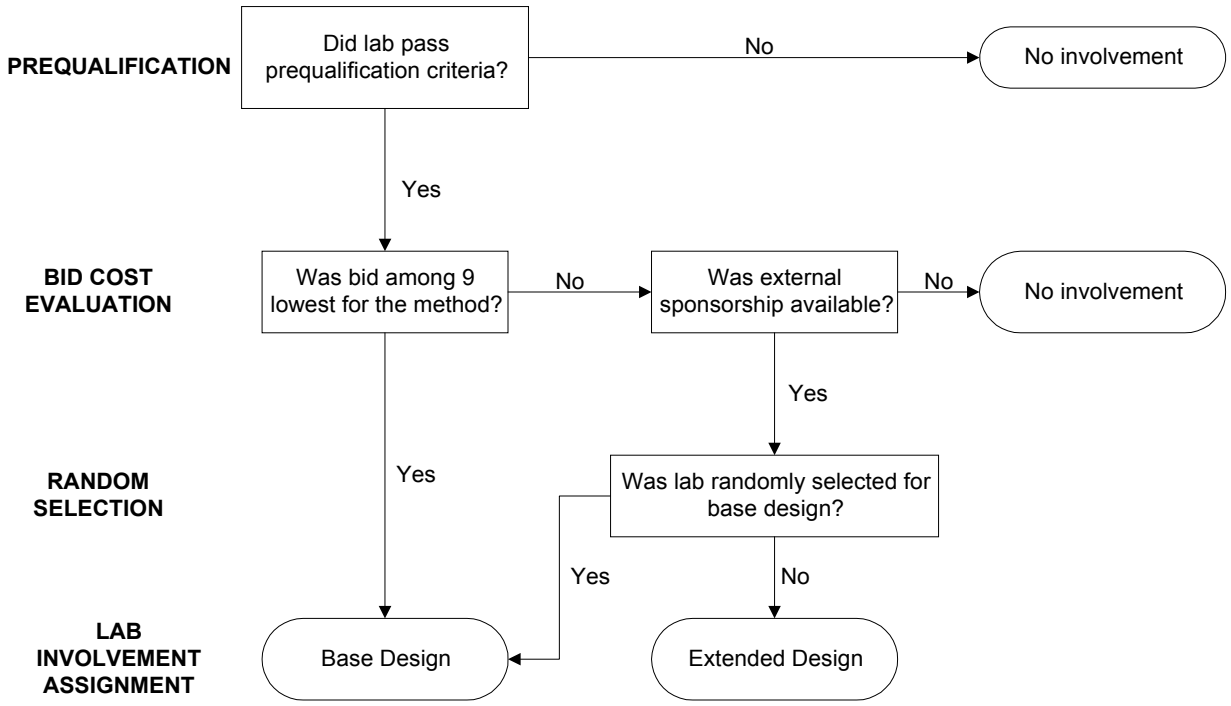

Figure 3.1 Participant laboratory selection process.

Table 3.3. Results of participant laboratory prequalification and selection.[a]

Test method	No. of labs solicited	No. of labs responding	No. of labs failing to prequalify	No. of labs selected for base study design		No. of labs selected for extended study design	Total no. of participant labs
				EPA sponsored labs	Non-EPA sponsored labs	Non-EPA sponsored labs	
Ceriodaphnia acute	319	48	2	9	11	8	28
Ceriodaphnia chronic	319	55	0	9	11	15	35
Fathead acute	319	50	1	9	11	9	29
Fathead chronic	319	49	0	9	11	7	27
Selenastrum chronic	319	16	2	9	2	0	11
Mysidopsis chronic	319	16	3	9	2	0	11
Sheepshead acute	319	7	0	7	0	0	7
Sheepshead chronic	319	9	2	7	0	0	7
Silverside acute	319	13	1	9	0	0	9
Silverside chronic	319	15	2	9	1	0	10
Champia chronic[b]	319	2	1	1	0	0	1
Holmesimysis acute[b]	319	2	0	2	0	0	2

[a] Appendix C contains an alphabetical list of the participant laboratories.
[b] Interlaboratory testing was canceled for this method due to insufficient participant laboratory support. Tests were performed by the referee laboratory only.

4.0 PRELIMINARY TESTING

Referee laboratories contracted to support each test method during the WET Variability Study were responsible for conducting preliminary testing of each sample type prior to interlaboratory testing. Preliminary testing was single-laboratory testing conducted at the referee laboratory to determine the appropriate composition of samples for use in the interlaboratory testing phase. A four-part preliminary testing plan was developed and instituted to provide background information on sample toxicity, necessary spiking concentrations, and the persistence of sample toxicity. Based on the results of preliminary testing, SCC selected appropriate sample sources and spiking levels for use in interlaboratory testing. The four parts of the preliminary testing plan consisted of the tests listed in Table 4.1 and accomplished the following specific objectives.

- **Part 1 - Background testing:** determine the suitability of effluent and receiving water sample matrices for use in the WET Variability Study through characterization of physical, chemical, and toxicological properties
- **Part 2 - Range-finding:** determine the appropriate spiking concentrations for the preparation of spiked effluent, receiving water, and reference toxicant sample types
- **Part 3 - Holding time testing:** determine the persistence of toxicity in spiked effluent and receiving water samples
- **Part 4 - Final preliminary testing:** assess whether test samples provided the desired range of toxicity following sample preparation, shipping, and handling

The specific requirements for each part of preliminary testing are described in Sections 4.1 to 4.4 below. These preliminary testing requirements were modified for the *Champia* chronic and *Holmesimysis* acute test methods after interlaboratory testing of these methods was canceled. With this cancellation, the objectives of any uncompleted preliminary tests were adjusted to better direct the use of preliminary test data toward assessing single-laboratory test precision rather than preparation for interlaboratory testing. Preliminary test results for these two methods are reported in Appendix D and summarized in Section 9 in lieu of interlaboratory test data. For all other methods, preliminary testing results (reported in Appendix D) were not used to assess test precision, but were used to support decisions regarding the selection of test samples for use in interlaboratory testing.

4.1 Part 1 - Background Testing

Part 1 of preliminary testing verified that selected effluent and receiving water sample matrix sources were acceptable for study use by assessing the physical, chemical, and toxicological characteristics of the samples. Referee laboratories were required to submit information on potential effluent and receiving water sample sources as part of their prequalification materials. EPA and SCC reviewed these materials, including historical information from the source, and made a preliminary selection of the effluent and receiving water sample sources for each test method. Following this determination, the referee laboratory initiated Part 1 of preliminary testing.

Table 4.1. Summary of preliminary testing requirements.

Test method	Part 1 - Background testing		Part 2 - Range-finding			Part 3 - Holding time testing		Part 4 - Final preliminary testing		
	Unspiked effluent	Unspiked receiving water	Spiked effluent	Spiked receiving water	Reference toxicant	Spiked effluent	Spiked receiving water	Spiked effluent	Spiked receiving water	Reference toxicant
Ceriodaphnia acute			✔	✔	✔	✔	✔	✔	✔	✔
Ceriodaphnia chronic	✔	✔	✔	✔	✔			✔	✔	✔
Fathead acute			✔	✔	✔	✔	✔	✔	✔	✔
Fathead chronic	✔	✔	✔	✔	✔			✔	✔	✔
Selenastrum chronic	✔[a]	✔[a]	✔[a]	✔[a]	✔[a]	✔[a]	✔[a]	✔[b]	✔[b]	✔[b]
Mysidopsis chronic	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
Sheepshead acute			✔	✔	✔	✔	✔	✔	✔	✔
Sheepshead chronic	✔	✔	✔	✔	✔			✔	✔	✔
Silverside acute			✔	✔	✔	✔	✔	✔	✔	✔
Silverside chronic	✔	✔	✔	✔	✔			✔	✔	✔
Champia chronic	✔	✔	✔[c]	✔	✔	✔[c]	✔	✔[c]	✔	✔
Holmesimysis acute	✔	✔	✔[c]	✔	✔	✔[c]	✔	✔[c]	✔	✔

[a] Test conducted with EDTA.
[b] Tests conducted with and without EDTA.
[c] Tests were conducted only on unspiked effluent samples.

Referee laboratories collected preliminary test samples according to Section 8 of the method manuals (USEPA, 1993; USEPA, 1994a; USEPA, 1994b). During Part 1 of preliminary testing, referee laboratories conducted physical and chemical analyses 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. For samples that were to be used in marine tests, salinity and copper also were measured. A smaller subset of parameters was analyzed for samples collected during Parts 2-4 of preliminary testing.

Following chemical and physical characterization of the sample matrices, a single background toxicity test using each of the test species was conducted on a sample from each effluent and receiving water source. If historical information (chemical analysis or toxicological analysis) on the effluent and receiving water matrix source was available, this information was evaluated along with results of background testing. Following completion of analysis and historical data gathering, a final determination of effluent and receiving water sample sources was made. The selection criteria for effluent and receiving water sample sources included the following elements.

- **Accessibility** - Selected effluent and receiving water sample sources were readily accessible to the referee laboratory. This included logistical accessibility as well as permission from the source provider to collect, test, and use the sample source in the WET Variability Study
- **Historic testing and experience** - It was important for referee laboratories to have significant experience in collecting and testing the selected effluent and receiving water samples. This experience and knowledge of historic testing allowed referee laboratories to identify conditions or characteristics of the source that could potentially pose problems in the WET Variability Study
- **Characterization** - Selected effluent and receiving water sample sources were well characterized by the referee laboratory through historic physical, chemical, and toxicological testing, as well as Part 1 of preliminary testing
- **Consistency** - Selected effluent and receiving water sample sources either provided a consistent level of toxicity or consistently produced no toxicity (in which case the sample could be spiked to achieve the desired effect level)

4.2 Part 2 - Range-finding

Part 2 of preliminary testing determined the range of spiking concentrations necessary to achieve a specific level of toxic effect for each sample type. This determination was critical to insuring that test concentrations used in interlaboratory testing bracketed the effect concentrations (LC50, IC25, IC50, and NOEC) evaluated in the WET Variability Study. During interlaboratory testing, participant laboratories were instructed to test each sample using a test concentration range of 6.25 - 100%, so it was important for test samples to produce measurable toxic effects within that test concentration range. Effluent and receiving water samples were spiked to produce target effect concentrations (LC50s for acute test methods and IC25s for chronic test methods) of 25% sample during interlaboratory testing. The reference toxicant sample was spiked to produce target effect concentrations of 50% sample during interlaboratory

testing. Spiking levels for the *Selenastrum* chronic test method were targeted to produce an IC50 of 38% sample during interlaboratory testing.

During Part 2 of preliminary testing, each matrix (effluent for the effluent sample, receiving water for the receiving water sample, and synthetic dilution water for the reference toxicant sample) was spiked at a range of concentrations estimated to encompass the desired effect concentrations (LC50s for acute test methods and IC25s for chronic test methods). Effluents and receiving water samples were not spiked if they possessed persistent toxicity and produced effect concentrations near the target effect level. Potassium chloride (KCl) was used as the spiking agent for freshwater methods, sheepshead acute and chronic methods, and the *Mysidopsis* chronic test method; copper sulfate ($CuSO_4$) was used as the spiking agent for silverside acute and chronic test methods, *Champia* chronic, and *Holmesimysis* acute test methods. Preliminary spiking levels for Part 2 testing were determined from referee laboratory reference toxicant testing databases, literature values, or range-finding tests conducted just prior to Part 2 testing.

Part 2 preliminary tests on spiked matrices were conducted as definitive tests according to the WET method manuals and specific requirements of the WET Variability Study plan (see Section 7). Part 2 testing was conducted for each test method and each sample matrix. If the results of Part 2 testing were not conclusive or if they differed greatly from historic reference toxicant testing conducted in the referee laboratory, Part 2 tests were repeated. Following Part 2 testing, appropriate spiking concentrations for interlaboratory testing were estimated. These spiking levels were estimated as the effect concentration determined in Part 2 testing divided by 25% (0.25) for effluent and receiving water samples and divided by 50% (0.5) for the reference toxicant sample. For example, if Part 2 testing for a given method determined an IC25 of 100 mg KCl/L in the effluent matrix, the final spiking concentration for the effluent sample type should be 400 mg KCl/L. This final spiking concentration was determined by dividing the Part 2 IC25 value (100 mg/L) by the target effect level of 25% (0.25) sample to obtain 400 mg KCl/L. When the final sample spiked at 400 mg KCl/L is diluted during interlaboratory testing using the standard 0.5 dilution factor (i.e., 100%, 50%, 25%, 12.5%, and 6.25% sample), the 25% sample test concentration should contain 100 mg KCl/L, and the test IC25 should be near the targeted 25% sample range.

4.3 Part 3 - Holding Time Testing

Part 3 of preliminary testing determined the persistence of toxicity in the effluent and receiving water samples. Excess volume of the spiked effluent and receiving water samples was retained from Part 2 testing and stored in the dark at 4°C. Following storage for seven days, a second test was conducted using the stored sample, and results were compared to that of the initial test. If effluent or receiving water samples were not spiked, Part 3 testing was conducted on excess unspiked effluent and receiving water sample collected in Part 1. For acute and chronic test methods using the same species, Part 3 testing was conducted using only the acute test method. The results of holding time testing provided valuable information on the persistence of sample toxicity. This information was useful in the timing and scheduling of referee laboratory sample preparation for interlaboratory testing. This information also

supported the assumption that sample toxicity remained constant when testing at a given participant laboratory was delayed due to problems with sample shipment or organism availability.

4.4 Part 4 - Final Preliminary Testing

Part 4 of preliminary testing validated that the samples and spiking concentrations (if applicable) selected for the WET Variability Study achieved the desired range of effect following sample preparation, shipping, and handling. Spiking concentrations for Part 4 testing were determined from the results of Part 2 testing with necessary adjustments to meet the target effect levels. Part 4 testing also served as a trial run for sample collection, preparation, packaging, and shipment in the interlaboratory testing phase. Each sample type that was used in final preliminary testing was collected, prepared, packaged, and shipped exactly as described for interlaboratory testing (see Sections 5 and 6). Final preliminary testing samples were shipped by the referee laboratory round-trip back to the referee laboratory (e.g., sent from the referee laboratory on one day for delivery back to that same facility the next day). Upon receipt, the referee laboratory conducted final preliminary tests as described for interlaboratory testing (see Section 7). Part 4 testing was used to determine if the selected spiking concentrations achieved the targeted effect levels following sample preparation and shipment. Spiking levels that produced effect concentrations within the range of 10-35% for effluent and receiving water samples or 35-60% for reference toxicant samples were considered appropriate for use in the interlaboratory testing phase. Based on the results from Part 4 preliminary testing, SCC selected the sample composition (matrix, spiking agent, and spiking levels) for use in interlaboratory testing. Referee laboratories then prepared (see Section 5) and shipped (see Section 6) samples to participant laboratories for interlaboratory testing (see Section 7).

5.0 SAMPLE PREPARATION

For each method, four test sample types were prepared in bulk by the referee laboratory, divided, and distributed to participant laboratories for testing. The four sample types included a blank sample, a reference toxicant sample, an effluent sample, and a receiving water sample. This section describes the preparation of these test samples for use in interlaboratory testing. The test sample types and appropriate spiking concentrations were selected based on preliminary testing conducted by the referee laboratories (see Section 4 and Appendix D).

5.1 Freshwater Methods

For the freshwater methods, Table 5.1 describes each of the sample types that were prepared and distributed for interlaboratory testing by the referee laboratory (EA Engineering, Science and Technology, Inc.). The blank sample type for all freshwater test methods, with the exception of the *Selenastrum* chronic method, consisted of moderately hard synthetic freshwater prepared according to Section 7 of the WET method manual (USEPA, 1994a). The blank sample type was prepared by adding the appropriate amounts of reagents (Section 7, USEPA, 1994a) to deionized water in cleaned and rinsed 5-gallon or 3-gallon (depending on the volume needed for interlaboratory testing) high-density polypropylene (HDPP) carboys. Following preparation, the bulk blank sample was thoroughly mixed and aerated for at least 24 hours (as required in Section 7, USEPA, 1994a) prior to removing aliquots for packaging and distribution to participant laboratories. For the *Selenastrum* chronic test method, the blank sample was prepared as deionized water.

The reference toxicant sample type for all freshwater test methods consisted of the blank sample matrix (moderately hard synthetic freshwater for *Ceriodaphnia* and fathead test methods and deionized water for the *Selenastrum* chronic method) spiked with KCl. Moderately hard synthetic freshwater was prepared by adding the appropriate amounts of reagents (Section 7, USEPA, 1994a) to deionized water in cleaned and rinsed 5-gallon or 3-gallon (depending on the volume needed for interlaboratory testing) HDPP carboys. The appropriate amount of reagent grade KCl was then added to the moderately hard synthetic freshwater (or deionized water for the *Selenastrum* chronic method) to achieve the spiking concentrations listed in Table 5.2. When ampule samples were reconstituted according to instructions provided in the participant laboratory SOPs (Appendix B), the resulting reconstituted sample yielded the spiking concentrations listed in Table 5.1.

Table 5.1. Description of samples used for freshwater methods in the WET Variability Study.

Test method	Sample type	Sample matrix	Spiking agent	Spiked concentration[a] (mg/L as KCl)	Collection date	Preparation date	Shipment date
Ceriodaphnia acute	Blank	MHSF[b]	None	-	-	11/06/99	11/08/99, 11/10/99
	Reference toxicant	MHSF[b]	KCl	1000	-	11/06/99	11/08/99, 11/10/99
	Effluent	Municipal effluent	KCl	2680	11/08/99	11/09/99	11/10/99
	Receiving water	River water	KCl	1800	11/05/99	11/06/99	11/08/99
Ceriodaphnia chronic	Blank	MHSF[b]	None	-	-	10/09/99	10/11/99, 10/25/99
	Reference toxicant	MHSF[b]	KCl	270	-	10/09/99	10/11/99, 10/25/99
	Effluent	Municipal effluent	KCl	2100	10/22/99	10/24/99	10/25/99
	Receiving water	River water	KCl	1200	10/08/99	10/10/99	10/11/99
Fathead acute	Blank	MHSF[b]	None	-	-	10/19/99	10/20/99, 11/03/99
	Reference toxicant	MHSF[b]	KCl	2200	-	10/19/99	10/20/99, 11/03/99
	Effluent	Municipal effluent	KCl	5328	11/01/99	11/02/99	11/03/99
	Receiving water	River water	KCl	5000	10/18/99	10/19/99	10/20/99
Fathead chronic	Blank	MHSF[b]	None	-	-	09/24/99	09/27/99, 10/04/99
	Reference toxicant	MHSF[b]	KCl	1220	-	09/24/99	09/27/99, 10/04/99
	Effluent	Municipal effluent	KCl	3600	10/01/99	10/03/99	10/04/99
	Receiving water	River water	KCl	2400	09/24/99	09/26/99	09/27/99
Selenastrum chronic	Blank	Deionized water	None	-	-	03/14/00	03/15/00, 03/22/00, 03/29/00
	Reference toxicant	Deionized water	KCl	5655	-	03/07/00	03/08/00, 03/15/00, 03/22/00, 03/29/00
	Effluent	Municipal effluent	KCl	11540	03/06/00	03/07/00	03/08/00, 03/15/00
	Receiving water	River water	KCl	11713	03/20/00	03/21/00	03/22/00

[a] Spiking concentrations are nominal values. Spiked concentrations for reference toxicant samples represent concentrations after proper reconstitution of the ampule sample. See Table 5.2 for a description of ampule sample contents.

[b] Moderately hard synthetic freshwater prepared according to Section 7 of the WET method manual (USEPA, 1994a).

Table 5.2. Spiking concentrations in reference toxicant ampule samples.

Test method[a]	Reference toxicant	Spiked conc. in prepared ampule sample (mg/L)[b]	Volume of ampule sample added to reconstituted sample (mL)	Reconstituted sample volume (L)	Resulting conc. in reconstituted sample (mg/L)[b]
Ceriodaphnia acute	KCl	10,000	100	1	1,000
Ceriodaphnia chronic	KCl	8,100	100	3	270
Fathead acute	KCl	88,000	100	4	2,200
Fathead chronic	KCl	97,600	100	8	1,220
Selenastrum chronic	KCl	113,100	200	4	5,655
Mysidopsis chronic	KCl	108,000	100	9	1,200
Sheepshead acute	KCl	25,280	500	4	3,160
Sheepshead chronic	KCl	90,000	500	15	3,000
Silverside acute	$CuSO_4$	40.0	100	4	1.0
Silverside chronic	$CuSO_4$	210	100	21	1.0

[a] Interlaboratory testing was not conducted for the *Champia* chronic and *Holmesimysis* acute test methods.
[b] Spiking concentrations are nominal values.

The effluent sample type for all freshwater test methods consisted of a freshwater municipal effluent spiked with KCl. The effluent was collected from a municipal wastewater treatment plant that is designed to treat 180 mgd, is able to handle peak flows of 400 mgd, and currently treats 140 to 150 mgd. The facility employs tertiary treatment for biological nutrient removal including single-stage nitrification/ denitrification, sand filtration, chlorination/dechlorination, and anaerobic digestion. Effluent was collected at this site from a small access pipe through which effluent was pumped from the main discharge outfall. Sample was collected from this access pipe using a funnel and tubing to fill 5-gallon HDPP carboys. Sample was then immediately transported to the referee laboratory at ambient temperature. Upon arrival, the referee laboratory stored the sample in the dark at <4°C. Table 5.3 lists the volume of effluent collected for each test method. The effluent sample was then homogenized in 50-gallon HDPP containers. If the volume of the sample collected for interlaboratory testing was greater than 50 gallons for a given test method, the sample was stored and homogenized in multiple 50-gallon containers connected with piping. Submersible pumps were used to circulate and homogenize the sample among individual containers. Following homogenization, the effluent sample for each test method was spiked at the appropriate concentration with KCl (Table 5.1). The appropriate amount of KCl was initially dissolved in a small volume of effluent and then added to the bulk effluent sample. When multiple containers were used, equal amounts of KCl were added to each container.

The receiving water sample type for all freshwater test methods consisted of a natural river water spiked with KCl. The receiving water was collected from the Gunpowder River near Falls Road, in Baltimore County, Maryland. Receiving water was collected by filling 1-gallon HDPP containers under the surface of the water. Receiving water sample was then transferred to 5-gallon HDPP containers and immediately transported to the referee laboratory at ambient temperature. Upon arrival at the referee laboratory, the sample was stored at <4°C in the dark. Table 5.4 lists the volume of receiving water collected for each

test method. The receiving water sample was then homogenized and spiked as described in the preceding paragraph for the effluent sample type.

Bulk samples for all sample types were prepared in cleaned and rinsed containers. Containers were cleaned with detergent, hydrochloric acid, acetone, and rinsed with deionized water. All bulk sample preparations were mixed thoroughly prior to spiking, following spiking, and prior to removing aliquots for distribution to participant laboratories. All bulk samples were stored in the dark at <4°C prior to shipment to participant laboratories.

Table 5.3. Effluent sample volumes collected for interlaboratory testing.

Test method[a]	Volume required per sample (L)	Number of participant lab samples required	Minimum required volume (L)[b]	Collected volume (L)
Ceriodaphnia acute	1	28	28	45
Ceriodaphnia chronic	7	28	196	270
Fathead acute	4	28	112	150
Fathead chronic	17.5	28	490	660
Selenastrum chronic	4	16	64	100
Mysidopsis chronic	21	16	336	450
Sheepshead acute	4	8	32	60
Sheepshead chronic	35	8	280	375
Silverside acute	4	13	52	60
Silverside chronic	49	14	686	1000

[a] Interlaboratory testing was not conducted for the *Champia* chronic and *Holmesimysis* acute test methods.
[b] Minimum required volume = Volume per sample X number of samples.

Table 5.4. Receiving water sample volumes collected for interlaboratory testing.

Test method[a]	Volume required per sample (L)	Number of participant lab samples required	Minimum required volume (L)[b]	Collected volume (L)
Ceriodaphnia acute	1	14	14	19
Ceriodaphnia chronic	7	14	98	135
Fathead acute	4	14	56	90
Fathead chronic	17.5	14	245	345
Selenastrum chronic	4	9	36	60
Mysidopsis chronic	21	9	189	278
Sheepshead acute	4	8	32	75
Sheepshead chronic	35	8	280	389
Silverside acute	4	7	28	36
Silverside chronic	49	8	392	540

[a] Interlaboratory testing was not conducted for the *Champia* chronic and *Holmesimysis* acute test methods.
[b] Minimum required volume = Volume per sample X number of samples.

5.2 Marine Methods

For the marine methods, Table 5.5 describes each of the sample types that were prepared and distributed for interlaboratory testing by the referee laboratory (EA Engineering, Science and Technology, Inc. for the *Mysidopsis* chronic and sheepshead acute and chronic test methods; Ogden Environmental and Energy Services, Inc. for the silverside acute and chronic test methods).

5.2.1 *Mysidopsis* Chronic and Sheepshead Acute and Chronic Test Methods

The blank sample type for the *Mysidopsis* chronic, sheepshead acute, and sheepshead chronic test methods consisted of synthetic seawater prepared at a salinity of 25 ppt. The blank sample type was prepared by adding the appropriate amounts of bioassay grade Forty Fathoms® artificial sea salts to deionized water in cleaned and rinsed 5-gallon or 3-gallon (depending on the volume needed for interlaboratory testing) HDPP carboys. Following preparation, the bulk blank sample was thoroughly mixed to dissolve the added reagents.

The reference toxicant sample type for the *Mysidopsis* chronic, sheepshead acute, and sheepshead chronic test methods consisted of deionized water spiked with KCl. For these test methods, deionized water rather than artificial seawater was spiked with KCl to improve the solubility of KCl in the highly concentrated reference toxicant ampule samples. The appropriate amount of reagent grade KCl was added to deionized water in cleaned and rinsed 5-gallon or 3-gallon (depending on the volume needed for interlaboratory testing) HDPP carboys to achieve the spiking concentrations listed in Table 5.2 for the reference toxicant samples. When ampule samples were reconstituted according to instructions provided in the participant laboratory SOPs (see Appendix B), the resulting reconstituted sample yielded the spiking concentrations listed in Table 5.5.

The effluent sample type for the *Mysidopsis* chronic, sheepshead acute, and sheepshead chronic test methods consisted of a municipal effluent spiked with KCl. The municipal effluent described previously for freshwater methods (see Section 5.1) also was used for the *Mysidopsis* chronic, sheepshead acute, and sheepshead chronic test methods. The effluent was collected and transported to the referee laboratory as previously described for the freshwater methods (see Section 5.1). Table 5.3 lists the volume of effluent collected for each test method. The effluent sample was then homogenized in 50-gallon HDPP containers. If the volume collected and necessary for interlaboratory testing was greater than 50 gallons for a given test method, the sample was stored and homogenized in multiple 50-gallon containers connected with piping. Submersible pumps were used to circulate and homogenize sample among individual containers. Following homogenization, the salinity of the effluent sample was adjusted by the addition of bioassay grade Forty Fathoms® artificial sea salts. Salinity of the effluent sample was adjusted to 23 ppt, 20 ppt, and 21 ppt for the *Mysidopsis* chronic, sheepshead acute, and sheepshead chronic test methods, respectively. Since the addition of the KCl spike increased salinity, the initial salinity levels were selected to achieve a final salinity of 25 ppt in the effluent sample following spiking with KCl. Following salinity adjustment, the effluent sample was spiked at the appropriate concentration with KCl (Table 5.5). The appropriate amount of KCl was initially dissolved in a small volume of

Table 5.5. Description of samples used for marine methods in the WET Variability Study.

Test method[a]	Sample type	Sample matrix	Spiking agent	Spiked concentration[b] (mg/L as KCl and as Cu)	Collection date	Preparation date	Shipment date
Mysidopsis chronic	Blank	synthetic seawater[c]	None	-	-	02/18/00	02/21/00, 02/28/00
	Reference toxicant	synthetic seawater[c]	KCl	1200	-	02/18/00	02/21/00, 02/28/00
	Effluent	municipal effluent	KCl	2000	02/16/00	02/18/00	02/21/00
	Receiving water	natural seawater	KCl	2400	02/24/00	02/25/00	02/28/00
Sheepshead acute	Blank	synthetic seawater[c]	None	-	-	03/03/00	03/06/00
	Reference toxicant	synthetic seawater[c]	KCl	3160	-	03/10/00	03/13/00
	Effluent	municipal effluent	KCl	5200	03/06/00	03/10/00	03/13/00
	Receiving water	natural seawater	KCl	5600	02/24/00	03/03/00	03/06/00
Sheepshead chronic	Blank	synthetic seawater[c]	None	-	-	03/17/00	03/20/00
	Reference toxicant	synthetic seawater[c]	KCl	3000	-	03/25/00	03/27/00
	Effluent	municipal effluent	KCl	4880	03/23/00	03/24/00	03/27/00
	Receiving water	natural seawater	KCl	4400	03/17/00	03/17/00	03/20/00
Silverside acute	Blank	synthetic seawater[c]	None	-	-	10/31/99	11/01/99, 11/08/99
	Reference toxicant	synthetic seawater[c]	$CuSO_4$	1.000	-	10/31/99	11/01/99, 11/08/99
	Effluent	industrial effluent	$CuSO_4$	0.922	11/03/99	11/07/99	11/08/99
	Receiving water	natural seawater	$CuSO_4$	0.565	10/30/99	10/31/99	11/01/99
Silverside chronic	Blank	synthetic seawater[c]	None	-	-	10/17/99	10/18/99, 10/25/99
	Reference toxicant	synthetic seawater[c]	$CuSO_4$	1.000	-	10/17/99	10/18/99
	Effluent	industrial effluent	$CuSO_4$	0.800	10/20/99	10/23/99	10/25/99
	Receiving water	natural seawater	$CuSO_4$	0.494	10/16/99	10/17/99	10/18/99

[a] Interlaboratory testing was not conducted for the *Champia* chronic and *Holmesimysis* acute test methods.
[b] Spiking concentrations are nominal values. Spiked concentrations for reference toxicant samples represent concentrations after proper reconstitution of the ampule sample. See Table 5.2 for a description of ampule sample contents.
[c] Synthetic seawater was prepared using bioassay grade Forty Fathoms® added to deionized water.

effluent and then added to the bulk effluent sample. When multiple containers were used, equal amounts of KCl were added to each container.

The receiving water sample type for the *Mysidopsis* chronic, sheepshead acute, and sheepshead chronic test methods consisted of a natural seawater spiked with KCl. Receiving water was collected from Manasquan Inlet, in Manasquan, Monmouth County, New Jersey. Seawater was collected within approximately 1 hour of high tide using a submersible pump, and transferred into 5-gallon HDPP carboys for transport. The seawater sample collected for each test method was filtered through a 5-µm filter either at the time of collection or upon receipt at the referee laboratory. The receiving water sample was transported to the referee laboratory at ambient temperature. Upon arrival, the referee laboratory stored the sample in the dark at <4°C. Table 5.4 lists the volume of receiving water collected for each test method. The receiving water sample was then homogenized and spiked as described in the preceding paragraph for the effluent sample type.

Bulk samples for all sample types were prepared in cleaned and rinsed containers. Containers were cleaned with detergent, hydrochloric acid, acetone, and rinsed with deionized water. All bulk sample preparations were mixed thoroughly prior to spiking, following spiking, and prior to removing aliquots for distribution to participant laboratories. All bulk samples were stored in the dark at <4°C prior to shipment to participant laboratories.

5.2.2 Silverside Acute and Chronic Test Methods

The blank sample type for the silverside acute and chronic test methods consisted of synthetic seawater prepared at a salinity of 25 ppt. The blank sample type was prepared by adding the appropriate amounts of bioassay grade Forty Fathoms® artificial sea salts to deionized water in polycarbonate carboys. Following preparation, the bulk blank sample was thoroughly mixed to dissolve the added reagents.

The reference toxicant sample type for the silverside acute and chronic test methods consisted of the blank sample matrix (synthetic seawater at 25 ppt salinity) spiked with $CuSO_4$. The appropriate volume of a concentrated $CuSO_4$ stock solution was added to synthetic seawater in 10-L polycarbonate containers to achieve the spiking concentrations listed in Table 5.2 for the reference toxicant ampule sample. When ampule samples were reconstituted according to instructions provided in the participant laboratory SOPs, the resulting reconstituted sample yielded the spiking concentrations listed in Table 5.5.

