State of Wisconsin

Aquatic Life Toxicity Testing Methods Manual

2nd Edition

Wisconsin Department of Natural Resources
Bureau of Watershed Management
P.O. Box 7921
Madison, WI 53707-7921

PUB-WT-797

November 2004

For more information on Whole Effluent Toxicity testing program, visit our website at:
http://dnr.wi.gov/topic/wastewater/WET.html
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Special thanks to Dawn Karner, UW State Lab of Hygiene, for her assistance in developing electronic versions of the WET Test Report Form for this 2nd Edition.

ACKNOWLEDGEMENTS

The Department and the Bureau of Watershed Management's Biomonitoring Team would like to express our sincere appreciation to the UW-Madison State Lab of Hygiene Biomonitoring Lab (SLH) and CT Laboratories (CT), for their contributions to the development of the 2nd Edition of this Methods Manual. We would especially like to thank Steve Geis from the SLH and Eric Korthals from CT, whose willingness to participate on the team that created this edition, collect data and conduct necessary research used to develop test methods, and participate in public meetings, workshops, and hearings, went beyond the call of duty and were critical to the quality and completion of this edition.

The authors would also like to thank the following individuals and companies for their participation in the creation of Edition #2 of this Methods Manual. The time and effort spent reviewing and commenting on the technical and scientific merits of this document and their advice regarding test method development was especially important and valuable to the Methods Manual Team. *Thank you!*

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EXECUTIVE SUMMARY

The United States Environmental Protection Agency (USEPA) promulgated regulations concerning the use of whole effluent toxicity (WET) methods to protect aquatic life in National Pollutant Discharge Elimination System (NPDES) permits in 1995 (60 FR 53529, October 16, 1995). In these rules, WET is defined as the aggregate toxic effect of an effluent or receiving water as measured with a toxicity test. The USEPA-approved WET methods are specified in the "Guidelines Establishing Test Procedures for the Analysis of Pollutants", 40 CFR 136.3, Tables IA and II, of the Clean Water Act. These WET methods employ standardized, freshwater, marine, and estuarine vertebrates, invertebrates, and plants to directly measure acute and chronic effects of effluents and receiving waters monitored under NPDES permits. On November 19, 2002, USEPA revised and made available updated method manual editions. Procedures for conducting USEPA WET methods are included in the following documents:

- *United States Environmental Protection Agency. 2002. Methods for Measuring the Acute Toxicity of Effluent to Freshwater and Marine Organisms, 5th ed. USEPA, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio. EPA-821-R-02-012.*

- *United States Environmental Protection Agency. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Surface Waters to Freshwater Organisms, 4th ed. USEPA, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio. EPA-821-R-02-013.*

As regulations, adherence to the specific test procedures outlined in these USEPA documents is required when monitoring WET under the NPDES program. Of course, the extent that such procedures are "requirements" depends on the text of the documents themselves (i.e., words of obligation, such as "must" or "shall" indicate a required procedure; "may" or "should" provide flexibility so that states and laboratories may optimize test methods for specific situations). The *"State of Wisconsin Aquatic Life Toxicity Testing Methods Manual"* (Methods Manual) is intended to comply with the requirements of 40 CFR part 136, while providing testing and laboratory procedures specific to those performing WET testing for the Wisconsin Pollutant Discharge Elimination System (WPDES) program. EPA's methods, out of necessity, include many provisions which allow different protocols to be followed, depending on the intended use of the test results and the area of the country in which the test is to be applied. Wisconsin's Methods Manual eliminates many of these optional parameters in order to insure the consistency of methods used by Wisconsin labs and permittees and, where possible, to improve upon available WET methods and make them more appropriate for use by Wisconsin permittees.

This 2nd edition (PUBL-WT-797) replaces the 1st edition of the Methods Manual (PUBL-WW-033-96). The Methods Manual is referenced in ss. NR 219.04 (Table A), Wis. Adm. Code, and all permittees and laboratories must follow this document in order to submit tests for compliance with a WPDES permit and/or to maintain laboratory certification or registration. All site-specific conditions such as sampling schedules or instream waste concentrations, which are different for each permittee, are specified in the individual WPDES permit.

The "permittee" is the holder of the WPDES permit and is ultimately responsible for the submission of test results and adherence to the requirements in their permit and for all data submitted to the Department. Permittees must follow the procedures listed here for report submittal and permit compliance requirements. Tests submitted for determining compliance with a WPDES permit must be performed according to this manual.

The "laboratory" is actually performing the WET tests for WPDES compliance (can be a contract laboratory, a permittee's in-house laboratory, etc.) and is responsible for adherence to laboratory procedures and requirements in order to be granted and maintain certification or registration for WPDES compliance testing. Laboratories must follow these procedures in order to be granted and maintain certification or registration for WET testing under s. NR 149, Wis. Adm. Code.

All WET tests conducted in accordance with the requirements set forth in a WPDES permit shall be performed by laboratories certified or registered by the Department. The following procedures shall be followed when performing WET tests in conjunction with WPDES permits. Deviations from these procedures are acceptable when conducting WET tests that are not required by, or specified in, a WPDES permit. In formulating these procedures, an attempt was made to balance scientific, practical, and cost considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be applied.

INTRODUCTION

Why Does The WDNR Require Toxicity Testing?

The Clean Water Act (CWA, 1972) sets up basic requirements for regulating toxic substances discharged to waters of the United States, stating that "*no toxics in toxic amounts*" may be discharged. The CWA established the National Pollutant Discharge Elimination System (NPDES) permits program. It is from this program that the Wisconsin Department of Natural Resources (WDNR) has derived its WPDES permits program. When limits were first placed in permits, they were based on physical factors such as biological oxygen demand (BOD) and suspended solids. Years later, water quality based effluent limits were placed in permits for "priority pollutants". However water quality criteria, and therefore effluent limits, exist for only a few of the thousands of chemicals in use today. Therefore, another mechanism is needed to predict the effects of the thousands of chemicals which do not have water quality criteria and to insure that the "*no toxics in toxic amounts*" goal of the CWA is met.

Federal policies recommend an integrated approach for controlling toxic pollutants that use whole effluent toxicity (WET) testing and chemical-specific analyses to protect aquatic life. The use of WET testing is necessary in addition to chemical-specific testing due to several factors, including: 1) the limitations of chemical analysis methods (for instance, limits of detection may not be low enough to show whether standards are being met), 2) inadequate toxicity data for some chemicals, and 3) the inability to predict the toxicity of chemicals when combined in an effluent (that is, limits for individual toxics provide protection against these compounds individually, but do not account for the effects they may have when combined).

In 1988 the WDNR began using WET testing, in addition to chemical-specific testing, to measure, predict, and control the discharge of materials that may be harmful to aquatic life. Recognizing that no single test or organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the WDNR requires a battery of aquatic toxicity tests which are broadly accepted and that measure different toxic effects using organisms representing different trophic levels and taxonomic groups.

This Methods Manual is intended to comply with the requirements of 40 CFR part 136, while providing testing and lab procedures specific to those performing WET tests for the Wisconsin Pollutant Discharge Elimination System (WPDES) program. EPA's methods, out of necessity, include many provisions which allow different protocols to be followed, depending on the intended use of the test results and the area of the country in which the test is to be applied. Wisconsin's Methods Manual eliminates many of these optional parameters in order to insure the consistency of methods used by Wisconsin labs and permittees and, where possible, to improve upon available WET methods and make them more appropriate for use by Wisconsin permittees.

What Are WET Tests?

In WET tests, organisms are exposed to effluent samples for a specific time period. Test treatments consist of different solutions containing different proportions of effluent. A control treatment (an exposure of the test organisms to dilution water with no effluent added) is used to measure the acceptability of the test by showing the quality of the organisms and the suitability of the dilution water, test conditions, and handling procedures. The organisms most commonly used in Wisconsin are *Pimephales promelas* (fathead minnow), *Ceriodaphnia dubia* (waterflea) and *Selenastrum capricornutum* (green algae).

There are two types of WET tests - acute and chronic. Acute tests typically last 48 to 96 hours and the objective is to determine the concentration of effluent that causes organisms to die during a short-term exposure under controlled conditions. Chronic tests estimate the effluent concentration that interferes with the normal growth or reproductive potential of test organisms. During a chronic test, several life stages of the organism are continuously exposed to the test material at various concentrations. WPDES-required chronic tests last about 7 days (4 days for *S. capricornumtum*). Responses such as growth, reproduction, and survival are measured. The fathead minnow test, which targets the <24 hour old fish, seeks to also use the most sensitive life stage of the organism. The *C. dubia* test encompasses the entire life cycle of the organism and therefore the most sensitive stages. There are other, longer-term chronic tests such as early life stage and embryo-larval tests which are available for use, but are usually not required in Wisconsin due to the high costs associated with these tests.

Why Require The Use Of These Organisms?

Species used for WET tests must be sensitive to toxic substances, necessary for the overall health of the food chain, and representative of the indigenous population present in the possible area of impact of the test material. These so called "indicator organisms" are used to estimate what may be happening in the environment when the effluent is introduced. All of the species required in this manual have been used in toxicity tests for many years.

The species *Ceriodaphnia dubia* belongs to a group of freshwater microcrustaceans, commonly referred to as water fleas, which are a major component of freshwater zooplankton and are the dominant planktivorous (plankton-eating) herbivores in lakes. They are abundant in ponds, quiescent sections of streams and rivers, and lakes throughout North America. The selection of *C. dubia* for routine use in toxicity testing is appropriate for a number of reasons, including: 1) they are broadly distributed and present throughout a wide range of habitats, 2) they are an important link in aquatic food chains and a significant source of food for small fish, 3) they have a short life cycle and are easy to culture in the laboratory, 4) they are sensitive to a broad range of contaminants, and 5) their small size requires small volumes of test water, leading to ease in sampling and transportation of wastewater samples.

Fathead minnows belong to the family Cyprinidae (the family which includes carps and minnows), the dominant freshwater family in terms of number of species. The fathead minnow is native to much of North America and thrives in ponds, lakes, ditches, and streams. Fathead minnows are good laboratory fish, taking readily to that life and adapting well to the dry commercial fish food, brine shrimp, etc., that is necessary for laboratory culturing.

Selenastrum capricornutum is a freshwater green algae, representative of higher order vascular plants. Like other species used in WET tests, *S. capricornutum* was chosen because of its importance in the food chain and its ability to represent other species in its trophic level. Use of *S. capricornutum* is appropriate for a number of reasons, including: 1) they are broadly distributed throughout a wide range of habitats, 2) they are an important link in aquatic food chains and are a significant source of food for higher organisms, 3) they have a short life cycle and are easy to culture in the laboratory, 4) they are sensitive to a broad range of contaminants, and 5) their small size requires small volumes of test water, leading to ease in sampling and transportation of wastewater samples.

Why Is My Facility Required To Perform WET Tests?

Since the promulgation of chs. NR 105 and NR 106, Wis. Adm. Code, WET testing has become a major part of the WDNR's water pollution control program. All surface water dischargers are evaluated to determine if WET testing should be included in their permit (see Chapter 1.3 of the "Whole Effluent Toxicity (WET) Program Guidance Document" for guidance used to make WET monitoring and limits decisions).

Factors that are considered in determining whether WET ***monitoring*** is required include, but are not limited to: 1) site-specific concerns, 2) designated uses of the receiving water, 3) dilution considerations, 4) facility type, 5) effluent variability, 6) chemical-specific data, 7) interactions between measured and unmeasured chemical constituents in the effluent, and 8) historical WET data. WET monitoring is used to determine whether a discharge has the potential to impact the aquatic life in the receiving waters.

WET ***limits*** are considered when the WDNR determines through previous WET monitoring that there is evidence that the discharge may be harmful to the receiving water's aquatic life (see Chapter 1.3, of the "Whole Effluent Toxicity (WET) Program Guidance Document"). Once a limit is in a permit, any failure of a WET test may be considered a violation of a permit condition and be subject to enforcement (see Chapter 1.8, of the "Whole Effluent Toxicity (WET) Program Guidance Document" for guidance on enforcement).

The "Whole Effluent Toxicity (WET) Program Guidance Document"

The "Whole Effluent Toxicity (WET) Program Guidance Document" is intended to assist staff when determining permit requirements, and to assist permittees and labs when conducting WET tests in accordance with these permits. Part I is intended for WDNR staff use when making permit decisions; Part II contains guidance and clarification of existing requirements for permittees and others. The Guidance Document is updated as needed, usually about once annually. It can be found at http://dnr.wi.gov/topic/wastewater/WET.html or from the Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

Laboratory Certification

The WDNR is authorized under state statute to certify and register laboratories to perform environmental analysis for covered programs. The laboratory certification and registration program was established to ensure that environmental monitoring data reported to the Department meets specific, consistent quality standards. All Wisconsin and out-of-state laboratories performing chemical and toxicological testing for compliance monitoring under WDNR's covered programs are required to be certified or registered under ch. NR 149, Wis. Adm. Code. This requirement applies to any laboratory that performs testing for the administrative codes and environmental programs listed below:

Administrative Codes and Programs Requiring Certification or Registration

NR 110	Sewerage Systems	NR 113	Servicing Septic Systems
NR 123	Well Compensation Program	NR 131	Metallic Mineral Prospecting
NR 132	Metallic Mineral Mining	NR 140	Groundwater Quality
NR 145	Private Wells	NR 150	Env. Analysis and Review Procedures
NR 157	Management of PCBs	NR 158	Hazardous Substance Discharge Notification
NR 182	Metallic Mining Waste	NR 206	Land Disposal of Municipal & Domestic Wastewaters
NR 210	Sewage Treatment Works	NR 211	General Pretreatment Requirements[A]
NR 212	Wasteload Allocated Effluent Limits	NR 214	Land Treatment of Industrial Waste
NR 216	Stormwater Management	**NR 219**	**Analytical Test Methods for Wastewater[B]**
NR 347	Sediment Sampling and Analysis	NR 507	Environmental Monitoring for Landfills
NR 605	Identification of Hazardous Wastes	NR 610	Small Quantity Generator Standards
NR 615	Large Quantity Generator Standards	NR 630	Storage, Treatment, & Disposal Facilities
NR 635	Groundwater Leachate Monitoring	NR 700	General Requirements for Investigation & Remediation
NR 712	Environmental Response Actions	NR 716	Site Investigation
NR 809	Safe Drinking Water	NR 811	Design of Community Water Supplies
NR 845	County Administration of NR 812 (private wells)	HFS 46	Group Day Care Center for Children

[A] If data is reported directly to the Department or a pretreatment ordinance mandates it.