The effluent sample type for the silverside acute and chronic test methods consisted of an industrial effluent spiked with $CuSO_4$. The effluent was collected from an industrial wastewater treatment facility that treats wastes from an oil refinery. Effluent was collected using an automatic sampler set to collect a single grab sample. Sample was collected and stored in 5-gallon buckets with 4mm polyethylene liners and plastic lids for transport to the referee laboratory. Upon arrival, the referee laboratory stored the sample in the dark at <4°C. Table 5.3 lists the volume of effluent collected for each test method. The effluent sample was then homogenized in 250-L polycarbonate containers using a mechanical mixer. The volume required for the silverside chronic test method necessitated the preparation of separate batches of

effluent sample prior to shipment of test samples for test initiation, the first renewal shipment, and the second renewal shipment. For each batch, it was necessary to mix and hold sample in two 250-L containers. The sample was homogenized among the two containers by repeatedly (six times) adding 40% of one container to the other and mixing each time. For the silverside acute test method, all effluent sample was mixed and prepared in a single 250-L container. Following homogenization, the salinity of the effluent sample was adjusted to 25 ppt by the addition of bioassay grade Forty Fathoms® artificial sea salts. The effluent sample was then spiked by adding the appropriate volume of a concentrated $CuSO_4$ stock solution to achieve the spiking concentrations listed in Table 5.5.

The receiving water sample type for the silverside acute and chronic test methods consisted of a natural seawater spiked with $CuSO_4$. Natural seawater was collected from the Scripps Institution of Oceanography seawater system in La Jolla, CA. Seawater was pumped from a fixed collection site 320m offshore of La Jolla, CA, filtered through a sand filter, and trucked to the referee laboratory. At the referee laboratory, the seawater was incorporated into the laboratory's flow-through seawater system that includes two 2,200-gallon storage tanks, an in-line 20-µm filter, and an in-line heater/chiller unit. The salinity of receiving water (initially 34 ppt) was adjusted to 25 ppt with the addition of deionized water. The bulk receiving water sample then was homogenized in 250-L polycarbonate containers and spiked by adding the appropriate volume of a concentrated $CuSO_4$ stock solution to achieve the spiking concentrations listed in Table 5.5.

Bulk samples for all sample types were prepared in cleaned and rinsed containers. Containers were cleaned with detergent, rinsed with tap water, then deionized water, and rinsed again with sample. All bulk sample preparations were mixed thoroughly prior to spiking, following spiking, and prior to removing aliquots for distribution to participant laboratories. All bulk samples were stored in the dark at <4°C prior to shipment to participant laboratories.

5.3 Problems Encountered in Sample Preparation

The reference toxicant sample prepared for the *Ceriodaphnia* chronic test method produced toxicity in only some of the participant laboratories (see Section 9.3). The referee laboratory also did not detect toxicity in this sample during the interlaboratory testing phase. It was determined that the reference toxicant sample prepared for interlaboratory testing was spiked at a level that was only slightly toxic and very near the minimum detection level (100% sample). Depending on the sensitivity of test organisms at individual laboratories, some laboratories identified the sample as toxic, while other laboratories did not. The spiking level used for this sample was based on preliminary testing results from three tests that indicated an IC25 of 138, 132, and 134 mg KCl/L. The average of these tests (135 mg KCl/L) was multiplied by two to obtain the target spiking level of 270 mg KCl/L. It should be noted that one of the preliminary tests produced an IC25 of 320 mg/L KCl, indicating that 135 mg KCl/L may have been a low estimate of the IC25 for KCl.

The reference toxicant sample for the silverside acute test also did not produce a toxic response in interlaboratory testing. This was caused by precipitation of the spiked copper in the liquid ampule

sample. Precipitation of copper in seawater samples also was observed in preliminary testing for the *Mysidopsis* and sheepshead test methods, so the spiking agent for these methods was changed (see Appendix D, Section D.9). For the silverside methods, this potential problem was not identified during preliminary testing so no change in the spiking agent was made prior to interlaboratory testing. Part 4 preliminary testing of the reference toxicant sample did not reveal copper precipitation and produced an LC50 of 0.29 mg Cu/L. The same spiking levels used in Part 4 testing were used for the interlaboratory sample, yet results for that sample showed precipitation and no toxicity. The referee laboratory discovered that in Part 4 preliminary testing, the reference toxicant ampule sample was prepared using deionized water, but the interlaboratory sample was prepared using synthetic seawater. To confirm that this difference in sample matrix resulted in the non-toxic interlaboratory sample, the referee laboratory prepared two additional reference toxicant samples at the same spiking level used for interlaboratory testing. One sample was prepared using deionized water and one was prepared using synthetic seawater. Testing results from the sample prepared using deionized water were consistent with Part 4 preliminary testing results (LC50 of 25.5% sample), and results from the ampule sample prepared using synthetic seawater were consistent with interlaboratory testing results (LC50 >100% sample). These additional tests confirmed that preparation of the reference toxicant ampule sample using synthetic seawater caused the precipitation of the spiked copper and produced a nontoxic sample for interlaboratory testing.

6.0 PACKAGING AND DISTRIBUTION OF TEST SAMPLES

6.1 Sample Distribution Scheme

Laboratories participating in the base study design (see Sections 2.2.3 and 3.5) each received four blind test samples; laboratories participating in the extended study design each received three blind test samples. The sample distribution scheme in Table 6.1 shows the number and type of samples distributed to each laboratory according to the laboratory's assigned position in the study. EPA-sponsored laboratories were randomly assigned among positions 1-9, non-EPA sponsored laboratories in the base study design were randomly assigned among positions 10-20, and laboratories participating in the extended study design were randomly ordered in positions 21- the total number of laboratories. As noted in Table 6.1, an alternate sample distribution scheme was used for the sheepshead acute and chronic test methods. Since fewer than nine laboratories participated in these methods, the sample distribution scheme would not have met the data quality objective of obtaining six data sets for each test method and sample type. For these test methods, one of each of the sample types was distributed to each of the seven participant laboratories.

6.2 Packaging and Shipment of Samples

After bulk test samples were prepared according to Section 5, each bulk test sample was divided into individual test sample aliquots for shipment to participant laboratories. Test sample aliquots were divided into containers appropriate for the individual test sample volumes. High-density polypropylene sample containers, of the appropriate size (refer to Tables 6.2 and 6.3) were pre-rinsed, filled with the sample, and sealed with zero head-space. All blank and reference toxicant samples were prepared and packaged as ampule samples. Ampule samples were small volume (generally 100 mL) liquid samples that were reconstituted at participant laboratories to provide the necessary test sample volume. For the *Selenastrum* chronic test and the sheepshead acute and chronic tests, larger volumes (200 mL, 500 mL, and 500 mL, respectively) were used for ampule samples to reduce the concentration of KCl in the reference toxicant ampule sample and avoid potential solubility problems. All ampule samples (blank and reference toxicant sample types) for a given test method were shipped in the same container style and size. Effluent and receiving water samples were prepared and packaged as whole volume samples in HDPP cubitainers. All whole volume samples for a given test method were shipped in the same container style and size. Tables 6.2 and 6.3 show the volumes and numbers of samples prepared for freshwater and marine test methods, respectively.

For chronic test methods that required daily renewal, samples were packaged and shipped in three separate aliquots. The first aliquot (Initiation) was received by the participant laboratory on test Day 0 and was used for test initiation on Day 0 and test renewal on Day 1. The second aliquot (Renewal 1) was received on test Day 2 and used for test renewals on Day 2 and Day 3. The final aliquot (Renewal 2) was received on test Day 4 and used for test renewal on Day 4, Day 5, Day 6, and Day 7 if necessary (for the *Ceriodaphnia* chronic test method). For ampule samples, all three separate aliquots were received on test Day 0, and participant laboratories were instructed to reconstitute the samples following the scheme

described above (separate aliquots reconstituted on test Days 0, 2, and 4). For all acute test methods, a single aliquot of sample was received and used for test initiation and any required renewals.

Table 6.1. Sample distribution scheme for the WET Variability Study.[a]

Participant laboratory assigned position		Number of samples of each type received			
		Blank	Effluent	Receiving water	Reference toxicant
Base study design EPA-sponsored	1	1	1	1	1
	2	1	2	1	0
	3	1	0	1	2
	4	1	1	0	2
	5	0	2	1	1
	6	1	1	0	2
	7	0	2	1	1
	8	1	1	0	2
	9	0	2	1	1
Base study design non-EPA sponsored	10	1	1	1	1
	11	1	2	1	0
	12	1	0	1	2
	13	0	2	0	2
	14	0	2	0	2
	15	1	2	1	0
	16	1	0	1	2
	17	0	2	0	2
	18	0	2	0	2
	19	1	2	1	0
	20	1	0	1	2
Extended study design non-EPA sponsored	21 - up (odd #s)	2	0	0	1
	21 - up (even #s)	1	0	0	2

[a] This sample distribution scheme was used for interlaboratory testing of all test methods except the sheepshead acute and chronic test methods. For these test methods, one of each of the sample types was distributed to each of the seven participant laboratories.

Samples were cooled to <4°C prior to shipment and then packed in coolers (e.g., 28, 48, 54-qt) containing wet ice. Depending on the test method performed by an individual participant laboratory, multiple test samples were shipped in one cooler if possible to reduce the number of coolers shipped. Test sample volumes that exceeded the maximum weight limit for overnight shipping were divided into separate coolers for shipment. Duplicate test sample aliquots were shipped in the same cooler whenever possible; if test sample volume prohibited shipping duplicates in the same cooler, they were shipped under the same airbill to ensure they were shipped together. All samples were shipped FedEx Priority Overnight for delivery on the day of scheduled testing (see Section 2.2.4). Referee laboratories conducted testing

simultaneously with participant laboratories on samples prepared identically to those for interlaboratory testing and shipped round-trip back to the referee laboratory.

6.3 Sample Tracking

Each WET test method received an EPA episode number to designate samples prepared for that test method. Each sample aliquot that was prepared and shipped was assigned a unique sample number and was accompanied by an EPA traffic report form. Duplicate samples received different sample numbers to retain the blind sample aspect of the study design. For chronic test methods that required additional shipments for sample renewal, the sample number remained 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 was clearly and permanently marked on each container and the accompanying EPA traffic report form. Sample numbers for each test method are given in Table 6.4. Following completion of the study, each test sample was assigned a sample code (in addition to the sample number previously assigned) as an alternate unique identifier. For the results section of this report, samples are identified by sample codes to aid in blinding the identity of individual participant laboratories.

Referee laboratories included an EPA traffic report form with each sample that was shipped to document the chain-of-custody for that sample. The traffic report form (see Appendix B) identified 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 was placed in a waterproof enclosure (e.g., Ziploc® bag) and packed in the cooler with the respective sample. Each cooler used in the study was 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.

For each shipment event, the referee laboratory also completed a sample shipment documentation form. The referee laboratory faxed this form to SCC immediately after sample pickup by FedEx. The sample shipment form documented the following information for each shipping event:

- Sample number - the unique identifying number for each sample aliquot
- Sample description - identified the sample as either blank, spiked effluent, spiked receiving water, or reference toxicant
- Participant laboratory name - name of the laboratory to which the sample was shipped
- Airbill number - the overnight shipping service's number that identified each individual shipment
- Size of test containers - the volume of the test container in which the sample was shipped
- Cooler number - a unique identifying number for the cooler in which the sample was shipped.
- Comments - any miscellaneous information related to sample shipment.

Table 6.2. Number and volume of samples required for freshwater methods in the WET Variability Study.

Test method	Sample type	Ampule or whole volume sample	Sample volume[a]	Number of samples required[b]			
				Initiation	Renewal 1	Renewal 2	Total
Ceriodaphnia acute	Blank	Ampule	100 mL	34	-	-	34
	Reference toxicant	Ampule	100 mL	32	-	-	32
	Effluent	Whole volume	1 L	28	-	-	28
	Receiving water	Whole volume	1 L	14	-	-	14
Ceriodaphnia chronic	Blank	Ampule	100 mL	35	35	35	105
	Reference toxicant	Ampule	100 mL	49	49	49	147
	Effluent	Whole volume	2 - 3 L	28	28	28	84
	Receiving water	Whole volume	2 - 3 L	14	14	14	42
Fathead acute	Blank	Ampule	100 mL	28	-	-	28
	Reference toxicant	Ampule	100 mL	39	-	-	39
	Effluent	Whole volume	4 L	30	-	-	30
	Receiving water	Whole volume	4 L	14	-	-	14
Fathead chronic	Blank	Ampule	100 mL	25	25	25	75
	Reference toxicant	Ampule	100 mL	38	38	38	114
	Effluent	Whole volume	5 - 7.5 L	28	28	28	84
	Receiving water	Whole volume	5 - 7.5 L	14	14	14	42
Selenastrum chronic	Blank	Ampule	200 mL	9	-	-	9
	Reference toxicant	Ampule	200 mL	14	-	-	14
	Effluent	Whole volume	4 L	16	-	-	16
	Receiving water	Whole volume	4 L	9	-	-	9

[a] For chronic test methods, sample volumes ranged depending upon the aliquot. More volume was required for the Renewal 2 aliquot than the Initiation or Renewal 1 aliquots.
[b] Number of samples includes samples shipped round-trip back to referee laboratory.

Table 6.3. Number and volume of samples required for marine methods in the WET Variability Study.

Test method[a]	Sample type	Ampule or whole volume sample	Sample volume[b]	Number of samples required[c]			
				Initiation	Renewal 1	Renewal 2	Total
Mysidopsis chronic	Blank	Ampule	100 mL	9	9	9	27
	Reference toxicant	Ampule	100 mL	14	14	14	42
	Effluent	Whole volume	6 - 9 L	16	16	16	48
	Receiving water	Whole volume	6 - 9 L	9	9	9	27
Sheepshead acute	Blank	Ampule	500 mL	8	-	-	8
	Reference toxicant	Ampule	500 mL	8	-	-	8
	Effluent	Whole volume	4 L	8	-	-	8
	Receiving water	Whole volume	4 L	8	-	-	8
Sheepshead chronic	Blank	Ampule	500 mL	8	8	8	24
	Reference toxicant	Ampule	500 mL	8	8	8	24
	Effluent	Whole volume	10 - 15 L	8	8	8	24
	Receiving water	Whole volume	10 - 15 L	8	8	8	24
Silverside acute	Blank	Ampule	100 mL	7	-	-	7
	Reference toxicant	Ampule	100 mL	13	-	-	13
	Effluent	Whole volume	4 L	13	-	-	13
	Receiving water	Whole volume	4 L	7	-	-	7
Silverside chronic	Blank	Ampule	100 mL	8	8	8	24
	Reference toxicant	Ampule	100 mL	14	14	14	42
	Effluent	Whole volume	14 - 21 L	14	14	14	42
	Receiving water	Whole volume	14 - 21 L	8	8	8	24

[a] Interlaboratory testing was not conducted for the *Champia* chronic and *Holmesimysis* acute test methods.
[b] For chronic test methods, sample volumes ranged depending upon the aliquot. More volume was required for the Renewal 2 aliquot than the Initiation or Renewal 1 aliquots.
[c] Number of samples includes samples shipped round-trip back to referee laboratory.

SCC entered shipment information into a database and used this information on the day of expected sample arrival to track the delivery of each sample through the FedEx automated shipment tracking system. If sample shipment problems were encountered, SCC notified participant laboratories of the problem and instructed the laboratory how to proceed. The instructions provided by SCC were specific to the individual case, but laboratories were generally instructed to initiate testing on the day of sample arrival if the sample was delivered prior to the close of business. If the delivery of renewal samples was delayed, laboratories were instructed to renew the test on time with remaining sample from the previous shipment. If sample shipments could not be located or if tracking did not indicate progress of the sample, the referee laboratory was instructed to resend the sample (prepared from the remaining bulk sample) for delivery the following day.

Upon receipt of each sample, participant laboratories were 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 faxed the completed traffic report form to SCC immediately upon sample receipt and retained a copy for inclusion in the data report. SCC's faxed receipt of the completed traffic report form from the participant laboratory served as the notification that the sample had arrived in good condition at the participant laboratory.

For ampule samples, participant laboratories were not required to measure post-shipment sample water quality. To avoid possible contamination between the highly concentrated reference toxicant ampule samples and blank samples, no direct measurements were made on the ampule samples. Temperature was measured in a temperature check sample that was included with each cooler containing ampule samples. This sample, which was clearly marked as a temperature check, contained tap water in the same volume and container as ampule samples.

Table 6.4. Episode numbers and sample numbers used in the WET Variability Study.

Test method	Episode number	Sample number range	Sample code range
Ceriodaphnia acute	6207	03000 - 03107	9217 - 9324
Ceriodaphnia chronic	6208	04000 - 04128	9325 - 9453
Fathead acute	6205	01000 - 01110	9001 - 9111
Fathead chronic	6206	02000 - 02104	9112 - 9216
Selenastrum growth	6209	05000 - 05047	9454 - 9501
Mysidopsis chronic	6216	12000 - 12047	9650 - 9697
Sheepshead acute	6214	10000 - 10031	9586 - 9617
Sheepshead chronic	6215	11000 - 11031	9618 - 9649
Silverside acute	6210	06000 - 06041	9502 - 9541
Silverside chronic	6211	07000 - 07043	9542 - 9585

6.4 Problems Encountered in Sample Distribution

On the first shipment day for the *Ceriodaphnia* acute test method (11/08/99), the referee laboratory inadvertently switched the blank and reference toxicant ampule samples. All participant laboratories that were intended to receive blank samples received reference toxicant samples, and all laboratories that were intended to receive reference toxicant samples received blank samples. This error was not noticed at the time, and the referee laboratory incorrectly reported sample types on the sample shipment documentation form faxed to SCC. Since all samples were received by participant laboratories as blind test samples, this error had no effect on the participant laboratory testing of samples. This error did alter the intended sample distribution scheme (Table 6.1); however, the number of blank and reference toxicant samples were approximately equal so effects on the study design were minimal. The error was first identified by SCC as a result of participant laboratory data reports. Test results and conductivity measurements on the two samples indicated that the samples were switched. Since KCl was used as the reference toxicant, conductivity measurements were used to properly identify the blank and reference toxicant samples. Conductivity of the reconstituted blank sample was approximately 300 μmhos/cm, and conductivity of the reconstituted reference toxicant sample was approximately 2900 μmhos/cm. The referee laboratory determined that the error was caused by inadvertently filling ampules intended for blank samples with the reference toxicant bulk sample and filling ampules intended for reference toxicant samples with the blank bulk sample.

Due to weather or other circumstances, sample shipments occasionally failed to arrive at the participant laboratory on time. Of the 1438 sample aliquots shipped in the WET Variability Study, 1412 (or 98%) successfully arrived on the intended delivery date. Tests that were initiated on samples greater than 36 hours old are identified with a data qualifier flag in the "Results" section of this report (Section 9). No participant laboratory tested samples that were greater than 72 hours old.

Samples also occasionally arrived at participant laboratories at temperatures above 4°C. Only 7.4% of the 1438 sample aliquots arrived at above the recommended sample shipment temperature. This also had little effect on test results in the WET Variability Study since sample characteristics were known and selected toxicants were not likely to be altered as a result of slight temperature fluctuations.

On seven occasions, participant laboratories noted that sample labels were smeared by melting ice in the coolers and were difficult to read. This problem was addressed by placing the ice in double-lined plastic bags within the coolers. On all occasions the sample numbers were identified using the accompanying traffic report form. Participant laboratories then re-labeled the sample.

7.0 INTERLABORATORY TESTING

Interlaboratory testing was conducted to obtain data from multiple laboratories on the same test sample. These data were used to evaluate the performance of the WET test methods. Prior to interlaboratory testing of each test method, SCC provided participant laboratories with method-specific SOPs documenting participant laboratory requirements (see Appendix B). These SOPs described the shipment and tracking of test samples, provided instructions for any necessary pre-test sample adjustments (including preparation of reconstituted ampule samples), provided general and method-specific testing requirements as described in Section 7.1 and 7.2, and described data reporting requirements. Participant laboratories were then provided with samples (prepared according to Section 5) for immediate testing. Interlaboratory testing was conducted according to the schedule provided in Section 2.2.4 for the *Ceriodaphnia* acute and chronic, fathead acute and chronic, *Selenastrum* chronic, *Mysidopsis* chronic, sheepshead acute and chronic, and silverside acute and chronic test methods. Interlaboratory testing was canceled for the *Champia* chronic and *Holmesimysis* acute test methods (see Section 2.1).

7.1 General Testing Requirements

Except where indicated in the SOPs provided to participant laboratories, each test was conducted in accordance with the general guidance and method-specific requirements for effluent testing included in the WET methods manuals. Additional general WET test requirements that were listed in participant laboratory SOPs are provided below:

(1) Tests were required to be conducted by the same laboratory personnel that routinely conduct WET tests at that laboratory facility and who were identified in the prequalification materials. The laboratory was required to contact SCC if these individuals could not be available during any part of the study. Personnel conducting the tests were to be identified clearly and consistently in records.

(2) To coordinate testing at participant laboratories, testing of each sample with each method was required to be initiated on the precise day specified in the study schedule. The study schedule was distributed to participating laboratories prior to commencement of each study round and in ample time to prepare for testing. Laboratories were required to test samples within 36 hours from the time of sample preparation (determined in the WET Variability Study as the time at which individual sample aliquots were divided from the bulk test sample for distribution to participant laboratories). Laboratories were required to report deviations from the study schedule to SCC immediately for approval.

(3) Laboratories were required to conduct tests within the physical and chemical water quality ranges specified in the 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) were provided to the testing laboratories prior to test initiation. Laboratories were required to refrigerate (at 4°C ± 2°C) test samples

immediately upon receipt and throughout the period of testing. Routine or continuous monitoring of refrigeration temperature was recommended to ensure that these sample holding requirements were met.

(4) Laboratories were required to measure test conditions (pH, conductivity or salinity, total alkalinity, total hardness, and dissolved oxygen) in each test according to guidance in the WET method manuals.

(5) Laboratories were required to use the dilution and control waters specified in Tables 7.1 - 7.12 for each test method. Laboratories were required to prepare these dilution waters according to instructions in Section 7 of the method manuals. For marine test methods, laboratories were required to prepare dilution waters that meet the salinity ranges specified in Tables 7.6 - 7.12.

(6) Laboratories were required to conduct all tests as definitive tests with a control and a minimum of five test concentrations prepared using a dilution factor of 0.5.

(7) Laboratories were required to conduct all tests using the number of replicates and number of test containers per concentration as specified in Tables 7.1 - 7.12.

(8) For a given test method, laboratories were required to use the same type, size, shape, and material for all test chambers. The test chamber material used had to be allowed by the WET method manuals.

(9) Laboratories were required to randomize test vessels in accordance with the WET method manuals. In addition, block randomization and use of known parentage were required for the *Ceriodaphnia* chronic test method as described in the method manual. The Agency plans to amend Method 1002.0 (*Ceriodaphnia* chronic test method) to require that test organisms be allocated among test replicates so that offspring of each female are evenly distributed among test replicates ("blocking by known parentage").

(10) While the method manual requires the termination of the *Ceriodaphnia* chronic test after the production of three broods in 60% of the controls, laboratories were required to conduct the *Ceriodaphnia* chronic test for eight days. Laboratories were required to record the survival, number of young per day, and number of broods at the end of Day 6, 7, and 8 (specifically, at 144 hours, at 168 hours, and at 192 hours, respectively, from test initiation). This was done to assess the effect of the three brood test acceptance criterion on test results. No test was allowed to be terminated prior to Day 8 for any reason, including a failure to meet test acceptance criteria. Laboratories were required to include the additional measurements on Days 6, 7, and 8 as raw data in the final data report. However, laboratories were required to analyze data from the *Ceriodaphnia* chronic test using the three brood approach as specified in the method manual.

(11) Laboratories were required to conduct the *Selenastrum* chronic test simultaneously with and without EDTA for each sample. For laboratories participating in the base study design (refer to Sections 2.2.3 and 3.5), four samples were tested with and without EDTA for a total of eight analyses.

(12) Laboratories were required to observe mortality and remove dead organisms in each test daily, except for the *Selenastrum* chronic and *Champia* chronic test methods. For the *Ceriodaphnia* chronic test method, laboratories were required to count young daily and determine the number of broods at each count.

(13) Laboratories were required to contact SCC immediately if test results indicated extreme toxicity (i.e., control mortalities, or complete mortality in all concentrations). Laboratories were then required to 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 initiated test failed to be completed for any reason, the laboratory was required to contact SCC immediately for problem resolution and scheduling of additional testing. In this case, laboratories were required to report the incomplete test data and fully document the reason for not completing the test.

(15) Laboratories were required to report all data obtained during the course of testing, including the response of organisms in control treatments.

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

(17) Laboratories were required to perform a reference toxicant QC test for each test method in the month that interlaboratory testing occurred. Results of this test had to be submitted with the final data package.

(18) Laboratories were required to submit hard copies of all data from laboratory bench sheets and statistical analyses, including but not limited to all bench sheets, raw data, sample tracking forms, and chemical analysis data. Laboratories also were required to submit data electronically according to the electronic template (Microsoft Excel® spreadsheet) that was provided by SCC prior to test initiation.

(19) Laboratories were required to analyze data in accordance with the statistical programs specified in the WET method manuals. Statistical methods and programs used had to be reported along with sample calculations.

(20) Laboratories were required to report a LC50 for each acute test. A NOEC and LC50 for survival, and a NOEC and IC25 for growth/reproduction were required as appropriate for each short-term chronic test as described in the method manuals and Table 2.3 of this report. Laboratories were required to report individual toxicity results and were not allowed to average or perform other data manipulations unless required by the WET method manual.

7.2 Method-Specific Requirements

EPA acknowledges that the promulgated WET methods distinguish between requirements (indicated by the compulsory terms "must" and "shall") and recommendations and guidance (indicated by discretionary terms "should" and "may"). The latter terms indicate that the analyst has flexibility to optimize successful test completion. Additionally, the WET method manuals allow variations of the methods that are typically fixed in the permit; therefore, for the purposes of this study, a set of test condition variables were defined by EPA (for example, dilution water, salinity, and acute test duration).

The summary of test conditions for the 12 WET methods evaluated in the WET Variability Study are provided in Tables 7.1 - 7.12. These tables were extracted from the summary test condition tables in the WET method manuals and modified to fit the scope of this study. Items shown in bold italic in these tables represent conditions standardized for the purposes of this study where WET method manuals provide a range. These test conditions were reiterated in participant laboratory SOPs (Appendix B).

Table 7.1. Summary of test conditions and test acceptability criteria for the *Ceriodaphnia* acute test method.

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

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

Table 7.2. Summary of test conditions and test acceptability criteria for the *Ceriodaphnia* chronic test method.

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

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

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

Table 7.3. Summary of test conditions and test acceptability criteria for the fathead acute test method.

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

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

Table 7.4. Summary of test conditions and test acceptability criteria for the fathead chronic test method.

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

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

Table 7.5. Summary of test conditions and test acceptability criteria for the *Selenastrum* chronic test method.

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 interlaboratory study testing schedule***
21. Sample volume required:	***2 L***

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

Table 7.6. Summary of test conditions and test acceptability criteria for the *Mysidopsis* chronic test method.

1. Test type:	Static renewal
2. Salinity:	***25‰ (± 2‰)***
3. Temperature:	26 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (50-100 ft-c.) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness, with phase in/out period
7. Test chamber:	8 oz plastic disposable cups, or 400 mL glass beakers
8. Test solution volume:	150 mL per replicate
9. Renewal of test solutions:	Daily
10. Age of test organisms:	7 days
11. No. organisms per test chamber:	***5***
12. No. replicate chambers per concentration:	***8***
13. No. larvae per concentration:	***40***
14. Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old)
15. Feeding regime:	Feed 150 24 h old nauplii per *Mysidopsis* 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‰) salinity synthetic seawater prepared with Bioassay Grade Forty Fathoms® artificial sea salts and MILLIPORE MILLI-Q® or equivalent deionized water (see WET 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 interlaboratory study testing schedule***
25. Sample volume required:	3 L/day

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

Table 7.7. Summary of test conditions and test acceptability criteria for the sheepshead acute test method.

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

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

Table 7.8. Summary of test conditions and test acceptability criteria for the sheepshead chronic test method.

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

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

Table 7.9. Summary of test conditions and test acceptability criteria for the silverside acute test method.

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

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

Table 7.10. Summary of test conditions and test acceptability criteria for the silverside chronic test method.

1. Test type:	Static renewal
2. Salinity:	***25‰ (± 2‰)***
3. Temperature:	25 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	l0-20 µE/m^2/s (50-100 ft-c) (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	***1 L containers***
8. Test solution volume:	***750 mL/replicate (loading and DO restrictions must be met)***
9. Renewal of test solutions:	Daily
10. Age of test organisms:	7-11 days post hatch; 24-h range in age
11. No. larvae per test chamber:	***10***
12. No. replicate chambers per concentration:	***4***
13. No. larvae per concentration:	***40***
14. Source of food:	Newly hatched *Artemia* nauplii (survival of 7-9 days old *Menidia beryllina* larvae improved by feeding 24 h old *Artemia*)
15. Feeding regime:	Feed 0.10 g wet weight *Artemia* nauplii per replicate on days 0-2; Feed 0.15 g wet weight *Artemia* nauplii per replicate on days 3-6
16. Cleaning:	Siphon daily, immediately before test solution renewal and feeding
17. Aeration:	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min.
18. Dilution water:	***25‰ (± 2‰) salinity synthetic seawater prepared with Bioassay Grade Forty Fathoms® artificial sea salts and 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 interlaboratory study testing schedule***
25. Sample volume required:	6 L/day

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

Table 7.11. Summary of test conditions and test acceptability criteria for the *Champia* chronic test method.

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:	***Samples treated as effluent samples for NPDES monitoring. Samples are to be used on the day specified by the interlaboratory study testing schedule***
19. Sample volume required:	2 L per test

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

Table 7.12. Summary of test conditions and test acceptability criteria for the *Holmesimysis* acute test method. The acute test procedure described in the Acute Methods Manual for *Mysidopsis bahia* will be used for this test with a salinity of 32‰ (± 2‰) and a temperature of 12 °C ± 1 °C.

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

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

8.0 DATA REPORTING AND EVALUATION

8.1 Report Submission

Within 30 days following the completion of interlaboratory testing for a given method, each laboratory was required to submit a data report detailing the conduct and results of WET testing completed on each sample. Table 8.1 lists the report due dates and number of reports received for each test method. Reports were received for all samples from all participating laboratories with the exception of one participant laboratory (laboratory ID #3) for the *Ceriodaphnia* chronic method. Participant laboratory #3 received samples for the *Ceriodaphnia* chronic method and initiated testing. During testing of the final two of three samples received, an overnight power failure caused the malfunction of water baths containing test chambers. The malfunctioning water baths over-heated test chambers, killing all test organisms (including controls) and terminating the test prematurely. The laboratory was unable to retest within reasonable sample holding times, so all further testing of this method was canceled at this laboratory. The laboratory was non-EPA-sponsored, and the sponsor declined to reimburse the laboratory for testing costs since all tests were not completed. In turn, the laboratory declined to submit a data report for this test method. As a result, interlaboratory data for the *Ceriodaphnia* chronic method include results from one referee laboratory and 34 of the 35 participant laboratories.

According to the Participant Laboratory Statement of Work and SOP (see Appendix B), each data report was required to consist of:

- **Narrative summary of testing** - The narrative summary was intended to quickly and clearly identify the laboratory, test method, samples tested, summarized test results, any problems associated with the samples or conduct of the tests, any modifications from approved procedures, and any laboratory comment on the performance of the method.
- **Hardcopy results** - This deliverable consisted of the items outlined in Table 8.2, all raw data (biological and chemical), all laboratory benchsheets, all pertinent sample information including copies of all completed EPA traffic report forms, and all pertinent quality assurance information including results of the monthly QA/QC reference toxicant tests.
- **Electronic results** - Laboratories also were required to submit selected raw and summarized data electronically using method-specific Microsoft Excel® spreadsheet templates that were provided to participant laboratories by SCC. Electronic data included general information, sample collection/receipt information, test condition information, raw biological data, raw water quality data, and summarized test results. Electronic data submission facilitated automated review and statistical analysis of study results.

Table 8.1. Report due dates.

Due date	Test method	Number of reports received[a]
11/12/99	Fathead chronic	28
12/02/99	Silverside chronic	11
12/03/99	*Ceriodaphnia* chronic	35
12/08/99	Fathead acute	30
12/13/99	*Ceriodaphnia* acute	29
12/13/99	Silverside acute	10
04/06/00	*Mysidopsis* chronic	12
04/17/00	Sheepshead acute	8
05/03/00	*Selenastrum* chronic	12
05/04/00	Sheepshead chronic	8

[a] The number of laboratory reports received includes participant and referee laboratories. One referee laboratory report was received for each method.

Table 8.2. Data reporting elements.