[B] Includes whole effluent toxicity (WET) testing requirements

Because high quality data is important to making sound environmental decisions the laboratory certification and registration program establishes minimum requirements for laboratories that when followed, result in traceable and well documented data. The requirements of this program are contained in s. 299.11, Wis. Stats., and ch. NR 149, Wis. Adm. Code.

In order for a laboratory to apply for certification or registration for acute and chronic whole effluent toxicity testing, the laboratory must submit a completed application to the laboratory certification program and complete an on-site evaluation. For additional information on laboratory certification and registration, contact the Bureau of Integrated Science Services' Laboratory Certification Program, 101 S. Webster St., Madison, WI 53707, (608) 267-7633 or via email at LabCert@dnr.state.wi.us.

A list of certified WET labs can be found at http://dnr.wi.gov/topic/wastewater/WET.html, or from the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

NOTE: Although the taxonomic name of S. capricornutum has changed frequently in recent years, this Methods Manual will refer to this species as S. capricornutum to maintain consistency with previous and current EPA versions of this method.

SECTION 1 - HEALTH AND SAFETY

1.1 INTRODUCTION

1.1.1 The purpose of this section is to provide guidance for insuring good laboratory safety practices. It is understood that the recommendations in this section may not be appropriate for all situations, are not intended to be all-encompassing, and are for advisory purposes only. Laboratories and/or permittees should consult other sources, such as OSHA (29 CFR 1910), in order to insure compliance with other agencies' regulations.

1.2 GENERAL PRECAUTIONS

1.2.1 Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management, and includes 1) the appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program, 2) the preparation of a formal, written health and safety plan, which is provided to each laboratory staff member, 3) an ongoing training program on laboratory safety, and 4) regularly scheduled, documented safety inspections.

1.2.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, electrocution, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

1.2.3 Prior to sample collection and laboratory work, personnel should determine that all required safety equipment and materials have been obtained and are in good condition.

1.2.4 Guidelines for the handling and disposal of hazardous materials should be strictly followed.

1.3 PERSONAL SAFETY EQUIPMENT

1.3.1 Personnel should use appropriate safety equipment, such as rubber or plastic aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes.

1.4 LABORATORY SAFETY EQUIPMENT

1.4.1 Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, and eye fountains.

1.4.2 Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

1.5 GENERAL LABORATORY AND FIELD OPERATIONS

1.5.1 Guidance in Material Safety Data Sheets should be followed for reagents and other chemicals purchased from supply houses. Incompatible materials should not be stored together.

1.5.2 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials. Personnel collecting samples and performing toxicity tests should not work alone.

1.5.3 Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the test areas should be used whenever necessary.

1.5.4 It is advisable to cleanse and disinfect exposed parts of the body immediately after collecting effluent samples.

1.5.5 All containers should be adequately labeled to indicate their contents.

1.5.6 Strong acids and volatile organic solvents employed in glassware cleaning should be used in a fume hood or under an exhaust canopy over the work area.

1.5.7 Only electrical equipment and extension cords bearing the approval of Underwriter Laboratories should be used. Ground fault interrupters should be installed in all wet laboratories where electrical equipment is used.

1.5.8 Mobile laboratories should be properly grounded to protect against electrical shock.

1.5.9 Compressed gas cylinders should be secured at all times in appropriate restraining equipment. All connections should be inspected for leakage periodically, since CO_2 leakage may displace normal atmosphere within non-ventilated areas.

1.6 DISEASE PREVENTION

1.6.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, and polio.

1.7 SAFETY MANUALS

1.7.1 For guidance on safe practices when collecting effluent samples and conducting toxicity tests, personnel should consult general industrial safety manuals, including USEPA (1986) and Walters and Jameson (1984).

1.8 WASTE DISPOSAL

1.8.1 Wastes generated during toxicity testing should be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state, and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in, performing testing activities. Local fire officials should be notified of any potentially hazardous conditions.

SECTION 2 - EFFLUENT/RECEIVING WATER SAMPLING & SAMPLE HANDLING

2.1 INTRODUCTION

2.1.1 The purpose of this section is to provide requirements for the collection and handling of effluent and receiving water samples that will ensure the proper completion of permit-required toxicity tests. It is understood that all of the conditions and requirements described herein may not be appropriate for all WPDES-permitted discharge situations. Therefore, deviations from such conditions may be allowed if approved by the Department prior to use. Sampling requirements stated in this section are the responsibility of those individuals taking the samples, whether permittee, laboratory personnel, consultant, or other.

2.1.2 Efforts should be made to insure that WET tests completed for WPDES compliance are representative of normal effluent and operating conditions. The Department should be notified if upset conditions occur during WET testing and appropriate information should be included on all report forms. Receiving water samples are collected to represent ambient water quality conditions at the time of effluent sampling. The Department may require the use of grab or composite effluent samples depending on the likelihood for variation in effluent quality. Conversely, the Department usually requires the use of grab samples for receiving water assuming that ambient water quality conditions are not highly variable within a sampling day.

2.1.3 Additional guidance regarding WET sampling procedures and sample handling can be found in Chapter 1.1 of the "Whole Effluent Toxicity (WET) Program Guidance Document", available at http://dnr.wi.gov/topic/wastewater/WET.html or from the Biomonitoring Coordinator at Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

2.2 EFFLUENT SAMPLING

2.2.1 The effluent sampling point and type is specified in the WPDES discharge permit.

2.2.2 Two samples collected over a three day (72-h) period will be necessary to complete an acute test and three samples collected over a six day (144-h) period will be necessary to complete a chronic test. Some situations (e.g., intermittent, fill & draw dischargers) may require deviations from this schedule, holding times, or other requirements. When possible, deviations should be specified in the WPDES permit. Written approval from the Department is required whenever sample type, number of samples, holding times or other changes are needed which deviate from WPDES permit requirements. Guidance regarding WET sampling schedules for intermittent dischargers can be found in Chapter 1.6 of the "Whole Effluent Toxicity (WET) Program Guidance Document", available at http://dnr.wi.gov/topic/wastewater/WET.html or from the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

2.2.3 A representative effluent sample shall be collected as a grab or composite as specified in the WPDES permit. Grab and composite samples shall be collected according to s. NR 218.04(12), Wis. Adm. Code, unless an alternate sample type is approved in writing by the Department.

2.2.4 Effluent samples must be collected with clean equipment (e.g., pumphead, tubing, transfer apparatus, containers, etc.) that has been rinsed with sample prior to collection. For equipment cleaning guidance, see Section 3.12, "*Quality Assurance, Labware Cleaning*", on page 22 of this manual.

2.2.5 The head space above the sample should be held to a minimum. Air which enters a container should be expelled by compressing the container before reclosing, if possible (i.e., where a Cubitainer® is used), or by using an appropriate discharge valve.

2.2.6 In order to ensure well-mixed samples, containers used for sample collection should be inverted gently several times prior to dispensing into a container used for shipping if the two containers are different.

2.2.7 Samples shall be chilled with ice or other means of refrigeration during and immediately after collection. Every effort should be made to achieve a sample temperature of $\leq 4^{o}C$ (without freezing).

2.2.8 Sample temperature shall be measured upon termination of the collection period. Temperature shall be measured in an aliquot removed from the sample container to avoid possible contamination from the temperature recording device.

2.2.9 Details of sample type, sample temperature, date, time, location, duration, name of collector, and procedures used for effluent sample collection should be recorded on chain-of-custody forms. **Any unusual condition (e.g., plant upsets, slug loads, weather conditions, etc.) should be noted on chain-of-custody and "Whole Effluent Toxicity Test Report Forms" (see Section 6).**

2.3 RECEIVING WATER SAMPLING

2.3.1 A minimum of one representative grab sample shall be collected upstream of the permittee's discharge, from the receiving water specified in the WPDES permit. When collecting samples from flowing waters, samples should be collected from a point that is well-mixed. For river situations, this is often at a mid-stream and mid-depth location which may require a boat and specialized sampling equipment (e.g., horizontal Kemmerer bottle, etc.). Attempts should be made not to collect samples in stagnant areas or near sediment.

2.3.2 Receiving water samples must be collected with clean equipment that has been rinsed with sample prior to collection. For equipment cleaning guidance, see Section 3.12, "*Quality Assurance, Labware Cleaning*", on page 22 of this manual.

2.3.3 The head space above the sample should be held to a minimum. Air which enters a container should be expelled by compressing the container before reclosing, if possible (i.e., where a Cubitainer® is used), or by using an appropriate discharge valve.

2.3.4 Receiving water samples shall not be collected from any point in contact with the permittee's mixing zone and every attempt shall be made to avoid contact with any other discharge's mixing zone.

2.3.5 In order to ensure well-mixed samples, containers used for sample collection should be inverted gently several times prior to dispensing into a container used for shipping, if the two containers are different.

2.3.6 Samples shall be chilled with ice or other means of refrigeration immediately after collection. Every effort should be made to prevent a receiving water sample from exceeding the ambient sample temperature at the time of collection.

2.3.7 Temperature of the sample shall be measured upon termination of the collection period. Temperature shall be measured in an aliquot removed from the sample container to avoid possible contamination from the temperature recording device.

2.3.8 Details of sample temperature, date, time, location, name of collector, and procedures used for receiving water sample collection shall be recorded on chain-of-custody forms. Any unusual condition (e.g., weather conditions, flooding, algal blooms, etc.) should be noted on the chain-of-custody and "Whole Effluent Toxicity Test Report Forms" (see Section 6).

2.3.9 If a natural water other than the receiving water is to be used for dilution as approved by the Department, it shall be collected as a grab sample as specified in part 2.3.1 of this section.

2.3.10 Additional guidance regarding receiving water use can be found in Chapter 1.2 of the WET Program Guidance Document, available at http://dnr.wi.gov/topic/wastewater/WET.html or from the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

2.4 SAMPLE HANDLING, PRESERVATION, AND SHIPPING

2.4.1 EFFLUENT SAMPLE HOLDING TIME

2.4.1.1 The following holding times shall apply to all effluent samples. Sample holding time begins when a grab sample is collected or when a composite sampling period is completed.

2.4.1.2 Maximum holding time prior to the initial use of an effluent sample for toxicity testing shall be 36-h after the completion of sample collection. Time of "initial use" is defined as the point in time when organisms have been introduced into test chambers for all tests.

2.4.1.3 Maximum holding time prior to final use of an effluent sample for solution renewal purposes shall be 96-h after initial use of a sample.

2.4.1.4 An individual sample may be conditionally acceptable if holding time requirements described in this section fall outside specifications, depending on the degree of the departure and the objectives of the tests. The acceptability of the sample will depend on the experience and professional judgment of the Department's reviewing staff. Any deviation from holding time requirements must be clearly described on the "Whole Effluent Toxicity Test Report Form" (see Section 6) sent to the Department for the test.

2.4.1.5 An extension of the holding time limit can be requested by the permittee. The request for an extension in sample holding time must include supportive data which show that the characteristics of toxicity of the effluent sample is not changed by extending the holding time. In no case should more than 72-h elapse between collection and initial use of the sample.

2.4.1.5.1 Where an extension in holding time for initial use (>36-h, but <72-h) is requested by a permittee, information on the effects of the extension in holding time on the toxicity of the samples must be obtained by comparing the results of multi-concentration acute toxicity tests performed on effluent samples held 36-h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test must be held under the same conditions as during shipment and holding.

2.4.1.5.2 An extension must be approved in writing by the Department prior to using an alternate holding time on any permit-required toxicity test. The holding time extension may be granted for the duration of the permit depending on the stability of operating conditions. The Department must be notified of changes in production and/or treatment operations which may necessitate additional studies to show extended holding times remain valid.

2.4.2 RECEIVING WATER SAMPLE HOLDING TIME

2.4.2.1 The following holding times shall apply to all receiving water samples. Sample holding time begins when a grab sample is collected or when a composite sampling period is completed.

2.4.2.2 Maximum holding time prior to the initial use of a receiving water sample for toxicity testing shall be 36-h after the completion of sample collection. Time of "initial use" is defined as the point in time when organisms have been introduced into test chambers for all tests.

2.4.2.3 There is no maximum holding time prior to final use for receiving water samples. However,

samples shall be stored at $\leq 4^{o}C$ (without freezing), in the dark until used and when not in use. A receiving water sample may be used throughout the test as long as the sample is first used within the 36-h holding time specified above.

2.4.2.4 An individual sample may be conditionally acceptable if holding time requirements described in this section fall outside specifications, depending on the degree of the departure and the objectives of the tests. The acceptability of the sample will depend on the experience and professional judgment of the Department's reviewing staff. Any deviation from holding time requirements must be clearly described on the "Whole Effluent Toxicity Test Report Form" (see Section 6) sent to the Department for the test.

2.4.2.5 An extension of the holding time limit can be requested by the permittee. The request for an extension in sample holding time must include supportive data which show that the characteristics of the receiving water sample is not changed by extending the holding time.

2.4.3 SAMPLE SHIPPING

2.4.3.1 Several sample shipping options are available, including express mail, air express, bus, and courier service. Permittees and laboratories should consult with private carriers for carrier's rules and shipping and receiving schedules.

2.4.3.2 Effluent samples shall be shipped with ice or under other refrigerated conditions to the performing laboratory.

2.4.3.2.1 Every effort should be made to maintain a sample temperature of $\leq 4^{o}C$ (without freezing), which may require samples to be pre-chilled in a refrigerator prior to shipment. At a minimum, adequate refrigeration (ice or otherwise) shall be provided with the sample in the shipping container to ensure that the sample temperature does not exceed $10^{o}C$ (without freezing) upon arrival at the performing laboratory, unless otherwise allowed as specified in part 2.4.3.2. It is acceptable to place the sample containers in plastic bags to preserve sample and label integrity.

2.4.3.2.2 Effluent samples should be shipped in new polyethylene containers (e.g. Cubitainers® or new plastic jugs). All sample containers should be rinsed with sample before being filled. Once used, effluent sample containers shall not be reused for samples collected in support of a WPDES permit-required toxicity test.

2.4.3.3 Receiving water samples shall be shipped with ice or under other refrigerated conditions to the performing laboratory.

2.4.3.3.1 Every effort should be made to prevent a receiving water sample from exceeding the ambient sample temperature at the time of collection.

2.4.3.3.2 Receiving water samples should be shipped in new polyethylene containers (e.g. Cubitainers® or new plastic "milk" jugs). All sample containers should be rinsed with sample before being filled. Once used, receiving water sample containers shall not be reused for samples collected in support of a WPDES permit-required toxicity test, unless they have been cleaned in accordance with Section 3.12, "*Quality Assurance, Labware Cleaning*", page 22 of this manual.

2.4.4 SAMPLE RECEIVING

2.4.4.1 Upon receiving effluent or receiving water samples in the laboratory, the temperature and pH of the sample, date and time of receipt, and the initials of the laboratory personnel receiving the sample shall be recorded. If the sample is received on ice then "received on ice" must also be recorded. “Received on ice “ means that sample containers are surrounded by an ice slurry, or

crushed, cubed or chipped ice at the time of receipt by the laboratory.

2.4.4.2 All samples shall be stored at $\leq 4^{o}C$ (without freezing), in the dark until used and when not in use.

2.4.4.3 Any effluent sample shall be rejected and arrangements made for a replacement if any of the following conditions are not met:

2.4.4.3.1 Effluent and receiving water samples must arrive with evidence of having been "received on ice" (as defined in Section 2.4.4.1) or under other refrigerated conditions.

2.4.4.3.2 Effluent samples must be $\leq 10°C$ (without freezing), unless the sample is received within 4 hours of the end of the collection period and the sample is used for testing upon arrival or stored immediately in the dark at $\leq 4°C$ (without freezing).

2.4.4.3.3 Effluent samples must comply with the maximum holding time prior to initial use specified in section 2.4.1.2, above.

SECTION 3 - QUALITY ASSURANCE

3.1 INTRODUCTION

3.1.1 All whole effluent toxicity (WET) tests conducted required by a WPDES permit shall be performed by laboratories certified or registered according to s. NR 149, Wis. Adm. Code. The following procedures shall be followed when performing WET tests for WPDES permits. Deviations from these procedures are acceptable when conducting WET tests that are not required by, or specified in, a WPDES permit.

3.1.2 Development and maintenance of a laboratory Quality Assurance (QA) program requires an ongoing commitment by laboratory management, and includes the following: (1) appointment of a laboratory QA officer with the responsibility and authority to develop and maintain a QA program; (2) preparation of a QA plan with data quality objectives; (3) preparation of written descriptions of laboratory standard operating procedures (SOP); and (4) provision of adequate, qualified staff and suitable space and equipment to assure reliable data.

3.1.3 The SOP document shall contain information describing all procedures associated with effluent toxicity testing, including but not limited to: equipment maintenance, sample collection, sample chain of custody, chemical analyses, organism culturing, toxicity testing, and data analysis. Because maintenance of certification or registration requires adherence to the SOP, this document shall be approved by the Department prior to certification or registration. Should modification of this document be necessary (changes in testing procedures, laboratory control water, sources of organisms, etc.), a summary of all changes shall be submitted to the Department's Biomonitoring Coordinator for review. The SOP document shall be signed by the QA manager or laboratory supervisor/manager. At a minimum, the laboratory shall maintain a copy of the latest version of the approved SOP document. The SOP shall be available for use by laboratory personnel. Management shall ensure that SOP procedures are communicated to, understood, and implemented by all laboratory personnel concerned.

3.1.4 Quality assurance practices within a toxicity test laboratory must address all activities that affect the quality of the final effluent toxicity data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition and operation of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

3.1.5 Quality control (QC) practices consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of QA, and general guidance on good laboratory practices related to toxicity testing, personnel may consult: FDA, 1978; USEPA, 1975; USEPA, 1979a; USEPA, 1980a; USEPA, 1980b; USEPA, 1991a; DeWoskin, 1984; and Taylor, 1987.

3.2 FACILITIES AND EQUIPMENT

3.2.1 Facilities must be well ventilated and free of toxic fumes. Sample preparation, culturing, and toxicity testing areas must be separated to avoid cross contamination of cultures or toxicity test solutions with toxic fumes. Laboratory ventilation systems should be designed and operated to ensure that return air from chemistry laboratories and/or sample handling areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely-fitting doors.

3.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent.

3.2.3 A good quality laboratory grade deionized water, providing a resistance of 18 megaohm-cm, must be available in the laboratory and in sufficient quantity for laboratory needs. Deionized water may be obtained

from MILLIPORE®, Milli-Q®, or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with reconditioned water from a CULLIGAN®, CONTINENTAL®, or equivalent mixed-bed water treatment system.

3.2.4 Air used for aeration must be free of oil and fumes. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters (Balston, Inc., Lexington, MA), and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

3.2.5 Materials used for exposure chambers, tubing, etc., that come in contact with the effluent and dilution water should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances, and may be reused after cleaning. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used to ship, store, and transfer effluents and receiving waters, but they should not be reused, because they could carry over adsorbed toxicants from one test to another. However, these containers may be reused if previously used only to store uncontaminated waters such as deionized or laboratory-prepared dilution waters. Glass or disposable polystyrene containers can be used as test chambers.

3.2.6 New plastic products should be tested for toxicity before general use by exposing organisms to them under ordinary test conditions.

3.2.7 Equipment which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below in Section 3.12. All material should be flushed or rinsed thoroughly with dilution water before using in the test.

3.2.8 Copper, galvanized material, rubber, brass, and lead must not come in contact with culture or dilution waters, effluent samples, or test solutions. Some materials, such as neoprene rubber (commonly used for stoppers), may be toxic and should be tested before use.

3.2.9 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

3.3 REAGENTS AND CONSUMABLES

3.3.1 Reagent water is defined as Type I water (APHA, 1992). Type I reagent water must have resistivity >>18 MΩ-cm at 25º and should contain <10 CFU/ml bacteria. It is advisable to provide a preconditioned feed water (reverse osmosis, distilled, or commercial deionizer) in front of the deionizing system to extend the life of the cartridges. The recommended four cartridge deionizing system configuration is: (1) ion exchange, (2) ion exchange, (3) carbon, (4) organic clean-up (ORGANEX-Q^{R}), followed by a 0.20 µm filter. Laboratories must verify and document that water meets the criteria for conductivity each day that tests are conducted.

3.3.2 Surface waters or dilution waters that contain undesirable organisms that may attack the test organisms should be filtered through a fine mesh net (60 µm or smaller openings).

3.3.3 Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.

3.3.4 Reagents for hardness and alkalinity tests (see Methods 130.1, 130.2, 310.1, 310.2; EPA 1979b).

3.3.5 Standard pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for instrument calibration (see USEPA Method 150.1; USEPA 1979b).

3.3.6 Specific conductivity and salinity standards (see USEPA Method 120.1; USEPA 1979b).

3.3.7 Laboratory QC check samples and standards for the above chemistry methods as required by ch. NR 149, Wis. Adm. Code.

3.3.8 Reference toxicant solutions (NaCl).

3.3.9 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1; USEPA 1979b), or reagents for modified Winkler analysis.

3.4 LABORATORY WATER USED FOR CULTURING & TEST DILUTION WATER

3.4.1 The quality of dilution water used for testing or culturing must be sufficient to allow satisfactory survival, growth, and reproduction of the test species as demonstrated by routine reference tests and negative control performance. Water used for culturing and testing shall be analyzed for toxic metals and organics whenever the minimum acceptability criteria for control survival, growth or reproduction are not met and no other cause, such as contaminated glassware or poor stock, can be identified. It is recognized that the analyte of some methods manuals may not include all potential toxicants, are based on estimates of chemical toxicity available at the time of publication and may specify detection limits which are not achievable in all matrices. However, for those analytes not listed, or for which the measured concentration or detection limit is greater than the method-specific limit, the laboratory must demonstrate that the analyte at the measured concentration or reported detection limit does not exceed one tenth the expected chronic value for the most sensitive species tested and/or cultured. The expected chronic value is based on professional judgment and the best available scientific data. The "USEPA Ambient Water Quality Criteria Documents", ch. NR 105, Wis. Adm. Code, and the USEPA AQUIRE database provide guidance and data on acceptability and toxicity of individual metals and organic compounds. Generally, the concentration of the metals Al, As, Cr, Co, Cu, Fe, Pb, Ni, and Zn, expressed as total metal, should not exceed 1 ug/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide concentrations should not exceed USEPA's Ambient Water Quality chronic criteria values, where available.

3.5 TEMPERATURE

3.5.1 Temperature monitoring shall be performed daily in testing units, culture areas, and sample storage areas. Temperature shall be monitored either continuously or observed and recorded twice daily (a.m. and p.m.) for at least two locations in the environmental control system. The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. At a minimum, temperature measurements representative of test conditions shall be recorded daily in the a.m. and again in the p.m. with a minimum of 2 hours between measurements. Thermometers shall have a scale with $\leq$ 0.1°C subdivisions. Continuous recording electronic-chart thermometers or bulb thermographs capable of documenting $\leq$ 0.1°C change are preferred. The use of a maximum/minimum temperature device is acceptable, however any deviation from the allowable testing temperature range will require the test to be restarted.

3.6 QUALITY OF TEST ORGANISMS

3.6.1 The test organisms used in the procedures described in this manual should be disease-free and positively identified to species. The laboratory or a contracted outside expert shall positively identify test organisms to species on an annual basis. They should appear healthy, behave normally, feed well, and have low mortality in cultures and test controls.

3.6.2 Where acute or chronic tests are performed with effluents or receiving waters using organisms obtained from outside the laboratory, toxicity tests of the same type must be performed with a reference toxicant

according to Schedule A in Section 3.15.2.2, unless the organism supplier provides control chart data from at least the last five monthly tests using the same reference toxicant and test conditions. These reference toxicant demonstrations are not necessary when organisms are used only to supplement or add diversity to existing lab cultures. The supplier must certify the species identification of the test organisms and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

3.6.3 If the laboratory maintains its own stock cultures, the sensitivity of the offspring should be determined in a reference toxicant test performed concurrently or at least once each month (see Section 3.15). If a test organism produced by in-house cultures is used less than monthly in effluent toxicity tests, a reference toxicant test must be performed concurrently with each effluent toxicity test.

3.6.4 If a routine reference toxicant test fails to meet acceptability criteria, the reference toxicant test must be immediately repeated. If the failed referenced toxicant test was being performed concurrently with an effluent or receiving water toxicity test, both tests must be repeated (see Section 3.15).

3.7 FOOD QUALITY

3.7.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests.

3.7.2 Problems with the nutritional suitability of food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the affect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in chronic toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration chronic tests, using the reference toxicant regularly employed in the laboratory QA program.

3.7.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. If the concentration of total organochlorine pesticides exceeds 0.15 mg/g wet weight, or the concentration of total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, Cr, Cd, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used. For foods (e.g., such as YCT) which are used to culture and test organisms, the quality of the food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 3.4 above.

3.8 ACCEPTABILITY OF ACUTE TOXICITY TEST RESULTS

3.8.1 A primary control (an exposure of the test organisms to dilution water with no effluent added) must be included in each acute test. The primary control must be compared to effluent treatments in the calculation and reporting of test results. A second control (e.g., an exposure of the test organisms to culture or laboratory water with no effluent added) which evaluates test organism health is also required. (See Section 4.4.)

3.8.2 For acute test results to be acceptable for determining permit compliance, primary control survival must be $\geq$90%. In the event that the primary control does not meet this criteria, the secondary control must have $\geq$ 90% survival in order for the test to be acceptable for permit compliance. If neither the primary nor the secondary control has survival $\geq$ 90%, the test must be repeated with the species which experienced the unacceptable controls within 30 days of the original test's end.

3.8.3 An individual test may be conditionally acceptable if certain QA/QC conditions (temperature, light, dissolved oxygen, pH) fall outside specifications, depending on the degree of the departure and the objectives of the tests. The acceptability of the test will depend on the experience and professional judgment of the Department's reviewing staff. Any deviation from test specifications must be clearly described on the "Whole Effluent Toxicity Test Report Form" (see Section 6) sent to the Department for the test.

TABLE 3.2 ACCEPTABILITY CRITERIA FOR STATIC RENEWAL ACUTE TESTS

PRETEST REQUIREMENTS (REQUIREMENTS FOR CULTURE ACCEPTABILITY)	
***C. dubia*:**	
Average number of neonates in 3 broods	$\geq$ 15
Mean survival	$\geq$ 80%
Number of neonates in each brood used	$\geq$ 8
Age of organism	$\leq$ 24-h.
Fathead Minnow:	
Age of organism	4-14 days
SAMPLE REQUIREMENTS	
Holding time	$\leq$ 36-h
Temperature during collection & prior to shipping	chilled w/ice or other refrigeration; should be $\leq 4^{o}$C (without freezing)
Refrigeration/chilling	Samples must be shipped w/ice or otherwise refrigerated (see 2.4.3.2)
Temperature upon arrival at the laboratory	$\leq$ 10 ° C (see Section 2.4.3, for exceptions)
TEST REQUIREMENTS (REQUIREMENTS FOR TEST ACCEPTABILITY)	
Temperature	20 $\pm$ 1 °C
Dissolved Oxygen	> 4.0 mg/L
Effluent – pH	$\geq$ 6.0 and $\leq$ 9.0 s.u.
Control survival	$\geq$ 90%
REFERENCE TOXICANT TESTING (RTT)	
Concurrent testing	if fails, repeat RTT & WET test (Section 3.15)
Monthly testing	if 2 consecutive fail, repeat & explain (see Section 3.15)

3.9 ACCEPTABILITY OF CHRONIC TOXICITY TESTS

3.9.1 A primary control (an exposure of the test organisms to dilution water with no effluent added) must be included in each chronic test. The primary control must be compared to effluent treatments in the calculation and reporting of test results. A second control (e.g., an exposure of the test organisms to culture or laboratory water with no effluent added) which evaluates test organism health is also required. (See Section 4.4.)

3.9.2 For *Ceriodaphnia dubia* chronic test results to be acceptable for determining permit compliance, the primary control must meet all of the following test acceptability criteria at test end:

- Survival must equal or exceed 80%
- $\geq$ 80% of the surviving females must have produced their third brood
- The mean number of young per surviving female must be $\geq$ 15

- The CV (standard deviation/mean) between replicates must be ≤ 40% (reproduction endpoint only, excluding young from dead adults)
- Replicates must contain < 20% males (see 3.9.2.1)

3.9.2.1 A test is unacceptable and must be repeated if populations in both controls contain > 20% males or if there are > 20% males over all test concentrations. At the end of the test, if 50% or more of the surviving organisms in a block are identified as males, the entire block must be excluded from data analysis for the reproductive endpoint. For blocks having fewer than 50% of surviving organisms identified as males, the males (not the entire block) must be excluded from the analysis of reproduction. In addition to these test acceptability criteria, if fewer than eight replicates in the control remain after excluding males and blocks with 50% or more of surviving organisms identified as males, the test is invalid and must be repeated with a newly collected sample within 30 days of the original test's end.