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

Table 8.2. Data reporting elements. (continued)

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 note 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 results (LC50, IC25, NOEC for each endpoint) and confidence limits (where applicable)
6.4 Statistical methods and software used to calculate results
6.5 Summary table of physical and chemical data

8.2 Data Review

8.2.1 Data Package Receipt and Gross Completeness Check

Data reports from all laboratories were submitted to SCC for review and verification of test results. Upon receipt, SCC personnel date-stamped data packages and performed an initial review to ensure that all required information was provided. Most laboratories reported results using their standard reporting formats rather than the suggested format in Table 8.2. This was acceptable provided that all pertinent information was included. If necessary information was not provided in the data report, SCC personnel contacted the laboratory and asked them to supply the additional information.

8.2.2 Data Accuracy and Quality Check

Following initial review of data packages for completeness, SCC personnel performed a detailed review of data reports to ensure that data were accurate and generated in accordance with the required procedures. The following review steps were completed for each data report:

- **Cross reference of raw data** - Raw data on submitted electronic benchsheets were compared to hardcopy laboratory benchsheets to ensure that no transcription or data entry errors occurred. Every entry of biological raw data (including individual replicate values of daily survival, daily reproduction, and weight) was cross-referenced against hardcopy benchsheets to ensure accuracy. Sample collection, test condition, and water quality raw data entered on electronic benchsheets were spot-checked against hardcopy benchsheets. If errors were found during the spot-check, more intensive review was initiated. All data fields that triggered automated data qualifier flags in the electronic benchsheet were also individually compared to hardcopy benchsheets to ensure accuracy. When errors or inconsistencies were identified, the electronic benchsheet was corrected to match the hardcopy laboratory benchsheets.

- **Narrative summary and hardcopy report review** - SCC reviewed the narrative summary and hardcopy report to ensure that tests were conducted in accordance with the WET method manuals, the WET Variability Study plan, and guidance provided in method-specific SOPs. If any deviations from the required or recommended procedures were identified, SCC data reviewers verified that those deviations were captured by automated or manual data qualifier flags in the electronic benchsheet.

- **Data qualifier flags** - The electronic benchsheets for each method were programmed to automatically identify and flag deviations in test conditions, sample holding times, sample temperatures, test acceptability criteria, or test water quality. For all flags that were automatically identified, SCC evaluated the electronic benchsheet and hardcopy report to ensure that the flag was warranted. Additional parameters, test conditions, and comments that were unable to be programmed for automated review were checked and flagged manually if necessary. Table 8.3 lists and describes the categories of data qualifier flags used in the WET Variability Study.

  Tests that were flagged for a failure to meet test acceptability criteria (flags a_1, a_2, a_3, a_4, a_6, and a_7) were considered invalid and were not included in the analysis of method performance (see Section 9.1.1). The large number of other flags (b_1 - g_{12}) described in Table 8.3 and identified for particular tests in

Section 9, emphasizes the extensive nature of test review rather than deficiencies in test conduct or quality control. While the presence of these flags may indicate a deviation from optimal test conditions, their presence alone was not used to invalidate and exclude test data from the analysis of method performance. Rather, these flags were used to identify potential causes for aberrant test results and to support inclusion or exclusion of data in outlier analyses (see Section 9.1.4).

- **Reference toxicant test review -** Laboratories were required to conduct a reference toxicant test within the month of testing for the WET Variability Study and provide the test results and current control chart with the data report. SCC data review staff verified that a reference toxicant test was conducted during the required time frame and reviewed the laboratory's control chart to ensure that the current test result fell within the control chart limits (2 standard deviations for point estimates and 1 concentration interval for hypothesis testing results). If the reference toxicant test fell outside of the control chart limits or was not conducted during the required time frame, a data qualifier flag for reference toxicant testing was associated (in the results database) with all test results from this laboratory for the given method.

Table 8.3. Test data qualifier flags.

Flag code	Flag Description
Test acceptability	
a_1	**Survival** of control organisms failed to meet the minimum test acceptability criteria for the method.
a_2	**Growth** of control organisms (measured as the mean weight of surviving control organisms) failed to meet the minimum test acceptability criteria for the method.
a_3	**Reproduction** of control organisms in the *Ceriodaphnia* chronic test failed to meet the minimum test acceptability criteria requiring that 60% of surviving control organisms have 3 broods prior to test termination at 8 days.
a_4	**Mean reproduction** of surviving control organisms in the *Ceriodaphnia* chronic test failed to meet the minimum test acceptability criteria of 15 neonates.
a_5	**Fecundity** endpoints were not generated because less than 50% of control females in the *Mysidopsis* chronic test produced eggs.
a_6	**Mean algal growth** of control organisms in the *Selenastrum* chronic test did not meet the minimum test acceptability criteria requiring a mean cell density of $1x10^6$ cells/mL with EDTA or $2x10^5$ cells/mL without EDTA.
a_7	**Algal growth variability** between control replicates in the *Selenastrum* chronic test did not meet the minimum test acceptability criteria requiring less than 20% variability (measured as % CV).
Sample Receipt	
b_1	**Sample temperature** was >4°C upon arrival at the participant lab.
b_2	**Sample was >36 hr old** at the time of test initiation.
b_3	**Sample was >72 hr old** at the time of test initiation.
b_4	**Sample was aerated** upon receipt due to over saturation of dissolved oxygen.
b_5	**Sample was inadvertently delivered** to and opened by a laboratory not involved in the study. The sample was quickly rerouted to the correct laboratory and tests were initiated on time.

Table 8.3. Test data qualifier flags (continued)

Flag code	Flag Description
	Dilution Water
c_1	**Dilution water used** for the test was different from that required in the study.
c_2	**Alkalinity** of dilution water was >10% outside of recommended ranges for moderately hard synthetic water.
c_3	**Hardness** of dilution water was >10% outside of recommended ranges for moderately hard synthetic water.
c_4	**pH** of dilution water was >10% outside of recommended ranges for moderately hard synthetic water.
c_5	**Salinity** of dilution water was outside of the range required in the study.
	Water Quality
d_1	**Temperature** of one or more test concentrations was outside of range required in the study.
d_2	**Dissolved oxygen (DO)** was less than 4 mg/L in one or more test concentrations.
d_3	**Aeration** was not provided in test when DO was <4 mg/L.
d_4	**pH** was <6 or >9 in one or more test concentrations.
d_5	**Salinity** of one or more test concentrations was outside of the range required in the study.
d_6	**Salinity was adjusted** during the test to compensate for evaporation due to test aeration.
d_7	**Total ammonia** was >5 mg/L in one or more test concentrations.
	Test Conditions
e_1	**Number of organisms per test container** differed from the required number of organisms due to accidental loss of one or more test organisms.
e_2	**Test chamber size** was different from that required in the study.
e_3	**Test solution volume** in test containers was outside of the range required in the study.
e_4	**Test renewals** were conducted more than 2 hours outside of the required time for test renewal.
e_5	**Feeding schedule** used during the test differed from feeding schedule recommended in the method manuals.
e_6	**Test termination** was conducted more than 2 hours prior to proper test termination time.
e_7	**Dilution series used** for testing was 12.5, 25, 50, 75, and 100% instead of the standard dilution series required in the study (6.25, 12.5, 25, 50, and 100%).
e_8	**Lighting cycle** was interrupted for 2 hours during power outage.
e_9	**Continuous shaking rate** of 100 cpm was not used.
e_{10}	**Initial cell density average** was more than 10% outside of required 10,000 cell/mL inoculation level.
e_{11}	**Initial cell density variability** among replicates was greater than a CV of 10%.
	Organisms
f_1	**Age of organism** was outside of range required in the study.
f_2	**Organism culture contaminated** with rotifers.
f_3	**Organism culture crashed** just prior to testing.
f_4	**Males** were identified in the test.
f_5	**Organisms** were cultured at 20°C and directly transferred to test temperature of 25°C.

Table 8.3. Test data qualifier flags (continued)

Flag code	Flag Description
	Quality control
g_1	**Reference toxicant test** conducted during the month of study testing was outside of the 2 standard deviation control chart limits or the test was not conducted.
g_2	**Percent minimum significant difference (PMSD)** was greater than the recommended criteria for this method (USEPA, 2000d).
g_3	**ASTM h statistic** for this test was greater than the recommended criteria, indicating that this test may be an outlier.
g_4	**ASTM k statistic** for this test was greater than the recommended criteria, indicating that this test may be an outlier.
g_5	**Interrupted concentration - response relationship** was observed (i.e., a test concentration was determined to be significantly different from the control, while one or more higher test concentrations were not significantly different from the control).
g_6	**One or multiple test replicates** were lost due to laboratory error.
g_7	**Two cell count methods** were used. Test failed test acceptability criteria for growth using coulter counter method, but passed test acceptability criteria for growth using Hemacytometer method.
g_8	**Test was repeated** due to laboratory error. Initial test was incorrectly terminated at 48 hours.
g_9	**Test was repeated** due to laboratory error. Initial test was incorrectly renewed with the wrong sample.
g_{10}	**Two sets of controls** were conducted for this test, and one did not meet test acceptability criteria for reproduction.
g_{11}	**Cell density** was measured using turbidity technique instead of cell counts.
g_{12}	**Referee laboratory test repeated**. Initial test failed test acceptability criteria for survival.

8.2.3 Effect Concentration Recalculation and Verification

To confirm that test results were calculated correctly and according to WET method manual requirements for statistical data analysis, all test results were recalculated by SCC using reviewed raw data from the electronic benchsheets. SCC conducted statistical analysis of WET test data using ToxCalc version 5.0 (Tidepool Scientific, 1996). Biological test data were electronically copied from electronic benchsheets directly into the ToxCalc software to avoid additional data transcription or data entry errors. Statistical methods for analysis were selected according to the WET method manuals using the EPA flowchart option in the ToxCalc software. Test results for the endpoints listed in Table 2.3 were calculated for the respective test methods. Any error messages that were produced by the software were noted and evaluated to ensure that the software defaulted to the correct alternate statistical method.

As part of the effect concentration recalculation and verification process, SCC reviewed the concentration-response curve generated for each sample and endpoint. SCC reported test results for each sample in accordance with EPA's guidance on the evaluation of concentration-response relationships (USEPA, 2000a). When SCC observed unexpected concentration-response patterns, EPA's guidance (USEPA, 2000a) was followed for determining whether the derived effect concentration was reliable and should be reported, the effect concentration was anomalous and should be explained, or the test was inconclusive and the sample should be retested. When EPA's concentration-response relationship guidance recommended retesting of a sample,

SCC reported the result for this sample as inconclusive. Table 8.4 lists the samples that produced unexpected concentration-response curves and were affected by the concentration-response guidance.

After test results were recalculated by SCC, these results were compared to results as reported by the participant laboratory. If recalculated and laboratory-reported results differed, the summarized test data and statistical analyses used for calculation were reviewed to isolate the source of the deviation. Following recalculation and review, test results were incorporated into a results database for the WET Variability Study.

Table 8.4. Sample results affected by EPA guidance on concentration-response relationships (USEPA, 2000a).

Method	Sample code	Concentration-response pattern observed[a]	Effect on reported test result
Ceriodaphnia chronic	9328	4	Reproduction NOEC and IC25 were reported as inconclusive since test percent minimum significant difference (PMSD) was greater than recommended criterion
	9332	4	Calculated IC25 was determined to be anomalous due to ICp smoothing; IC25 was adjusted to >100% since mean response in the 100% treatment was within 25% of control mean
	9333	5	6.25% treatment was determined to be anomalous and reproduction NOEC was reported as highest concentration not significantly different from control
	9341	5	6.25% and 12.5% treatments were determined to be anomalous and reproduction NOEC was reported as highest concentration not significantly different from control
	9343	6	Reproduction NOEC was reported as concentration below the LOEC
	9379	5	6.25% treatment was determined to be anomalous and survival NOEC was reported as highest concentration not significantly different from control
	9380	6	Survival NOEC was reported as inconclusive since test PMSD was greater than recommended criterion
	9392	6	Reproduction NOEC was reported as concentration below the LOEC
	9408	4	Calculated IC25 was determined to be anomalous due to ICp smoothing; IC25 was adjusted to >100% since mean response in the 100% treatment was within 25% of control mean
	9415	5	Survival NOEC was reported as inconclusive since test PMSD was greater than recommended criterion
Fathead chronic	9122	5	6.25% treatment was determined to be anomalous and growth NOEC was reported as highest concentration not significantly different from control
	9129	6	Growth NOEC was reported as concentration below the LOEC
	9145	5	25% treatment was determined to be anomalous and survival NOEC was reported as highest concentration not significantly different from control
	9161	6	Growth NOEC was reported as concentration below the LOEC
	9168	5	6.25% treatment was determined to be anomalous and growth NOEC was reported as highest concentration not significantly different from control
	9193	6	Growth NOEC was reported as concentration below the LOEC

Table 8.4. Sample results affected by EPA guidance on concentration-response relationships (USEPA, 2000a). (continued)

Method	Sample code	Concentration-response pattern observed[a]	Effect on reported test result
Fathead chronic (continued)	9194	6	Growth NOEC was reported as concentration below the LOEC
	9209	5	Growth NOEC was reported as inconclusive since test PMSD was greater than recommended criterion
	9212	6	Survival and growth NOECs were reported as concentration below the LOEC
Selenastrum chronic	9454 (w/o EDTA)	4	Growth NOEC, IC25, and IC50 were reported as inconclusive since test PMSD was greater than recommended criterion
	9455 (w/ EDTA)	4	Growth NOEC, IC25, and IC50 were reported as inconclusive since control response was marginal and below laboratory's normal range of control performance
	9455 (w/o EDTA	4	Calculated IC25 was determined to be anomalous due to ICp smoothing; IC25 was adjusted to >100% since mean response in the 100% treatment was within 25% of control mean
	9468 (w/ EDTA)	5	50% treatment was determined to be anomalous and growth NOEC was reported as highest concentration not significantly different from control
	9468 (w/o EDTA)	5	Growth NOEC was reported as inconclusive since test PMSD was greater than recommended criterion
	9473 (w/o EDTA)	5	Growth NOEC was reported as inconclusive since test PMSD was greater than recommended criterion
Mysidopsis chronic	9682	5	12.5% treatment was determined to be anomalous and fecundity NOEC was reported as highest concentration not significantly different from control
	9694	5	12.5% treatment was determined to be anomalous and fecundity NOEC was reported as highest concentration not significantly different from control
	9696	5	12.5% treatment was determined to be anomalous and growth NOEC was reported as highest concentration not significantly different from control
Silverside chronic	9545	4	Growth NOEC and IC25 were reported as inconclusive since test PMSD was greater than recommended criterion
	9556	5	12.5% treatment was determined to be anomalous and growth NOEC was reported as highest concentration not significantly different from control

[a] Concentration-response patterns are numbered as identified in Chapter 4 of USEPA, 2000a.

9.0 RESULTS

9.1 Analysis of Results

SCC personnel entered recalculated and verified test results for each sample tested into a results database along with associated data qualifier flags, sample information, and summary test information (e.g., control mean, control CV, test minimum significant difference, etc.). Information in the results database was used to evaluate the test completion rate, false positive rate, and precision for each test method. All calculated test results presented in this section were rounded to three significant figures for consistency. A formal analysis of measurement error for each data type was not conducted; however, three significant figures is believed to be consistent with most WET test measurements (e.g., test concentrations, weights, counts). Summary statistics presented in this section (e.g., mean, standard deviation, CV) were calculated from test results prior to rounding (so summary statistics of rounded results may differ slightly).

9.1.1 Valid Tests

Only valid tests were used in the determination of false positive rates and precision. A valid test was defined as a test that met the required test acceptability criteria for the method as stated in the WET method manuals. Tests that deviated from specified test conditions were identified with data qualifier flags (see Section 8.2.2) but were not excluded as invalid tests. The WET method manuals state that tests that deviate from specified test conditions may be conditionally acceptable depending on the degree of the departure and the objectives of the test. Based on the study objectives of assessing the performance of WET test methods, these tests were included in the analysis of false positive rates and precision unless the combined results of test review (see Section 8.2) and outlier analysis (see Section 9.1.4) indicated that the test condition deviations significantly affected test results.

When EPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a) recommended retesting of a sample, the test result in question was identified as inconclusive and was not included in the analysis of false positive rates and precision for the methods. Also, test results from referee laboratories were excluded from determinations of successful test completion rates, false positive rates, and precision for the methods. While referee laboratory testing was conducted similarly to and simultaneously with participant laboratory testing, the identity of samples was not blinded to the referee laboratory. Appendix F summarizes study results when referee laboratory data are included in the analysis of successful test completion rates, false positive rates, and precision.

9.1.2 Successful Test Completion Rate

The successful test completion rate was calculated independently for each test method as the percentage of initiated and properly terminated tests that met the test acceptability criteria as specified in the WET method manuals. Participant laboratories that failed to complete tests due to reasons unrelated to the test methods themselves (i.e., laboratory error) were not included in the test completion rate calculations or statistical analyses. This occurred for only four samples (9586, 9587, 9589, and 9618). In three cases a 96-hour test was incorrectly terminated at 48 hours, and in one case the test was renewed using the wrong sample. In each case, the referee laboratory sent a new sample aliquot from the original bulk sample preparation for retesting at the

participant laboratory. Results from the repeated tests are presented in this report and were used in the determination of successful test completion rates, false positive rates, and precision.

9.1.3 False Positive Rate

The false positive rate was calculated independently for each test method and for each endpoint and effect concentration reported (LC50, survival NOEC, IC25 for growth, IC25 for reproduction, NOEC for growth, and NOEC for reproduction). The false positive rate was determined as the rate at which test results indicated toxicity (i.e., a calculated effect concentration <100% sample) in blank samples, and was calculated as:

$$\frac{\text{Number of valid tests indicating toxicity in blank samples}}{\text{Total number of valid tests conducted on blank samples}} \times 100\%$$

9.1.4 Precision

Precision estimates were generated independently for each test method, point estimate, and sample type tested (except for the blank sample type). For sample types that were tested using within-laboratory replication, estimates were provided for within-laboratory precision (based on the within-laboratory variance component), between-laboratory precision (based on the between-laboratory variance component), and total precision (based on the total variance). For sample types that were not tested using within-laboratory replication, a single precision estimate was generated based on the total variance.

When test results were calculated as outside of the test concentration range (i.e., >100% or <6.25%), these censored values were set to the limits of the test concentration range for the purposes of calculating summary statistics and estimating precision. Censored values of >100% were set to 100%, and censored values of <6.25% were set to 6.25%. Censored IC25 values of >12.5%, >25%, and >50% also were possible for the *Mysidopsis* chronic fecundity endpoint. These censored values were set to 12.5%, 25%, and 50%, respectively. A large proportion of censored values (e.g., >100% or <6.25%) within a data set, was evidence that the sample type failed to produce toxicity that could be definitively measured within the test concentration range. Because the study was designed to characterize the precision WET methods within their measurement range, precision estimates were not calculated for sample types that failed to produce toxicity that could be definitively measured within the test concentration range. As a result, precision estimates were not calculated for the blank sample type for all test methods, the reference toxicant sample type for the *Ceriodaphnia* chronic and silverside acute test methods (see Section 5.3), and all sample types for the *Mysidopsis* chronic fecundity endpoint.

Only participant laboratory results from valid tests (see Section 9.1.1) were included in the calculation of precision estimates; invalid tests were excluded. SCC conducted an outlier analysis of valid test results to determine if any additional test results should be excluded from the analysis of precision. SCC used the calculation of ASTM's h and k statistics (ASTM, 1997) to evaluate data consistency and identify potential outliers. ASTM h statistics were used to examine the consistency of test results from laboratory to laboratory. ASTM k statistics were used to examine the consistency of within-laboratory precision from laboratory to laboratory. For each test method, ASTM h statistics were calculated for each laboratory and each sample type (except the blank sample type) using the equation below.

$$h = \frac{\bar{x} - \bar{\bar{x}}}{\sqrt{\sum_{1}^{p} (\bar{x} - \bar{\bar{x}})^2 \Big/ (p-1)}}$$

where, $\bar{x}$ = a laboratory's average test result for a given sample type (if the laboratory only tested one sample of a given sample type, that individual result was used)

$\bar{\bar{x}}$ = the average of individual laboratory averages $(\sum_{1}^{p} \bar{x} / p)$

p = number of laboratories testing a given sample type

For each test method, ASTM k statistics were calculated for each laboratory and each sample type (except the blank sample type) using the equation below. The k statistic was not calculable for laboratories that did not test replicate samples of the same sample type.

$$k = \frac{s}{\sqrt{\sum_{1}^{p} s^2 \Big/ p}}$$

where, $s = \sqrt{\sum_{1}^{n} \left(x - \bar{x}\right)^2 \Big/ (n-1)}$

p = number of laboratories testing a given sample type
x = an individual test result
$\bar{x}$ = a laboratory's average test result for a given sample type
n = number of test results for a given sample type from a single laboratory

For each test method and sample type (excluding the blank sample type), SCC compared the h and k statistics calculated for each laboratory to critical values of h and k statistics at the recommended 0.5% significance level (see ASTM, 1997 for table of critical values). Test results from laboratories with a calculated h statistic above the critical value were significantly different (at the 0.5% significance level) from results reported by other laboratories for the same sample type. These inconsistent test results were flagged (see Table 8.3) and identified as potential outliers. Laboratories with a calculated k statistic above the critical value experienced greater within-laboratory variability than other laboratories testing the same sample type. These inconsistent test results also were flagged (see Table 8.3) for further investigation.

Since estimates of coefficients of variation can be biased by extreme values and by small data sets, it was important to closely investigate individual data points before discarding them as outliers. An individual test result was only discarded as an outlier if the laboratory was identified by ASTM h statistics as an outlier and a reasonable cause for producing the aberrant result could be determined. The data qualifier flags associated with each test result were useful in this determination. In general, a very conservative approach to excluding outliers was taken. Only 15 tests in the entire study of 698 tests were identified by ASTM h statistics as potential

outliers. Two of these tests were also flagged for extreme ASTM k statistics. Of the 15 tests identified as potential outliers, only 8 were excluded from precision estimates based on the determination of a cause for inconsistent results. Table 9.1 shows the test results that were identified as potential outliers and provides a rationale for the inclusion or exclusion of these data points from precision estimates.

Precision was estimated by the coefficient of variation (CV) for point estimates. For NOEC values, precision was simply described by the range and distribution of NOEC values and the percentage of values falling within one concentration of the median (as described in the WET method manuals for evaluating routine reference toxicant test results using NOECs). The CV for point estimates was calculated as:

$$CV = \frac{\sqrt{S^2}}{\overline{X}} \times 100\%$$

where, S^2 = variance (S = the standard deviation)

$\overline{X}$ = mean of valid test results for a given method, endpoint, and sample type

For test methods and sample types that included within-laboratory replication (i.e., multiple tests on the same sample type from a given laboratory), the variance identified in the above equation was obtained by maximum likelihood estimation using the PROC MIXED procedure in SAS version 8 (SAS Institute, 2000). This procedure estimated the within-laboratory, between-laboratory, and total variance components. Each of these variance components were individually used to calculate within-laboratory, between-laboratory, and total CVs. The total CVs express the total interlaboratory variability of the results, including both within-laboratory and between-laboratory components of variability.

For test methods and sample types that did not include within-laboratory replication, the variance identified in the above equation was obtained by the following equation using the PROC MEANS procedure in SAS version 8 (SAS Institute, 2000).

$$S^2 = \frac{1}{n-1} \sum_{i=1}^{n} (X_i - \overline{X})^2$$

where, n = number of valid test results for a given method, endpoint, and sample type

X_i = individual result i (i ranging from 1 to n)

S^2 = variance of the n test results (S = the standard deviation)

$\overline{X}$ = mean of the n test results

Table 9.1. Test results identified as potential outliers by ASTM h statistics.

Test method	Lab ID	Sample code	Test endpoint	Included/excluded	Rationale
Ceriodaphnia chronic	42	9347	survival and reproduction	excluded	These duplicate samples were flagged for both h and k statistics, indicating that the laboratory's mean test result was significantly different from other laboratories and that within-lab variability was also significantly higher for this laboratory; this high within-lab variability could explain the inconsistent mean test result from this laboratory
	42	9348	survival and reproduction	excluded	
Fathead acute	62	9033	survival	included	No data qualifier flags
	62	9034	survival	included	
	205	9065	survival	included	Only data qualifier flag was for test chamber size
	205	9066	survival	included	
Fathead chronic	205	9177	growth	included	No data qualifier flags
	125	9162	growth	excluded	Dilution water quality was highly variable for this laboratory. For these two tests, dilution water hardness at test initiation was 111 mg/L, compared to the expected hardness range of 80-100 mg/L for moderately hard reconstituted water. For the remaining two fathead chronic tests conducted in this laboratory, dilution water alkalinity at test initiation was 45 mg/L, compared to the expected alkalinity range of 60-70 mg/L.
	125	9163	growth	excluded	
Selenastrum chronic	39	9468 (w/ EDTA)	growth	excluded	Cell growth was marginal and failed test acceptability criteria when measured using coulter counter; other flags included sample temperature, test temperature, test pH, continuous shaking rate, and interrupted concentration-response relationship
	125	9476 (w/o EDTA)	growth	excluded	Reference toxicant test was not conducted concurrently with tests or within the month of testing
Sheepshead acute	425	9617	survival	included	Data qualifier flags were observed for salinity of 30 ppt on test day 4 and for test termination at 93.7 hours (rather than 96); however, the data point was included due to the small size of the data set for this method (this result represented 14% of the data set)
	101	9600	survival	included	No data qualifier flags
Silverside chronic	421	9582	survival and growth	excluded	Reference toxicant test conducted concurrently with samples was outside of control chart limits
	421	9583	survival and growth	excluded	

9.2 *Ceriodaphnia* Acute Test Method Results

A total of 28 participant laboratories conducted the *Ceriodaphnia* acute test method in the WET Variability Study. These laboratories tested a total of 34 blank samples, 30 reference toxicant samples, 27 effluent samples, and 13 receiving water samples. For each sample tested, a 48-hour LC50 was generated as a test result. Results of *Ceriodaphnia* acute testing are shown in Tables 9.2 - 9.5 for each sample type.

The sample distribution scheme used for the *Ceriodaphnia* acute test method was inadvertently altered from the original study design (see Section 6.4) due to an error in sample distribution. On the first week of testing, all laboratories that were intended to receive blank samples were shipped reference toxicant samples, and all laboratories that were intended to receive reference toxicant samples were shipped blank samples. This error was not identified prior to the second week of testing, so shipments during the second week were conducted as planned. This caused some laboratories to receive three blank samples and no reference toxicant samples and other laboratories to receive three reference toxicant samples and no blank samples. This sample distribution error should not affect the evaluation of the *Ceriodaphnia* acute test method.

9.2.1 Successful Test Completion Rate

A total of 104 *Ceriodaphnia* acute tests were initiated by 28 participant laboratories. All 104 tests were completed; however, tests conducted on five samples (9232, 9222, 9233, 9234, and 9231) were invalid due to failure to meet test acceptability criteria for survival. The resulting successful test completion rate calculated in the WET Variability Study for the *Ceriodaphnia* acute test method was 95.2%. Four of the five invalid tests were conducted in a single laboratory (Lab 29). This laboratory failed to properly culture test organisms at the test temperature of 25°C. The laboratory transferred organisms cultured at 20°C to the test temperature of 25°C, causing significant mortality in all test treatments and failure of test acceptability criteria.

9.2.2 False Positive Rate

A total of 33 valid tests were completed on blank samples by 14 participant laboratories (Table 9.2). The LC50 calculated for all 33 blank samples was >100%, indicating no toxicity and no false positives. The resulting false positive rate calculated in the WET Variability Study for the *Ceriodaphnia* acute test method was 0.00%.

9.2.3 Precision

Precision of the *Ceriodaphnia* acute test method was estimated by calculating the CV of LC50 values obtained for the reference toxicant, effluent, and receiving water samples. Within-laboratory, between-laboratory, and total CVs were calculated for the reference toxicant and effluent samples. Only a total CV was calculated for the receiving water samples since no within-laboratory replication was provided for this sample type. All valid participant laboratory test data for the reference toxicant, effluent, and receiving water samples were used in estimating precision. No test results were identified by ASTM h statistics as possible outliers.

Table 9.6 summarizes the precision of point estimates from the *Ceriodaphnia* acute test method. Within-laboratory CVs ranged from 9.68% to 14.6%, between-laboratory CVs ranged from 15.2% to 32.8%, and total CVs ranged from 21.1% to 34.2%. Total CVs were lower for reference toxicant samples (21.1%) than for

effluent (34.2%) or receiving water (31.8%) samples. As expected, the majority of variability was due to the between-laboratory component, with within-laboratory CVs averaging 12.1% and between-laboratory CVs averaging 24.0%. Averaging the CVs based on total variance for the three sample types, a total CV of 29.0% was obtained for the *Ceriodaphnia* acute test method in the WET Variability Study.

Table 9.2. Results for *Ceriodaphnia* acute test method performed on blank samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
29	9232	11/09/99	Invalid[b]	5.00	41.8	a_1, e_2, f_5
6	9221	11/09/99	>100	100	0.00	
18	9224	11/09/99	>100	100	0.00	d_1, e_2
18	9225	11/11/99	>100	100	0.00	d_1, e_2
18	9226	11/11/99	>100	100	0.00	d_1, e_2
33	9235	11/09/99	>100	100	0.00	
33	9236	11/09/99	>100	100	0.00	
33	9237	11/11/99	>100	100	0.00	
46	9243	11/09/99	>100	100	0.00	c_4, d_4
46	9244	11/09/99	>100	100	0.00	c_4, d_4
46	9245	11/11/99	>100	100	0.00	c_4
62	9250	11/09/99	>100	100	0.00	
62	9251	11/09/99	>100	100	0.00	
69	9254	11/09/99	>100	100	0.00	
69	9255	11/09/99	>100	100	0.00	
70	9258	11/09/99	>100	100	0.00	c_2
70	9259	11/09/99	>100	90.0	11.2	c_2
70	9260	11/11/99	>100	90.0	11.2	c_2
73	9262	11/09/99	>100	100	0.00	
73	9263	11/09/99	>100	100	0.00	
73	9264	11/11/99	>100	100	0.00	
105	9270	11/09/99	>100	100	0.00	g_5
157	9283	11/09/99	>100	100	0.00	
157	9284	11/09/99	>100	100	0.00	
157	9285	11/11/99	>100	100	0.00	b_1
251	9295	11/09/99	>100	100	0.00	
251	9296	11/09/99	>100	100	0.00	
311	9299	11/09/99	>100	100	0.00	
311	9300	11/09/99	>100	100	0.00	
417	9315	11/09/99	>100	100	0.00	
417	9316	11/11/99	>100	100	0.00	
452	9322	11/09/99	>100	100	0.00	
452	9323	11/11/99	>100	95.0	9.26	
452	9324	11/11/99	>100	95.0	9.26	
Summary Statistics	**N**		**33**			
	Min		**>100**			
	Max		**>100**			
	Median		**>100**			
	Mean		**>100**			
	False positives		**0**			
	False positive rate		**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from invalid tests were excluded from summary statistics.