3.9.2.2 Determinations regarding test acceptability criteria for survival and reproduction must be made prior to the exclusion of any blocks. In the event that the primary controls do not meet all of the end of test criteria, the secondary controls must meet all of these criteria for the tests to be acceptable for determining permit compliance. If end of test acceptability criteria in neither the primary nor the secondary control meets all of these requirements, the test must be repeated within 30 days of the original test's end.

3.9.3 For fathead minnow chronic test results to be acceptable for determining permit compliance, primary control survival must be ≥ 80% and the primary control coefficient of variation (CV, calculated as standard deviation/mean) between replicates must be ≤ 40% (dry weight only, excluding dead adults). At the end of the test, the mean biomass of the surviving fathead minnows in the primary control must equal or exceed 0.25 mg (dry weight only, excluding dead adults). In the event that the primary controls do not meet all of these end of test criteria, the secondary controls must meet all of these criteria for the tests to be acceptable for determining permit compliance. A test is considered unacceptable and must be repeated (within 30 days of the original test's end) when both controls experience a failure of one or all of these test acceptability criteria.

3.9.4 For *Selenastrum capricornutum* chronic test results to be acceptable, the algal cell density in the primary control must be > 1 X 10^6 cells/ml at the end of the test, and the primary control coefficient of variation between replicates (CV, calculated as standard deviation/mean) must be ≤ 20%. In the event that the primary control does not meet both of these criteria, the secondary control must meet both of these criteria in order for the test to be acceptable. A test is considered unacceptable and should be repeated when both controls experience a failure of one or all of these test acceptability criteria.

TABLE 3.3 ACCEPTABILITY CRITERIA FOR STATIC/STATIC-RENEWAL CHRONIC TESTS

PRETEST REQUIREMENTS (REQUIREMENTS FOR CULTURE ACCEPTABILITY)	
***C. dubia*:**	
Average number of neonates	≥ 20
Mean survival	≥ 80%
Neonates used in test	Must be from 3rd or subsequent brood
Number of neonates in third or subsequent brood	≥ 8
Age of organism	≤ 24-h; released within same 8-h window
Fathead Minnow:	
Age of larvae	≤ 24-h
SAMPLE REQUIREMENTS	
Holding time	≤ 36-h

Temperature during collection and prior to shipping	≤ 4 °C
Refrigeration/chilling	Samples must be shipped w/ice or otherwise refrigerated (see 2.4.3.2)
Temperature upon arrival at the laboratory	≤ 10 °C (see Section 2.4.3 for exceptions)
TEST REQUIREMENTS (REQUIREMENTS FOR TEST ACCEPTABILITY)	
Temperature	25 ± 1 °C
Dissolved Oxygen	> 4.0 mg/L
Effluent – pH	≥ 6.0 and ≤ 9.0 s.u.
Control Variability – biomass (fathead minnow) & reproduction (*C. dubia*)	CV between replicates ≤ 40%; reproduction/growth endpoint only, excluding dead adults (or young from dead adults)
Control Variability – growth - *S. capricornutum*	CV between replicates ≤ 20%
Control survival	≥ 80%
C. dubia male production	≤20% in controls and ≤20% over all concentrations
C. dubia mean control reproduction	≥ 15 neonates/surviving females in 1st 3 broods; ≥~~60~~ 80% produce 3 broods
Fathead minnow mean control biomass	≥ 0.25 mg/fish
S. capricornutum control performance	cell density > 1 X 10^6 cells/ml at the end of the test
REFERENCE TOXICANT TESTING (RTT)	
Concurrent testing	if fails, repeat RTT and WET test (Section 3.15)
Monthly testing	if 2 consecutive fail, repeat & explain (see Section 3.15)

3.9.5 An individual test may be conditionally acceptable if certain QA/QC conditions (temperature, light, dissolved oxygen, pH) fall outside specifications, depending on the degree of the departure and the objectives of the tests. The acceptability of the test will depend on the experience and professional judgment of the Department's reviewing staff. Any deviation from test specifications must be clearly described on the "Whole Effluent Toxicity Test Report Form" (see Section 6) sent to the Department for the test.

3.10 ANALYTICAL METHODS

3.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions must include established QA practices (USEPA, 1979a; USEPA, 1993).

3.10.2 Reagent containers shall be dated when received from the supplier and the shelf life shall not be exceeded. Also, working solutions shall be labeled when prepared, and the recommended shelf life shall be observed.

3.11 QUALITY CONTROL REQUIREMENTS

3.11.1 Laboratories performing chemical analyses for whole effluent toxicity testing are required to meet the quality control requirements specified in ch. NR 149, Wis. Adm. Code. The laboratory's quality control program shall include provisions for **instrument calibration**, and the analysis of quality control elements- **method blanks**, **known standards**, and **replicate samples**. In addition, laboratories are required to determine the acceptability of each of these measures through use of control limits and to perform and document corrective action when any of these measures fails to meet these limits. Refer to ch. NR 149, Laboratory Certification and Registration, for specific quality control requirements for laboratory performing chemical analyses for WET testing.

3.11.1.1 **Instrument calibration** is required prior to use each day, when used for routine measurements of chemical and physical parameters such as DO, conductivity pH and total residual chlorine. Procedures are detailed in analytical methods or by the instrument manufacturer. If analyzing for ammonia using an ion selective electrode, the meter requires daily calibration. Calibration of the laboratory's pH meter is required for alkalinity determinations as well. Refer to *Standard Methods for the Examination of Water and Wastewater*, 20th edition (American Public Health Association, 1015 Fifteenth Street NW, Washington DC 2005, 1998) or EPA analytical test methods as cited in ch. NR 219,. Wis. Adm. Code, for additional information regarding calibration requirements.

Following daily instrument calibration, some analytical test methods require verification of calibration using a second-source initial calibration verification standard (ICV). If required by method, ICVs must meet criteria *before* samples can be analyzed.

3.11.1.2 **Method blanks** are samples of reagent water which is processed through all preparation steps and the analytical method at the same time and in the same manner as samples are processed. They are used to determine if contamination of samples can be attributed to the analytical process. A method blank shall be prepared and analyzed on each analysis day.

Method blanks are required for the following tests: alkalinity, ammonia, hardness and total residual chlorine.

Method blank results exceed control limits when results are higher than the highest of any of the following: 1. method detection limit, 2. five percent of the regulatory limit for that analyte or 3. five percent of the measured concentration in the sample. If the results of the method blank exceed control limits, the laboratory shall take corrective action and document its efforts. An acceptable method blank is required *before* samples can be analyzed.

3.11.1.3 **Known standards** are samples prepared or acquired by a laboratory with a known concentration of analyte used to calibrate or verify the calibration of an analytical system. A known standard is required to be analyzed each day and after each 20 samples, if more than 20 samples are analyzed daily. Prepared solutions for known standards are commonly available; alternatively, laboratories can prepare them from neat compounds from a lot different than that used to prepare calibration standards.

Known standards are required for: ammonia, conductivity, hardness and total residual chlorine.

The known standard results shall be within ± 10% of the true value. If the results of the known standard exceed ± 10%, the laboratory shall take corrective action and document its efforts. An acceptable known standard is required *before* samples can be analyzed.

3.11.1.4 **Replicate samples** are 2 equal aliquots taken from the same sample container and analyzed independently for the same constituent. They are used to assess a laboratory's precision in generating results. A replicate sample shall be analyzed after the analysis of 20 samples for each matrix type. Laboratories are encouraged to select samples that have quantifiable test results. Comparing non-detect results for replicate samples is strongly discouraged as it does not challenge the laboratory's ability to reproduce results.

Replicate samples are required for the following tests: alkalinity, ammonia, hardness and total residual chlorine.

The laboratory shall establish control limits for replicate analyses, using either default limits or deriving statistical limits from in-house data. Control limits for replicates are typically expressed as relative percent difference (RPD) or, to a lesser degree, concentration-dependent absolute range. Typical default

limits for chemical tests performed in WET laboratories are ≤ 10% RPD. If the results of the sample replicate exceed control limits, the laboratory shall take corrective action and document its efforts. If, upon reanalysis, results still exceed limits, the results may be qualified in accordance with ch. NR 149, Wis. Adm. Code.

The following table summarizes the frequency for quality control measures required for WET testing:

Test	Calibration	Known Standard	Method Blank	Replicate [1]
Alkalinity	None [2]	None	Daily [3]	1 in 20 samples
Ammonia	Daily	Daily	Daily [3]	1 in 20 samples
Conductivity	Daily	Daily	None	None
Dissolved Oxygen (DO)	Daily	None	None	None
Hardness	None	Daily	Daily [3]	1 in 20 samples
pH	Daily	None	None	None
Total Residual Chlorine [4]	Daily	Daily	Daily	1 in 20 samples

1. To ensure frequency for analysis of sample replicates is being met, and to minimize the potential volume of data that could require qualification, laboratories are encouraged to perform sample replicate analysis weekly (if laboratory does not routinely analyze 20 samples per week). This will enable to laboratory to collect sufficient data to in-house statistical control limits.
2. Daily calibration of the laboratory's pH meter is required when determining alkalinity by titration to pH = 4.5. The laboratory should verify a pH = 4 buffer to ensure accuracy near the end-point of this titration. If the laboratory prepares its own H_2SO_4 to be used as titrant, it must be standardized against a primary standard grade reagent to determine molarity. Purchased titrants do not require standardization. If autoanalyzer is used to determine alkalinity, follow reference method and NR 149 requirements for multi-point calibration.
3. Laboratory reagent water is to analyzed as method blank when required.
4. Quality control measures may vary, based on technique used to determine total residual chlorine. Refer to manufacturer's instructions, analytical method or contact your laboratory's auditor to ensure you are meeting these requirements.

3.11.2 **Thermometers, thermocouples and infrared guns.** Devices used to measure the temperature of culture chambers, water baths, refrigerators, freezers, samples received by the laboratory or elsewhere in support of WET testing shall be calibrated or verified every three months (quarterly) against thermometers traceable to the National Institute of Standards and Technology. Each device shall be assigned and labeled with a unique identifier, date of calibration and correction factor, if needed. Infrared guns, typically used for sample receipt, should be checked more frequently as they do not maintain calibration as well as other temperature-measuring devices. Temperature device calibration records shall be maintained separately in a permanent logbook.

3.11.3 **Light** intensity is required to be determined annually for ambient light, using a light meter, as specified in method requirements. **Photoperiod** shall be verified quarterly in accordance with methods. Algal tests require daily monitoring of light intensity. Quarterly and annual records of light intensity and photoperiod shall be maintained separately in a permanent logbook by the laboratory; daily measurements are to be included on benchsheets used for algal tests. When monitoring indicates intensity or photoperiod do not meet method requirements, the laboratory shall take corrective action and record effort to address failures in a permanent logbook.

3.12 LABWARE CLEANING

3.12.1 Disposable labware shall be used once and discarded. New plasticware used for effluent or dilution water collection or organism test chambers does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with sample dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and rinsed well in deionized water and dilution water.

3.12.2 All labware (other than single use disposable), sample containers, test vessels, tanks, and other equipment used or reused for toxicity testing and any receiving water sample containers that are reused shall be cleaned according to the following procedures, except where materials may not be compatible with acids or acetone, in which case the manufacturer's recommended cleaning procedures should be followed:

1. Soak 15 minutes and scrub with detergent in tap water, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals, and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water. ***Caution:*** *Concentrated HNO_3 is a strong oxidizer and may react and combust with acetone.*
4. Rinse twice with deionized water.
5. Rinse once with liberal amounts of fresh, full-strength, reagent grade acetone (or an alternate solvent approved for use by the Department) to remove organic compounds. Use a fume hood or canopy.
6. Rinse three times with distilled or deionized water.

3.12.3 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

3.12.4 Sterilization of labware is not required. However, when sterilization is necessary, the guidelines established in Chapter V, Section 4.1 of the Manual for the Certification of Laboratories Analyzing Drinking Waters (USEPA, 1997) should be followed.

3.13 REPLICATION AND TEST SENSITIVITY

3.13.1 The sensitivity of toxicity tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

3.14 VARIABILITY IN TOXICITY TEST RESULTS

3.14.1 Factors which can affect toxicity test variability, precision, and success include: the experience and skill of the laboratory analyst; test organism age, condition, and sensitivity; dilution water quality; temperature control; and the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. A discussion regarding WET test variability can be found in Chapter 2.9 of the WET Program Guidance Document, available at http://dnr.wi.gov/topic/wastewater/WET.html or from the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

3.14.2 Test precision can be estimated by using the same strain of organisms under the same test conditions, and employing a known reference toxicant. Intra- and inter-laboratory precision are described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation) of the calculated endpoints from the replicated tests. In order for toxicity tests to be acceptable for permit compliance, controls must meet certain variability criteria (see Sections 3.8 & 3.9).

3.14.3 The USEPA states that comparisons of WET method precision with method precision for analytes commonly limited in NPDES permits clearly demonstrates the variability of the promulgated USEPA WET methods is within the range of variability experienced in other types of analyses. Several independent researchers and studies also have concluded that method performance improves when prescribed methods are followed closely by experienced analysts (USEPA, 2000). Conclusions by DeGraeve et al. (1998) included the following:

"The project team believes that the results demonstrate that the test methods can be routinely completed successfully by well-trained, competent WET testing laboratories and that the results, considered collectively, suggest that the test methods that are being used to measure WET are technically sound."

3.14.4 Data on single-laboratory and multi-laboratory precision are described below. Single-laboratory precision is a measure of the reproducibility of results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Multi-laboratory precision is a measure of the reproducibility of test results from different laboratories using the same method and analyzing the same test material. Multi-laboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision includes both within-laboratory and between-laboratory components of variability.

3.14.4.1 In 2000, USEPA conducted an interlaboratory variability study of the Fathead Minnow, *Pimephales promela*s, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 27 participant laboratories tested blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. Of the 101 Fathead Minnow Larval Survival and Growth tests conducted in this study, 98.0% were successfully completed and met the required test acceptability criteria. Of 24 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 4.4%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Averaged across sample types, the total interlaboratory variability (expressed as CV) was 20.9% for IC_{25} results.