Table 9.3. Results for *Ceriodaphnia* acute test method performed on reference toxicant samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9307	11/09/99	40.6[b]	100	0.00	
Referee	9310	11/11/99	34.4[b]	100	0.00	
3	9217	11/09/99	31.9	100	0.00	b_4
3	9218	11/09/99	27.6	100	0.00	
3	9219	11/11/99	18.3	100	0.00	e_1
25	9227	11/09/99	34.4	100	0.00	e_3
25	9229	11/11/99	33.0	100	0.00	e_3
25	9230	11/11/99	33.0	100	0.00	e_3
42	9239	11/09/99	37.9	100	0.00	
42	9241	11/11/99	35.4	100	0.00	b_1
42	9242	11/11/99	50.0	100	0.00	b_1
60	9246	11/09/99	17.1	100	0.00	c_1, e_3
101	9265	11/09/99	21.7	100	0.00	e_2, e_3
101	9267	11/11/99	25.9	100	0.00	e_2, e_3
101	9268	11/11/99	27.7	100	0.00	e_2, e_3
113	9273	11/09/99	35.4	100	0.00	
113	9274	11/09/99	32.0	100	0.00	
113	9275	11/11/99	35.4	90.0	18.7	
125	9276	11/09/99	26.2	100	0.00	
125	9279	11/11/99	24.8	100	0.00	
141	9280	11/09/99	31.2	100	0.00	c_2
141	9281	11/11/99	32.7	100	0.00	c_2
141	9282	11/11/99	35.4	100	0.00	c_2
205	9287	11/09/99	39.2	100	0.00	c_1, c_2, d_1
238	9291	11/09/99	30.8	100	0.00	e_2, e_6
406	9303	11/09/99	31.8	100	0.00	c_1
416	9311	11/09/99	34.2	100	0.00	c_2, c_3
416	9312	11/11/99	24.1	100	0.00	c_2, c_3
416	9313	11/11/99	26.8	100	0.00	c_2, c_3
425	9318	11/09/99	35.4	100	0.00	c_1
425	9320	11/11/99	30.0	100	0.00	c_1
425	9321	11/11/99	28.9	100	0.00	c_1, g_5
Summary Statistics	**N**		**30**			
	Min		**17.1**			
	Max		**50.0**			
	Median		**31.8**			
	Mean		**30.9**			
	Within-lab	**STD**	**4.52**			
		CV%	**14.6%**			
	Between-lab	**STD**	**4.70**			
		CV%	**15.2%**			
	Total	**STD**	**6.52**			
		CV%	**21.1%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.4. Results for *Ceriodaphnia* acute test method performed on effluent samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9309	11/11/99	24.6[b]	100	0.00	
6	9222	11/11/99	Invalid[c]	40.0	59.5	a_1
29	9233	11/11/99	Invalid[c]	0.00	0.00	a_1, d_1, e_2, f_5
29	9234	11/11/99	Invalid[c]	0.00	0.00	a_1, d_1, e_2, f_5
6	9223	11/11/99	9.99	100	0.00	
33	9238	11/11/99	13.0	100	0.00	
60	9248	11/11/99	13.2	95.0	9.26	c_1, e_3
60	9249	11/11/99	10.3	95.0	9.26	c_1, e_3
62	9252	11/11/99	28.2	90.0	18.7	
62	9253	11/11/99	26.8	100	0.00	
69	9256	11/11/99	27.7	100	0.00	
69	9257	11/11/99	33.0	100	0.00	
70	9261	11/11/99	21.2	90.0	11.2	c_2
105	9271	11/11/99	23.6	100	0.00	
105	9272	11/11/99	23.1	100	0.00	
125	9278	11/11/99	20.3	100	0.00	
157	9286	11/11/99	35.4	100	0.00	b_1
205	9289	11/11/99	36.6	100	0.00	b_1, b_4, c_1, c_2, d_1
205	9290	11/11/99	30.8	100	0.00	b_1, b_4, c_1, c_2, d_1
238	9293	11/11/99	16.9	100	0.00	e_2
238	9294	11/11/99	16.5	100	0.00	e_2
251	9297	11/11/99	17.1	100	0.00	
251	9298	11/11/99	19.5	100	0.00	
311	9301	11/11/99	24.1	100	0.00	
311	9302	11/11/99	21.8	100	0.00	
406	9305	11/11/99	27.7	100	0.00	b_1, c_1, c_2
406	9306	11/11/99	27.0	100	0.00	c_1, c_2
417	9317	11/11/99	15.0	100	0.00	
Summary Statistics	**N**		**24**			
	Min		**9.99**			
	Max		**36.6**			
	Median		**22.4**			
	Mean		**22.5**			
	Within-lab	**STD**	**2.17**			
		CV%	**9.68%**			
	Between-lab	**STD**	**7.36**			
		CV%	**32.8%**			
	Total	**STD**	**7.68**			
		CV%	**34.2%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.5. Results for *Ceriodaphnia* acute test method performed on receiving water samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9308	11/9/99	30.8[b]	100	0.00	d_1
29	9231	11/9/99	Invalid[c]	5.00	41.8	a_1, e_2, f_5
6	9220	11/9/99	20.8	100	0.00	
25	9228	11/9/99	34.2	100	0.00	e_3
42	9240	11/9/99	26.8	100	0.00	
60	9247	11/9/99	16.5	100	0.00	c_1, e_3
101	9266	11/9/99	21.5	100	0.00	e_2, e_3
105	9269	11/9/99	11.1	100	0.00	
125	9277	11/9/99	33.2	90.0	11.2	
205	9288	11/9/99	34.2	100	0.00	b_1, b_4, c_1, c_2, d_1
238	9292	11/9/99	23.3	100	0.00	e_2, e_6
406	9304	11/9/99	19.9	100	0.00	c_1
417	9314	11/9/99	20.4	100	0.00	
425	9319	11/9/99	17.7	100	0.00	c_1
Summary Statistics	**N**		**12**			
	Min		**11.1**			
	Max		**34.2**			
	Median		**21.2**			
	Mean		**23.3**			
	STD		**7.40**			
	CV%		**31.8%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.6. Precision of point estimates from the *Ceriodaphnia* acute test method.

Sample type	**CV (%)**		
	Within-lab[a]	**Between-lab[a]**	**Total**
Reference toxicant	14.6	15.2	21.1
Effluent	9.68	32.8	34.2
Receiving water	-	-	31.8
Average	**12.1**	**24.0**	**29.0**

[a] Within and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.

9.3 *Ceriodaphnia* Chronic Test Method Results

A total of 35 participant laboratories conducted the *Ceriodaphnia* chronic test method in the WET Variability Study. One of the participant laboratories did not submit a data report (see Section 8.1), so summarized results are based on 34 participant laboratories. These laboratories tested a total of 34 blank samples, 48 reference toxicant samples, 27 effluent samples, and 13 receiving water samples. For each sample tested, a survival NOEC, a reproduction NOEC, a survival LC50, and a reproduction IC25 were generated as test results. While all *Ceriodaphnia* chronic tests performed in the WET Variability Study were conducted for eight days, results presented in this section were determined using the current WET method manual criterion for test termination. This criterion states that tests should be terminated when 60% of the surviving control females have produced their third brood, or at the end of 8 days, whichever occurs first. As described in the WET method manual, reproduction was measured as the total number of young produced per original female at the time of appropriate test termination. Organisms positively identified as males were excluded from the reproduction analysis. Also, test concentrations above the survival NOEC were excluded from hypothesis testing conducted on the reproduction endpoint. Results of *Ceriodaphnia* chronic testing are shown in Tables 9.7 - 9.10 for each sample type.

Precision estimates were not calculated for the reference toxicant sample type because this sample type failed to produce toxicity that could be definitively measured within the test concentration range. For the reference toxicant sample, 97.3% of LC50s were >100% sample; 72.2% of IC25s were >100% sample. This was caused by a reference toxicant sample that was only moderately toxic. The spiking concentration of KCl for this sample was selected to achieve an IC25 of approximately 50% sample based on preliminary testing. Despite preliminary testing efforts, the spiking level selected was insufficient to produce this targeted level of effect (see Section 5.3).

9.3.1 Successful Test Completion Rate

A total of 122 *Ceriodaphnia* chronic tests were initiated by 34 participant laboratories. All 122 tests were completed; however, tests conducted on 22 samples were invalid due to failure to meet test acceptability criteria for survival or reproduction. The resulting successful test completion rate calculated in the WET Variability Study for the *Ceriodaphnia* chronic test method was 82.0%. In addition, the reproduction results for sample 9328 and the survival NOEC results for samples 9415 and 9380 were reported as inconclusive based on an evaluation of the concentration-response relationship (see Table 8.4). If these tests are considered unsuccessful in addition to invalid tests (since the test would be repeated in a regulatory context if the test endpoint required in the permit produced an inconclusive result), the successful test completion rate becomes 79.5%.

Of the 34 participant laboratories, 24 produced valid results for all samples tested. The 22 invalid tests were concentrated in the remaining 10 laboratories. Of these 10 laboratories, 8 laboratories performed invalid tests on 50% or more of the samples tested. Two laboratories performed invalid tests on all samples tested. This attributed to the relatively low successful test completion rate achieved for the *Ceriodaphnia* chronic test method in the WET Variability Study.

9.3.2 False Positive Rate

A total of 27 valid tests were conducted on blank samples by 22 participant laboratories (Table 9.7). No false positives were observed for the survival endpoint. The survival NOEC was 100% for all 27 blank samples, and the LC50 was >100% for all 27 blank samples. One false positive was observed for sublethal endpoints. The reproduction NOEC for sample 9450 was 25%, and the reproduction IC25 for this sample was 15.9%. The resulting false positive rate calculated in the WET Variability Study for the *Ceriodaphnia* chronic test method was 3.70% for the reproduction endpoint. The one false positive that was observed for this method originated from a laboratory that failed all other *Ceriodaphnia* chronic tests conducted.

In addition to the false positive reported above, a participant laboratory reported a reproduction IC25 of less than 100% for sample 9332, indicating a false positive result. Based on EPA guidance for evaluating concentration-response relationships (USEPA, 2000a), this value was determined to be an anomalous result of the ICp (percentage inhibition concentration) smoothing procedure, and the IC25 was corrected to >100% (Table 8.4). A participant laboratory also reported a survival NOEC and reproduction NOEC of less than 100% for sample 9379, indicating a false positive result. This sample exhibited an interrupted concentration-response curve, and based on EPA guidance for evaluating concentration-response relationships, the survival NOEC and growth NOEC were recalculated and reported as 100% (Table 8.4). Sample 9341 also produced an interrupted concentration-response curve, but the reproduction NOEC was similarly recalculated and reported as 100% (Table 8.4).

9.3.3 Precision

Precision of the *Ceriodaphnia* chronic test method was estimated by calculating the CV of LC50 and IC25 values obtained for the effluent and receiving water samples. CVs were not calculated for the reference toxicant sample type because this sample type failed to produce toxicity that could be definitively measured within the test concentration range (see Sections 5.3 and 9.1.4). For the effluent sample type, within-laboratory, between-laboratory, and total CVs were calculated. Only a total CV was calculated for the receiving water samples since no within-laboratory replication was provided for this sample type. Survival and reproduction results from samples 9347 and 9348 were excluded from the analysis of precision. These test samples were identified by ASTM h statistics as possible outliers, and review of data qualifier flags revealed a possible cause (Table 9.1).

Table 9.11 summarizes the precision of point estimates from the *Ceriodaphnia* chronic test method. Within-laboratory and between-laboratory CVs for LC50 values were 7.09% and 21.8%, respectively. Total CVs ranged from 20.0% for the receiving water sample type to 23.0% for the effluent sample type. Averaging the CVs based on total variance for these two sample types, a total CV of 21.5% was obtained for the *Ceriodaphnia* chronic survival endpoint.

CVs for IC25 values were higher than those for LC50 values. Within-laboratory CVs for the IC25 were 17.4%, between-laboratory CVs were 27.6%, and total CVs ranged from 32.6% to 37.4%. Averaging the CVs for the IC25 based on total variance for the two sample types, a total CV of 35.0% was obtained for the *Ceriodaphnia* chronic reproduction endpoint.

The precision of NOEC values was determined by evaluating the range and distribution of NOEC values and the percentage of values falling within and beyond one concentration from the median. Table 9.12 describes the precision of NOEC values for the *Ceriodaphnia* chronic method. For the survival endpoint, NOEC values spanned two concentrations for the receiving water sample types and three concentrations for the reference toxicant and effluent sample types. The percentage of values within one concentration of the median was 97.2%, 91.3%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

Reproduction NOEC values were less precise for the reference toxicant sample type, spanning five concentrations, but were comparable for the effluent and receiving water sample types, spanning three and two concentrations, respectively. The percentage of values within one concentration of the median was 83.3%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. Of the eight results (for samples 9342, 9343, 9360, 9392, 9397, 9415, 9361, and 9362) that were beyond one concentration from the median, three (samples 9343, 9392, and 9415) were the result of interrupted concentration-response curves. Three results that were beyond one concentration from the median were from very statistically sensitive tests, with percent minimum significant differences (PMSDs) below EPA's recommended lower bound of 11% (USEPA, 2000d). The PMSDs for these three samples (9360, 9361, and 9362) were 8.7%, 8.1%, and 9.2%, respectively.

Table 9.7. Results for *Ceriodaphnia* chronic test method performed on blank samples.

			Survival Information			Reproduction Information					
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (neonates)**	**Control CV (%)**	**Day of proper test termination**	**Flags[a]**
Referee	9425	10/12/99	100[b]	>100[b]	87.5	100[b]	>100[b]	17.1	48.6	7	d_1, g_2, g_6
46	9352	10/26/99	Invalid[c]	Invalid[c]	70.0	Invalid[c]	Invalid[c]	2.40	79.1	8	a_1, a_3, a_4, c_4, d_1, d_4, e_5, f_3, g_2
69	9363	10/12/99	Invalid[c]	Invalid[c]	100	Invalid[c]	Invalid[c]	9.10	53.7	8	a_4, c_2, e_6, f_3, g_2
311	9411	10/26/99	Invalid[c]	Invalid[c]	90.0	Invalid[c]	Invalid[c]	9.50	43.9	8	a_3, a_4, c_2, g_2
311	9412	10/26/99	Invalid[c]	Invalid[c]	100	Invalid[c]	Invalid[c]	8.40	56.7	8	a_3, a_4, c_2, g_2
333	9420	10/26/99	Invalid[c]	Invalid[c]	70.0	Invalid[c]	Invalid[c]	26.1	69.7	7	a_1, e_2, e_3, e_6, g_2, g_5
406	9422	10/12/99	Invalid[c]	Invalid[c]	70.0	Invalid[c]	Invalid[c]	21.1	50.4	6	a_1, c_1, c_2, g_2
406	9423	10/12/99	Invalid[c]	Invalid[c]	77.8	Invalid[c]	Invalid[c]	21.6	54.9	6	a_1, c_1, c_2, g_2
6	9330	10/26/99	100	>100	100	100	>100	25.4	26.0	6	f_4
25	9332	10/12/99	100	>100	80.0	100	>100	19.2	63.0	7	e_6
27	9337	10/26/99	100	>100	100	100	>100	20.1	27.8	6	
27	9338	10/26/99	100	>100	100	100	>100	24.2	12.3	7	b_1
30	9340	10/26/99	100	>100	80.0	100	>100	16.0	58.6	7	g_2
30	9341	10/26/99	100	>100	100	100	>100	23.5	22.1	6	g_5
33	9344	10/26/99	100	>100	100	100	>100	15.8	51.7	6	g_2
44	9349	10/12/99	100	>100	100	100	>100	30.8	6.46	7	e_2
44	9350	10/12/99	100	>100	100	100	>100	29.5	4.86	7	e_2
49	9356	10/12/99	100	>100	90.0	100	>100	24.1	18.7	6	c_2
71	9367	10/13/99	100	>100	100	100	>100	23.1	15.1	7	
73	9371	10/12/99	100	>100	100	100	>100	21.5	20.5	6	
101	9376	10/26/99	100	>100	100	100	>100	20.4	17.2	7	
105	9379	10/26/99	100	>100	100	100	>100	24.9	27.4	6	c_2, e_6, g_5
113	9381	10/12/99	100	>100	100	100	>100	26.5	12.4	7	c_2, e_6

Table 9.7. Results for *Ceriodaphnia* chronic test method performed on blank samples. (continued)

			Survival Information			Reproduction Information					
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (neonates)**	**Control CV (%)**	**Day of proper test termination**	**Flags[a]**
113	9382	10/12/99	100	>100	100	100	>100	27.5	9.11	7	c_2, e_6
125	9384	10/12/99	100	>100	90.0	100	>100	17.3	53.1	7	c_2, g_2
231	9402	10/27/99	100	>100	100	100	>100	22.7	16.6	8	b_2
299	9409	10/27/99	100	>100	90.0	100	>100	22.2	12.2	6	b_2, d_1
299	9410	10/27/99	100	>100	100	100	>100	24.8	15.7	6	b_2, d_1
416	9429	10/12/99	100	>100	100	100	>100	31.6	15.6	7	c_2, c_3
417	9432	10/12/99	100	>100	90.0	100	>100	18.8	28.7	8	c_2
421	9436	10/12/99	100	>100	100	100	>100	30.0	32.2	7	d_1, e_6
425	9439	10/12/99	100	>100	100	100	>100	18.9	33.8	8	c_1
448	9445	10/26/99	100	>100	100	100	>100	23.6	4.09	6	
450	9446	10/12/99	100	>100	100	100	>100	22.2	47.9	6	c_2, g_2
452	9450	10/12/99	100	>100	90.0	25	15.9	19.4	73.9	7	f_2, g_1, g_2
Summary Statistics	**N**		**27**	**27**		**27**	**27**				
	Min		**100**	**>100**		**25**	**15.9**				
	Max		**100**	**>100**		**100**	**>100**				
	Median		**100**	**>100**		**100**	**>100**				
	Mean			**>100**			**96.9**				
	False positives		**0**	**0**		**1**	**1**				
	False positive rate		**0.00%**	**0.00%**		**3.70%**	**3.70%**				

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.8. Results for *Ceriodaphnia* chronic test method performed on reference toxicant samples.

			Survival Information			Reproduction Information					
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (neonates)**	**Control CV (%)**	**Day of proper test termination**	**Flags[a]**
Referee	9428	10/26/99	100[b]	>100[b]	100	100[b]	>100[b]	25.3	11.9	6	d_1
6	9329	10/12/99	Invalid[c]	Invalid[c]	100	Invalid[c]	Invalid[c]	10.8	59.0	8	a_4, g_2
46	9354	10/26/99	Invalid[c]	Invalid[c]	100	Invalid[c]	Invalid[c]	5.60	68.5	8	a_3, a_4, c_4, d_1, d_4, e_5, f_3, g_2
46	9355	10/26/99	Invalid[c]	Invalid[c]	100	Invalid[c]	Invalid[c]	5.00	21.1	8	a_3, a_4, c_4, d_1, d_4, e_5, f_3, g_2
105	9377	10/12/99	Invalid[c]	Invalid[c]	100	Invalid[c]	Invalid[c]	14.8	46.7	6	a_4, c_2, e_6, g_2
105	9378	10/12/99	Invalid[c]	Invalid[c]	60.0	Invalid[c]	Invalid[c]	7.30	95.1	7	a_1, a_4, c_2, e_6, g_2, g_5
238	9405	10/12/99	Invalid[c]	Invalid[c]	30.0	Invalid[c]	Invalid[c]	9.50	16.6	8	a_1, a_3, a_4, e_2
311	9413	10/26/99	Invalid[c]	Invalid[c]	100	Invalid[c]	Invalid[c]	6.30	65.3	8	a_3, a_4, c_2, g_2
333	9419	10/26/99	Invalid[c]	Invalid[c]	50.0	Invalid[c]	Invalid[c]	13.1	115	6	a_1, a_4, e_2, e_3, e_6, g_2
421	9438	10/26/99	Invalid[c]	Invalid[c]	77.8	Invalid[c]	Invalid[c]	28.0	53.7	7	a_1, c_3, d_1, e_6
452	9452	10/27/99	Invalid[c]	Invalid[c]	90.0	Invalid[c]	Invalid[c]	7.80	58.2	8	a_3, a_4, b_2, f_2, g_1, g_2
452	9453	10/27/99	Invalid[c]	Invalid[c]	80.0	Invalid[c]	Invalid[c]	9.00	35.1	8	a_3, a_4, b_2, f_2, g_1, g_2
6	9328	10/12/99	100	>100	100	Inconclusive[d]	Inconclusive[d]	15.9	48.6	6	g_2
27	9336	10/12/99	100	>100	100	100	>100	24.7	9.36	7	b_1
30	9339	10/12/99	100	>100	100	50	<6.25	25.8	38.7	6	g_2
33	9342	10/12/99	100	>100	100	25	28.1	19.2	39.0	6	
33	9343	10/12/99	100	>100	100	12.5	24.9	21.1	18.1	6	g_5
42	9345	10/12/99	100	>100	100	100	>100	18.0	13.4	7	
42	9346	10/12/99	100	>100	100	100	>100	17.2	12.2	7	
44	9351	10/26/99	100	>100	100	100	>100	32.8	13.2	7	e_2
49	9357	10/26/99	100	>100	90.0	100	>100	19.8	40.7	6	c_3
49	9358	10/26/99	100	>100	100	100	>100	21.5	14.7	6	c_3, f_4
62	9359	10/12/99	100	>100	100	100	>100	25.6	8.48	6	
62	9360	10/12/99	100	>100	100	6.25	>100	27.4	7.14	6	
73	9372	10/26/99	100	>100	100	100	>100	22.7	12.8	6	
73	9373	10/26/99	100	>100	90.0	100	>100	23.0	20.1	6	

Table 9.8. Results for *Ceriodaphnia* chronic test method performed on reference toxicant samples. (continued)

			Survival Information			Reproduction Information					
LabID	Sample code	Test date	NOEC (% sample)	LC50 (% sample)	Control mean (%)	NOEC (% sample)	IC25 (% sample)	Control mean (neonates)	Control CV (%)	Day of proper test termination	Flags[a]
101	9374	10/12/99	100	>100	100	100	>100	22.8	7.40	6	
101	9375	10/12/99	100	>100	100	100	>100	24.2	8.22	6	
113	9383	10/26/99	100	>100	100	100	>100	32.0	13.4	7	e_6
125	9386	10/26/99	100	>100	100	100	>100	22.6	16.7	6	c_2
125	9387	10/26/99	100	>100	100	100	>100	21.5	19.0	6	c_2
134	9389	10/12/99	100	>100	100	100	>100	25.2	29.1	6	f_4, g_2
141	9392	10/12/99	100	>100	100	12.5	93.6	20.7	13.3	7	c_2, c_3, d_4, g_5
141	9393	10/12/99	100	>100	100	50	>100	19.1	10.6	7	c_2, c_3
205	9397	10/12/99	25	55.8	100	6.25	<6.25	16.5	21.6	6	b_1, c_1, c_2
231	9400	10/12/99	100	>100	100	50	81.2	24.6	8.40	6	
231	9401	10/12/99	100	>100	90.0	50	91.4	24.9	12.6	6	
299	9408	10/12/99	100	>100	100	100	>100	24.0	33.0	6	d_1, e_6
313	9414	10/20/99	100	>100	100	100	85.6	23.3	58.0	7	g_2
313	9415	10/20/99	Inconclusive[d]	>100	100	12.5	7.67	19.4	40.9	6	g_2,g_5
406	9424	10/26/99	100	>100	100	100	>100	26.3	15.5	6	c_1, f_4
416	9430	10/26/99	100	>100	100	100	>100	24.7	26.0	6	c_2, c_3
416	9431	10/26/99	100	>100	100	100	>100	25.8	12.9	6	c_2, c_3
417	9434	10/26/99	100	>100	90.0	100	>100	17.5	43.8	8	c_2, g_2
417	9435	10/26/99	100	>100	100	100	82.7	19.6	22.8	8	c_2, f_4
421	9437	10/26/99	100	>100	100	100	>100	30.1	33.1	7	c_3, d_1, e_6
448	9443	10/12/99	100	>100	100	100	>100	21.0	21.2	6	
448	9444	10/12/99	100	>100	100	100	>100	21.4	8.87	6	
450	9449	10/26/99	100	>100	100	100	>100	24.3	15.2	6	c_2
Summary Statistics	**N**		**36**	**37**		**36**	**36**				
	Min		**25**	**55.8**		**6.25**	**<6.25**				
	Max		**100**	**>100**		**100**	**>100**				
	Median		**100**	**>100**		**100**	**>100**				
	Mean			**98.8**			**86.3**				

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.
[d] Results were excluded from summary statistics. Based on EPA guidance for evaluating concentration-response relationships (EPA, 2000a) the test result was inconclusive and the sample should be retested to obtain a reliable result.

Table 9.9. Results for *Ceriodaphnia* chronic test method performed on effluent samples.

			Survival Information			Reproduction Information					
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (neonates)**	**Control CV (%)**	**Day of proper test termination**	**Flags[a]**
Referee	9427	10/26/99	25[b]	33.0[b]	100	12.5[b]	18.5[b]	24.0	8.10	6	
6	9331	10/26/99	Invalid[c]	Invalid[c]	100	Invalid[c]	Invalid[c]	13.1	17.1	6	a_4
25	9334	10/26/99	25	33.4	100	12.5	21.1	22.7	13.0	8	
25	9335	10/26/99	25	33.7	90.0	25	21.9	20.2	36.7	8	
42	9347	10/26/99	50[e]	70.7[e]	100	50[e]	60.4[e]	15.7	18.0	7	g_3, g_4
42	9348	10/26/99	25[e]	50.0[e]	100	25[e]	37.8[e]	18.1	19.0	7	g_3, g_4
62	9361	10/26/99	6.25	11.8	90.0	6.25	8.96	18.8	11.2	6	b_1
62	9362	10/26/99	6.25	12.5	100	6.25	9.38	20.2	10.6	6	b_1, d_1
69	9365	10/26/99	25	35.4	100	25	31.3	19.9	14.9	7	c_2
69	9366	10/26/99	25	35.4	100	12.5	23.2	29.3	17.8	6	c_2
71	9369	10/26/99	25	35.4	100	25	30.2	20.5	26.8	6	
71	9370	10/26/99	25	35.4	100	25	29.5	22.2	24.3	6	
105	9380	10/26/99	Inconclusive[d]	27.9	90.0	12.5	14.1	32.4	40.3	8	c_2, e_6, g_2, g_5
134	9390	10/26/99	12.5	26.5	100	12.5	18.6	27.0	45.8	6	f_4, g_2
134	9391	10/26/99	25	26.0	100	12.5	17.0	38.4	9.94	6	f_4
141	9394	10/26/99	25	30.9	100	12.5	16.3	15.9	9.58	6	c_2, c_3
141	9395	10/26/99	12.5	26.8	100	12.5	16.2	15.8	7.78	6	c_2, c_3, d_4
205	9398	10/26/99	25	28.7	100	12.5	13.5	18.7	35.3	6	b_1, c_1, c_2
205	9399	10/26/99	12.5	26.8	100	12.5	14.3	17.8	25.2	6	b_1, c_1, c_2
231	9403	10/27/99	25	28.7	100	12.5	18.2	22.3	21.8	8	b_1, b_2
238	9406	10/26/99	12.5	16.5	100	12.5	15.3	15.7	25.0	7	b_1, c_2, e_2
238	9407	10/26/99	12.5	20.2	100	12.5	17.1	17.1	18.2	8	b_1, c_2, e_2
313	9416	10/26/99	25	33.0	100	12.5	20.9	24.9	18.5	6	
313	9417	10/26/99	25	28.7	100	12.5	16.7	19.1	33.3	6	
333	9421	10/26/99	25	27.3	80.0	12.5	17.0	20.5	57.0	6	e_2, e_3, e_6, g_2
425	9441	10/26/99	25	30.8	100	12.5	17.0	15.7	12.4	8	c_1, e_6

Table 9.9. Results for *Ceriodaphnia* chronic test method performed on effluent samples. (continued)

			Survival Information			Reproduction Information					
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (neonates)**	**Control CV (%)**	**Day of proper test termination**	**Flags[a]**
425	9442	10/26/99	25	26.0	100	6.25	<6.25	16.4	11.9	8	c_1, e_6, g_{10}
450	9448	10/26/99	12.5	21.9	88.9	12.5	16.4	21.8	40.0	6	c_2, g_6
Summary Statistics	**N**		**23**	**24**		**24**	**24**				
	Min		**6.25**	**11.8**		**6.25**	**<6.25**				
	Max		**25**	**35.4**		**25**	**31.3**				
	Median		**25**	**28.3**		**12.5**	**17.0**				
	Mean			**27.5**			**17.9**				
	Within-lab	**STD**		**1.95**			**3.13**				
		CV%		**7.09%**			**17.4%**				
	Between-lab	**STD**		**6.00**			**4.95**				
		CV%		**21.8%**			**27.6%**				
	Total	**STD**		**6.31**			**5.85**				
		CV%		**23.0%**			**32.6%**				

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.
[d] Results were excluded from summary statistics. Based on EPA guidance for evaluating concentration-response relationships (EPA, 2000a) the test result was inconclusive and the sample should be retested to obtain a reliable result.
[e] Results were identified as outliers, a probable cause was identified, and therefore results were excluded from summary statistics.

Table 9.10. Results for *Ceriodaphnia* chronic test method performed on receiving water samples.

			Survival Information			Reproduction Information					
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (neonates)**	**Control CV (%)**	**Day of proper test termination**	**Flags[a]**
Referee	9426	10/12/99	25[b]	35.4[b]	100	25[b]	31.3[b]	20.5	30.3	6	
46	9353	10/26/99	Invalid[c]	Invalid[c]	80.0	Invalid[c]	Invalid[c]	5.50	67.6	8	a_3, a_4, b_1, b_2, b_3, c_4, d_1, d_4, e_5, f_3, g_2
238	9404	10/12/99	Invalid[c]	Invalid[c]	40.0	Invalid[c]	Invalid[c]	7.60	29.2	7	a_1, a_4, b_1, e_2, g_2
452	9451	10/12/99	Invalid[c]	Invalid[c]	90.0	Invalid[c]	Invalid[c]	10.2	45.0	8	a_4, f_2, g_1, g_2
25	9333	10/12/99	50	59.0	100	25	53.8	26.6	10.7	6	e_6, g_5
69	9364	10/12/99	25	40.5	100	25	27.1	15.0	37.7	8	c_2, f_3, g_2
71	9368	10/13/99	25	40.6	100	12.5	23.0	20.7	20.1	6	
125	9385	10/12/99	25	35.4	90.0	25	31.3	17.3	53.1	7	c_2, g_2
134	9388	10/12/99	25	37.9	100	25	31.6	31.6	39.9	7	f_4
205	9396	10/12/99	25	34.6	100	12.5	10.5	17.2	34.1	6	b_1, c_1, c_2
333	9418	10/12/99	25	53.6	100	25	28.1	36.2	7.79	6	b_1, e_2, e_3, e_4
417	9433	10/12/99	25	40.8	100	25	32.6	18.4	40.5	8	c_2, f_4, g_2
425	9440	10/12/99	25	33.0	100	12.5	22.3	19.5	13.7	8	c_1
450	9447	10/12/99	25	44.3	80.0	25	33.4	21.0	77.3	7	c_2, d_1, g_2
Summary Statistics	**N**		**10**	**10**		**10**	**10**				
	Min		**25**	**33.0**		**12.5**	**10.5**				
	Max		**50**	**59.0**		**25**	**53.8**				
	Median		**25**	**40.6**		**25**	**29.7**				
	Mean			**42.0**			**29.4**				
	STD			**8.38**			**11.0**				
	CV%			**20.0%**			**37.4%**				

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.11. Precision of point estimates from the *Ceriodaphnia* chronic test method.

Sample type	CV (%)					
	LC50			IC25		
	Within-lab [a]	Between-lab [a]	Total	Within-lab [a]	Between-lab [a]	Total
Reference toxicant [b]	-	-	-	-	-	-
Effluent	7.09	21.8	23.0	17.4	27.6	32.6
Receiving water	-	-	20.0	-	-	37.4
Average	**7.09**	**21.8**	**21.5**	**17.4**	**27.6**	**35.0**

[a] Within- and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.
[b] Precision estimates were not calculated for the reference toxicant sample type because this sample type failed to produce toxicity that could be definitively measured within the test concentration range.

Table 9.12. Precision of NOEC values from the *Ceriodaphnia* chronic test method.

Sample type	Endpoint	NOEC Frequency						Median (% sample)	% within 1 conc. of median	% beyond 1 conc. of median
		<6.25%	6.25%	12.5%	25%	50%	100%			
Reference toxicant	Survival	0	0	0	1	0	35	100	97.2	2.78
	Reproduction	0	2	3	1	4	26	100	83.3	16.7
Effluent	Survival	0	2	6	15	0	0	25	91.3	8.70
	Reproduction	0	3	17	4	0	0	12.5	100	0.00
Receiving water	Survival	0	0	0	9	1	0	25	100	0.00
	Reproduction	0	0	3	7	0	0	25	100	0.00

9.4 Fathead Acute Test Method Results

A total of 29 participant laboratories conducted the fathead acute test method in the WET Variability Study. These laboratories tested a total of 27 blank samples, 38 reference toxicant samples, 29 effluent samples, and 13 receiving water samples. For each sample tested, a 96-hour LC50 was generated as a test result. Results of fathead acute testing are shown in Tables 9.13 - 9.16 for each sample type.

9.4.1 Successful Test Completion Rate

A total of 107 fathead acute tests were initiated by 29 participant laboratories. All 107 tests were completed and met test acceptability criteria. The resulting successful test completion rate calculated in the WET Variability Study for the fathead acute test method was 100%. Two tests conducted by the referee laboratory were invalid due to control survival of 65%. These two tests were initiated on the same day, so poor health of organisms used for testing on that day is a likely cause.

9.4.2 False Positive Rate

A total of 27 blank samples were analyzed by 22 participant laboratories (Table 9.13). The LC50 calculated for all 27 blank samples was >100%, indicating no toxicity and no false positives. The resulting false positive rate calculated in the WET Variability Study for the fathead acute test method was 0.00%.