3.14.4.2 In 2000, USEPA conducted an interlaboratory variability study of the *Ceriodaphnia dubi*a Survival and Reproduction Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 34 participant laboratories tested blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. Of the 122 *Ceriodaphnia dubia* Survival and Reproduction tests conducted in this study, 82.0% were successfully completed and met the required test acceptability criteria. Of 27 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 3.7%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Averaged across sample types, the total interlaboratory variability (expressed as CV) was 35% for IC_{25} results.

3.14.5 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-Based Toxics Control (see pp. 2-4, and 11-15; USEPA, 1991b).

3.15 REFERENCE TOXICANTS

3.15.1 Reference toxicant testing serves two purposes: 1) to determine the sensitivity of test organisms over time, and 2) to assess the comparability of within- and between-laboratory test results. Reference toxicant test results can be used to identify potential sources of variability, such as test organism health, difference among batches of organisms, changes in laboratory water or food quality, and performance by laboratory technicians. By standardizing reference toxicants, testing laboratories can compare test results, permittees and regulatory authorities can better compare and evaluate laboratories, and data can be used to quantify within- and between-laboratory test variability.

In order to demonstrate ongoing laboratory performance, reference toxicant tests shall be conducted with moderately hard synthetic water (mixed as described in USEPA, 2002a) and five NaCl treatments, according to Table 3.1. All reference toxicant tests required by this section shall be performed according to the conditions given in Table 3.1. The Department may approve a change of reference toxicant or other condition after results from a minimum of five tests performed with NaCl (all species and test types affected) has been submitted.

TABLE 3.1: STANDARD CONDITIONS FOR REFERENCE TOXICANT TESTS		
TEST TYPE	SPECIES	DILUTION SERIES* (g/L of NaCl)
Acute	*Ceriodaphnia dubia*	0.625, 1.25, 2.5, 5.0, 10.0
	Fathead Minnow	1.25, 2.5, 5.0, 10.0, 20.0
Chronic	*Ceriodaphnia dubia*	0.20, 0.40, 0.80, 1.6, 3.2
	Fathead Minnow	0.625, 1.25, 2.5, 5.0, 10.0
	Selenastrum capricornutum	0.625, 1.25, 2.5, 5.0, 10.0

*Labs may add a dilution to the required series, but must include the dilutions specified above unless use of a different dilution series is first obtained, in writing, from the Department.

3.15.2 In order to document variability in test organism sensitivity and the ability of laboratory personnel to generate consistent and precise test results, the following performance demonstrations are required:

3.15.2.1 **Initial Demonstration of Laboratory Performance**: It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. Prior to laboratory certification or registration, each laboratory shall initially establish an individual precision benchmark for each species and test type performed. To meet this requirement, the intra-laboratory precision, expressed as percent coefficient of variation (CV%) between replicates of the test endpoint (acute = LC_{50}; chronic = IC_{25} or IC_{50}), of each type of test to be used in a laboratory must be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant (NaCl), at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and same data analysis methods. Intra-laboratory precision benchmarks shall be re-established for each species and test type affected whenever a significant change occurs in laboratory culture or testing procedures (including, but not limited to when the source of culture water, food, or organisms is changed).

3.15.2.2 **Ongoing Laboratory Performance**: Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during that month. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test. Each laboratory's reference toxicity data may reflect conditions unique to that facility, however, each laboratory's reference toxicity results should reflect good repeatability. For each species and test type performed, the ongoing laboratory performance shall be continually evaluated according to one of the schedules listed below.

Schedule A: Perform a reference toxicant test with each WPDES test for each species and test type performed by the laboratory. Such a reference toxicant test shall be initiated within 7 calendar days prior to initiation or concurrently with an effluent toxicity test if organisms used in testing are cultured in the testing laboratory. If testing organisms are obtained from a source outside of the testing laboratory, reference toxicant testing shall be done concurrently with each effluent test. If a reference toxicant test result does not fall within the control limits as specified below, the results must be explained, if possible, and the reference toxicant and effluent toxicity tests repeated. The results of all reference toxicant tests started, valid and invalid, shall be reported to the Department with an explanation of the tests performed and results. Tests which are started and not completed must also be reported to the Department, with an explanation of reasons for not completing the test.

Schedule B: Perform reference toxicant tests on a regularly scheduled basis at a minimum frequency of once per month for each species and test type performed by the laboratory. If two or more consecutive reference toxicant test results fall outside of control limits as specified below, the results must be

explained and the reference toxicant test repeated immediately. If reference toxicant results cannot be explained to the satisfaction of the Department or if results cause reason for concern, the Department may require that all effluent toxicity tests generated during the period of the excursion(s) be repeated. The results of all reference toxicant tests started, valid and invalid, shall be reported to the Department with an explanation of the tests performed and results. Tests which are started and not completed must also be reported to the Department, with an explanation of reasons for not completing the test.

3.15.3 Acute Control Charts: A control chart (graphical **and** tabular) representing the mean LC_{50} and upper and lower control limits (mean $\pm$ 2 standard deviations (SD)) shall be established for each species as described below. The control chart shall be recalculated after the completion of each reference toxicant test using data from the previous 20 months. Any exceedance of either the upper or lower control limit after establishment of the control chart shall prompt a review of the culture and test systems. If an exceedance can be attributed to a specific problem in the culture or test system, that data should not be included in future calculations of the control limits. If the exceedance appears to represent normal variability, it shall be included in the data set. If the laboratory cannot associate the exceedance with either the culture or test system but believes the exceedance does not represent normal variability, the laboratory may submit to the Department for review the appropriate statistical analysis that identifies the data point as an outlier (See Section 5.3.2). Such data should be excluded from future calculations of the control limits. If an exceedance is attributed to the culture or test system, the laboratory shall report to the Department what corrective actions have been taken and the time period that the exceedance(s) may have influenced WPDES related effluent test results.

3.15.4 Chronic Control Charts: A control chart (graphical **and** tabular) representing the mean IC_{25} (IC_{50} for *S. capricornutum*) and upper and lower control limits (mean $\pm$ 2 SD) shall be established for each species as described below. The control chart shall be recalculated after the completion of each reference toxicant test using data from the previous 20 months. Any exceedance of either the upper or lower control limit after establishment of the control chart shall prompt a review of the culture and test systems. If an exceedance can be attributed to a specific problem in the culture or test system, that data should not be included in future calculations of the control limits. If the exceedance appears to represent normal variability, it shall be included in the data set. If the laboratory cannot associate the exceedance with either the culture or test system but believes the exceedance does not represent normal variability, the laboratory may submit to the Department for review the appropriate statistical analysis that identifies the data point as an outlier (See Section 5.3.2). Such data should be excluded from future calculations of the control limits. If an exceedance is attributed to the culture or test system, the laboratory shall report to the Department what corrective actions have been taken and the time period that the exceedance(s) may have influenced WPDES related effluent test results.

3.15.5 A control chart should be prepared for each combination of test species and test condition, as mentioned above. Toxicity endpoints from five tests are adequate for establishing the control charts. A running plot must be maintained for the toxicity values from successive tests with the reference toxicant, and endpoints are examined to determine if they are within prescribed limits. Control charts are used to evaluate the cumulative trend of results from a series of samples. The mean and upper and lower control limits ($\pm$ 2 S.D.) are re-calculated with each successive test result. After two years of data collection, or a minimum of 20 data points, the control chart should be maintained using only the 20 most recent data.

3.15.6 The data from each reference toxicant test shall be reviewed to insure that all test acceptability criteria are met (as required in 3.8 and 3.9), to identify unsuspected trends or patterns in the responses (i.e., to insure a proper concentration-response relationship), and to detect potential outliers in replicate data. Tests that do not meet all of these criteria should not be included in control charts and must be repeated.

3.15.7 Laboratories should compare the calculated CV (i.e., standard deviation/mean) of the LC_{50} for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-3 in USEPA, 2000). If the calculated CV exceeds the 75^{th} percentile of CVs reported nationally, the laboratory should use the 75^{th} and 90^{th} percentiles to calculate warning and control limits, respectively, and the laboratory should investigate options for reducing variability.

3.15.8 Outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, should be readily identified (See Section 5.3.2). If more than one out of 20 reference toxicant tests fall outside the control limits, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. In those instances when the laboratory can document the cause for the outlier (e.g., operator error, culture health or test system failure), the outlier should be excluded from the future calculations of the control limits. If two or more consecutive tests do not fall within the control limits, the results must be explained and the reference toxicant test must be immediately repeated. Actions taken to correct the problem must be reported. When two or more reference toxicant tests have fallen outside the control limits, the effluent toxicity tests conducted during the month in which the reference toxicant tests failed are suspect, and should be considered as provisional and subject to careful review.

3.15.9 If the toxicity value from a given test with the reference toxicant falls well outside the expected range for the test organisms when using the standard dilution water, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. When this occurs, the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects and may be required to be repeated with a different batch of test organisms.

3.15.10 Performance should improve with experience, and the control limits for point estimates should gradually narrow. However, control limits of ± 2 S.D., by definition, will be exceeded 5% of the time, regardless of how well a laboratory performs. Highly proficient laboratories which develop a very narrow control limit may be unfairly penalized if a test which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits will be considered by the Department in determining whether or not a reference toxicant test result falls "well" outside the expected range or whether a datum is an outlier and should be rejected. The width of the control limits may be evaluated by comparing the calculated CV of the LC/IC for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-3 in USEPA, 2000). In determining whether or not a reference toxicant test result falls "well" outside the expected range, the result also may be compared with upper and lower bounds for ±3S, as any result outside these control limits would be expected to occur by chance only 1 out of 100 tests. When a result from a reference toxicant test is outside the 99% confidence intervals, the laboratory must conduct an immediate investigation to assess the possible causes for the outlier.

3.15.11 Reference Toxicant Reporting: If conducting reference toxicant tests according to Schedule A, results shall be submitted at the WPDES testing frequency. If conducting reference toxicant tests according to Schedule B, results shall be submitted quarterly, due 30 days after the end of each quarter (on or before April 30, July 30, October 30, and January 30 of each year). The submittal shall include reference toxicant test results, presented graphically and tabularly, reflecting the previous 20 tests and shall be submitted to: WDNR, Biomonitoring Coordinator, Bureau of Watershed Management, P.O. Box 7921, Madison, WI 53707-7921. Reference toxicant test results may be emailed to the Biomonitoring Coordinator, however it is the responsibility of lab personnel to insure that these are received by the Department.

3.16 RECORD KEEPING

3.16.1 Complete records shall be maintained for each individual toxicity test or group of tests on closely related samples. This file must contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed or initialed and dated by the personnel performing the tests. Laboratories must collect and maintain all testing information for five years from the date of test completion in order to demonstrate

compliance with laboratory certification requirements. Permittees are required to retain records pertaining to WPDES permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

3.17 LABORATORY STAFF QUALIFICATIONS & TRAINING

3.17.1 Laboratory staff experience is one of the most important factors influencing WET test and laboratory performance. Grothe et al. (1996) stated that "*a number of problems with WET test are caused by misapplication of the tests, misinterpretation of the data, lack of competence of the laboratories conducting WET testing, poor condition/health of test organisms, and lack of training of laboratory personnel…*". The most important initial consideration in developing precise data is a laboratory's experience and success in performing a specific analysis. Most critical reviews of WET data precision emphasize this initial consideration. Experienced professionals most likely will be able to develop the most consistent and reliable information and can interpret anomalous conditions in the testing or results (USEPA, 2000).

3.17.2 The ability to successfully complete WET tests is a direct function of the training and expertise of lab staff. It is important for staff who are responsible for WET testing to have training in the biological sciences and practical experience in WET testing. Lack of experience can be a major source of WET test variability. These methods are restricted to use by, or under the supervision of, staff experienced in the use, conduct, and interpretation of WET tests. The laboratory must keep complete files documenting the training and experience of each staff member. Staff must be able to demonstrate the ability to generate acceptable test results using the procedures described in this Methods Manual.

3.17.3 The laboratory shall specify and document the responsibility, authority, and interrelationship of all personnel who manage, perform, or verify work affecting the quality of calibrations and tests. Such documentation shall include: 1) a clear description of the lines of responsibility in the laboratory and shall be proportioned such that adequate supervision is ensured, and 2) job descriptions for all positions. The laboratory shall also be responsible for ensuring that all personnel have demonstrated initial and ongoing proficiency in the activities for which they are responsible (see 3.17.4 below). Proper training of personnel must be kept up to date by ensuring the following: 1) Evidence must be on file that demonstrates that each employee has read, understood, and is using the latest version of the laboratory's in-house quality assurance documentation, which relates to his/her job responsibilities; and 2) Training courses or workshops on specific equipment, analytical techniques, or laboratory procedures shall be documented.

3.17.4 Analyst training shall be considered up to date if an employee training file contains a certification that technical personnel have read, understood, and agreed to perform the most recent version of the test methods given in this manual and documentation of continued proficiency by at least one of the following once per year: 1) acceptable performance of a blind sample (e.g., EPA's DMRQA program) or 2) another demonstration of capability (e.g., performance of a reference toxicant test).

3.17.5 The importance of a competent supervisor/manager and professional staff with relevant training and experience is necessary in order to generate valid toxicity testing data. The laboratory shall have sufficient personnel, having the necessary education, training, technical knowledge, and experience for their assigned functions. All personnel shall be responsible for complying with all quality assurance/quality control requirements that pertain to their organizational/technical function. Each technical staff member must have a combination of experience and education to adequately demonstrate a specific knowledge of their particular function (see 3.17.7 below) and a general knowledge of laboratory operations, analytical test methods, QA/QC procedures, and records management. Laboratory management shall certify that personnel with appropriate educational and/or technical background perform all tests for which the laboratory is accredited. Such certification shall be documented.

3.17.6 In laboratories with a specialized "work cell" (a group consisting of analysts with specifically defined tasks who together perform the test method), the group as a unit must meet the criteria established in this section and this demonstration of capability must be fully documented. When a work cell is employed and the members

of the cell change, the work cell must demonstrate continued proficiency through continuing performance checks (as described in 3.17.4).

3.17.7 Qualifications and responsibilities

3.17.7.1 Manager/Supervisor

3.17.7.1.1 Each lab must identify an individual or group who is responsible for the management of the toxicity testing lab. The manager(s) is (are) responsible for the overall performance of the laboratory in its execution and reporting of analyses.

3.17.7.1.2 The manager(s) must have sufficient academic training and experience to properly implement testing and a quality assurance program.

3.17.7.1.3 The manager(s) must have a minimum of a bachelor of science degree in biological sciences or closely related science curricula and at least three years laboratory experience in aquatic toxicity testing or a master of science degree in biological or closely related science curricula and at least one year laboratory experience in culturing and aquatic toxicity testing .

3.17.7.1.4 The manager(s) shall be responsible for defining the minimum level of qualification, experience, and skills necessary for all positions in the laboratory.

3.17.7.2 Professional Biologist/Analyst

3.17.7.2.1 The biologist/analyst performs toxicological tests with no or minimal supervision.

3.17.7.2.2 A professional biologist/analyst must have:

3.17.7.2.2.1 A minimum of a bachelor's degree in the areas of biology, zoology, fisheries, chemistry, environmental science, or related fields, and

3.17.7.2.2.2 A minimum of two weeks formal or on-the-job training from a federal agency, state agency, academic institution (or equivalent training) in culturing and toxicity testing of effluents and surface waters, and

3.17.7.2.2.3 At least one year of bench experience with no or minimal supervision from a professional biologist/analyst.

3.17.7.3 Biological Technician

3.17.7.3.1 The technician performs toxicity tests and culturing of aquatic organisms with supervision from a manager/supervisor or professional biologist/analyst.

3.17.7.3.2 A technician should have:

3.17.7.3.2.1 At least a high school education (two years of college in biology, zoology, chemistry, or related fields is recommended), and

3.17.7.3.2.2 One week of either formal (from a federal agency, state agency, or academic institution) or on-the-job training in culturing and toxicity testing of effluents and surface waters , and

3.17.7.3.2.3 At least one year of culturing aquatic organisms and bench experience in toxicity testing is recommended.

SECTION 4 - TOXICITY TEST PROCEDURES

4.1 INTRODUCTION

4.1.1 This manual describes toxicity tests for use in the Wisconsin Pollutant Discharge Elimination System (WPDES) permits program to identify effluents containing toxic materials in toxic concentrations. The methods included in this manual comply with the requirements of 40 CFR part 136, while providing testing and laboratory procedures specific to those performing whole effluent toxicity (WET) testing for the WPDES program and, therefore, constitute approved methods for acute and chronic toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a laboratory or on-site, by the Department or the permittee.

4.1.2 The data may be used for WPDES permits development and to determine compliance with permit requirements. The tests may be performed as a part of self-monitoring permit requirements, compliance inspections, toxics sampling inspections, and/or special investigations. Data from toxicity tests performed as part of permit requirements may be evaluated during compliance evaluation inspections and performance audit inspections.

4.1.3 Modifications of these tests may also be used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, or to compare and control the effectiveness of various treatment technologies.

4.1.4 The "Whole Effluent Toxicity (WET) Program Guidance Document" serves as a companion to this Methods Manual and provides guidance regarding test and sampling, toxicity reduction evaluations, and other WET-related topics. It can be found at http://dnr.wi.gov/topic/wastewater/WET.html or by contacting the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

4.1.5 Methods are presented in this manual for three species of freshwater organisms from three phylogenetic groups. The three species for which test methods are provided are the fathead minnow, *Pimephales promelas*; the daphnid, *Ceriodaphnia dubia*; and the green alga, *Selenastrum capricornutum*. The use of any species or test conditions other than those described in this manual shall be subject to the approval of the Department.

4.1.6 All WET tests conducted in accordance with the requirements set forth in a WPDES permit shall be performed by laboratories certified or registered by the Department, according to s. NR 149.22, Wis. Adm. Code. These methods are restricted to use by, or under the supervision of, analysts experienced in the use or conduct of aquatic toxicity tests and the interpretation of data from aquatic toxicity testing. Each analyst must demonstrate the ability to generate acceptable test results with these methods using the procedures described in this methods manual (Section 3.17).

4.1.7 The objective of effluent tests described in this manual is to estimate the "safe" concentration, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. Endpoints that have been considered in toxicity tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, terata, and others. Since it is not feasible to measure all of these (and other possible) effects on a routine basis, observations in the toxicity tests required by this manual have been limited to mortality, growth, and reproduction.

4.2 SCOPE AND APPLICATION

4.2.1 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

4.2.2 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long

sample collection period involved in composite sampling and because the test chambers are not sealed, highly degradable or highly volatile toxicants in the source, such as chlorine, may not be detected in the test.

4.3 INTERFERENCES

4.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment.

4.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 2 for sampling requirements).

4.3.3 The amount and type of natural food in the effluent or dilution water may confound test results.

4.3.4 Food added during the test may sequester metals and other toxic substances and confound test results. Daily renewal of solutions, however, will reduce the probability of reduction of toxicity caused by feeding.

4.3.5 Pathogenic, predatory and/or planktivorous organisms in the dilution water and effluent may affect test organism survival, reproduction and/or growth, and confound test results.

4.4 DILUTION WATER AND CONTROLS

4.4.1 If the objective of the test is to estimate the absolute toxicity of the effluent (as in most acute tests, see Section 4.4.4 below), a synthetic (standard) laboratory dilution water may be used. A second set of controls, using culture water, must also be included in the test (see Section 4.4.6 below).

4.4.2 If the objective of the test is to estimate the toxicity of the effluent in uncontaminated receiving water (as in most chronic tests, see Section 4.4.5 below), the test must be conducted using dilution water consisting of receiving water, collected upstream and out of the influence of the outfall, or with another uncontaminated surface water with similar characteristics as the receiving water, unless an alternate dilution water is approved by the Department prior to testing. A second set of controls, using culture water, must also be included in the test (see Section 4.4.6 below).

4.4.3 When dual controls (one control using dilution water and one control using culture water) are used, the dilution water control must be used to determine test acceptability. It is also the dilution water control that must be compared to effluent treatments in the calculation and reporting of test results. The secondary control is used only to evaluate the appropriateness of the dilution water source. Significant differences between organism responses in culture water and dilution water controls could indicate toxicity in the dilution water and may suggest an alternative dilution water source.

4.4.4 When a zone of initial dilution (ZID) has not been approved (i.e., when compliance is determined in 100% effluent), the dilution water used in acute tests may be a standard laboratory water or a grab sample collected from the receiving water. If a ZID has been approved, receiving water must be used for dilution, unless the use of another dilution water is approved by the Department prior to use. The receiving water used must be specified in the WPDES permit, and samples taken as specified in Section 2.3. Receiving water samples must be taken in a location where every attempt has been made to avoid contact with any portion of the mixing zone of the permittee's or any other permittees' discharge. Dilution water must be used as the primary control for the test. The dilution water control must be compared to effluent treatments in the calculation and reporting of test results. For guidance regarding dilution waters, see Chapter 2.11 of the "WET Guidance Document".

4.4.5 The dilution water used in chronic tests shall be a grab sample collected from the receiving water specified in the WPDES permit and shall be taken as specified in Section 2.3, unless the use of another dilution water is approved by the Department prior to use. The sample must be taken in a location where every attempt has been made to avoid contact with any portion of the mixing zone of the permittee's or any other permittees' discharge.

Dilution water must be used as the primary control for the test. The dilution water control must be compared to effluent treatments in the calculation and reporting of test results.

4.4.6 When receiving water is used for dilution, a second control which evaluates test organism health is also required. This control shall be culture water or standard dilution water. If an alternate water has been chosen for use as the dilution water, the secondary control must be the receiving water, if available.

4.4.7 If the Department has determined that the receiving water is inappropriate as a dilution water, an alternate dilution water may be used. This dilution water shall be uncontaminated surface water or standard dilution water having approximately the same characteristics as the receiving water. The hardness of this dilution water must comply with the following guidelines:

4.4.7.1 Laboratory water hardness is not required to be adjusted lower than 45 mg/L or greater than 250 mg/L. Receiving water hardness should be determined from the first receiving water sample that was collected along with the first effluent sample. Due to laboratory constraints to analyze receiving water samples for hardness before a test is initiated, an alternate historical receiving water hardness may be used provided the value is from the same calendar quarter as the current sample. The date, time, and location of this sample must be noted on the "Whole Effluent Toxicity Test Report Form" (see Section 6). The laboratory water hardness must be adjusted to comply with 4.4.7.2.1 or 4.4.7.2.2 below:

4.4.7.2 Laboratory water hardness shall be adjusted according to the following guidelines:

4.4.7.2.1 If the measured or historical hardness of the receiving water is <130 mg/L, moderately hard synthetic water (mixed as described in USEPA, 2002a, Table 7 or 8) shall be used for diluent. If the measured or historical hardness of the receiving water is $\geq$130 mg/L, hard synthetic water (mixed as described in USEPA, 2002a, Table 7 or 8) shall be used for diluent; OR

4.4.7.2.2 If the measured or historical hardness of the receiving water is below 100 mg/L, the laboratory water shall be adjusted to within $\pm$ 10 mg/L of that hardness. If the measured or historical hardness of the receiving water is greater than 100 mg/L, the laboratory water shall be adjusted to within $\pm$ 20 mg/L of that hardness.

4.4.8 Dilution Water Holding: a given batch of dilution water must not be used for more than 14 days following preparation because of the possible build-up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

4.5 PREPARATION OF SAMPLES FOR TOXICITY TESTS

4.5.1 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of the sample should be expelled by compressing the container before reclosing, if possible (i.e., where a Cubitainer® used), or by using an appropriate discharge valve.

4.5.2 It may be necessary to first coarse-filter receiving water samples through a sieve having 2 to 4 mm mesh openings to remove debris and/or break up large floating or suspended solids. If samples used in fathead minnow or *C. dubia* tests contain indigenous organisms that may attack or be confused with the test organisms, the samples may be filtered through a sieve with 60 µm mesh openings. (See Section 4.15.4 for additional requirements regarding filtration of effluent samples.) Effluent and receiving water samples used for *S. capricornutum* tests must be filtered through a GF/A or GF/C filter, or other filter providing 0.45µm particle size retention. Glass-fiber filters generally provide more rapid filtering rates and greater filtrate volume before plugging. Caution: filtration may remove some toxicity.

4.5.3 At a minimum, pH, conductivity, hardness, alkalinity, total ammonia and total residual chlorine must be

measured in the undiluted effluent. At a minimum, pH, conductivity, hardness, alkalinity, and total ammonia must be measured in the receiving water. These measurements are also recommended for the laboratory control. These measurements must be made of each sample upon arrival at the laboratory.

4.5.4 Test organisms (especially *C. dubia*) may experience reduced survival and/or reproduction if exposed to test waters that have hardness or alkalinity values significantly different from culture waters. Acclimation/culturing in one water and testing in another of significantly different hardness or alkalinity should be avoided to minimize stress due to routine water quality changes. If the lab must routinely test an effluent or receiving water that has significantly different hardness or alkalinity than normal lab culture waters, it may be necessary to include an additional control water (adjusted to simulate water quality conditions of concern) and/or provide organisms cultured under water quality conditions (e.g., hardness, alkalinity) that are similar to that of the effluent or receiving water.

4.6 MULTI-CONCENTRATION (DEFINITIVE) TOXICITY TESTS

4.6.1 The tests required for use in determining WPDES permit compliance are multi-concentration, or definitive, tests which provide a point estimate of effluent toxicity in terms of a Lethal Concentration (LC) or an Inhibition Concentration (IC). Tests may be static (as described in this manual for *S. capricornutum*), static renewal (as described in this manual for *C. dubia* & fathead minnow), or flow-through.

4.6.2 The test solutions must consist of at least five effluent treatments, a primary control, and a secondary control with the appropriate replicates (see Tables 4.1-4.4 & 4.7) in each treatment.

4.7 STATIC-RENEWAL TESTS

4.7.1 In static-renewal tests, test organisms are exposed to a fresh solution of the same concentration of sample every 24-hr or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

4.7.2 The volume of the effluent used must be sufficient to prepare all concentrations of effluent needed for the toxicity test and for routine chemical analysis.

4.7.3 Each dilution shall be prepared to provide sufficient material for toxicity testing and routine chemical analyses. The solutions should be well mixed with a glass rod, PFTE stir bar, or other means. The test solutions should then be brought to the required temperature, aliquots of each sample concentration delivered to the test chambers, the chambers arranged in random order, and the test organisms added. The point at which organisms are added to the test solutions must be within 36-h of the time at end of sample collection. The remaining volumes of each sample concentration should then be used, as necessary, for the chemical analyses.

4.8 FLOW-THROUGH TESTS

4.8.1 Two types of flow-through tests are in common use: 1) sample is pumped continuously from the sampling point directly to the dilutor system, and 2) grab or composite samples are collected periodically, placed in a tank adjacent to the test laboratory, and pumped continuously from the tank to the dilutor system. The flow-through method is the preferred method for on-site tests. Because of the large volume of effluent required for flow-through tests, it is generally considered too costly and impractical to conduct these tests off-site at a central laboratory. This manual does not provide comprehensive procedures for flow-through testing, due to its limited use. However, if flow-through testing is to be done, specific procedures and/or deviations from the procedures in this manual must be approved by the Department and specified in the WPDES permit.

4.8.2 Flow-through tests should be done with the same effluent concentrations used for static tests. Controls must consist of the same dilution water, test conditions, procedures, and organisms used in testing the effluent.

4.8.3 The dilutor system should be operated long enough prior to adding test organisms to calibrate the dilutor and make necessary adjustments in temperature, flow rate, and aeration. The flow rate through the dilutor must provide for a minimum of five 90% replacements of water volume in each chamber every 24 h. This replacement rate should provide sufficient flow to maintain an adequate concentration of DO. The dilutor should be capable of maintaining the concentration at each dilution within 5% of the starting concentration for the duration of the test. The calibration of the dilutor should be checked before the test begins to determine the volume of effluent and dilution water used in each portion of the delivery system and the flow rate through each test chamber. General operation of the dilutor should be checked at the beginning and end of each test day.

4.9 TEST ORGANISMS

4.9.1 The cladoceran, *Ceriodaphnia dubia,* and the fathead minnow, *Pimephales promelas*, are required in tests used for determining WPDES compliance. Other species may be substituted or added, after approval by the Department, and specified in the WPDES permit before use. It is essential that good quality test organisms be readily available throughout the year from in-house or commercial sources to meet WPDES monitoring requirements. The organisms used in toxicity tests must be identified to species. If there is any doubt as to the identity of the test organisms, representative specimens should be sent to a taxonomic expert to confirm the identification.

4.10 HANDLING OF TEST ORGANISMS

4.10.1 Test chambers must be positioned randomly. Organisms should not be subjected to changes of more than 3°C in water temperature in any 12 h period.