9.4.3 Precision

Precision of the fathead acute test method was estimated by calculating the CV of LC50 values obtained for the reference toxicant, effluent, and receiving water samples. Within-laboratory, between-laboratory, and total CVs were calculated for the reference toxicant and effluent samples. Only a total CV was calculated for the receiving water samples since no within-laboratory replication was provided for this sample type. All participant laboratory test data for the reference toxicant, effluent, and receiving water samples were used in estimating precision. While results on four test samples were identified by ASTM h statistics as possible outliers, these results were not excluded from the analysis of precision since a reasonable cause for the outliers could not be identified (Table 9.1).

Table 9.17 summarizes the precision of point estimates from the fathead acute test method. CVs were consistent among sample types, with within-laboratory CVs ranging from 7.62% to 10.3%, between-laboratory CVs ranging from 19.2% to 19.7%, and total CVs ranging from 17.2% to 21.8%. As expected, the majority of variability was due to the between-laboratory component, with within-laboratory CVs averaging 8.96% and between-laboratory CVs averaging 19.4%. Averaging the CVs based on total variance for the three sample types, a total CV of 20.0% was obtained for the fathead acute test method in the WET Variability Study.

Table 9.13. Results for fathead acute test method performed on blank samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9093	10/21/99	>100[b]	95.0	8.66	
3	9002	11/04/99	>100	90.0	17.1	
3	9003	11/04/99	>100	100	0.00	
6	9004	10/21/99	>100	100	0.00	
27	9012	10/21/99	>100	95.0	8.66	e_4
33	9018	11/04/99	>100	100	0.00	
42	9024	10/21/99	>100	100	0.00	b_5
42	9025	10/21/99	>100	95.0	8.66	b_5
46	9029	11/04/99	>100	100	0.00	c_4, d_4
60	9030	10/22/99	>100	90.0	0.00	b_2, c_1, d_7, e_2, e_3
60	9031	10/22/99	>100	100	0.00	b_2, c_1, d_7, e_2, e_3
70	9037	10/21/99	>100	100	0.00	
73	9041	10/21/99	>100	100	0.00	
101	9044	10/21/99	>100	100	0.00	
146	9056	10/21/99	>100	100	0.00	d_1, e_4
157	9061	11/04/99	>100	100	0.00	
157	9062	11/04/99	>100	100	0.00	
205	9063	10/21/99	>100	100	0.00	b_1, e_2
231	9069	11/04/99	>100	100	0.00	e_2, e_3
238	9073	11/04/99	>100	100	0.00	d_1, e_2
244	9077	11/04/99	>100	100	0.00	
251	9081	11/04/99	>100	100	0.00	
311	9082	10/21/99	>100	100	0.00	d_1, e_4
313	9085	10/21/99	>100	100	0.00	
406	9089	10/21/99	>100	95.0	8.66	c_1
416	9097	10/21/99	>100	100	0.00	
452	9109	10/21/99	>100	100	0.00	
452	9110	10/21/99	>100	100	0.00	
Summary Statistics	**N**		**27**			
	Min		**>100**			
	Max		**>100**			
	Median		**>100**			
	Mean		**>100**			
	False positives		**0**			
	False positive rate		**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.14. Results for fathead acute test method performed on reference toxicant samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9096	11/04/99	Invalid[b]	65.0	7.92	a_1
3	9001	10/21/99	41.8	95.0	8.66	
6	9006	11/04/99	43.6	100	0.00	
6	9007	11/04/99	46.7	100	0.00	
25	9008	10/21/99	35.4	100	0.00	g_1
25	9009	10/21/99	40.1	100	0.00	g_1
33	9017	10/21/99	32.3	100	0.00	
41	9020	10/21/99	26.8	100	0.00	e_2
41	9021	10/21/99	24.1	100	0.00	e_2
42	9026	11/04/99	31.9	100	0.00	
46	9027	10/21/99	23.4	95.0	8.66	c_4, d_4
46	9028	10/21/99	29.6	95.0	8.66	c_4, d_4
60	9032	11/04/99	33.9	100	0.00	c_1, d_2, d_3, e_2, e_3
62	9033	10/21/99	63.9	100	0.00	g_3
62	9034	10/21/99	55.5	100	0.00	g_3
73	9042	11/04/99	35.4	100	0.00	
73	9043	11/04/99	34.2	100	0.00	
101	9046	11/04/99	55.4	100	0.00	
101	9047	11/04/99	53.6	100	0.00	
105	9049	10/21/99	36.7	95.0	8.66	e_2
141	9052	10/21/99	34.0	95.0	8.66	
141	9053	10/21/99	37.9	95.0	8.66	
146	9059	11/04/99	32.7	100	0.00	d_1, e_4
157	9060	10/21/99	33.0	100	0.00	
231	9067	10/21/99	34.2	100	0.00	e_2, e_3
231	9068	10/21/99	38.2	100	0.00	e_2, e_3
238	9071	10/21/99	39.2	100	0.00	d_1, e_2
238	9072	10/21/99	46.7	100	0.00	d_1, e_2
244	9075	10/21/99	33.7	100	0.00	
244	9076	10/21/99	34.3	100	0.00	
251	9079	10/21/99	34.2	100	0.00	
251	9080	10/21/99	34.6	100	0.00	
311	9083	11/04/99	39.2	100	0.00	d_1
311	9084	11/04/99	42.0	100	0.00	d_1
313	9087	11/04/99	45.6	95.0	8.66	
313	9088	11/04/99	46.8	95.0	8.66	
417	9102	10/21/99	42.1	100	0.00	e_2, e_4

Table 9.14. Results for fathead acute test method performed on reference toxicant samples. (continued)

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
425	9106	10/21/99	43.5	100	0.00	
452	9111	11/04/99	30.9	100	0.00	e_1
Summary Statistics	**N**		**38**			
	Min		**23.4**			
	Max		**63.9**			
	Median		**36.0**			
	Mean		**38.6**			
	Within-lab	**STD**	**2.94**			
		CV%	**7.62%**			
	Between-lab	**STD**	**7.60**			
		CV%	**19.7%**			
	Total	**STD**	**8.15**			
		CV%	**21.1%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.15. Results for fathead acute test method performed on effluent samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9095	11/4/99	Invalid[b]	65.0	24.0	a_1, d_1
25	9010	11/4/99	21.8	100	0.00	g_1
25	9011	11/4/99	22.5	100	0.00	g_1
27	9014	11/4/99	18.9	100	0.00	
27	9015	11/4/99	19.8	100	0.00	
33	9019	11/4/99	19.6	100	0.00	
41	9022	11/4/99	16.2	95.0	8.66	e_2
41	9023	11/4/99	18.3	100	0.00	b_1, e_2
62	9035	11/4/99	27.7	100	0.00	e_4
62	9036	11/4/99	23.3	100	0.00	e_4
70	9039	11/4/99	21.8	100	0.00	e_1
70	9040	11/4/99	21.0	100	0.00	
105	9050	11/4/99	23.9	100	0.00	d_2, e_2
105	9051	11/4/99	15.8	100	0.00	d_2, e_2
141	9054	11/4/99	17.5	95.0	8.66	
141	9055	11/4/99	15.7	95.0	8.66	
146	9058	11/4/99	18.2	100	0.00	d_1, e_4
205	9065	11/4/99	37.7	100	0.00	e_2, g_3
205	9066	11/4/99	32.7	100	0.00	e_2, g_3
231	9070	11/4/99	18.9	100	0.00	e_2, e_3
238	9074	11/4/99	19.6	100	0.00	d_1, e_2
244	9078	11/4/99	19.6	100	0.00	
406	9091	11/4/99	27.7	100	0.00	c_1, e_4
406	9092	11/4/99	26.2	100	0.00	c_1, e_4
416	9099	11/4/99	22.3	95.0	8.66	
416	9100	11/4/99	23.1	100	0.00	
417	9103	11/4/99	18.2	100	0.00	e_2, e_4
417	9104	11/4/99	18.3	100	0.00	e_2, e_4
425	9107	11/4/99	19.7	95.0	8.66	d_1
425	9108	11/4/99	19.1	90.0	17.1	d_1
Summary Statistics	**N**		**29**			
	Min		**15.7**			
	Max		**37.7**			
	Median		**19.7**			
	Mean		**21.6**			
	Within-lab	**STD**	**2.22**			
		CV%	**10.3%**			
	Between-lab	**STD**	**4.14**			
		CV%	**19.2%**			
	Total	**STD**	**4.70**			
		CV%	**21.8%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.16. Results for fathead acute test method performed on receiving water samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9094	10/21/99	19.6[b]	95.0	8.66	
6	9005	10/21/99	17.7	100	0.00	
27	9013	10/21/99	17.7	100	0.00	e_4
33	9016	10/21/99	16.6	100	0.00	
70	9038	10/21/99	18.9	100	0.00	
101	9045	10/21/99	26.2	95.0	8.66	
105	9048	10/21/99	16.9	95.0	8.66	e_2
146	9057	10/21/99	20.3	100	0.00	d_1, e_4
205	9064	10/21/99	16.2	100	0.00	b_1, e_2
313	9086	10/21/99	23.9	100	0.00	
406	9090	10/21/99	19.1	100	0.00	c_1
416	9098	10/21/99	16.9	100	0.00	
417	9101	10/21/99	21.6	100	0.00	e_2, e_4
425	9105	10/21/99	25.0	100	0.00	
Summary Statistics	**N**		**13**			
	Min		**16.2**			
	Max		**26.2**			
	Median		**18.9**			
	Mean		**19.8**			
	STD		**3.41**			
	CV%		**17.2%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.17. Precision of point estimates from the fathead acute test method.

Sample type	**CV (%)**		
	Within-lab [a]	**Between-lab [a]**	**Total**
Reference toxicant	7.62	19.7	21.1
Effluent	10.3	19.2	21.8
Receiving water	-	-	17.2
Average	**8.96**	**19.4**	**20.0**

[a] Within- and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.

9.5 Fathead Chronic Test Method Results

A total of 27 participant laboratories conducted the fathead chronic test method in the WET Variability Study. These laboratories tested a total of 24 blank samples, 37 reference toxicant samples, 27 effluent samples, and 13 receiving water samples. For each sample tested, a 7-day survival NOEC, a 7-day growth NOEC, a 7-day survival LC50, and a 7-day growth IC25 were generated as test results. As described in the WET method manual, growth was measured as the total weight per replicate divided by the number of original organisms in that replicate. This definition provides a combined growth and survival endpoint that is more accurately termed biomass. Also, test concentrations above the survival NOEC were excluded from hypothesis testing conducted on the growth endpoint. Results of fathead chronic testing are shown in Tables 9.18 - 9.21 for each sample type.

9.5.1 Successful Test Completion Rate

A total of 101 fathead chronic tests were initiated by 27 participant laboratories. All 101 tests were completed; however, tests conducted on samples 9199 and 9118 were invalid due to failure to meet test acceptability criteria for growth and survival, respectively. The resulting successful test completion rate calculated in the WET Variability Study for the fathead chronic test method was 98.0%. In addition, the growth NOEC result for sample 9209 was reported as inconclusive based on an evaluation of the concentration-response relationship (Table 8.4). If this test is considered unsuccessful (since the test would be repeated in a regulatory context if the test endpoint required in the permit produced an inconclusive result), the successful test completion rate becomes 97.0%.

9.5.2 False Positive Rate

A total of 24 blank samples were analyzed by 20 participant laboratories (Table 9.18). No false positives were observed for the survival endpoint. The survival NOEC was 100% for all 24 blank samples, and the LC50 was >100% for all 24 blank samples. One false positive was observed for sublethal endpoints. The growth NOEC for sample 9158 was 50%, and the growth IC25 for this sample was 93.6%. The resulting false positive rate calculated in the WET Variability Study for the fathead chronic test method was 4.35% for the growth NOEC and 4.17% for the growth IC25. The one false positive that was observed was due to poor survival in a single replicate of the 100% test concentration treatment. For this sample, the survival in the 100% test concentration was 90%, 100%, 90%, and 50% for the 4 replicates, respectively. Disregarding replicate 4, the survival for this treatment would be identical to the control survival (95%).

In addition to the false positive reported above, participant laboratories reported a NOEC of less than 100% for two additional samples (9145 and 9209), indicating false positive results. These samples exhibited an interrupted concentration-response curve. Based on EPA guidance for evaluating concentration-response relationships, the growth and survival NOEC for sample 9145 was recalculated and reported as 100%, and the growth NOEC for sample 9209 was reported as inconclusive (Table 8.4).

9.5.3 Precision

Precision of the fathead chronic test method was estimated by calculating the CV of LC50 and IC25 values obtained for the reference toxicant, effluent, and receiving water samples. Within-laboratory, between-laboratory, and total CVs were calculated for the reference toxicant and effluent samples. Only a total CV was calculated for the receiving water samples since no within-laboratory replication was provided for this sample type. Results from two samples (9162 and 9163) were excluded from the analysis of precision. These test samples were identified by ASTM h statistics as possible outliers, and review of data qualifier flags revealed a possible cause (Table 9.1). One additional sample (9177) was identified as a possible outlier but was not excluded from the analysis of precision since a reasonable cause was not identified.

Table 9.22 summarizes the precision of point estimates from the fathead chronic test method. CVs for LC50 values were consistent among sample types, with within-laboratory CVs ranging from 6.59% to 9.16%, between-laboratory CVs ranging from 10.6% to 12.0%, and total CVs ranging from 12.5% to 15.1%. As expected, the majority of variability was due to the between-laboratory component, with within-laboratory CVs averaging 7.87% and between-laboratory CVs averaging 11.3%. Averaging the CVs for the LC50 based on total variance for the three sample types, a total CV of 13.4% was obtained for the fathead chronic survival endpoint.

CVs for IC25 values were higher than those for LC50 values. Within-laboratory CVs for the IC25 ranged from 10.0% to 19.1%, between-laboratory CVs ranged from 12.9% to 17.2%, and total CVs ranged from 19.8% to 23.1%. Within-laboratory CVs (averaging 14.6%) were only slightly lower than between-laboratory CVs (averaging 15.0%). Averaging the CVs for the IC25 based on total variance for the three sample types, a total CV of 20.9% was obtained for the fathead chronic growth endpoint.

The precision of NOEC values was determined simply by evaluating the range and distribution of NOEC values and the percentage of values falling within and beyond one concentration from the median. Table 9.23 describes the precision of NOEC values for the fathead chronic method. For the survival endpoint, NOEC values spanned four concentrations for the reference toxicant sample type and two concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 97.2%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

Growth NOEC values were less precise, spanning five concentrations for the reference toxicant sample type and four concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 86.1%, 91.7%, and 76.9% for the reference toxicant, effluent, and receiving water sample types, respectively. Of the 10 results (for samples 9150, 9177, 9193, 9194, 9212, 9129, 9166, 9161, 9176, and 9180) that were beyond 1 concentration from the median, 5 results (samples 9193, 9194, 9212, 9129, and 9161) were the consequence of interrupted concentration-response curves. For each of these samples, test concentrations higher than the reported NOEC were not significantly different from the control, but the NOEC was reported as the concentration below the LOEC based on EPA's concentration-response guidance (Table 8.4). Three results that were beyond one concentration from the median were from very statistically sensitive tests, with PMSDs near or below EPA's recommended lower bound of 9.4% (USEPA, 2000d). The PMSDs for these three samples (9150, 9166, and 9180) were 9.2%, 9.7%, and 10.3%, respectively. The

remaining two samples (9176 and 9177) that were beyond one concentration from the median were from the same laboratory (205) and were conducted on the same day, indicating that test organisms or specific test procedures in this laboratory may have produced conditions that caused greater sensitivity.

Table 9.18. Results for fathead chronic test method performed on blank samples.

			Survival Information				Growth Information				
LabID	Sample code	Test date	NOEC (% sample)	LC50 (% sample)	Control mean (%)	Control CV (%)	NOEC (% sample)	IC25 (% sample)	Control mean (mg)	Control CV (%)	Flags[a]
Referee	9201	09/28/99	100[b]	>100[b]	100	0.00	100[b]	>100[b]	0.477	5.23	d_1, e_1
3	9113	10/05/99	100	>100	100	0.00	100	>100	0.381	7.59	
3	9114	10/05/99	100	>100	95.0	11.4	100	>100	0.365	11.7	
6	9117	10/05/99	100	>100	97.5	5.94	100	>100	0.371	8.46	
25	9119	09/28/99	100	>100	100	0.00	100	>100	0.511	4.90	e_3
27	9123	09/28/99	100	>100	100	0.00	100	>100	0.788	4.50	b_1
33	9131	09/29/99	100	>100	100	0.00	100	>100	0.383	16.1	b_2
42	9135	09/28/99	100	>100	90.0	9.94	100	>100	0.471	10.6	b_1
42	9136	09/28/99	100	>100	92.5	6.32	100	>100	0.506	5.02	b_1, d_7
46	9138	09/28/99	100	>100	96.7	6.93	100	>100	0.300	12.0	c_4, d_4, f_1
49	9142	09/28/99	100	>100	95.2	6.73	100	>100	0.360	9.88	e_1
49	9143	09/28/99	100	>100	97.5	5.94	100	>100	0.379	3.77	e_1
62	9145	09/28/99	100	>100	95.0	7.07	100	>100	0.293	18.2	d_1, g_5
73	9151	10/05/99	100	>100	100	0.00	100	>100	0.807	8.70	
101	9152	09/28/99	100	>100	100	0.00	100	>100	0.375	22.9	
105	9158	10/05/99	100	>100	95.0	11.4	50	93.6	0.392	12.5	c_4, e_2
125	9160	09/28/99	100	>100	92.5	6.32	100	>100	0.382	16.0	c_2, g_2
231	9182	10/05/99	100	>100	97.5	5.94	100	>100	0.663	6.38	e_2, e_3
238	9186	10/05/99	100	>100	95.0	7.07	100	>100	0.373	13.4	c_2, e_2, e_6, g_2
299	9188	09/28/99	100	>100	100	0.00	100	>100	0.770	2.93	d_1, e_1, e_2, e_6
311	9192	09/28/99	100	>100	100	0.00	100	>100	0.503	4.58	
333	9196	10/05/99	100	>100	97.5	5.94	100	>100	0.602	5.75	
333	9197	10/05/99	100	>100	95.0	11.4	100	>100	0.693	17.2	e_1, e_6
406	9198	09/28/99	100	>100	90.0	9.94	100	>100	0.262	9.51	b_1, c_1, f_1
425	9209	09/28/99	100	>100	100	0.00	Inconclusive[c]	>100	0.643	6.89	c_2, g_2, g_5

Table 9.18. Results for fathead chronic test method performed on a blank samples. (continued)

			Survival Information				Growth Information				
LabID	Sample code	Test date	NOEC (% sample)	LC50 (% sample)	Control mean (%)	Control CV (%)	NOEC (% sample)	IC25 (% sample)	Control mean (mg)	Control CV (%)	Flags[a]
Summary Statistics	N		24	24			23	24			
	Min		100	>100			50	93.6			
	Max		100	>100			100	>100			
	Median		100	>100			100	>100			
	Mean			>100				99.7			
	False positives		0	0			1	1			
	False positive rate		0.00%	0.00%			4.35%	4.17%			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results were excluded from summary statistics. Based on EPA guidance for evaluating concentration-response relationships (EPA, 2000a) the test result was inconclusive and the sample should be retested to obtain a reliable result.

Table 9.19. Results for fathead chronic test method performed on reference toxicant samples.

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9204	10/05/99	50[b]	70.7[b]	100	0.00	50[b]	63.3[b]	0.435	14.3	d_1, e_1
406	9199	10/05/99	Invalid[c]	Invalid[c]	95.0	7.07	Invalid[c]	Invalid[c]	0.230	9.51	a_2, c_1, e_6, f_1, g_5
3	9112	09/29/99	50	83.7	100	0.00	50	64.2	0.534	4.26	b_2, c_2, f_1
6	9115	09/29/99	50	70.7	97.5	5.94	50	62.5	0.250	15.3	b_2
6	9116	09/29/99	50	66.2	97.5	5.94	50	56.7	0.274	7.97	b_2
25	9122	10/05/99	50	51.4	100	0.00	50	51.4	0.467	9.59	e_3, g_5
27	9125	10/05/99	25	40.6	100	0.00	25	33.5	0.671	7.57	b_1
27	9126	10/05/99	25	54.9	100	0.00	25	47.5	0.692	7.40	b_1
30	9127	09/28/99	25	53.2	95.0	7.07	25	26.8	0.687	32.5	
30	9128	09/28/99	25	58.4	100	0.00	25	45.0	0.574	14.2	
42	9137	10/05/99	50	66.4	95.0	7.07	50	57.1	0.506	7.33	b_1
49	9144	10/05/99	50	68.5	85.0	10.9	50	58.4	0.386	7.55	d_1
62	9147	10/05/99	50	67.9	92.5	6.32	50	53.6	0.275	4.70	
62	9148	10/05/99	50	62.1	90.0	14.0	50	54.2	0.303	16.9	
73	9149	09/28/99	50	64.0	97.5	5.94	25	51.5	0.754	7.83	
73	9150	09/28/99	25	65.0	100	0.00	12.5	56.8	0.697	6.87	
105	9156	09/29/99	50	63.8	92.5	6.32	50	58.2	0.246	14.1	b_2, d_2, e_2
105	9157	09/29/99	50	65.5	100	0.00	50	58.9	0.258	17.0	b_2, d_2, e_2
134	9165	09/28/99	50	65.0	100	0.00	25	49.4	0.617	14.1	
141	9168	09/28/99	50	69.5	100	0.00	50	57.4	0.433	15.6	c_2, c_3, g_5
141	9169	09/28/99	50	73.7	100	0.00	50	63.2	0.433	15.6	c_2, c_3
157	9173	09/28/99	50	66.8	95.0	7.07	25	53.4	0.505	11.0	c_3
205	9177	09/28/99	50	69.0	97.5	5.94	6.25	20.2	0.478	7.13	g_3
231	9181	09/28/99	50	68.3	100	0.00	50	59.1	0.490	9.53	e_2, e_3
238	9184	09/28/99	50	73.7	100	0.00	50	59.4	0.442	18.1	e_2

Table 9.19. Results for fathead chronic test method performed on reference toxicant samples. (continued)

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
238	9185	09/28/99	50	74.5	100	0.00	50	56.3	0.532	16.1	c_3, e_2, e_6
299	9190	10/05/99	25	59.5	100	0.00	25	35.7	0.743	2.97	d_1, e_2, e_6
299	9191	10/05/99	25	49.6	100	0.00	25	34.0	0.686	9.18	d_1, e_2, e_6
311	9193	10/05/99	50	68.8	100	0.00	<6.25	56.6	0.413	9.46	g_5
311	9194	10/05/99	50	69.5	100	0.00	12.5	58.0	0.392	3.76	g_5
333	9195	09/28/99	50	70.7	92.5	11.3	50	59.8	0.632	10.3	
406	9200	10/05/99	50	60.9	90.0	9.94	50	58.8	0.235	12.2	c_1, e_6, f_1
416	9205	09/28/99	50	71.2	100	0.00	50	58.9	0.454	2.22	c_2, c_3
416	9206	09/28/99	50	70.7	100	0.00	50	63.1	0.414	9.56	c_2
425	9211	10/05/99	25	62.7	100	0.00	25	57.0	0.563	4.44	c_2
425	9212	10/05/99	6.25	54.3	100	0.00	6.25	50.4	0.493	9.14	c_2, g_5
452	9213	09/29/99	50	69.5	97.5	5.94	50	60.9	0.588	12.8	b_2, e_2
452	9214	09/29/99	50	71.2	100	0.00	50	57.3	0.710	6.65	b_2, e_2
Summary Statistics	**N**		**36**	**36**			**36**	**36**			
	Min		**6.25**	**40.6**			**<6.25**	**20.2**			
	Max		**50**	**83.7**			**50**	**64.2**			
	Median		**50**	**66.6**			**50**	**56.9**			
	Mean			**65.0**				**52.9**			
	Within-lab	**STD**		**4.29**				**5.30**			
		CV%		**6.59%**				**10.0%**			
	Between-lab	**STD**		**6.87**				**9.10**			
		CV%		**10.6%**				**17.2%**			
	Total	**STD**		**8.10**				**10.5**			
		CV%		**12.5%**				**19.9%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.20. Results for fathead chronic test method performed on effluent samples.

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (%sample)**	**IC25 (%sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9203	10/05/99	25[b]	31.1[b]	97.5	5.94	12.5[b]	26.9[b]	0.430	10.7	e_1
6	9118	10/05/99	Invalid[c]	Invalid[c]	70.0	23.8	Invalid[c]	Invalid[c]	0.348	27.4	a_1
25	9121	10/05/99	12.5	27.8	92.5	6.32	12.5	20.6	0.448	11.6	e_3
30	9129	10/05/99	12.5	21.6	92.5	11.3	<6.25	16.1	0.490	10.9	d_2, g_5
30	9130	10/05/99	12.5	19.9	85.0	20.2	12.5	16.1	0.375	29.9	d_2
33	9133	10/05/99	12.5	24.5	95.0	11.4	12.5	14.9	0.686	20.7	b_1, g_2
33	9134	10/05/99	12.5	24.5	87.5	20.0	12.5	21.2	0.644	16.6	b_1
46	9140	10/05/99	12.5	19.3	95.0	11.4	12.5	16.1	0.345	16.8	c_4, d_4, f_1
46	9141	10/05/99	12.5	20.0	97.5	5.94	12.5	16.3	0.395	14.7	c_4, d_4, f_1
101	9154	10/05/99	25	32.1	97.5	5.94	25	29.2	0.490	14.3	
101	9155	10/05/99	25	30.5	97.5	5.94	12.5	20.5	0.528	5.66	
105	9159	10/05/99	12.5	26.3	95.0	11.4	12.5	18.4	0.366	5.22	d_2, e_2
125	9162	10/05/99	12.5	21.1	92.5	11.3	<6.25[d]	7.04[d]	0.390	8.36	c_3, g_3
125	9163	10/05/99	25	27.6	92.5	11.3	<6.25[d]	<6.25[d]	0.390	8.36	c_3, g_3
134	9166	10/05/99	12.5	30.3	100	0.00	<6.25	18.4	0.607	7.78	
134	9167	10/05/99	12.5	31.3	100	0.00	6.25	26.1	0.593	3.49	d_1
141	9170	10/05/99	12.5	29.7	100	0.00	12.5	23.8	0.435	4.79	c_2, c_3, d_2
141	9171	10/05/99	12.5	27.6	100	0.00	6.25	15.6	0.435	4.79	c_2, c_3, d_2
157	9174	10/05/99	12.5	29.6	97.5	5.94	12.5	29.0	0.545	12.4	
157	9175	10/05/99	25	28.4	95.0	11.4	25	26.8	0.494	13.7	
205	9178	10/05/99	12.5	27.7	100	0.00	<12.5	20.5	0.403	6.53	b_1, b_4, e_7
205	9179	10/05/99	12.5	25.4	100	0.00	12.5	17.3	0.420	15.4	b_1, b_4, e_7
231	9183	10/05/99	12.5	24.1	100	0.00	12.5	19.0	0.545	6.81	b_1, e_2, e_3
238	9187	10/05/99	12.5	24.2	100	0.00	12.5	16.9	0.451	6.61	e_2
416	9207	10/05/99	12.5	21.0	100	0.00	12.5	14.8	0.703	9.21	c_2, c_3, f_1
416	9208	10/05/99	25	28.8	97.5	5.94	12.5	23.7	0.698	15.2	c_2, c_3, f_1

Table 9.20. Results for fathead chronic test method performed on effluent samples. (continued)

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (%sample)**	**IC25 (%sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
452	9215	10/05/99	12.5	30.4	100	0.00	12.5	26.2	0.461	10.0	e_2
452	9216	10/05/99	25	34.1	95.0	11.4	25	29.8	0.420	6.90	e_2
Summary Statistics	**N**		**26**	**26**			**24**	**24**			
	Min		**12.5**	**19.3**			**<6.25**	**14.8**			
	Max		**25**	**34.1**			**25**	**29.8**			
	Median		**12.5**	**27.6**			**12.5**	**19.8**			
	Mean			**26.5**				**20.7**			
	Within-lab	**STD**		**2.42**				**3.96**			
		CV%		**9.16%**				**19.1%**			
	Between-lab	**STD**		**3.16**				**2.68**			
		CV%		**12.0%**				**12.9%**			
	Total	**STD**		**3.99**				**4.78**			
		CV%		**15.1%**				**23.1%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.
[d] Results were identified as outliers, a probable cause was identified, and therefore results were excluded from summary statistics.

Table 9.21. Results for fathead chronic test method performed on receiving water samples.

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (%sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9202	9/28/99	25[b]	34.9[b]	97.5	5.94	25[b]	29.5[b]	0.473	4.34	e_1
25	9120	9/28/99	25	31.2	95.0	7.07	25	29.3	0.510	5.48	e_3
27	9124	9/28/99	12.5	28.7	100	0.00	12.5	28.0	0.655	8.70	
33	9132	9/29/99	25	34.2	100	0.00	25	29.3	0.381	13.9	b_2
46	9139	9/28/99	12.5	28.0	100	0.00	6.25	19.0	0.563	11.8	c_4, d_1, d_4, e_6, f_1
62	9146	9/28/99	25	32.5	95.0	7.07	25	29.2	0.285	8.35	d_1
101	9153	9/28/99	12.5	31.2	97.5	5.94	12.5	25.9	0.353	23.2	
125	9161	9/28/99	25	37.4	92.5	6.32	<6.25	28.6	0.382	16.0	c_2, g_5
134	9164	9/28/99	25	35.4	97.5	5.94	12.5	25.4	0.575	5.88	
157	9172	9/28/99	25	34.1	90.0	14.0	25	29.8	0.545	14.4	
205	9176	9/28/99	25	41.8	97.5	5.94	<6.25	16.1	0.433	17.7	b_1, b_4, d_2
231	9180	9/28/99	25	37.2	100	0.00	<6.25	22.6	0.506	8.43	b_1, e_2, e_3
299	9189	9/28/99	12.5	27.7	100	0.00	12.5	18.8	0.719	10.2	e_2, e_6
425	9210	9/28/99	25	37.1	100	0.00	25	32.4	0.675	12.7	c_2
Summary Statistics	**N**		**13**	**13**			**13**	**13**			
	Min		**12.5**	**27.7**			**<6.25**	**16.1**			
	Max		**25**	**41.8**			**25**	**32.4**			
	Median		**25**	**34.1**			**12.5**	**28.0**			
	Mean			**33.6**				**25.7**			
	STD			**4.22**				**5.08**			
	CV%			**12.6%**				**19.8%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.22. Precision of point estimates from the fathead chronic test method.

Sample type	CV (%)					
	LC50			IC25		
	Within-lab [a]	Between-lab [a]	Total	Within-lab [a]	Between-lab [a]	Total
Reference toxicant	6.59	10.6	12.5	10.0	17.2	19.9
Effluent	9.16	12.0	15.1	19.1	12.9	23.1
Receiving water	-	-	12.6	-	-	19.8
Average	**7.87**	**11.3**	**13.4**	**14.6**	**15.0**	**20.9**

[a] Within- and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.

Table 9.23. Precision of NOEC values from the fathead chronic test method.

Sample type	Endpoint	NOEC Frequency						Median (% sample)	% within 1 conc. of median	% beyond 1 conc. of median
		<6.25%	6.25%	12.5%	25%	50%	100%			
Reference toxicant	Survival	0	1	0	8	27	0	50	97.2	2.78
	Growth	1	2	2	10	21	0	50	86.1	13.9
Effluent	Survival	0	0	20	6	0	0	12.5	100	0.00
	Growth	2	3	16	3	0	0	12.5	91.7	8.33
Receiving water	Survival	0	0	4	9	0	0	25	100	0.00
	Growth	3	1	4	5	0	0	12.5	76.9	23.1

9.6 *Selenastrum* Chronic Test Method Results

A total of 11 participant laboratories conducted the *Selenastrum* chronic test method in the WET Variability Study. These laboratories tested a total of 8 blank samples, 13 reference toxicant samples, 15 effluent samples, and 8 receiving water samples. Each of these samples was tested with and without the addition of EDTA to the sample and dilution water. For each test conducted, a 96-hour growth NOEC, a 96-hour growth IC25, and a 96-hour growth IC50 were generated as test results. The growth endpoint was measured as cell density (cells per mL). Test acceptability criteria were assessed independently for tests conducted with EDTA (minimum control cell density of $1x10^6$ cells/mL) and without EDTA (minimum control cell density of $2x10^5$ cells/mL). The test acceptability criteria for control variability was determined by calculating the CV of growth in control replicates (control CV must be less than 20% to meet test acceptability criteria). Results of *Selenastrum* chronic testing are shown in Tables 9.24 - 9.31 for each sample type tested with and without the addition of EDTA.

9.6.1 Successful Test Completion Rate

A total of 44 *Selenastrum* chronic tests were initiated with the addition of EDTA, and 44 tests were initiated without the addition of EDTA. All 88 tests were completed; however, 16 tests conducted with EDTA and 15 tests conducted without EDTA were invalid due to failure to meet test acceptability criteria for control growth or control variability. The resulting successful test completion rate calculated in the WET Variability Study for the *Selenastrum* chronic test method was 63.6% with EDTA and 65.9% without EDTA. In addition, growth endpoints for samples 9473 without EDTA, 9455 with EDTA, 9454 without EDTA, and 9468 without EDTA were reported as inconclusive based on an evaluation of the concentration-response relationship (Table 8.4). If these tests are considered unsuccessful (since the tests would be repeated in a regulatory context if the test endpoint required in the permit produced an inconclusive result), the successful test completion rate becomes 61.4% with EDTA and 59.1% without EDTA.

Two distinct patterns of test failures were observed for laboratories conducting the *Selenastrum* chronic test method. The first pattern involved the failure of both tests (with and without EDTA) conducted on a given sample and a given day. This pattern was observed in laboratories 39, 299, 33, and 459. All but 2 of the 18 test failures from these laboratories were observed to occur for tests conducted with and without EDTA on a given sample. For instance, laboratory 39 failed tests with and without EDTA on samples 9466 and 9467, but passed tests with and without EDTA on samples 9468 and 9469. The second pattern of test failures involved the failure of all tests conducted with a given nutrient type (with or without EDTA). Laboratories 33, 406, and 209 exhibited this pattern. Laboratories 33 and 406 failed all tests (8 of 8) with EDTA and passed all but 1 (7 of 8) test without EDTA. Laboratory 209 failed all tests (4 of 4) without EDTA and passed all tests with EDTA. This pattern was further explained by the culturing methods and general testing procedures used in each laboratory. Laboratories 33 and 406, which failed all tests with EDTA, normally cultured organisms and conducted tests without the addition of EDTA. Laboratory 209, which failed all tests without EDTA, normally cultured organisms and conducted tests with EDTA.

9.6.2 False Positive Rate

A total of five valid tests were conducted on blank samples with the addition of EDTA (Table 9.24). No false positives were observed for the growth NOEC, IC25, or IC50. The growth NOEC was 100% for all 5 blank

samples tested with EDTA, and the IC25 and IC50 values were >100% for all 5 blank samples tested with EDTA. The resulting false positive rate calculated in the WET Variability Study for the *Selenastrum* chronic test method conducted with the addition of EDTA was 0.00%.

A total of six valid tests were conducted on blank samples without the addition of EDTA (Table 9.25). For the growth NOEC, one false positive was observed and one test result was determined as inconclusive due to evaluation of the concentration-response relationship (Table 8.4). The growth NOEC for sample 9457 tested without EDTA was reported as 6.25%. Two false positives were observed for the IC25. The IC25 for samples 9457 and 9473 were reported as 11.7% and 24.6%, respectively. No false positives were observed for the IC50. The resulting false positive rate calculated in the WET Variability Study for the *Selenastrum* chronic test method conducted without the addition of EDTA was 20.0%, 33.3%, and 0.00% for the growth NOEC, IC25, and IC50, respectively.

9.6.3 Precision

Precision of the *Selenastrum* chronic test method was estimated by calculating the CV of IC25 and IC50 values obtained for the reference toxicant, effluent, and receiving water samples. Within-laboratory, between-laboratory, and total CVs were calculated for the reference toxicant and effluent samples. Only a total CV was calculated for the receiving water samples since no within-laboratory replication was provided for this sample type. Results from two samples (9468 w/ EDTA and 9476 w/o EDTA) were excluded from the analysis of precision. These test samples were identified by ASTM h statistics as possible outliers, and review of data qualifier flags revealed a possible cause (Table 9.1).

Table 9.32 summarizes the precision of point estimates from the *Selenastrum* chronic test method conducted with EDTA. CVs for IC25 values ranged from 10.9% to 39.5% for the within-laboratory variability component, 8.48% to 20.8% for the between-laboratory variability component, and 23.5% to 40.4% for the total variability component. Uncharacteristically, within-laboratory CVs (averaging 25.2%) were higher than between-laboratory CVs (averaging 14.6%). This is likely due to the unusually large within-laboratory CV for the effluent sample type. Averaging the CVs based on total variance for the three sample types, a total CV of 34.3% was obtained for the IC25 in the *Selenastrum* chronic test method conducted with EDTA. IC50 values were slightly more precise than IC25 values. For the IC50, CVs averaged 5.82%, 13.2%, and 32.2% for the within-laboratory, between-laboratory, and total variability components, respectively.

Table 9.33 summarizes the precision of point estimates from the *Selenastrum* chronic test method conducted without EDTA. The *Selenastrum* test method was less precise when conducted without the addition of EDTA. CVs for IC25 values ranged from 21.0% to 25.6% for the within-laboratory variability component, 60.3% to 83.6% for the between-laboratory variability component, and 24.1% to 87.5% for the total variability component. Within-laboratory CVs averaged 23.3%, and between-laboratory CVs averaged 72.0%. Averaging the CVs based on total variance for the three sample types, a total CV of 58.5% was obtained for the IC25 in the *Selenastrum* chronic test method conducted without EDTA. IC50 values were again slightly more precise than IC25 values. For the IC50, CVs averaged 14.5%, 43.9%, and 58.5% for the within-laboratory, between-laboratory, and total variability components, respectively.

The precision of NOEC values was determined by evaluating the range and distribution of NOEC values and the percentage of values falling within and beyond one concentration from the median. Table 9.34 describes the precision of NOEC values for the *Selenastrum* chronic method. For tests conducted with EDTA, NOEC values spanned three concentrations for the effluent sample type and four concentrations for the reference toxicant and receiving water sample types. The percentage of values within one concentration of the median was 85.7%, 100%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively.

For tests conducted without EDTA, NOEC values spanned six concentrations for the reference toxicant sample type, four concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 40%, 50%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

Table 9.24. Results for *Selenastrum* chronic test method performed on blank samples with EDTA.

				Growth Information					
LabID	**Sample Code**	**Nutrient**	**Test date**	**NOEC (% sample)**	**IC25 (% sample)**	**IC50 (% sample)**	**Control Mean (Cells/mL)**	**Control CV (%)**	**Flags[a]**
Referee	9497	EDTA	03/30/00	Invalid[b]	Invalid[b]	Invalid[b]	1.71e+06	28.0	a_7, d_4, g_2
33	9459	EDTA	03/16/00	Invalid[c]	Invalid[c]	Invalid[c]	2.28e+05	3.25	a_6
333	9489	EDTA	03/30/00	Invalid[c]	Invalid[c]	Invalid[c]	1.66e+06	29.6	a_7, d_4, g_2
406	9491	EDTA	03/23/00	Invalid[c]	Invalid[c]	Invalid[c]	6.03e+05	26.7	a_6, a_7, d_1, d_4, e_9, g_1, g_2
3	9457	EDTA	03/30/00	100	>100	>100	4.79e+06	9.77	
36	9463	EDTA	03/16/00	100	>100	>100	1.32e+06	9.53	d_4
62	9473	EDTA	03/30/00	100	>100	>100	2.98e+06	15.2	d_4, g_2
209	9481	EDTA	03/30/00	100	>100	>100	3.51e+06	12.6	e_9, g_{11}
459	9499	EDTA	03/16/00	100	>100	>100	1.94e+06	5.50	d_4, g_2
Summary Statistics	**N**			**5**	**5**	**5**			
	Min			**100**	**>100**	**>100**			
	Max			**100**	**>100**	**>100**			
	Median			**100**	**>100**	**>100**			
	Mean				**>100**	**>100**			
	False positives			**0**	**0**	**0**			
	False positive rate			**0.00%**	**0.00%**	**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.25. Results for *Selenastrum* chronic test method performed on blank samples without EDTA.

				Growth Information					
LabID	**Sample Code**	**Nutrient**	**Test date**	**NOEC (% sample)**	**IC25 (% sample)**	**IC50 (% sample)**	**Control Mean (Cells/mL)**	**Control CV (%)**	**Flags[a]**
Referee	9497	w/o EDTA	03/30/00	Invalid[b]	Invalid[b]	Invalid[b]	1.64e+06	27.8	a_7, d_1, d_4
209	9481	w/o EDTA	03/30/00	Invalid[c]	Invalid[c]	Invalid[c]	4.38e+05	39.2	a_7, e_9, g_2, g_5, g_{11}
333	9489	w/o EDTA	03/30/00	Invalid[c]	Invalid[c]	Invalid[c]	9.59e+05	28.8	a_7, g_2
3	9457	w/o EDTA	03/30/00	6.25	11.7	>100	2.65e+05	7.29	
33	9459	w/o EDTA	03/16/00	100	>100	>100	4.89e+05	4.35	
36	9463	w/o EDTA	03/16/00	100	>100	>100	1.44e+06	17.9	d_4, g_6
62	9473	w/o EDTA	03/30/00	Inconclusive[d]	24.6	>100	3.68e+06	8.72	d_4, g_2, g_5
406	9491	w/o EDTA	03/23/00	100	>100	>100	5.65e+05	16.2	d_1, d_4, e_9, g_1, g_2
459	9499	w/o EDTA	03/16/00	100	>100	>100	7.75e+05	8.70	d_4, g_2
Summary Statistics	**N**			**5**	**6**	**6**			
	Min			**6.25**	**11.7**	**>100**			
	Max			**100**	**>100**	**>100**			
	Median			**100**	**>100**	**>100**			
	Mean				**72.7**	**>100**			
	False positives			**1**	**2**	**0**			
	False positive rate			**20.0%**	**33.3%**	**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.
[d] Results were excluded from summary statistics. Based on EPA guidance for evaluating concentration-response relationships (EPA, 2000a) the test result was inconclusive and the sample should be retested to obtain a reliable result.

Table 9.26. Results for *Selenastrum* chronic test method performed on reference toxicant samples with EDTA.

				Growth Information					
LabID	**Sample Code**	**Nutrient**	**Test date**	**NOEC (% sample)**	**IC25 (% sample)**	**IC50 (% sample)**	**Control Mean (Cells/mL)**	**Control CV (%)**	**Flags[a]**
Referee	9495	EDTA	03/16/00	<6.25[b]	8.40[b]	35.5[b]	1.98e+06	4.43	d_1, d_4
33	9460	EDTA	03/23/00	Invalid[c]	Invalid[c]	Invalid[c]	2.70e+05	5.60	a_6
33	9461	EDTA	03/30/00	Invalid[c]	Invalid[c]	Invalid[c]	4.37e+05	3.46	a_6
299	9485	EDTA	03/30/00	Invalid[c]	Invalid[c]	Invalid[c]	1.50e+05	14.4	a_6, d_1, d_4, e_3, e_8, e_9, e_{10}, e_{11}, g_2
406	9492	EDTA	03/23/00	Invalid[c]	Invalid[c]	Invalid[c]	5.68e+05	17.9	a_6, d_1, d_4, e_9, g_1, g_2
406	9493	EDTA	03/30/00	Invalid[c]	Invalid[c]	Invalid[c]	6.18e+05	13.8	a_6, b_1, d_1, e_9, g_1, g_2
3	9455	EDTA	03/16/00	Inconclusive[d]	Inconclusive[d]	Inconclusive[d]	1.53e+06	11.1	d_4
36	9465	EDTA	03/30/00	25	39.4	68.4	1.29e+06	17.6	d_4, e_9
39	9469	EDTA	03/30/00	25	40.7	73.2	1.13e+06	7.19	d_4, e_2, e_3, e_9, g_1
125	9477	EDTA	03/30/00	6.25	19.9	54.8	2.19e+06	5.07	g_1
209	9478	EDTA	03/09/00	50	42.8	68.1	2.87e+06	19.6	e_9, g_2, g_{11}
209	9479	EDTA	03/17/00	25	39.6	65.5	2.27e+06	2.65	d_1, e_9, g_{11}
459	9500	EDTA	03/23/00	25	43.0	75.6	2.24e+06	2.70	
459	9501	EDTA	03/30/00	50	50.8	71.7	1.43e+06	11.8	d_4, g_2, g_6
Summary Statistics	**N**			**7**	**7**	**7**			
	Min			**6.25**	**19.9**	**54.8**			
	Max			**50**	**50.8**	**75.6**			
	Median			**25**	**40.7**	**68.4**			
	Mean				**39.5**	**68.2**			
	Within-lab	**STD**			**4.30**	**2.39**			
		CV%			**10.9%**	**3.51%**			
	Between-lab	**STD**			**8.20**	**6.36**			
		CV%			**20.8%**	**9.33%**			
	Total	**STD**			**9.26**	**6.80**			
		CV%			**23.5%**	**9.97%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.
[d] Results were excluded from summary statistics. Based on EPA guidance for evaluating concentration-response relationships (EPA, 2000a) the test result was inconclusive and the sample should be retested to obtain a reliable result.

Table 9.27. Results for *Selenastrum* chronic test method performed on reference toxicant samples without EDTA.

				Growth Information					
LabID	**Sample Code**	**Nutrient**	**Test date**	**NOEC (% sample)**	**IC25 (% sample)**	**IC50 (% sample)**	**Control Mean (Cells/mL)**	**Control CV (%)**	**Flags[a]**
Referee	9495	w/o EDTA	03/16/00	6.25[b]	10.2[b]	37.6[b]	1.85e+06	9.08	d_1, d_4, g_6
209	9478	w/o EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	1.48e+06	102	a_7, e_9, g_2, g_{11}
209	9479	w/o EDTA	03/17/00	Invalid[c]	Invalid[c]	Invalid[c]	1.00e+04	0.00	a_6, d_1, e_9, g_2, g_{11}
299	9485	w/o EDTA	03/30/00	Invalid[c]	Invalid[c]	Invalid[c]	1.71e+05	22.3	a_6, a_7, d_1, e_3, e_8, e_9, e_{11}, g_2
3	9455	w/o EDTA	03/16/00	100	>100	>100	2.71e+05	3.13	
33	9460	w/o EDTA	03/23/00	100	>100	>100	2.25e+05	0.915	
33	9461	w/o EDTA	03/30/00	100	>100	>100	4.77e+05	7.23	
36	9465	w/o EDTA	03/30/00	12.5	15.7	21.5	2.02e+06	6.99	d_4, e_9
39	9469	w/o EDTA	03/30/00	12.5	22.7	57.3	1.93e+06	11.4	d_4, e_2, e_3, e_9, g_1
125	9477	w/o EDTA	03/30/00	6.25	13.2	23.7	1.18e+06	5.49	g_1
406	9492	w/o EDTA	03/23/00	25	19.4	33.4	3.33e+05	6.67	d_1, d_4, e_9, g_1, g_2
406	9493	w/o EDTA	03/30/00	6.25	12.9	22.0	4.48e+05	9.19	b_1, d_1, e_9, g_1
459	9500	w/o EDTA	03/23/00	<6.25	9.69	24.7	2.27e+06	4.18	
459	9501	w/o EDTA	03/30/00	25	35.9	53.9	1.27e+06	14.7	g_2
Summary Statistics	**N**			**10**	**10**	**10**			
	Min			**<6.25**	**9.69**	**21.5**			
	Max			**100**	**>100**	**>100**			
	Median			**18.75**	**21.0**	**43.7**			
	Mean				**42.9**	**53.7**			
	Within-lab	**STD**			**11.0**	**12.8**			
		CV%			**25.6%**	**23.8%**			
	Between-lab	**STD**			**35.9**	**29.9**			
		CV%			**83.6%**	**55.6%**			
	Total	**STD**			**37.6**	**32.5**			
		CV%			**87.5%**	**60.5%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.28. Results for *Selenastrum* chronic test method performed on effluent samples with EDTA.

				Growth Information					
LabID	**Sample Code**	**Nutrient**	**Test date**	**NOEC (% sample)**	**IC25 (% sample)**	**IC50 (% sample)**	**Control Mean (Cells/mL)**	**Control CV (%)**	**Flags[a]**
Referee	9494	EDTA	03/09/00	<6.25[b]	10.7[b]	29.9[b]	2.18e+06	4.45	d_1
33	9458	EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	2.52e+05	6.19	a_6, g_5
39	9466	EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	1.27e+06	28.9	a_7, b_1, d_4, e_9, g_2
39	9467	EDTA	03/16/00	Invalid[c]	Invalid[c]	Invalid[c]	5.02e+05	21.2	a_6, a_7, d_1, d_4, e_9, g_2
299	9482	EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	2.75e+06	45.4	a_7, d_4, e_3, e_9, e_{10}, e_{11}, g_2
299	9483	EDTA	03/16/00	Invalid[c]	Invalid[c]	Invalid[c]	4.35e+05	30.4	a_6, a_7, d_1, d_4, e_3, e_9, e_{10}, e_{11}, g_2
333	9487	EDTA	03/16/00	Invalid[c]	Invalid[c]	Invalid[c]	2.64e+06	27.4	a_7, b_1, d_1, d_4, g_2
406	9490	EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	7.58e+05	21.2	a_6, a_7, b_1, d_1, d_4, e_9, g_1, g_2
459	9498	EDTA	03/10/00	Invalid[c]	Invalid[c]	Invalid[c]	5.90e+05	17.0	a_6, b_1, b_2, g_2
3	9454	EDTA	03/09/00	<6.25	17.7	45.6	4.66e+06	3.87	d_4
36	9462	EDTA	03/09/00	6.25	13.3	34.2	1.94e+06	18.9	d_1, d_4, e_6
62	9470	EDTA	03/09/00	12.5	27.2	54.9	3.37e+06	4.29	d_4
62	9471	EDTA	03/16/00	12.5	16.7	46.7	3.90e+06	8.29	d_4
125	9474	EDTA	03/09/00	12.5	36.5	62.6	2.30e+06	10.0	g_1
125	9475	EDTA	03/16/00	6.25	22.6	60.9	2.33e+06	7.95	g_1
333	9486	EDTA	03/09/00	6.25	10.3	58.3	3.54e+06	8.28	d_1, d_4
Summary Statistics	**N**			**7**	**7**	**7**			
	Min			**<6.25**	**10.3**	**34.2**			
	Max			**12.5**	**36.5**	**62.6**			
	Median			**6.25**	**17.7**	**54.9**			
	Mean				**20.6**	**51.9**			
	Within-lab	**STD**			**8.15**	**4.22**			
		CV%			**39.5%**	**8.14%**			
	Between-lab	**STD**			**1.75**	**8.91**			
		CV%			**8.48%**	**17.2%**			
	Total	**STD**			**8.33**	**9.86**			
		CV%			**40.4%**	**19.0%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.29. Results for *Selenastrum* chronic test method performed on effluent samples without EDTA.

				Growth Information					
LabID	**Sample Code**	**Nutrient**	**Test date**	**NOEC (% sample)**	**IC25 (% sample)**	**IC50 (% sample)**	**Control Mean (Cells/mL)**	**Control CV (%)**	**Flags[a]**
Referee	9494	w/o EDTA	03/09/00	6.25[b]	8.85[b]	19.7[b]	1.44e+06	18.2	d_1
39	9466	w/o EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	1.07e+05	75.0	a_6, a_7, b_1, d_4, e_9, g_2
39	9467	w/o EDTA	03/16/00	Invalid[c]	Invalid[c]	Invalid[c]	3.10e+05	29.0	a_7, d_1, d_4, e_9, g_2, g_5
299	9482	w/o EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	6.28e+04	62.9	a_6, a_7, d_1, d_4, e_3, e_9, g_2
299	9483	w/o EDTA	03/16/00	Invalid[c]	Invalid[c]	Invalid[c]	4.00e+04	41.7	a_6, a_7, d_1, d_4, e_3, e_9, e_{11}, g_2
333	9486	w/o EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	2.61e+06	20.9	a_7, d_1, d_4
333	9487	w/o EDTA	03/16/00	Invalid[c]	Invalid[c]	Invalid[c]	1.44e+06	53.8	a_7, b_1, d_1, d_4, g_2
406	9490	w/o EDTA	03/09/00	Invalid[c]	Invalid[c]	Invalid[c]	5.43e+05	26.9	a_7, b_1, d_1, d_4, e_9, g_1, g_2
459	9498	w/o EDTA	03/10/00	Invalid[c]	Invalid[c]	Invalid[c]	6.23e+05	26.2	a_7, b_1, b_2, g_2
3	9454	w/o EDTA	03/09/00	Inconclusive[d]	Inconclusive[d]	Inconclusive[d]	5.11e+05	8.03	d_4, g_2
33	9458	w/o EDTA	03/09/00	50	65.1	80.2	2.53e+05	6.51	
36	9462	w/o EDTA	03/09/00	12.5	20.1	43.6	2.23e+06	10.2	d_1, d_4
62	9470	w/o EDTA	03/09/00	6.25	11.9	35.1	4.11e+06	14.1	d_4
62	9471	w/o EDTA	03/16/00	12.5	10.3	37.5	3.93e+06	16.4	d_4
125	9474	w/o EDTA	03/09/00	50	57.6	74.0	8.38e+05	18.5	g_1, g_2
125	9475	w/o EDTA	03/16/00	25	43.2	68.6	1.03e+06	12.3	g_1
Summary Statistics	**N**			**6**	**6**	**6**			
	Min			**6.25**	**10.3**	**35.1**			
	Max			**50**	**65.1**	**80.2**			
	Median			**18.75**	**31.7**	**56.1**			
	Mean				**34.7**	**56.5**			
	Within-lab	**STD**			**7.27**	**2.97**			
		CV%			**21.0%**	**5.25%**			
	Between-lab	**STD**			**20.9**	**18.1**			
		CV%			**60.3%**	**32.1%**			
	Total	**STD**			**22.2**	**18.4**			
		CV%			**63.9%**	**32.5%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.
[d] Results were excluded from summary statistics. Based on EPA guidance for evaluating concentration-response relationships (EPA, 2000a) the test result was inconclusive and the sample should be retested to obtain a reliable result.

Table 9.30. Results for *Selenastrum* chronic test method performed on receiving water samples with EDTA.

				Growth Information					
LabID	**Sample Code**	**Nutrient**	**Test date**	**NOEC (% sample)**	**IC25 (% sample)**	**IC50 (% sample)**	**Control Mean (Cells/mL)**	**Control CV (%)**	**Flags[a]**
Referee	9496	EDTA	03/23/00	<6.25[b]	<6.25[b]	26.8[b]	1.94e+06	8.48	d_1, d_4
3	9456	EDTA	03/23/00	6.25	17.2	35.8	4.00e+06	2.65	d_4
36	9464	EDTA	03/23/00	25	24.7	38.0	2.33e+06	17.1	d_4, e_9
39	9468	EDTA	03/23/00	100[c]	>100[c]	>100	1.35e+06	9.47	b_1, d_1, d_4, e_9, g_3, g_5, g_7
62	9472	EDTA	03/24/00	6.25	20.2	36.8	3.39e+06	3.66	b_2, d_4, f_1
125	9476	EDTA	03/23/00	12.5	24.7	64.5	2.46e+06	5.59	g_1
209	9480	EDTA	03/23/00	12.5	11.1	18.1	2.28e+06	15.6	e_9, g_2, g_{11}
299	9484	EDTA	03/23/00	<6.25	<6.25	<6.25	2.53e+06	19.8	d_1, d_4, e_3, e_9, e_{10}, g_6
333	9488	EDTA	03/23/00	25	19.7	41.2	2.79e+06	18.7	b_1, d_1, d_4
Summary Statistics	**N**			**7**	**7**	**8**			
	Min			**<6.25**	**<6.25**	**<6.25**			
	Max			**25**	**24.7**	**>100**			
	Median			**12.5**	**19.7**	**37.4**			
	Mean				**17.7**	**42.6**			
	STD				**6.88**	**28.8**			
	CV%				**38.9%**	**67.6%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results were identified as outliers, a probable cause was identified, and therefore results were excluded from summary statistics.

Table 9.31. Results for *Selenastrum* chronic test method performed on receiving water samples without EDTA.

				Growth Information					
LabID	**Sample Code**	**Nutrient**	**Test date**	**NOEC (% sample)**	**IC25 (% sample)**	**IC50 (% sample)**	**Control Mean (Cells/mL)**	**Control CV (%)**	**Flags[a]**
Referee	9496	w/o EDTA	03/23/00	12.5[b]	15.8[b]	39.2[b]	1.36e+06	14.3	d_1, d_4
209	9480	w/o EDTA	03/23/00	Invalid[c]	Invalid[c]	Invalid[c]	2.07e+05	23.0	a_7, e_9, g_2, g_{11}
299	9484	w/o EDTA	03/23/00	Invalid[c]	Invalid[c]	Invalid[c]	1.12e+05	39.0	a_6, a_7, d_1, e_3, e_9, e_{10}
3	9456	w/o EDTA	03/23/00	<6.25	<6.25	39.1	2.88e+05	12.2	
36	9464	w/o EDTA	03/23/00	6.25	10.1	18.4	1.50e+06	13.8	d_4, e_9
39	9468	w/o EDTA	03/23/00	Inconclusive[d]	10.3	>100	1.06e+06	2.89	b_1, d_1, d_4, e_9, g_2, g_5, g_6
62	9472	w/o EDTA	03/24/00	6.25	9.76	15.0	3.49e+06	11.1	b_2, d_4, f_1
125	9476	w/o EDTA	03/23/00	<6.25[e]	32.1[e]	55.9	1.38e+06	6.96	g_1, g_3
333	9488	w/o EDTA	03/23/00	6.25	6.34	14.6	1.14e+06	7.77	b_1, d_1, g_2
Summary Statistics	**N**			**4**	**5**	**6**			
	Min			**<6.25**	**<6.25**	**14.6**			
	Max			**6.25**	**10.3**	**>100**			
	Median			**6.25**	**9.76**	**28.8**			
	Mean				**8.54**	**40.5**			
	STD				**2.05**	**33.4**			
	CV%				**24.1%**	**82.5%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.
[d] Results were excluded from summary statistics. Based on EPA guidance for evaluating concentration-response relationships (EPA, 2000a) the test result was inconclusive and the sample should be retested to obtain a reliable result.
[e] Results were identified as outliers, a probable cause was identified, and therefore results were excluded from summary statistics.

Table 9.32. Precision of point estimates from the *Selenastrum* chronic test method conducted with EDTA.

Sample type	CV (%)					
	IC25			IC50		
	Within-lab [a]	Between-lab [a]	Total	Within-lab [a]	Between-lab [a]	Total
Reference toxicant	10.9	20.8	23.5	3.51	9.33	9.97
Effluent	39.5	8.48	40.4	8.14	17.2	19.0
Receiving water	-	-	38.9	-	-	67.6
Average	**25.2**	**14.6**	**34.3**	**5.82**	**13.2**	**32.2**

[a] Within and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.

Table 9.33. Precision of point estimates from the *Selenastrum* chronic test method conducted without EDTA.

Sample type	CV (%)					
	IC25			IC50		
	Within-lab [a]	Between-lab [a]	Total	Within-lab [a]	Between-lab [a]	Total
Reference toxicant	25.6	83.6	87.5	23.8	55.6	60.5
Effluent	21.0	60.3	63.9	5.25	32.1	32.5
Receiving water	-	-	24.1	-	-	82.5
Average	**23.3**	**72.0**	**58.5**	**14.5**	**43.9**	**58.5**

[a] Within- and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.

Table 9.34. Precision of NOEC values from the *Selenastrum* chronic test method.

Nutrient	Sample type	NOEC Frequency						Median	% within 1 conc. of median	% beyond 1 conc. of median
		<6.25%	6.25%	12.5%	25%	50%	100%			
With EDTA	Reference toxicant	0	1	0	4	2	0	25	85.7	14.3
	Effluent	1	3	3	0	0	0	6.25	100	0.00
	Receiving water	1	2	2	2	0	0	12.5	85.7	14.3
Without EDTA	Reference toxicant	1	2	2	2	0	3	18.75	40.0	60.0
	Effluent	0	1	2	1	2	0	18.75	50.0	50.0
	Receiving water	1	3	0	0	0	0	6.25	100	0.00

9.7 *Mysidopsis* Chronic Test Method Results

A total of 11 participant laboratories conducted the *Mysidopsis* chronic test method in the WET Variability Study. These laboratories tested a total of 8 blank samples, 13 reference toxicant samples, 15 effluent samples, and 8 receiving water samples. For each sample tested, a 7-day survival NOEC, a 7-day growth NOEC, a 7-day fecundity NOEC, a 7-day LC50, a 7-day growth IC25, and a 7-day fecundity IC25 were generated as test results. As described in the WET method manual, growth was measured as the total weight per replicate divided by the number of original organisms in that replicate. This definition provides a combined growth and survival endpoint that is more accurately termed biomass. The fecundity endpoint was measured as the percentage of females with eggs. Replicates without identified females were excluded from the analysis of fecundity. Results for the fecundity endpoint were not calculated if less than 50% of control females produced eggs. Also, test concentrations above the survival NOEC were excluded from hypothesis testing conducted on growth and fecundity endpoints. Results of *Mysidopsis* chronic testing are shown in Tables 9.35 - 9.38 for each sample type.

9.7.1 Successful Test Completion Rate

A total of 44 *Mysidopsis* chronic tests were initiated by 11 participant laboratories. All 44 tests were completed; however, the test conducted on sample 9690 was invalid due to failure to meet test acceptability criteria for survival. The resulting successful test completion rate calculated in the WET Variability Study for the *Mysidopsis* chronic test method was 97.7%. One test in the referee laboratory was also invalid due to failure to meet test acceptability criteria for survival. Of the 44 *Mysidopsis* chronic tests initiated, laboratories were able to report results for the fecundity endpoint in only 22 (or 50% of) tests. The remaining 22 tests did not meet the minimum control fecundity necessary to report fecundity results. The fecundity endpoint is an optional endpoint in the *Mysidopsis* chronic test, so failure to generate fecundity data does not invalidate a test.

9.7.2 False Positive Rate

A total of seven valid tests were conducted by seven participant laboratories on blank samples (Table 9.35). No false positives were observed for survival, growth, or fecundity endpoints. The survival NOEC was 100% for all 7 blank samples, and the LC50 was >100% for all 7 blank samples. The NOEC for growth was 100% for all 7 blank samples, and the IC25 was >100% for all 7 blank samples. The fecundity endpoint was only calculable for four samples. The NOEC for fecundity was 100% for all 4 samples, and the IC25 for fecundity was >100% for all 4 samples. The resulting false positive rate calculated in the WET Variability Study for the *Mysidopsis* chronic test method was 0.00%.

A false positive growth NOEC result was reported by the participant laboratory for sample 9658. Upon SCC recalculation and verification, the growth NOEC was properly reported as 100%. SCC test review discovered that the participant laboratory had calculated growth based on the weight per surviving *Mysidopsis* rather than per original *Mysidopsis*. Also, sample 9696 produced an interrupted concentration-response curve; however, based on EPA guidance for evaluating concentration-response relationships (USEPA, 2000a), the NOEC for this sample should be reported as 100%.

9.7.3 Precision

Precision of the *Mysidopsis* chronic test method was estimated by calculating the CV of LC50 and growth IC25 values obtained for the reference toxicant, effluent, and receiving water samples. Precision estimates were not calculated for fecundity IC25 values because results for this endpoint could not be consistently and definitively measured within the test concentration range (as evidenced by a large proportion of censored data (e.g., >12.5%, >25%, or >50%) for this endpoint). Within-laboratory, between-laboratory, and total CVs were calculated for the reference toxicant and effluent samples. Only a total CV was calculated for the receiving water samples since no within-laboratory replication was provided for this sample type. All participant laboratory test data for the reference toxicant, effluent, and receiving water samples were used in estimating precision. No test results were identified by ASTM h statistics as possible outliers.

Table 9.39 summarizes the precision of point estimates from the *Mysidopsis* chronic test method. CVs for LC50 values were consistent among sample types; within-laboratory CVs ranged from 6.09% to 7.06%, between-laboratory CVs ranged from 24.6% to 30.0%, and total CVs ranged from 25.6% to 37.4%. As expected, the majority of variability was due to the between-laboratory component, with within-laboratory CVs averaging 6.57% and between-laboratory CVs averaging 27.3%. Averaging the CVs for the LC50 based on total variance for the three sample types, a total CV of 31.2% was obtained for the *Mysidopsis* chronic survival endpoint.

CVs for growth IC25 values were higher than those for LC50 values. Within-laboratory CVs for the growth IC25 ranged from 5.26% to 8.69%, between-laboratory CVs ranged from 36.6% to 40.0%, and total CVs ranged from 37.0% to 45.9%. Within-laboratory CVs (averaging 6.98%) were much lower than between-laboratory CVs (averaging 38.3%). Averaging the CVs for the IC25 based on total variance for the three sample types, a total CV of 41.3% was obtained for the *Mysidopsis* chronic growth endpoint.