4.10.2 The number of organisms of a given species required for static renewal tests are given in Tables 4.1-4.4 & 4.7. Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch dry surfaces or are injured during handling must be discarded. Small fish and invertebrates should be captured with 4 to 8 mm inside diameter pipettes. Organisms larger than 10 mm may be captured by dip net. To avoid carryover of excess culture water in transferring small organisms to the test chambers, it may be advantageous to distribute daphnids and larval fish first to small holding vessels, such as weighing boats, petri dishes, or small beakers. The water in the intermediary holding vessels is then drawn down to a small volume and the entire lot is transferred to a test chamber. In the case of daphnids, both excessive handling and carryover of culture water can be avoided by placing the tip of the transfer pipette below the surface of the water in the test chambers and allowing the organisms to swim out of the pipette without discharging the contents.

4.10.3 Crowding should be avoided. The DO must be maintained at a minimum of 4.0 mg/L. The solubility of oxygen depends on temperature, salinity, and altitude. Aerate only if necessary. (See Section 4.15.5 for additional requirements regarding filtration of effluent samples.)

4.11 REPLICATE TEST CHAMBERS

4.11.1 For static renewal tests, four or more test chambers must be provided for each effluent concentration and the controls (see Tables 4.1-4.4 & 4.7). Although the data from replicate chambers are usually combined to determine the LC_{50}, IC_{25} (IC_{50} for *S. capricornutum*), and confidence intervals, the practice of dividing the test population for each effluent concentration between two or more replicate chambers has several advantages and is considered good laboratory practice because it: 1) permits easier viewing and counting of test organisms; 2) more easily avoids possible violations of loading limits, which might occur if all of the test organisms are placed in a single test vessel; and 3) ensures against the invalidation of the test which might result from accidental loss of a test vessel, where all of the test organisms for a given treatment are in a single chamber.

4.12 DILUTION SERIES

4.12.1 Tests consist of a control and a minimum of five effluent concentrations. This manual gives standard dilution series for each test type (see Tables 4.1-4.4 & 4.7), however, test concentrations may be selected independently for individual situations based on the objective of the test, the expected range of toxicity, the instream waste concentration (IWC), and any available historical testing information on the effluent. The dilution series to be used must be specified in the WPDES permit.

4.12.2 The dilution series given in this manual (see Tables 4.1-4.4 & 4.7) are intended for use as a default when little information is known about the effluent being tested and when the existing data suggests that the concentration of interest (e.g., ZID concentration for acute, IWC for chronic) is within the range of the dilution series given. In some situations, a more appropriate dilution series may be necessary based on experience from past testing of a given effluent. The appropriate selection of a dilution series can be important for accurately identifying concentration-response relationships and increasing the precision of effect concentrations estimated from those relationships. Alternate dilution series may be used if specified in the WPDES permit.

4.12.3 When tests are to be used in determining compliance with chronic testing requirements, it is often preferable to select effluent test concentrations which bracket the instream waste concentration (IWC). This can be achieved by selecting effluent test concentrations in the following manner: 1) 100% effluent, 2) [IWC+100] / 2, 3) IWC, 4) IWC/2, 5) IWC/4. For example, where the IWC=50%, appropriate effluent concentrations would be 100%, 75%, 50%, 25%, and 12.5%. For additional guidance on selecting appropriate dilution series, see Chapter 2.11 of the "Whole Effluent Toxicity (WET) Program Guidance Document".

4.12.4 The dilution series that is required for testing must be specified in the WPDES discharge permit. Permittees or laboratories may choose to add effluent concentrations to the WPDES permit-required dilution series, in order to collect additional information.

4.13 LOADING OF TEST ORGANISMS

4.13.1 A limit is placed on the loading (weight) of organisms per liter of test solution to minimize the depletion of dissolved oxygen, the accumulation of injurious concentrations of metabolic waste products, and/or stress induced by crowding, any of which could significantly affect the test results. However, the probability of exceeding loading limits is greatly reduced with the use of very young test organisms.

4.13.2 For static renewal tests, loading in the test solutions must not exceed the following live weights: 0.65 g/L at 20°C (acute) or 0.40 g/L at 25°C (chronic).

4.13.3 For flow-through tests, the live weight of organisms in the test chambers must not be > 2.5 g/L at 25°C.

4.14 FEEDING

4.14.1 Where indicated in the test summary tables (see Tables 4.1-4.4 & 4.7), food is made available to test organisms while holding before they are placed in the test chambers or before test renewal. Fish should be fed as much as they will eat at least once a day with live or frozen brine shrimp or dry food (frozen food should be completely thawed before use). Brine shrimp can be supplemented with commercially prepared flake food.

4.14.2 Problems caused by feeding, such as the possible alteration of the toxicant concentration, the buildup of food and metabolic wastes and resulting oxygen demand, are common in static test systems. Excess food and fecal material should be removed daily by aspirating with a pipette.

4.14.3 Feeding does not cause the above problems in flow-through systems. However, it is advisable to remove excess food, fecal material, and any particulate matter that settles from the effluent, from the bottom of the test vessels daily by aspirating with a pipette.

4.14.4 Organisms should be observed carefully each day for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed. It is not uncommon to have some fish mortality (5-10%) in a holding tank during the first 48 h because of individuals that refuse to eat artificial food. A daily record of feeding, behavioral observations, and mortality should be maintained.

4.15 EFFLUENT SAMPLE MANIPULATION

4.15.1 Manipulation of effluent samples (aeration, filtration, addition of chemicals, etc.) is not allowed in most situations and should be minimized or avoided whenever possible. No substance should be introduced into the sample unless absolutely necessary for a successful toxicity test. Any sample manipulation (aeration, filtration, addition of chemicals, etc.) that is determined to be necessary for successful completion of a toxicity test, must be noted on the "Whole Effluent Toxicity Test Report Form" (see Section 6) submitted for that test.

4.15.2 If sample manipulation is determined to be necessary, parallel tests of adjusted and unadjusted effluent must be included to demonstrate what, if any, affect the manipulation may have had on the test. Parallel tests must be similar in every way other than the adjustment being demonstrated (same dilutions and under the same test conditions). Controls must be conducted that show the adjustment itself has not caused toxicity. No more chemical should be introduced into the sample than is absolutely necessary for a successful toxicity test. The adjustment chemicals themselves might be toxic or enhance the toxicity of other substances.

4.15.3 Dechlorination: Effluent samples shall not be dechlorinated prior to toxicity testing, unless done as part of a parallel test of adjusted and unadjusted effluent. If there is reason to demonstrate that chlorine is the sole cause of toxicity (for example, if samples are taken after chlorination, but before dechlorination) this may be demonstrated by conducting parallel tests of adjusted and unadjusted effluent.

4.15.4 Filtration

4.15.4.1 No filtration of effluent samples used in fathead minnow and *Ceriodaphnia dubia* tests is allowed, unless samples are taken from lagoon systems or the necessity for filtration has been documented. (See Section 4.5.2 for requirements regarding filtration of receiving water samples). Justification for filtration of effluent samples should be based on the observation of organisms that may attack, be confused with test organisms, or otherwise interfere with the test. Most effluent samples taken from non-lagoon wastewater treatment systems do not contain indigenous organisms that would attack or be confused with test organisms. Unless the "Whole Effluent Toxicity Test Report Form" (see Section 6) contains good justification, tests on filtered samples will be rejected. If a lab can demonstrate that a particular effluent contains organisms that interfere with toxicity testing, then samples of that effluent may be filtered. Filter pore sizes should be no smaller than is necessary to remove the unwanted organisms. Pore diameters must never be > 60µm.

4.15.4.2 Effluent and receiving water samples used for *S. capricornutum* tests must be filtered through a filter providing 0.45µm particle size retention before they are used in toxicity tests (see Section 4.20.6.3).

4.15.5 Aeration: No aeration of effluent or receiving water samples is allowed prior to testing unless dissolved oxygen is < 4.0 mg/L. The manipulation of test solutions alone has been shown to often remove or dilute supersaturation sufficiently. Supersaturation of dissolved gases in a sample would justify aeration only after preparation of test concentrations and pouring of replicates have been shown to not remove or dilute excess gases adequately. Replicates for the highest effluent concentration should be prepared first so they can equilibrate while the rest of the dilution series is prepared and other replicates poured. If the manipulation of test solutions alone does not remove or dilute supersaturation sufficiently, and available information suggests that supersaturation may negatively affect test organisms, then the test chambers may be aerated. If this procedure often does not work for a given effluent, then future effluent samples from this discharge may be aerated prior to test setup.

4.15.6 pH Adjustment: If an effluent pH is outside of the range of 6.0 to 9.0 s.u. upon arrival at the lab, then

the permittee may be in violation of a WPDES permit limit for pH. The lab should immediately alert the permittee to a potential problem if this occurs. As stated in Tables 3.1 and 3.2, effluent samples are not acceptable for use in toxicity tests when pH values are outside the range of 6.0 to 9.0 s.u. Therefore, it may be necessary to restart tests or take alternative samples when pH is outside of this range. Labs are not allowed to add acids or bases to samples used in permit-required WET tests. Acids and bases might themselves be toxic or change the toxicity of other substances.

4.15.7 pH Control

4.15.7.1 During the conduct of static-renewal tests, the pH in test containers may fluctuate or drift from the initial pH value. This pH drift can be upwards or downwards, depending on test conditions and sample characteristics, but usually is an upward drift. For instance, the addition of food substances such as algae may cause a decrease in pH, while the loss of carbon dioxide (CO_2) from supersaturated effluent samples often causes an increase in pH.

4.15.7.2 While pH drift is relatively mild for some samples, many effluent samples routinely exhibit a greater degree of pH drift. For example, many municipal effluents are typically discharged at a pH of 7.2-7.4, but the pH may equilibrate in WET tests without pH control and stabilize at 8.0-8.5. This degree of pH drift will interfere with test results if the sample contains a compound with toxicity that is pH-dependent at a concentration that is near the toxicity threshold. Compounds with pH-dependent toxicity are those with chemical characteristics that allow sufficient differences in dissociation, solubility, or speciation to occur within a certain pH range. It is a common assumption and misconception that only ammonia is affected by pH drift in WET tests - other examples of pH-dependent toxicants include metals, hydrogen sulfide, cyanide, and ionizable organics.

4.15.7.3 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases, so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper have been shown to be more toxic at a lower pH, while nickel and zinc are more toxic at a higher pH. A change in pH during testing means that an effluent sample might be tested for toxicity at a pH different than what is actually present at the point of discharge. Under certain circumstances, this pH drift could influence sample toxicity and be considered a test interference. For these reasons, pH control measures are required in all acute tests and recommended in most chronic tests completed for WPDES permit compliance, as specified below in 4.15.7.5.

4.15.7.4 Artifactual toxicity caused by a shift in pH during testing can be reduced or eliminated by exposing the test chambers to a CO_2 controlled atmosphere. An alternate approach would be to conduct onsite flow-through toxicity tests with a turnover rate in the test chamber which maintains the pH of the test solution to that of the effluent.

4.15.7.5 All static-renewal acute tests must be conducted in a CO_2 atmosphere (a 2.5% mixture, or an equivalent mixture shown to work successfully in the lab) or under flow-through conditions that maintain the pH at a level no lower than the measured effluent pH at the time of discharge. Static and static-renewal chronic tests are not required to be conducted in a CO_2 atmosphere or under flow-through conditions, but if pH control measures are used, the pH shall be maintained at a level no lower than the receiving water pH. Test chambers should be monitored closely to insure that pH levels do not fall below these thresholds. (Warning: samples with alkalinity < 50 mg/L may experience significant decreases in pH when CO_2 is used. Labs should be aware of this potential problem and take care to prevent significant drops in pH.) Tests may be completed without pH control, with Department approval, if it can be demonstrated that pH drops of > 1.0 s.u. have occurred in previous tests with the effluent when using CO2 entrapment methods.

4.15.7.6 Advantages of using CO_2 include less alteration of normal test solution chemistry and use of a natural buffer system to achieve ongoing pH control. For guidance on the use of these methods, personnel

may consult Mount et al. (1992) which gives several methodological variations, along with example applications of the technique. Additional guidance is also given in Section 4.15.8 below and in Chapter 2.8 of the "Whole Effluent Toxicity (WET) Program Guidance Document", available from the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921; or at http://dnr.wi.gov/topic/wastewater/WET.html.

4.15.8 Recommended CO_2 Procedure

4.15.8.1 Tests conducted in a pH controlling CO_2 environment should be contained in an air tight Plexiglas® chamber. The chamber should be large enough to hold all containers from a single test. The top of the chamber should be lined with an air tight gasket such as window and door insulating foam strips. Any breaks between insulation strips should be sealed with silicone caulk. A framed piece of safety glass slightly larger than the Plexiglas chamber should be used as a cover. Vacuum grease between the glass cover and the gasket should be used to provide an airtight seal.

4.15.8.2 A gas cylinder containing carbon dioxide and air (2.5% CO_2, balanced air, is recommended) should be used to purge the atmosphere of the Plexiglas chamber. Tubing connected to PVC spigots or valves inserted into opposite ends of the chamber should be used to allow the CO_2 mixture to enter the chamber. The chamber should be purged for 30 seconds at 10 psi (this should be adequate for controlling the pH drift of most environmental samples). The test chamber should be purged with CO_2 every 24 hours and whenever the glass cover is removed.

4.16 LIGHT, PHOTOPERIOD, AND TEMPERATURE

4.16.1 For fathead minnow and *Ceriodaphnia dubia* tests, the light quality and intensity should be at ambient laboratory levels, approximately 10-20 uE/m2/s, or 50 to 100 foot candles (ft-c), with a photoperiod of 16-h of light and 8-h of darkness. For *Selenastrum capricornutum* tests, test chambers must be incubated under continuous illumination at 400±40 ft-c or 4,300 ± 430 lux, at 25 ± 1°C

4.16.2 Light intensity and photoperiod shall be maintained as specified in Tables 4.1-4.4 & 4.7. Ambient lab light measurements shall be made and recorded on a yearly basis. Photoperiod shall be documented at least quarterly. For algal tests, the light intensity in testing areas shall be measured by placing a light meter at the level of test chambers and recorded at the start of each test.

4.16.3 Test temperature will depend on test type (see Tables 4.1-4.4 & 4.7). Maintenance of temperature can be accomplished in static and static renewal tests by use of a water bath or environmental chamber, and in flow-through tests by passing the effluent and/or dilution water through separate coils immersed in a heating or cooling water bath prior to entering the dilutor system.