The precision of NOEC values was determined by evaluating the range and distribution of NOEC values and the percentage of values falling within and beyond one concentration from the median. Table 9.40 describes the precision of NOEC values for the *Mysidopsis* chronic test method. For the survival endpoint, NOEC values spanned three concentrations for all three sample types, and 100% of NOEC values were within one concentration of the median. Growth endpoints performed similarly, with the exception of the reference toxicant sample. Growth NOEC values spanned four concentrations for the reference toxicant sample type, and 7.69% were beyond one concentration from the median. The data set for the fecundity endpoint was much smaller than that for the survival and growth endpoints, since only 50% of tests achieved the necessary fecundity in controls (egg production in 50% of control females). In this reduced data set, fecundity NOEC values spanned three concentrations for each sample type and 75.0%, 87.5%, and 66.7% of values were within one concentration of the median for the reference toxicant, effluent, and receiving water sample types, respectively.

Table 9.35. Results for *Mysidopsis* chronic test method performed on blank samples.

			Survival Information				Growth Information				Fecundity Information				
LabID	**Sample Code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9687	02/29/00	Invalid[b]	Invalid[b]	76.3	21.5	Invalid[b]	Invalid[b]	0.322	21.9	Invalid[b]	Invalid[b]	85.7	17.2	a_1, d_2, d_3, d_5, e_1, e_2, g_1
417	9690	02/22/00	Invalid[c]	Invalid[c]	78.1	20.9	Invalid[c]	Invalid[c]	0.224	45.4	-	-	-	-	a_1, a_5, d_5, e_1, g_2
22	9650	02/22/00	100	>100	95.0	8.57	100	>100	0.194	16.1	-	-	-	-	a_5, b_1, d_5, e_2, e_3, g_1
39	9658	02/22/00	100	>100	90.0	10.4	100	>100	0.403	18.1	-	-	-	-	a_5, d_5
73	9664	02/29/00	100	>100	100	0.716	100	>100	0.417	7.68	100	>100	72.6	31.5	e_1
77	9666	02/22/00	100	>100	100	0.00	100	>100	0.278	8.75	-	-	-	-	a_5
101	9672	02/29/00	100	>100	100	0.00	100	>100	0.360	7.86	100	>100	96.9	7.53	e_1
125	9676	02/29/00	100	>100	92.5	9.81	100	>100	0.621	43.9	100	>100	77.1	23.4	g_2
420	9696	02/29/00	100	>100	100	0.00	100	>100	0.354	10.7	100	>100	85.4	13.5	e_1, e_2, g_5
Summary Statistics	**N**		**7**	**7**			**7**	**7**			**4**	**4**			
	Min		**100**	**>100**			**100**	**>100**			**100**	**>100**			
	Max		**100**	**>100**			**100**	**>100**			**100**	**>100**			
	Median		**100**	**>100**			**100**	**>100**			**100**	**>100**			
	Mean			**>100**				**>100**				**>100**			
	False positives		**0**	**0**			**0**	**0**			**0**	**0**			
	False positive rate		**0.00%**	**0.00%**			**0.00%**	**0.00%**			**0.00%**	**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.36. Results for *Mysidopsis* chronic test method performed on reference toxicant samples.

			Survival Information				Growth Information				Fecundity Information				
LabID	Sample Code	Test date	NOEC (% sample)	LC50 (% sample)	Control mean (%)	Control CV (%)	NOEC (% sample)	IC25 (% sample)	Control mean (mg)	Control CV (%)	NOEC (% sample)	IC25 (% sample)	Control mean (%)	Control CV (%)	Flags[a]
Referee	9689	02/22/00	50[b]	66.9[b]	87.1	13.8	50[b]	36.4[b]	0.180	40.2	50[b]	>50[b]	59.5	36.3	d_1, e_1, e_2, g_1, g_2
22	9652	02/29/00	25	42.5	97.5	6.40	25	30.8	0.211	23.6	-	-	-	-	a_5, b_1, c_5, d_5, e_2, e_3, g_1, g_2
22	9653	02/29/00	25	43.9	95.0	8.57	6.25	26.4	0.228	22.4	-	-	-	-	a_5, b_1, c_5, d_5, e_2, e_3, g_1
36	9657	02/29/00	25	54.8	97.5	6.40	25	38.2	0.304	18.0	-	-	-	-	a_5, e_1, e_6
39	9660	02/29/00	25	35.4	82.5	16.5	12.5	19.5	0.419	32.3	-	-	-	-	a_5, d_2, d_3, d_5, e_1
39	9661	02/29/00	25	35.4	80.0	15.6	25	26.7	0.415	34.4	-	-	-	-	a_5, d_2, d_3, d_5
77	9668	02/29/00	25	57.3	100	0.00	25	39.3	0.259	14.9	-	-	-	-	a_5
77	9669	02/29/00	50	63.7	100	0.00	25	38.4	0.251	21.5	-	-	-	-	a_5
101	9670	02/22/00	12.5	29.2	100	0.00	12.5	21.0	0.275	17.3	12.5	12.7	85.4	13.8	
101	9671	02/22/00	12.5	24.0	100	0.00	12.5	21.4	0.255	13.7	12.5	>25	84.6	14.7	
181	9681	02/29/00	50	70.7	87.5	14.4	50	60.9	0.173	11.4	-	-	-	-	a_5, g_1
238	9685	02/29/00	25	41.6	95.0	8.57	25	36.4	0.194	9.81	25	46.4	80.6	19.2	d_5, e_2, e_3
417	9693	02/29/00	50	59.5	100	0.940	25	27.6	0.283	40.6	-	-	-	-	a_5, d_5, e_1, g_2
420	9695	02/22/00	50	70.7	100	0.00	50	61.8	0.338	6.84	50	>50	81.3	24.5	e_1, e_2
Summary Statistics	**N**		**13**	**13**			**13**	**13**			**4**	**4**			
	Min		**12.5**	**24.0**			**6.25**	**19.5**			**12.5**	**12.7**			
	Max		**50**	**70.7**			**50**	**61.8**			**50**	**>50**			
	Median		**25**	**43.9**			**25**	**30.8**			**18.75**	**35.7**			
	Mean			**48.4**				**34.5**				**33.5**			
	Within-lab	**STD**		**2.94**				**3.00**							
		CV%		**6.09%**				**8.69%**							
	Between-lab	**STD**		**14.5**				**13.8**							
		CV%		**30.0%**				**40.0%**							
	Total	**STD**		**14.8**				**14.1**							
		CV%		**30.6%**				**40.9%**							

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.37. Results for *Mysidopsis* chronic test method performed on effluent samples.

			Survival Information				Growth Information				Fecundity Information				
LabID	Sample Code	Test date	NOEC (% sample)	LC50 (% sample)	Control mean (%)	Control CV (%)	NOEC (% sample)	IC25 (% sample)	Control mean (mg)	Control CV (%)	NOEC (% sample)	IC25 (% sample)	Control mean (%)	Control CV (%)	Flags[a]
Referee	9686	02/22/00	25[b]	33.4[b]	95.0	8.34	25[b]	30.2[b]	0.323	22.7	25[b]	>25[b]	77.1	16.6	d_1, e_1, e_2, g_1
22	9651	02/22/00	12.5	21.0	87.5	10.3	12.5	16.5	0.175	17.4	-	-	-	-	a_5, b_1, d_5, e_2, e_3, g_1
36	9654	02/23/00	25	30.3	87.5	14.4	12.5	19.5	0.251	18.8	-	-	-	-	a_5, b_2, e_1, e_6
36	9655	02/23/00	12.5	27.5	100	0.00	12.5	16.9	0.289	17.7	-	-	-	-	a_5, b_2, e_6
39	9659	02/22/00	12.5	18.4	82.5	7.41	6.25	9.32	0.408	18.5	-	-	-	-	a_5, b_1, d_5
73	9662	02/22/00	25	34.7	100	0.940	25	31.1	0.338	5.25	25	>25	71.9	16.2	e_1
73	9663	02/22/00	25	35.2	100	0.940	25	30.8	0.430	6.34	25	>25	71.9	25.3	e_1
77	9667	02/22/00	12.5	26.2	100	0.00	12.5	18.3	0.292	22.0	-	-	-	-	a_5
125	9674	02/22/00	6.25	12.1	90.0	17.3	6.25	10.3	0.622	67.9	6.25	>12.5	92.7	11.4	d_4, g_2
125	9675	02/22/00	12.5	15.6	90.0	17.3	12.5	8.66	0.622	67.9	12.5	>12.5	92.7	11.4	d_4, g_2
181	9678	02/22/00	12.5	27.2	95.0	8.57	12.5	20.2	0.193	16.2	-	-	-	-	a_5, g_1
181	9679	02/22/00	12.5	26.9	95.0	12.6	12.5	21.5	0.202	17.6	12.5	>25	69.4	33.1	g_1
238	9682	02/22/00	25	35.4	100	0.00	25	31.3	0.206	11.1	25	>25	93.8	10.5	d_5, e_2, e_3, g_1, g_5
238	9683	02/22/00	25	31.3	100	0.00	25	30.1	0.205	11.7	25	>25	89.6	14.9	d_5, e_2, e_3, g_1
417	9691	02/22/00	25	30.0	95.0	12.6	25	28.1	0.288	28.8	-	-	-	-	a_5, d_5, e_1
420	9694	02/22/00	25	35.4	100	0.00	25	30.0	0.339	11.2	25	8.45	83.3	19.0	e_2, g_5
Summary Statistics	**N**		**15**	**15**			**15**	**15**			**8**	**8**			
	Min		**6.25**	**12.1**			**6.25**	**8.66**			**6.25**	**8.45**			
	Max		**25**	**35.4**			**25**	**31.3**			**25**	**>25**			
	Median		**12.5**	**27.5**			**12.5**	**20.2**			**25**	**>25**			
	Mean			**27.1**				**21.5**				**19.8**			
	Within-lab	**STD**		**1.92**				**1.13**							
		CV%		**7.06%**				**5.26%**							
	Between-lab	**STD**		**6.67**				**7.88**							
		CV%		**24.6%**				**36.6%**							
	Total	**STD**		**6.94**				**7.96**							
		CV%		**25.6%**				**37.0%**							

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.38. Results for *Mysidopsis* chronic test method performed on receiving water samples.

			Survival Information				Growth Information				Fecundity Information				
LabID	**Sample Code**	**Test date**	**NOEC (% samplle)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9688	02/29/00	25[b]	33.6[b]	86.9	14.8	12.5[b]	23.5[b]	0.337	13.5	-	-	-	-	a_5, d_2, d_3, e_1, e_2, g_1
36	9656	02/29/00	12.5	21.2	97.5	6.40	6.25	14.6	0.323	9.03	12.5	>25	50.0	38.6	e_6
73	9665	02/29/00	25	33.5	100	1.05	12.5	25.3	0.494	8.76	25	>25	78.6	32.9	e_1
101	9673	02/29/00	6.25	13.0	97.5	6.40	6.25	10.2	0.350	10.1	6.25	>12.5	95.8	10.5	
125	9677	02/29/00	6.25	9.69	92.5	9.81	6.25	8.02	0.621	43.9	6.25	8.53	77.1	23.4	g_2
181	9680	02/29/00	12.5	26.5	97.5	6.40	12.5	23.2	0.195	18.3	-	-	-	-	a_5, g_1
238	9684	02/29/00	12.5	30.2	97.5	6.40	12.5	31.3	0.209	11.0	6.25	12.5	100	6.86	b_1, d_5, e_2, e_3
417	9692	02/29/00	25	28.2	100	1.83	12.5	15.2	0.235	23.1	-	-	-	-	a_5, d_5, e_1
420	9697	02/29/00	25	34.4	100	0.00	25	31.3	0.342	11.3	25	>25	75.0	29.5	e_1, e_2
Summary Statistics	**N**		**8**	**8**			**8**	**8**			**6**	**6**			
	Min		**6.25**	**9.69**			**6.25**	**8.02**			**6.25**	**8.53**			
	Max		**25**	**34.4**			**25**	**31.3**			**25**	**>25**			
	Median		**12.5**	**27.3**			**12.5**	**19.2**			**9.375**	**18.8**			
	Mean			**24.6**				**19.9**				**18.1**			
	STD			**9.20**				**9.12**							
	CV%			**37.4%**				**45.9%**							

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.39. Precision of point estimates from the *Mysidopsis* chronic test method.

Sample type	CV (%)					
	LC50			IC25		
	Within-lab [a]	Between-lab [a]	Total	Within-lab [a]	Between-lab [a]	Total
Reference toxicant	6.09	30.0	30.6	8.69	40.0	40.9
Effluent	7.06	24.6	25.6	5.26	36.6	37.0
Receiving water	-	-	37.4	-	-	45.9
Average	**6.57**	**27.3**	**31.2**	**6.98**	**38.3**	**41.3**

[a] Within and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.

Table 9.40. Precision of NOEC values from the *Mysidopsis* chronic test method.

Sample type	Endpoint	NOEC Frequency						Median (% sample)	% within 1 conc. of median	% beyond 1 conc. of median
		<6.25%	6.25%	12.5%	25%	50%	100%			
Reference toxicant	Survival	0	0	2	7	4	0	25	100	0.00
	Growth	0	1	3	7	2	0	25	92.3	7.69
	Fecundity	0	0	2	1	1	0	18.75	75.0	25.0
Effluent	Survival	0	1	7	7	0	0	12.5	100	0.00
	Growth	0	2	7	6	0	0	12.5	100	0.00
	Fecundity	0	1	2	5	0	0	25	87.5	12.5
Receiving water	Survival	0	2	3	3	0	0	12.5	100	0.00
	Growth	0	3	4	1	0	0	12.5	100	0.00
	Fecundity	0	3	1	2	0	0	9.375	66.7	33.3

9.8 Sheepshead Acute Test Method Results

A total of seven participant laboratories conducted the sheepshead acute test method in the WET Variability Study. These laboratories tested a total of seven blank samples, seven reference toxicant samples, seven effluent samples, and seven receiving water samples. For each sample tested, a 96-hour LC50 was generated as a test result. Results of sheepshead acute testing are shown in Tables 9.41 - 9.44 for each sample type.

9.8.1 Successful Test Completion Rate

A total of 28 samples were tested at 7 participant laboratories. All 28 tests were completed and met test acceptability criteria. The resulting successful test completion rate calculated in the WET Variability Study for the sheepshead acute test method was 100%. Testing on two samples (9586 and 9589) was repeated due to laboratory error. The laboratory terminated the initial tests after 48 hours rather than the required 96 hours. Due to this error, the laboratory retested samples the following week at their expense. Because this error was not a result of the test method or test method performance, it was not included in the calculation of the successful test completion rate.

9.8.2 False Positive Rate

A total of seven blank samples were analyzed by seven participant laboratories (Table 9.41). The LC50 calculated for all 7 blank samples was >100%, indicating no toxicity and no false positives. The resulting false positive rate calculated in the WET Variability Study for the sheepshead acute test method was 0.00%.

9.8.3 Precision

Precision of the sheepshead acute test method was estimated by calculating the CV of LC50 values obtained for the reference toxicant, effluent, and receiving water samples. Since no within-laboratory replication was provided for this method, CVs were calculated based on total variability and no estimates for precision were available for within-laboratory and between-laboratory components of variability. All participant laboratory test data for the reference toxicant, effluent, and receiving water samples were used in estimating precision. While results on two test samples (9617 and 9600) were identified by ASTM h statistics as possible outliers, these results were not excluded from the analysis of precision due to the small size of the data set. Exclusion of either result would translate to exclusion of 14% of the respective data set.

Table 9.45 summarizes the precision of point estimates from the sheepshead acute test method. CVs based on total variance ranged from 19.4% to 32.5% with an average CV of 26.0% obtained for the sheepshead acute test method in the WET Variability Study.

Table 9.41. Results for sheepshead acute test method performed on blank samples.

			Survival Information			
LabID	**Sample Code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9606	03/07/00	>100[b]	100	0.00	d_5
22	9586	03/17/00	>100	100	0.00	d_5, e_2, e_3, e_4, e_5, g_8
29	9590	03/07/00	>100	95.0	8.66	d_1, e_2
73	9594	03/07/00	>100	100	0.00	
101	9598	03/07/00	>100	100	0.00	
238	9602	03/07/00	>100	100	0.00	e_2, e_3
420	9610	03/07/00	>100	100	0.00	e_2
425	9614	03/07/00	>100	100	0.00	d_5, e_6
Summary Statistics	**N**		**7**			
	Min		**>100**			
	Max		**>100**			
	Median		**>100**			
	Mean		**>100**			
	False positives		**0**			
	False positive rate		**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.42. Results for sheepshead acute test method performed on reference toxicant samples.

			Survival Information			
LabID	**Sample Code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9609	03/14/00	40.6[b]	100	0.00	d_1, d_2, d_5
22	9589	03/17/00	37.6	100	0.00	d_5, e_2, e_3, e_4, e_5, g_8
29	9593	03/14/00	46.7	100	0.00	d_2, d_3, d_5, e_2
73	9597	03/14/00	37.2	100	0.00	
101	9601	03/14/00	35.4	100	0.00	
238	9605	03/14/00	35.4	100	0.00	e_2, e_3, e_4
420	9613	03/14/00	39.2	100	0.00	e_2
425	9617	03/14/00	66.0	100	0.00	d_5, e_6, g_3
Summary Statistics	**N**		**7**			
	Min		**35.4**			
	Max		**66.0**			
	Median		**37.6**			
	Mean		**42.5**			
	STD		**11.1**			
	CV%		**26.0%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.43. Results for sheepshead acute test method performed on effluent samples.

			Survival Information			
LabID	**Sample Code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9608	03/14/00	35.4[b]	100	0.00	d_2, d_5
22	9588	03/14/00	33.4	100	0.00	d_5, e_2, e_3, e_4, e_5
29	9592	03/14/00	35.4	100	0.00	d_2, e_2
73	9596	03/14/00	35.4	100	0.00	
101	9600	03/14/00	18.3	100	0.00	g_3
238	9604	03/14/00	35.4	100	0.00	e_2, e_3, e_4, e_6
420	9612	03/14/00	35.4	100	0.00	e_2
425	9616	03/14/00	34.2	100	0.00	d_5, e_6
Summary Statistics	**N**		**7**			
	Min		**18.3**			
	Max		**35.4**			
	Median		**35.4**			
	Mean		**32.5**			
	STD		**6.29**			
	CV%		**19.4%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.44. Results for sheepshead acute test method performed on receiving water samples.

			Survival Information			
LabID	**Sample Code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9607	03/07/00	25.9[b]	100	0.00	d_2, d_5
22	9587	03/17/00	20.3	100	0.00	d_5, e_2, e_3, e_4, e_5, g_8
29	9591	03/07/00	17.7	100	0.00	d_1, d_2, e_2
73	9595	03/07/00	35.4	100	0.00	
101	9599	03/07/00	15.5	100	0.00	
238	9603	03/07/00	22.5	100	0.00	e_2, e_3
420	9611	03/07/00	35.4	100	0.00	e_2
425	9615	03/07/00	27.7	100	0.00	d_5, e_6
Summary Statistics	**N**		**7**			
	Min		**15.5**			
	Max		**35.4**			
	Median		**22.5**			
	Mean		**24.9**			
	STD		**8.10**			
	CV%		**32.5%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.45. Precision of point estimates from the sheepshead acute test method.

Sample type	CV (%)		
	Within-lab[a]	Between-lab[a]	Total
Reference toxicant	-	-	26.0
Effluent	-	-	19.4
Receiving water	-	-	32.5
Average	**-**	**-**	**26.0**

[a] Within and between-laboratory components of variability were not calculated for this method since no within-laboratory replication was provided.

9.9 Sheepshead Chronic Test Method Results

A total of seven participant laboratories conducted the sheepshead chronic test method in the WET Variability Study. These laboratories tested a total of seven blank samples, seven reference toxicant samples, seven effluent samples, and seven receiving water samples. For each sample tested, a 7-day survival NOEC, a 7-day growth NOEC, a 7-day LC50, and a 7-day growth IC25 were generated as test results. As described in the WET method manual, growth was measured as the total weight per replicate divided by the number of original organisms in that replicate. This definition provides a combined growth and survival endpoint that is more accurately termed biomass. Also, test concentrations above the survival NOEC were excluded from hypothesis testing conducted on the growth endpoint. Results of sheepshead chronic testing are shown in Tables 9.46 - 9.49 for each sample type.

9.9.1 Successful Test Completion Rate

A total of 28 samples were tested at 7 participant laboratories. All 28 tests were completed and met test acceptability criteria. The resulting successful test completion rate calculated in the WET Variability Study for the sheepshead chronic test method was 100%. Testing on sample 9618 was repeated due to laboratory error. The laboratory inadvertently renewed the test on Day 6 with the wrong sample. Due to this error, the laboratory retested the sample the following week at their expense. Because this error was not a result of the test method or test method performance, it was not included in the calculation of the successful test completion rate.

9.9.2 False Positive Rate

A total of seven blank samples were analyzed by seven participant laboratories (Table 9.46). No false positives were observed for survival or growth endpoints. The survival NOEC was 100% for all 7 blank samples, and the LC50 was >100% for all 7 blank samples. The NOEC for growth was 100% for all 7 blank samples, and the IC25 was >100% for all 7 blank samples. The resulting false positive rate calculated in the WET Variability Study for the sheepshead chronic test method was 0.00%.

9.9.3 Precision

Precision of the sheepshead chronic test method was estimated by calculating the CV of LC50 and IC25 values obtained for the reference toxicant, effluent, and receiving water samples. Since no within-laboratory replication was provided for this method, CVs were calculated based on total variability and no estimates for precision were available for within-laboratory and between-laboratory components of variability. All participant laboratory test data for the reference toxicant, effluent, and receiving water samples were used in estimating precision. No test results were identified by ASTM h statistics as possible outliers.

Table 9.50 summarizes the precision of point estimates from the sheepshead chronic test method. CVs for LC50 values were extremely low for effluent (2.33%) and receiving water (1.63%) sample types and higher for the reference toxicant sample type (22.2%). This trend was also observed for CVs of IC25 values, which were 18.4%, 6.12%, and 7.15% for the reference toxicant, effluent, and receiving water sample types, respectively. The higher variability associated with the reference toxicant sample could be due to the added step of reconstituting the reference toxicant ampule sample in each laboratory, or it could be an anomaly associated with the small data set for this method. Averaging the CVs based on total variance for the three sample types, total CVs of 8.73% for the LC50 and 10.5% for the IC25 were obtained for the sheepshead chronic test method in the WET Variability Study.

The precision of NOEC values was determined by evaluating the range and distribution of NOEC values and the percentage of values falling within and beyond one concentration from the median. Table 9.51 describes the precision of NOEC values for the sheepshead chronic method. For survival and growth endpoints, NOEC values spanned only two concentrations or did not vary at all between laboratories. All NOEC values (100%) were within one concentration of the median.

Table 9.46. Results for sheepshead chronic test method performed on blank samples.

			Survival Information				Growth Information				
LabID	**Sample Code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9642	03/21/00	100[b]	>100[b]	100	0.00	100[b]	>100[b]	1.21	3.06	d_1, e_5
22	9618	03/29/00	100	>100	100	0.00	100	>100	0.825	8.30	b_2, d_5, e_2, e_3, e_5, g_1, g_9
73	9622	03/21/00	100	>100	100	0.00	100	>100	0.751	7.08	
101	9626	03/21/00	100	>100	100	0.00	100	>100	0.908	5.35	e_2, e_5
181	9630	03/21/00	100	>100	100	0.00	100	>100	0.913	11.5	e_5
238	9634	03/21/00	100	>100	100	0.00	100	>100	0.669	14.8	d_5, e_2
273	9638	03/21/00	100	>100	100	0.00	100	>100	1.61	6.56	d_2, d_3
420	9646	03/21/00	100	>100	100	0.00	100	>100	1.92	6.78	e_2, e_5
Summary Statistics	**N**		**7**	**7**			**7**	**7**			
	Min		**100**	**>100**			**100**	**>100**			
	Max		**100**	**>100**			**100**	**>100**			
	Median		**100**	**>100**			**100**	**>100**			
	Mean			**>100**				**>100**			
	False positives		**0**	**0**			**0**	**0**			
	False positive rate		**0.00%**	**0.00%**			**0.00%**	**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.47. Results for sheepshead chronic test method performed on reference toxicant samples.

			Survival Information				Growth Information				
LabID	**Sample Code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9645	03/28/00	50[b]	64.4[b]	100	0.00	50[b]	54.3[b]	0.889	8.31	d_1, d_5, e_5
22	9621	03/28/00	25	45.1	100	0.00	25	31.1	0.844	3.65	c_5, d_5, e_2, e_3, e_5, g_1
73	9625	03/28/00	50	68.9	97.5	5.94	25	44.9	0.654	12.9	
101	9629	03/28/00	25	37.2	100	0.00	25	29.6	1.26	3.06	d_2, e_2, e_5
181	9633	03/28/00	25	51.1	100	0.00	25	40.6	0.760	9.77	e_5
238	9637	03/28/00	50	66.0	100	0.00	25	49.3	0.641	2.73	d_5, e_2
273	9641	03/28/00	50	69.5	100	0.00	25	43.1	1.60	5.47	d_2
420	9649	03/28/00	25	61.6	100	0.00	25	37.3	2.10	2.15	e_2, e_5
Summary Statistics	**N**		**7**	**7**			**7**	**7**			
	Min		**25**	**37.2**			**25**	**29.6**			
	Max		**50**	**69.5**			**25**	**49.3**			
	Median		**25**	**61.6**			**25**	**40.6**			
	Mean			**57.0**				**39.4**			
	STD			**12.7**				**7.24**			
	CV%			**22.2%**				**18.4%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.48. Results for sheepshead chronic test method performed on effluent samples.

			Survival Information				Growth Information				
LabID	**Sample Code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9644	03/28/00	25[b]	34.1[b]	100	0.00	25[b]	29.4[b]	0.994	4.11	d_1, e_5
22	9620	03/28/00	25	33.6	100	0.00	12.5	27.1	0.896	6.51	c_5, d_5, e_2, e_3, e_5, g_1
73	9624	03/28/00	25	34.2	100	0.00	12.5	26.8	0.653	13.7	
101	9628	03/28/00	25	33.7	100	0.00	25	28.7	1.23	10.0	d_2, e_2, e_5
181	9632	03/28/00	25	34.3	97.5	5.94	25	29.7	0.768	15.5	e_5
238	9636	03/28/00	25	35.4	100	0.00	25	30.5	0.626	2.62	b_1, d_5, e_2
273	9640	03/28/00	25	35.4	100	0.00	12.5	27.9	1.56	2.26	d_2
420	9648	03/28/00	25	35.4	100	0.00	12.5	25.6	2.02	10.1	d_5, e_2, e_5
Summary Statistics	**N**		**7**	**7**			**7**	**7**			
	Min		**25**	**33.6**			**12.5**	**25.6**			
	Max		**25**	**35.4**			**25**	**30.5**			
	Median		**25**	**34.3**			**12.5**	**27.9**			
	Mean			**34.5**				**28.0**			
	STD			**0.805**				**1.72**			
	CV%			**2.33%**				**6.12%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.49. Results for sheepshead chronic test method performed on receiving water samples.

			Survival Information				Growth Information				
LabID	**Sample Code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9643	03/21/00	25[b]	35.4[b]	100	0.00	6.25[b]	27.5[b]	1.20	1.12	d_1, e_1, e_5, g_6
22	9619	03/21/00	25	35.4	92.5	11.3	25	28.7	1.08	4.51	c_5, d_5, e_2, e_3, e_5, g_1
73	9623	03/21/00	25	35.3	100	0.00	12.5	27.9	0.841	6.93	
101	9627	03/21/00	25	34.2	100	0.00	25	29.8	0.933	6.63	e_2, e_5
181	9631	03/21/00	25	34.2	100	0.00	12.5	27.3	1.05	3.90	e_5
238	9635	03/21/00	25	35.4	100	0.00	12.5	27.8	0.637	5.79	b_1, d_5, e_2
273	9639	03/21/00	25	35.3	100	0.00	12.5	25.4	1.61	3.44	d_2, d_3
420	9647	03/21/00	25	35.4	100	0.00	12.5	24.0	2.08	5.03	e_2, e_5
Summary Statistics	**N**		**7**	**7**			**7**	**7**			
	Min		**25**	**34.2**			**12.5**	**24.0**			
	Max		**25**	**35.4**			**25**	**29.8**			
	Median		**25**	**35.3**			**12.5**	**27.8**			
	Mean			**35.0**				**27.3**			
	STD			**0.569**				**1.95**			
	CV%			**1.63%**				**7.15%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.50. Precision of point estimates from the sheepshead chronic test method.

Sample type	CV (%)					
	LC50			IC25		
	Within-lab[a]	Between-lab[a]	Total	Within-lab[a]	Between-lab[a]	Total
Reference toxicant	–	–	22.2	–	–	18.4
Effluent	–	–	2.33	–	–	6.12
Receiving water	–	–	1.63	–	–	7.15
Average	**–**	**–**	**8.73**	**–**	**–**	**10.5**

[a] Within- and between-laboratory components of variability were not calculated for this method since no within-laboratory replication was provided.

Table 9.51. Precision of NOEC values from the sheepshead chronic test method.

Sample type	Endpoint	NOEC Frequency						Median (% sample)	% within 1 conc. of median	% beyond 1 conc. of median
		<6.25%	6.25%	12.5%	25%	50%	100%			
Reference toxicant	Survival	0	0	0	4	3	0	25	100	0.00
	Growth	0	0	0	7	0	0	25	100	0.00
Effluent	Survival	0	0	0	7	0	0	25	100	0.00
	Growth	0	0	4	3	0	0	12.5	100	0.00
Receiving water	Survival	0	0	0	7	0	0	25	100	0.00
	Growth	0	0	5	2	0	0	12.5	100	0.00

9.10 Silverside Acute Test Method Results

A total of nine participant laboratories conducted the silverside acute test method in the WET Variability Study. These laboratories tested a total of 6 blank samples, 12 reference toxicant samples, 12 effluent samples, and 6 receiving water samples. For each sample tested, a 96-hour LC50 was generated as a test result. Results of silverside acute testing are shown in Tables 9.52 - 9.55 for each sample type.

Precision estimates for the silverside acute test were not calculated for the reference toxicant sample type because this sample type failed to produce toxicity that could be definitively measured within the test concentration range. All LC50s for the reference toxicant sample were >100% sample. This was caused by precipitation of the spiked copper in the liquid ampule sample (see Section 5.3).

9.10.1 Successful Test Completion Rate

A total of 36 silverside acute tests were initiated by 9 participant laboratories. Of the 36 tests initiated, 2 tests (samples 9507 and 9506) failed to meet test acceptability criteria for control survival. The resulting successful test completion rate calculated in the WET Variability Study for the silverside acute test method was 94.4%. The two invalid tests were initiated in the same laboratory (33) on the same day and were likely due to poor health of test organisms supplied on that day. In addition, the referee laboratory failed to meet the test acceptability criteria for control survival in three tests (samples 9531, 9532, and 9533). The referee laboratory successfully repeated these tests, and results from the repeated tests are shown in Tables 9.52 - 9.54.

9.10.2 False Positive Rate

A total of six blank samples were analyzed by six participant laboratories (Table 9.52). The LC50 calculated for all 6 blank samples was >100%, indicating no toxicity and no false positives. The resulting false positive rate calculated in the WET Variability Study for the silverside acute test method was 0.00%.

9.10.3 Precision

Precision of the silverside acute test method was estimated by calculating the CV of LC50 values obtained for the effluent and receiving water samples. Precision estimates were not calculated for the reference toxicant sample type because this sample type failed to produce toxicity that could be definitively measured within the test concentration range (see Sections 5.3 and 9.1.4). Within-laboratory, between-laboratory, and total CVs were calculated for the effluent samples. Only a total CV was calculated for the receiving water samples since no within-laboratory replication was provided for this sample type. All participant laboratory test data for the effluent and receiving water samples were used in estimating precision. No results were identified by ASTM h statistics as possible outliers.

Table 9.56 summarizes the precision of point estimates from the silverside acute test method. As expected, the majority of variability was due to the between-laboratory component, with a within-laboratory CV of 9.91% and a between-laboratory CV of 49.7%. Total CVs were much higher for the effluent sample type (50.7%) than the receiving water sample type (26.3%). Averaging the CVs based on total variance for the two sample types, a total CV of 38.5% was obtained for the silverside acute test method in the WET Variability Study.

Table 9.52. Results for silverside acute test method performed on blank samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9532	12/03/99	>100[b]	100	0.00	b_2, b_3, e_2, e_4, g_1, g_{12}
29	9504	11/09/99	>100	100	0.00	e_1, e_2
36	9512	11/09/99	>100	100	0.00	d_2, d_6, e_4
209	9518	11/02/99	>100	100	0.00	
244	9528	11/09/99	>100	100	0.00	e_4
425	9536	11/09/99	>100	95.0	8.66	d_1, e_6
459	9538	11/03/99	>100	100	0.00	b_2, d_2
Summary Statistics	**N**		**6**			
	Min		**>100**			
	Max		**>100**			
	Median		**>100**			
	Mean		**>100**			
	False positives		**0**			
	False positive rate		**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.53. Results for silverside acute test method performed on reference toxicant samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9531	12/03/99	>100[b]	100	0.00	b_2, b_3, e_2, e_4, g_1, g_{12}
33	9507	11/02/99	Invalid[c]	55.0	8.52	a_1
29	9502	11/02/99	>100	100	0.00	b_1, e_2
29	9503	11/02/99	>100	100	0.00	b_1, e_2
36	9510	11/02/99	>100	95.0	8.66	c_5, d_2, d_5, d_6
36	9511	11/02/99	>100	95.0	8.66	d_2, d_5, d_6
125	9515	11/02/99	>100	100	0.00	
209	9520	11/09/99	>100	95.0	8.66	
209	9521	11/09/99	>100	90.0	0.00	
221	9523	11/02/99	>100	95.0	8.66	
244	9526	11/02/99	>100	95.0	8.66	e_1, e_4
244	9527	11/02/99	>100	95.0	8.66	e_4
425	9535	11/02/99	>100	100	0.00	
Summary Statistics	**N**		**11**			
	Min		**>100**			
	Max		**>100**			
	Median		**>100**			
	Mean		**>100**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.54. Results for silverside acute test method performed on effluent samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9533	12/03/99	28.6[b]	90.0	0.00	b_2, b_3, e_2, e_4, g_1, g_{12}
29	9505	11/09/99	42.0	100	0.00	d_2, e_2
33	9508	11/09/99	20.4	90.0	0.00	
33	9509	11/09/99	17.9	90.0	17.1	
36	9513	11/09/99	40.8	90.0	0.00	d_2, d_7, e_4
125	9516	11/09/99	23.9	100	0.00	
125	9517	11/09/99	18.3	100	0.00	
221	9524	11/09/99	68.2	90.0	0.00	d_1, e_4
221	9525	11/09/99	60.3	95.0	8.66	d_1, e_4
244	9529	11/09/99	84.7	100	0.00	e_4
425	9537	11/09/99	37.7	100	0.00	
459	9540	11/09/99	38.2	100	0.00	d_2
459	9541	11/09/99	33.0	100	0.00	d_2
Summary Statistics	**N**		**12**			
	Min		**17.9**			
	Max		**84.7**			
	Median		**38.0**			
	Mean		**40.4**			
	Within-lab	**STD**	**4.01**			
		CV%	**9.91%**			
	Between-lab	**STD**	**20.1**			
		CV%	**49.7%**			
	Total	**STD**	**20.5**			
		CV%	**50.7%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.55. Results for silverside acute test method performed on receiving water samples.

			Survival Information			
LabID	**Sample code**	**Test date**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**Flags[a]**
Referee	9530	11/02/99	47.3[b]	95.0	8.66	e_2, g_1
33	9506	11/02/99	Invalid[c]	65.0	24.0	a_1
125	9514	11/02/99	21.5	100	0.00	
209	9519	11/02/99	28.7	100	0.00	
221	9522	11/02/99	44.3	90.0	17.1	d_1
425	9534	11/02/99	35.4	100	0.00	d_5
459	9539	11/03/99	39.0	100	0.435	b_2, d_2, e_1
Summary Statistics	**N**		**5**			
	Min		**21.5**			
	Max		**44.3**			
	Median		**35.4**			
	Mean		**33.8**			
	STD		**8.90**			
	CV%		**26.3%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results from invalid tests were excluded from summary statistics.

Table 9.56. Precision of point estimates from the silverside acute test method.

Sample type	**CV (%)**		
	Within-lab[a]	**Between-lab[a]**	**Total**
Reference toxicant[b]	-	-	-
Effluent	9.91	49.7	50.7
Receiving water	-	-	26.3
Average	**9.91**	**49.7**	**38.5**

[a] Within- and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.
[b] Precision estimates were not calculated for the reference toxicant sample type because this sample type failed to produce toxicity that could be definitively measured within the test concentration range (see Section 5.3 and 9.1.4).

9.11 Silverside Chronic Test Method Results

A total of 10 participant laboratories conducted the silverside chronic test method in the WET Variability Study. These laboratories tested a total of 7 blank samples, 13 reference toxicant samples, 13 effluent samples, and 7 receiving water samples. For each sample tested, a 7-day survival NOEC, a 7-day growth NOEC, a 7-day LC50, and a 7-day growth IC25 were generated as test results. As described in the WET method manual, growth was measured as the total weight per replicate divided by the number of original organisms in that replicate. This definition provides a combined growth and survival endpoint that is more accurately termed biomass. Also, test concentrations above the survival NOEC were excluded from hypothesis testing conducted on the growth endpoint. Results of silverside chronic testing are shown in Tables 9.57 - 9.60 for each sample type.

9.11.1 Successful Test Completion Rate

A total of 40 samples were tested at 10 participant laboratories. All 40 tests were completed and met test acceptability criteria. The resulting successful test completion rate calculated in the WET Variability Study for the silverside chronic test method was 100%. The growth endpoint results for sample 9545 were reported as inconclusive based on an evaluation of the concentration-response relationship (Table 8.4). If this test is considered unsuccessful (since the test would be repeated in a regulatory context if the test endpoint required in the permit produced an inconclusive result), the successful test completion rate becomes 97.5%.

9.11.2 False Positive Rate

A total of seven blank samples were analyzed by seven participant laboratories (Table 9.57). No false positives were observed for survival or growth endpoints. The survival NOEC was 100% for all 7 blank samples, and the LC50 was >100% for all 7 blank samples. The growth NOEC was 100% for all 7 blank samples, and the IC25 was >100% for all 7 blank samples. The resulting false positive rate calculated in the WET Variability Study for the silverside chronic test method was 0.00%.

A participant laboratory reported a growth NOEC of less than 100% for sample 9556, indicating a false positive. This sample exhibited an interrupted concentration-response curve, with only the 12.5% treatment producing a significant difference from the control. Based on EPA guidance for evaluating concentration-response relationships, the growth NOEC for sample 9556 was recalculated and reported as 100% (Table 8.4).

9.11.3 Precision

Precision of the silverside chronic test method was estimated by calculating the CV of LC50 and IC25 values obtained for the reference toxicant, effluent, and receiving water samples. Within-laboratory, between-laboratory, and total CVs were calculated for the reference toxicant and effluent samples. Only a total CV was calculated for the receiving water samples since no within-laboratory replication was provided for this sample type. Results from two samples (9582 and 9583) were excluded from the analysis of precision. Samples 9582 and 9583 were identified by ASTM h statistics as possible outliers, and review of data qualifier flags revealed a possible cause (Table 9.1).

Table 9.61 summarizes the precision of point estimates from the silverside chronic test method. For LC50 values, within-laboratory CVs ranged from 9.17% to 12.2%, between-laboratory CVs ranged from 32.2% to 46.8%, and total CVs ranged from 33.5% to 48.4%. CVs were generally higher for the effluent sample (48.4%) than for reference toxicant (33.5%) or receiving water (40.0%) samples. As expected, the majority of variability was due to the between-laboratory component, with within-laboratory CVs averaging 10.7% and between-laboratory CVs averaging 39.5%. Averaging the CVs for the LC50 based on total variance for the three sample types, a total CV of 40.6% was obtained for the silverside chronic survival endpoint.

CVs for IC25 values were slightly higher than those for LC50 values. Within-laboratory CVs for the IC25 ranged from 7.24% to 22.0%, between-laboratory CVs ranged from 29.1% to 55.5%, and total CVs ranged from 36.4% to 56.0%. As expected, the majority of variability was due to the between-laboratory component, with within-laboratory CVs averaging 14.6% and between-laboratory CVs averaging 42.3%. Averaging the CVs for the IC25 based on total variance for the three sample types, a total CV of 43.8% was obtained for the silverside chronic growth endpoint.

The precision of NOEC values was determined by evaluating the range and distribution of NOEC values and the percentage of values falling within and beyond one concentration from the median. Table 9.62 describes the precision of NOEC values for the silverside chronic method. For the survival endpoint, NOEC values spanned four concentrations for the reference toxicant sample type, five concentrations for the effluent sample type, and three concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 90.9%, 84.6%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. Growth NOEC values spanned four concentrations for the reference toxicant and effluent sample types and spanned three concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 90.9%, 91.7%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. Of the four results (for samples 9561, 9545, 9564, and 9562) that were beyond one concentration from the median, two (samples 9564 and 9562) were from the same laboratory (125). During tests on both samples, the pH in control replicates and lower test concentrations exceeded 9.0.

Table 9.57. Results for silverside chronic test method performed on blank samples.

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9581	10/26/99	100[b]	>100[b]	97.5	5.94	100[b]	>100[b]	0.647	5.66	e_5
22	9544	10/26/99	100	>100	97.5	5.94	100	>100	0.566	8.50	b_1, c_5, d_5, e_5
36	9550	10/19/99	100	>100	97.5	5.94	100	>100	1.28	12.0	
39	9556	10/26/99	100	>100	97.5	5.94	100	>100	0.783	5.75	g_5
124	9558	10/19/99	100	>100	100	0.00	100	>100	2.33	5.48	e_5, e_6
221	9570	10/19/99	100	>100	95.0	7.07	100	>100	0.896	16.2	d_1, d_2, e_5
333	9576	10/26/99	100	>100	95.0	7.07	100	>100	1.46	7.76	d_1, e_1, e_6, g_1
421	9584	10/27/99	100	>100	97.5	5.94	100	>100	1.07	14.4	d_1, d_5, e_6, g_1
Summary Statistics	**N**		**7**	**7**			**7**	**7**			
	Min		**100**	**>100**			**100**	**>100**			
	Max		**100**	**>100**			**100**	**>100**			
	Median		**100**	**>100**			**100**	**>100**			
	Mean			**100**				**100**			
	False positives		**0**	**0**			**0**	**0**			
	False positive rate		**0.00%**	**0.00%**			**0.00%**	**0.00%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.58. Results for silverside chronic test method performed on reference toxicant samples.

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9578	10/19/99	6.25[b]	17.1[b]	97.5	5.94	6.25[b]	15.3[b]	0.603	13.1	b_1, e_5, e_6
22	9543	10/19/99	12.5	28.1	97.5	5.94	12.5	18.1	0.951	28.7	b_1, c_5, d_5, e_5
33	9547	10/19/99	12.5	23.5	100	0.00	12.5	17.8	0.941	14.2	g_1
36	9552	10/26/99	12.5	24.3	100	0.00	12.5	26.1	1.41	3.36	b_1, e_6
36	9553	10/26/99	12.5	21.1	97.5	5.94	12.5	17.3	1.36	4.08	b_1, e_6
39	9554	10/19/99	12.5	27.3	95.0	7.07	12.5	17.7	1.06	13.9	
39	9555	10/19/99	12.5	23.7	95.0	7.07	12.5	16.9	1.06	13.9	
124	9561	10/26/99	<6.25	11.0	100	0.00	<6.25	6.95	2.81	10.4	e_5
125	9563	10/19/99	6.25	10.0	80.0	9.48	6.25	8.02	1.34	8.50	d_4, e_5
209	9567	10/19/99	12.5	26.7	100	0.00	12.5	20.6	1.57	13.5	
333	9574	10/19/99	12.5	34.6	100	0.00	12.5	30.5	1.92	6.56	g_1
333	9575	10/19/99	25	37.0	100	0.00	25	25.6	2.09	3.06	g_1
421	9582	10/20/99	25[c]	65.6[c]	100	0.00	25[c]	53.7[c]	1.25	14.9	b_2, d_1, e_6, g_1, g_3
421	9583	10/20/99	100[c]	>100[c]	95.0	7.07	100[c]	>100[c]	0.915	8.44	b_2, d_1, e_6, g_1, g_3
Summary Statistics	**N**		**11**	**11**			**11**	**11**			
	Min		**<6.25**	**9.96**			**<6.25**	**6.95**			
	Max		**25**	**37.0**			**25**	**30.5**			
	Median		**12.5**	**24.3**			**12.5**	**17.8**			
	Mean			**24.3**				**18.7**			
	Within-lab	**STD**		**2.23**				**4.10**			
		CV%		**9.17%**				**22.0%**			
	Between-lab	**STD**		**7.82**				**5.43**			
		CV%		**32.2%**				**29.1%**			
	Total	**STD**		**8.13**				**6.80**			
		CV%		**33.5%**				**36.4%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results were identified as outliers, a probable cause was identified, and therefore results were excluded from summary statistics.

Table 9.59. Results for silverside chronic test method performed on effluent samples.

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9580	10/26/99	12.5[b]	28.9[b]	92.5	11.3	12.5[b]	27.8[b]	0.561	11.1	e_5
22	9545	10/26/99	100	>100	97.5	5.94	Inconclusive[c]	Inconclusive[c]	0.613	24.8	b_1, c_5, d_5, e_5, g_2
33	9548	10/27/99	12.5	57.7	100	0.00	12.5	53.7	0.867	21.7	b_1, b_2, g_1
33	9549	10/27/99	25	75.1	97.5	5.94	25	56.9	1.02	7.85	b_1, b_2, g_1
39	9557	10/26/99	25	43.2	97.5	5.94	25	32.5	0.783	5.75	b_1, d_7
124	9560	10/26/99	25	48.2	100	0.00	25	36.8	2.86	7.31	e_5
125	9564	10/26/99	6.25	16.4	100	0.00	6.25	8.74	0.984	13.0	d_4, e_5
125	9565	10/26/99	12.5	20.9	100	0.00	12.5	16.1	0.984	13.0	d_4, e_5
209	9568	10/26/99	25	35.6	92.5	6.32	25	31.1	1.29	5.27	d_7
209	9569	10/26/99	12.5	32.0	100	0.00	12.5	30.8	1.19	14.9	d_1, d_7
221	9572	10/26/99	50	66.1	92.5	16.1	50	63.0	0.602	7.37	d_1, e_5
221	9573	10/26/99	50	70.7	100	0.00	50	67.3	0.607	13.3	d_1, e_5
333	9577	10/26/99	25	47.2	95.0	11.4	25	40.4	1.58	2.01	d_1, g_1
421	9585	10/27/99	50	>100	100	0.00	50	>100	0.921	21.8	d_1, d_7, e_6, g_1, g_2
Summary Statistics	**N**		**13**	**13**			**12**	**12**			
	Min		**6.25**	**16.4**			**6.25**	**8.74**			
	Max		**100**	**>100**			**50**	**>100**			
	Median		**25**	**48.2**			**25**	**38.6**			
	Mean			**54.9**				**44.8**			
	Within-lab	**STD**		**6.70**				**3.24**			
		CV%		**12.2%**				**7.24%**			
	Between-lab	**STD**		**25.7**				**24.9**			
		CV%		**46.8%**				**55.5%**			
	Total	**STD**		**26.5**				**25.1**			
		CV%		**48.4%**				**56.0%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.
[c] Results were excluded from summary statistics. Based on EPA guidance for evaluating concentration-response relationships (EPA, 2000a) the test result was inconclusive and the sample should be retested to obtain a reliable result.

Table 9.60. Results for silverside chronic test method performed on receiving water samples.

			Survival Information				Growth Information				
LabID	**Sample code**	**Test date**	**NOEC (% sample)**	**LC50 (% sample)**	**Control mean (%)**	**Control CV (%)**	**NOEC (% sample)**	**IC25 (% sample)**	**Control mean (mg)**	**Control CV (%)**	**Flags[a]**
Referee	9579	10/19/99	12.5[b]	26.2[b]	85.0	6.95	12.5[b]	19.1[b]	0.644	9.60	b_1, e_5, e_6
22	9542	10/19/99	25	52.7	95.0	7.07	25	36.0	0.840	21.3	b_1, c_5, d_5, e_5
33	9546	10/19/99	25	43.8	100	0.00	25	36.0	0.941	14.2	b_1, g_1
36	9551	10/19/99	12.5	38.4	100	0.00	12.5	29.3	1.34	8.12	
124	9559	10/19/99	12.5	26.5	100	0.00	12.5	18.2	2.33	5.48	e_5, e_6
125	9562	10/19/99	6.25	11.5	80.0	9.48	6.25	9.35	1.34	8.45	d_4, e_5
209	9566	10/19/99	25	55.8	97.5	5.94	25	41.6	1.58	9.46	
221	9571	10/19/99	25	39.2	100	0.00	25	32.8	1.18	12.2	d_1, d_2, e_5
Summary Statistics	**N**		**7**	**7**			**7**	**7**			
	Min		**6.25**	**11.5**			**6.25**	**9.35**			
	Max		**25**	**55.8**			**25**	**41.6**			
	Median		**25**	**39.2**			**25**	**32.8**			
	Mean			**38.3**				**29.0**			
	STD			**15.3**				**11.4**			
	CV%			**40.0%**				**39.1%**			

[a] Data qualifier flags are described in Table 8.3.
[b] Results from the referee laboratory were excluded from summary statistics.

Table 9.61. Precision of point estimates from the silverside chronic test method.

Sample type	CV (%)					
	LC50			IC25		
	Within-lab [a]	Between-lab [a]	Total	Within-lab [a]	Between-lab [a]	Total
Reference toxicant	9.17	32.2	33.5	22.0	29.1	36.4
Effluent	12.2	46.8	48.4	7.24	55.5	56.0
Receiving water	-	-	40.0	-	-	39.1
Average	**10.7**	**39.5**	**40.6**	**14.6**	**42.3**	**43.8**

[a] Within- and between-laboratory components of variability were not calculated for the receiving water sample type since no within-laboratory replication was provided for this sample type.

Table 9.62. Precision of NOEC values from the silverside chronic test method.

Sample type	Endpoint	NOEC Frequency						Median (% sample)	% within 1 conc. of median	% beyond 1 conc. of median
		<6.25%	6.25%	12.5%	25%	50%	100%			
Reference toxicant	Survival	1	1	8	1	0	0	12.5	90.9	9.09
	Growth	1	1	8	1	0	0	12.5	90.9	9.09
Effluent	Survival	0	1	3	5	3	1	25	84.6	15.4
	Growth	0	1	3	5	3	0	25	91.7	8.33
Receiving water	Survival	0	1	2	4	0	0	25	85.7	14.3
	Growth	0	1	2	4	0	0	25	85.7	14.3

9.12 *Champia* Chronic Preliminary Testing Results

No interlaboratory test data were obtained for the *Champia* chronic test method (see Section 2.1); however, preliminary testing conducted by the referee laboratory (EnviroSystems, Inc.) provided limited single-laboratory data on the precision of the method. The results of preliminary testing are detailed in Appendix D and summarized here.

In total, 17 *Champia* chronic tests were conducted by the referee laboratory during preliminary testing. Three tests were conducted on reference toxicant samples (filtered, sterilized natural seawater spiked with $CuSO_4$), four tests were conducted on spiked receiving water samples (unfiltered and unsterilized natural seawater spiked with $CuSO_4$), seven tests were conducted on unspiked effluent samples (municipal wastewater treatment plant effluent), and three tests were conducted on unspiked receiving water samples (unfiltered and unsterilized natural seawater). For the three reference toxicant tests conducted between 7/27/99 and 5/16/00, the IC25 averaged 0.228 μg Cu/L with a CV of 27.6%. For the four tests conducted on spiked receiving water, a CV of 49.7% was achieved. For the unspiked effluent and unspiked receiving water sample types, method precision could only be assessed in the testing of duplicate samples. Results from effluent and receiving water samples collected on different dates would reflect effluent and receiving water variability as well as method variability. Testing of duplicate effluent samples collected on 5/9/00 yielded a CV of 50.0%. Testing of duplicate receiving water samples collected on 5/23/00 could not be adequately compared due to an inconclusive test result from one of the duplicate samples (see Appendix D).

9.13 *Holmesimysis* Acute Preliminary Testing Results

No interlaboratory test data were obtained for the *Holmesimysis* acute test method (see Section 2.1); however, preliminary testing conducted by the referee laboratory (MEC Analytical, Inc.) provided limited single-laboratory data for this method. The results of preliminary testing are detailed in Appendix D and summarized here.

In total, five *Holmesimysis* acute tests were conducted by the referee laboratory during preliminary testing. Of these five tests, three exhibited control survival of 80% and failed to meet test acceptability criteria. It is believed that poor control survival in these tests was due to the use of a synthetic seawater rather than a natural seawater for organism holding and test dilution. The two remaining tests met test acceptability criteria for control survival, but test organisms were field-collected and their ages could not be confirmed as meeting the required test conditions (1-5 days; 24-h range in age). Additional tests were not conducted by the referee laboratory due to difficulties in obtaining field-collected gravid females (used to produce juveniles for test use) during the winter and spring months of the WET Variability Study (see Appendix D).

9.14 Results Summary

This section summarizes the results of interlaboratory testing conducted during the WET Variability Study. In this study, EPA assessed the performance of the WET test methods by calculating successful test completion rates, false positive rates, and precision (i.e., CVs for point estimates and ranges for NOEC values) for each test method evaluated in interlaboratory testing.

9.14.1 Successful Test Completion Rate

The successful test completion rate for each WET test method was calculated as the percentage of initiated and properly terminated tests that met the test acceptability criteria as specified in the WET method manuals (see Section 9.1.2). Table 9.63 summarizes the successful test completion rates achieved in the WET Variability Study. Successful test completion rates were 100% for 4 of the 10 test methods, indicating that no invalid tests were conducted for those methods. The successful test completion rate was above 94% for 8 of the 10 methods. Only the *Ceriodaphnia* chronic and *Selenastrum* chronic test methods achieved successful test completion rates below 90%.

Table 9.63. Successful test completion rates for test methods evaluated in the WET Variability Study.

Test method	N	No. of invalid tests	Successful test completion rate (%)
Ceriodaphnia acute	104	5	95.2
Ceriodaphnia chronic	122	22	82.0
Fathead acute	107	0	100
Fathead chronic	101	2	98.0
Selenastrum chronic (with EDTA)	44	16	63.6
Selenastrum chronic (without EDTA)	44	15	65.9
Mysidopsis chronic	44	1	97.7
Sheepshead acute	28	0	100
Sheepshead chronic	28	0	100
Silverside acute	36	2	94.4
Silverside chronic	40	0	100

The successful test completion rate for the *Ceriodaphnia* chronic test method (82.0%) was suppressed by poor performance in a subset of laboratories (see Section 9.3.1). Only 10 of the 34 participant laboratories performed invalid tests, but 8 of these laboratories performed invalid tests on 50% or more of the samples tested. The low rate of successful test completion in these 8 laboratories may have been influenced by the study's strict testing schedule, which required each test to be conducted on a given day and all tests to be conducted within a 15-day time period (see Section 2.2.5 and Table 2.4). When invalid tests conducted in a given laboratory were due to marginal or poor health of the test organism cultures, then it was logical that the laboratory would fail a high percentage of tests during this study because culture health was unlikely to fully recover within 15 days.

Successful test completion rates for the *Selenastrum* chronic test method were 63.6% and 65.9% for tests conducted with and without EDTA, respectively. The lower successful test completion rates for this method appeared to be partially attributable to laboratory inexperience in using both the with and without EDTA techniques (see Section 9.6.1). Two laboratories that cultured organisms without EDTA and generally

conducted tests without EDTA showed poor successful test completion (failing eight of eight tests) when EDTA was used. These laboratories failed all tests (8) conducted with EDTA and passed all but one test (7) without EDTA. Another laboratory that cultured organisms with EDTA and generally conducted tests with EDTA showed poor successful test completion (failing four of four tests) when the without EDTA procedure was used. This laboratory failed all tests (4 of 4) without EDTA and passed all tests (4 of 4) with EDTA.

9.14.2 False Positive Rate

False positive rates were calculated as the percentage of valid tests showing toxicity in blank samples (see Section 9.1.3). Table 9.64 summarizes the false positive rates observed for test methods evaluated in the WET Variability Study. For the survival endpoint, false positive rates were 0.00% for all test methods evaluated. False positive rates for the reproduction endpoint were 3.70% for the *Ceriodaphnia* chronic test method and 0.00% for the *Mysidopsis* chronic test method.

Table 9.64. False positive rates for test methods evaluated in the WET Variability Study.

Test method	N	False positive rate (%)					
		Survival endpoint		Growth endpoint		Reproduction endpoint	
		NOEC	LC50	NOEC	IC25	NOEC	IC25
Ceriodaphnia acute	33	-	0.00	-	-	-	-
Ceriodaphnia chronic	27	0.00	0.00	-	-	3.70	3.70
Fathead acute	27	-	0.00	-	-	-	-
Fathead chronic	24	0.00	0.00	4.35[a]	4.17	-	-
Selenastrum chronic (with EDTA)	5	-	-	0.00	0.00	-	-
Selenastrum chronic (without EDTA)	6	-	-	20.0[b]	33.3	-	-
Mysidopsis chronic	7	0.00	0.00	0.00	0.00	0.00[c]	0.00[c]
Sheepshead acute	7	-	0.00	-	-	-	-
Sheepshead chronic	7	0.00	0.00	0.00	0.00	-	-
Silverside acute	6	-	0.00	-	-	-	-
Silverside chronic	7	0.00	0.00	0.00	0.00	-	-

[a] N for the growth NOEC was 23.
[b] N for the growth NOEC was 5.
[c] N for the fecundity endpoint was 4.

For the growth endpoint, false positive rates were 0.00% for all test methods except the fathead chronic test method and the *Selenastrum* chronic test method conducted without EDTA. For the fathead chronic test method, false positive rates were 4.17% for the growth IC25 and 4.35% for the growth NOEC. For the *Selenastrum* chronic test method conducted without EDTA, false positive rates were 33.3% for the growth IC25 and 20.0% for the growth NOEC. These relatively high false positive rates for the *Selenastrum* chronic test method may be due in part to a small sample size. The false positive rate of 33.3% reflects only 2 false

positives out of 6 valid test results, and the 20.0% false positive rate reflects only 1 false positive out of 5 valid tests. When the growth IC50 was calculated for the same 6 tests, no false positives were observed. Also, no false positives were observed for the *Selenastrum* chronic test performed with the addition of EDTA.

In summary, false positives were observed for only 3 of the 10 test methods (*Ceriodaphnia* chronic, fathead chronic, and *Selenastrum* chronic performed without EDTA), and the rate of false positives was below the theoretical false positive rate of 5% (based on the recommended 0.05 alpha level for hypothesis testing) for all test methods except for the *Selenastrum* chronic test method performed without EDTA.

9.14.3 Precision

The precision of test methods evaluated in the WET Variability Study was estimated by calculating a CV for point estimates (i.e., LC50s and IC25s). For test methods that included within-laboratory replication of sample types, CVs were calculated based on within-laboratory, between-laboratory, and total variance (see Section 9.1.4). These CVs were calculated independently for each sample type and averaged to obtain final estimates of within-laboratory, between-laboratory, and total variability. Table 9.65 shows the CVs calculated for each test method based on within-laboratory, between-laboratory, and total variability. As expected, the within-laboratory variability observed for most test methods was much lower than between-laboratory variability. Within-laboratory CVs ranged from 6.57% to 12.1% for LC50 values and from 6.98% to 25.2% for IC25 values. Within-laboratory variability was highest for the *Selenastrum* chronic test method, with CVs of 25.2% and 23.3% for growth IC25 results from tests conducted with and without EDTA, respectively. The within-laboratory variability for this method was much lower when calculated for the growth IC50 values. CVs for the *Selenastrum* growth IC50 were 5.82% with EDTA and 14.5% without EDTA. Within-laboratory CVs calculated in the WET Variability Study represent the variability of results between replicate samples tested simultaneously in a given laboratory. Therefore, these within-laboratory CVs are expected to be lower than previously reported within-laboratory CVs based on reference toxicants tested over time in a given laboratory (as in USEPA, 2000d).

Between-laboratory variability was higher than within-laboratory variability for all test methods with the exception of the *Selenastrum* chronic test method performed with EDTA. Between-laboratory variability observed in the WET Variability Study ranged from 11.3% to 49.7% for LC50 values and from 14.6% to 72.0% for IC25 values. Similarly to within-laboratory CVs, the growth IC50 for the *Selenastrum* chronic test method was less variable than the growth IC25. The between-laboratory CV for the growth IC50 was 13.2% (versus 14.6% for the IC25) when the *Selenastrum* chronic test was conducted with EDTA and 43.9% (versus 72.0% for the IC25) when the *Selenastrum* chronic test was conducted without EDTA.

The CVs calculated based on total variance were used to summarize the precision of test methods evaluated in the WET Variability Study. These precision estimates are averaged across sample types and incorporate both within-laboratory and between-laboratory components of method variability. Summarized precision estimates based on total variance are presented in Table 9.66. CVs for acute WET test methods ranged from 20.0% to 38.5%. CVs for chronic WET test methods ranged from 8.73% to 40.6% for LC50 values and from 10.5% to 58.5% for IC25 values. These CVs are well within the range of CVs previously reported for WET test methods. USEPA (1988) reported multilaboratory precision (CVs) of 22-167% (with a weighted mean of 50%) for acute WET methods testing reference toxicants. USEPA (1991) reported interlaboratory precision (CVs) of acute

methods as 34.6% to 50.1% for the sheepshead acute method and 22.3% to 59.5% for the *Mysidopsis* acute method. Interlaboratory precision (CVs) of chronic methods was previously reported as 20.5% to 41.1% for the *Ceriodaphnia* chronic test method, 31% for the fathead chronic method, and 44.2% for the sheepshead chronic method (USEPA, 1991). EPA intends to further comment on the results of the WET Variability Study and the significance of these results in subsequent rulemaking.

Table 9.65. Within-laboratory, between-laboratory, and total variability observed for test methods evaluated in the WET Variability Study.

Test method	CV (%)[a]					
	LC50			IC25		
	Within-laboratory	Between-laboratory	Total[b]	Within-laboratory	Between-laboratory	Total[b]
Ceriodaphnia acute	12.1	24.0	29.0	-	-	-
Ceriodaphnia chronic	7.09	21.8	21.5	17.4	27.6	35.0
Fathead acute	8.96	19.4	20.0	-	-	-
Fathead chronic	7.87	11.3	13.4	14.6	15.0	20.9
Selenastrum chronic (with EDTA)	-	-	-	25.2	14.6	34.3
Selenastrum chronic (without EDTA)	-	-	-	23.3	72.0	58.5
Mysidopsis chronic[c]	6.57	27.3	31.2	6.98	38.3	41.3
Sheepshead acute[d]	-	-	26.0	-	-	-
Sheepshead chronic[d]	-	-	8.73	-	-	10.5
Silverside acute	9.91	49.7	38.5	-	-	-
Silverside chronic	10.7	39.5	40.6	14.6	42.3	43.8

[a] Within-laboratory, between-laboratory, and total CVs presented are averaged across sample types. No within-laboratory replication was provided for the receiving water sample type, so CVs based on within and between-laboratory variance are averaged across only the reference toxicant and effluent sample types; CVs based on total variance are averaged across the reference toxicant, effluent, and receiving water sample types. See Sections 9.2 - 9.11 for precision estimates calculated independently for each sample type and variance component.

[b] CVs based on total variance may not necessarily be greater than CVs based on within and between-laboratory variance because the CVs presented are averaged across sample types.

[c] For the *Mysidopsis* chronic test method, CVs for the IC25 represent results for the growth endpoint.

[d] Within and between-laboratory components of variability were not estimated for the sheepshead acute and chronic test methods because no within-laboratory replication was provided for these methods.

Table 9.66. Summarized precision estimates (CVs) for test methods evaluated in the WET Variability Study.

Test method	CV (%)[a]	
	LC50	IC25
Ceriodaphnia acute	29.0	-
Ceriodaphnia chronic	21.5	35.0
Fathead acute	20.0	-
Fathead chronic	13.4	20.9
Selenastrum chronic (with EDTA)	-	34.3
Selenastrum chronic (without EDTA)	-	58.5
Mysidopsis chronic[b]	31.2	41.3
Sheepshead acute	26.0	-
Sheepshead chronic	8.73	10.5
Silverside acute	38.5	-
Silverside chronic	40.6	43.8

[a] CVs presented are based on total variance and averaged across sample types.
[b] For the *Mysidopsis* chronic test method, CVs for the IC25 represent results for the growth endpoint.

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