4.17 STRESS

4.17.1 Minimize stress on organisms by avoiding unnecessary disturbances. They are delicate and should be handled as carefully and as little as possible so that they are not unnecessarily stressed. Daphnids should be transferred with a pipette that is approximately 1 1/2 the size of the organism, taking care to release them under the surface of the water. Any organisms that are injured or dropped during handling must be discarded.

4.18 DISSOLVED OXYGEN CONCENTRATION

4.18.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required dissolved oxygen (DO) (see Section 4.14.5 for requirements regarding aeration prior to testing). Aeration can reduce the apparent toxicity of the test solutions by stripping them of volatile toxic substances, or change its toxicity by altering the pH. The DO in test solutions should not be allowed to fall below 4.0 mg/L.

4.18.2 In static tests, low DOs may occur in higher concentrations of wastewater. Aeration may be accomplished by bubbling air through a pipette at the rate of ≤ 100 bubbles/min. If aeration is necessary, all test solutions (effluent and controls) must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8-h after the test is started. Unless aeration is initiated during the first 8-h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

4.18.3 In most flow-through tests, DO depletion is not a problem in the test chambers because aeration occurs as the liquids pass through the dilutor system. If the DO decreases to a level that would be a source of additional stress, the turnover rate of the solutions in the test chambers must be increased sufficiently to maintain acceptable DO levels. If the increased turnover rate does not maintain adequate DO levels, aerate the dilution water prior to the addition of the effluent, and aerate all test solutions. To reduce the potential for driving off volatile compounds in the wastewater, aeration may be accomplished by bubbling air through a 1-ml pipette at a rate of no more than 100 bubbles/min, using an air valve to control the flow.

4.18.4 Caution must be exercised to avoid excessive aeration. Turbulence caused by aeration should not result in a physical stress to the test organisms. When aeration is used, the methodology must be detailed on the "Whole Effluent Toxicity Test Report Form" (see Section 6). For safety reasons, pure oxygen should not be used to aerate test solutions.

4.19 ROUTINE CHEMICAL AND PHYSICAL DETERMINATIONS

4.19.1 Chemical and physical data should be measured according to methods listed in s. NR 219.04, Wis. Adm. Code. Refer to section 3.11 of this manual, Quality Control Requirements, for the specific quality control measures required for each chemical test performed by the laboratory . At a minimum, the following measurements shall be made:

4.19.1.1 DO and pH shall be measured at the beginning and end of each 24-h exposure period in the test concentrations and in the control.

4.19.1.2 Temperature shall be monitored either continuously or observed and recorded twice daily (a.m. and p.m.) for at least two locations in the environmental control system.

4.19.1.3 Conductivity, alkalinity, and hardness shall be measured in each new sample and in the controls. Conductivity is recommended to be measured daily in all test concentrations. It is a useful parameter to identify if toxicity from total dissolved solids (TDS) is an issue for *C. dubia* and it is an excellent check on whether the test dilutions were prepared and labeled correctly.

4.20 STATIC RENEWAL TEST CONDITIONS FOR ACUTE TESTS

4.20.1 The following tables summarize methods to be used to measure the acute toxicity of effluents to *Ceriodaphnia dubia* during a 48-h static renewal exposure and to the fathead minnow (*Pimephales promelas*) in a 96-h static renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms. The State of Wisconsin regulates acute toxicity at end of pipe conditions in most situations.

4.20.2 Requirements for static renewal tests using *Ceriodaphnia dubia* and fathead minnows (*Pimephales promelas*) are provided in Tables 4.1 and 4.2 , respectively.

TABLE 4.1 ACUTE TEST CONDITIONS FOR *C. DUBIA* STATIC RENEWAL TESTS	
1. Test type:	Static renewal

TABLE 4.1 ACUTE TEST CONDITIONS FOR *C. DUBIA* STATIC RENEWAL TESTS	
2. Test duration:	48-h
3. Temperature (°C):	20 ± 1°C, temperatures must not deviate (i.e., maximum minus minimum) by more than 3°C during the test
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	50-100 ft-c (540-1075 lux)
6. Photoperiod:	16-h light, 8-h darkness
7. Test chamber size:	30 ml
8. Test solution volume:	15 ml minimum
9. Renewal of test concentrations:	Daily
10. Age of test organisms:	< 24-h old
11. No. organisms per test chamber:	Minimum 5
12. No. replicate chambers per concentration:	Minimum 4
13. No. organisms per concentration:	Minimum 20
14. Feeding regime:	Feed only while holding prior to test; newly released young should have food available a minimum of 2-h prior to use in the test; additives are allowed in food
15. Cleaning:	Cleaning not required
16. Aeration:	None, unless DO < 40% saturation. Rate should not exceed 100 bubbles/min
17. Dilution water:	Receiving water or synthetic water (see Section 4.4)
18. Dilution series:	5 effluent concentrations and dual controls (minimum);100, 50, 25, 12.5, 6.25% + any selected by permittee; alternate series may be selected at permit reissuance (see 4.12)
19. Test acceptability:	Survival ≥ 90% in controls
20. Sampling requirement:	Specified in WPDES permit and Section 2

TABLE 4.2 ACUTE TEST CONDITIONS FOR FATHEAD MINNOW (*PIMEPHALES PROMELAS*) STATIC RENEWAL TESTS	
1. Test type:	Static renewal
2. Test duration:	96-h
3. Temperature (°C):	20 ± 1°C, temperatures must not deviate (i.e., maximum minus minimum) by more than 3°C during the test
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	50-100 ft-c (540-1075 lux)
6. Photoperiod:	16-h light, 8-h darkness
7. Test chamber size:	250 ml
8. Test solution volume:	200 ml minimum
9. Renewal of test concentrations:	Daily
10. Age of test organisms:	~~1 – 14~~ 4-14 days; ≤ 24-h range in age required
11. No. organisms per test chamber:	Minimum 10
12. No. replicate chambers per concentration:	Minimum 4
13. No. organisms per concentration:	Minimum 40
14. Feeding regime:	Must feed while holding prior to test or add 0.2 ml *Artemia* nauplii concentrate 2-h prior to renewal at 48-h; additives are allowed only to food added prior to test
15. Cleaning:	Cleaning not required
16. Aeration:	None, unless DO < 40% saturation. Rate should not exceed 100 bubbles/min
17. Dilution water:	Receiving water or synthetic water (see Section 4.4)
18. Dilution series:	5 effluent concentrations and dual controls (minimum); 100, 50, 25, 12.5, 6.25% + any selected by permittee; alternate series may be selected at permit reissuance (see 4.12)
19. Test acceptability:	Survival ≥ 90% in controls
20. Sampling requirement:	Specified in WPDES permit and Section 2

4.21 STATIC-RENEWAL TEST CONDITIONS FOR CHRONIC TESTS

4.21.1 The following methods measure the sub-lethal effects of effluents and receiving water to the cladoceran, *Ceriodaphnia dubia*, during a three-brood, static renewal exposure (Section 4.21.3), fathead minnow (*Pimephales promelas*) larvae, in a seven-day, static renewal exposure (Section 4.21.4), and the freshwater green alga, *Selenastrum capricornutum*, in a four-day static exposure (Section 4.21.5). These tests are intended to measure the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

4.21.2 In fathead minnow and *Ceriodaphnia dubia* tests, test solutions are renewed daily, using the most recently collected sample. Most fathead minnow and *Ceriodaphnia dubia* chronic tests will include a suite of three samples, with two of the samples used for two days and one sample used for three days (see Section 2.2.2). Samples may be used for a maximum of three consecutive days. After samples are first used, they must be held over in the dark at $\leq$ 4°C (without freezing) for use on the following day(s). *Selenastrum capricornutum* static tests require only one sample and may be completed with any of the samples collected for the fathead minnow and *Ceriodaphnia dubia* portion. Any tests started and not completed must be reported to the Department.

4.21.3 *CERIODAPHNIA DUBIA* CHRONIC STATIC RENEWAL TEST PROCEDURES

4.21.3.1 SUMMARY OF METHOD

4.21.3.1.1 *Ceriodaphnia dubia* are exposed in a static renewal system to concentrations of effluent and receiving water, until $\geq$80% of surviving females in the primary control have 3 broods of offspring or 8 days, whichever comes first. Test results are based on survival and reproduction. If the test is conducted as described, 80% or more of surviving females should have 3 broods, and a total of 15 or more young/female. For guidance on *C. dubia* culturing methods, refer to "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (USEPA, 2002b).

4.21.3.2 START OF THE TEST

4.21.3.2.1 Neonates less than 24-h old, released within the same 8-h period, are required to begin the test. The neonates must be obtained from individual cultures using brood boards. Neonates are taken only from females that have eight or more young in their third or subsequent broods. These females can be used as brood stock until they are 14 days old. If the neonates are held more than one or two hours before using in the test, they should be fed (0.1 ml YCT and 0.1 ml algal concentrate is recommended).

4.21.3.2.2 The test is started with new disposable polystyrene cups or clean 30-mL borosilicate glass beakers that are labeled and color-coded with tape. Each following day, a new set of plastic cups or clean glass beakers is prepared, labeled, and color-coded with tape similar to the original set. New solutions are placed in the new set of test chambers, and the test organisms are transferred from the original test chambers to the new ones with corresponding labels and color-codes. Each day, previously used glass beakers are cleaned for the following day, and previously used plastic cups are discarded.

4.21.3.2.3 Neonates from mass cultures are not to be used directly in toxicity tests. Individual cultures are used as the immediate source of neonates for toxicity tests.

4.21.3.2.4 To facilitate identification and permit future reference, labs should maintain a record of the animal(s) used to start *C. dubia* cultures (an individual which has been sacrificed after producing young, mounted on a microscope slide, and retained as a permanent slide mount). Species identification of the stock culture must be verified annually by preparing slide mounts (for guidance on how to do this, refer to section 13.6.16.4 of "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms", USEPA, 2002b).

4.21.3.2.5 Test solutions must be block randomized using a template or a table of random numbers. When using the randomized block design, chambers are randomized once, at the beginning of the test. A number of different templates should be prepared, so that the same template is not used for every test.

4.21.3.2.6 Ten brood cups, each with 8 or more young, are randomly selected from a brood board for use in setting up a test. To start the test, neonates from these ten brood cups are distributed to each test chamber on the test board (one per test chamber). Test organisms must be assigned to test chambers using a block randomization procedure, such that offspring from a single female are distributed evenly among the treatments, appearing once in every test concentration. This arrangement is referred to as "blocking by known parentage". One effective technique is to block randomize the test board and transfer one neonate from the first brood cup to each of the six test chambers in the first row on the test board. One neonate from the second brood cup is then transferred to each of the six test chambers in the second row on the test board. This process is continued until each of the 70 test chambers contains one neonate. The set of seven test chambers (one for each test treatment and primary control) containing organisms derived from a single female parent is referred to as a block. When using this technique, each row of the test board will represent a block.

4.21.3.2.7 This blocking procedure allows the performance of each female test organism to be tracked to its parent culture organism. This technique ensures that any brood effects (i.e., differences in test organism fecundity or sensitivity attributable to the source of parentage) are evenly distributed among the test treatments. Also, by knowing the parentage of each test organism, blocks consisting largely of males can be omitted from all test treatments at the end of the test (see section 5.7.1.3.), decreasing variability among replicates.

4.21.3.2.8 In general, the occurrence of males in healthy, well-maintained individual cultures is rare. In interlaboratory testing of *Ceriodaphnia dubia*, males were identified in only 7% (9 of 126 tests) of tests conducted (USEPA, 2001a). The number of males identified in these tests ranged from 1 to 12. In five tests containing a large number of males (4-12), laboratories conducting those tests also noted that organism cultures were experiencing or recovering from some stress. Since male production in cladoceran populations is generally associated with conditions of environmental stress, culture conditions should be examined whenever males are identified in a test.

4.21.3.3 DISSOLVED OXYGEN (DO)

4.21.3.3.1 Low DO concentrations may affect test results when performing effluent toxicity tests. Although aeration is not practical for the chronic *C. dubia* test, it may be necessary in some instances. If the DO in the effluent and/or dilution water is low (< 4.0 mg/L), aerate before preparing the test solutions. If aeration of the test vessels is necessary, all treatments and controls must be aerated equally. (See Sections 4.14.4 & 4.17)

4.21.3.4 FEEDING

4.21.3.4.1 The organisms must be fed when the test is initiated, and daily thereafter. Food should be added to the fresh medium immediately before or immediately after the adults are transferred. Recommended feeding rates are 0.1 ml YCT/15 ml test solution and 0.1 ml *S. capricornutum* concentrate/15 ml test solution (0.1 ml of algal concentrate containing 3.0-3.5 X 10^7 cells/ml will provide 2-2.3 X 10^5 cells/ml in the test chamber).

4.21.3.5 OBSERVATIONS DURING THE TEST

4.21.3.5.1 Tests shall be terminated when 80% or more of the surviving females in the primary control have produced their third brood, or at the end of 8 days (whichever comes first). Three broods are usually

obtained in the controls within seven-days with tests conducted at 25 ± 1°C. A brood is a group of offspring released from the female over a short period of time when the carapace is discarded during molting. The number of young in each brood should increase over the period of the test. Animals may be transferred to fresh test solution before completing the release of a brood, resulting in split broods. Care is needed when interpreting the results to determine the number of broods released during the test. In this three brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

4.21.3.5.2 Each day, live adults shall be transferred to fresh test solutions in new plastic or properly cleaned glass test chambers, and the numbers of live young recorded. The young are discarded after counting.

4.21.3.5.3 Some of the effects caused by toxic substances may include: 1) a reduction in the number of young produced, 2) young developed in the brood pouch of the adults, but not released during the exposure period, and 3) partially or fully developed young released, but all dead at the end of the 24-h period. Such effects must be noted on data sheets and on the "Whole Effluent Toxicity Test Report Form" (see Section 6) submitted to the Department.

4.21.3.6 TERMINATION OF THE TEST

4.21.3.6.1Tests shall be terminated when 80% or more of the surviving *C. dubia* in the primary control have produced their third brood or at the end of 8 days, whichever comes first. Once this test endpoint has been reached, only neonates produced in the first three broods shall be counted. Because of the rapid rate of development of *C. dubia*, at test termination all observations on organism survival and numbers of offspring must be completed within two hours. An extension of more than a few hours in the test period would be a significant part of the brood production cycle of the animals, and could result in additional broods. A test is considered unacceptable and must be repeated when less than 80% of the surviving *C. dubia* in the controls fail to produce 3 broods in 8 days (see 3.9.3).

4.21.3.6.2 Any animal not producing young should be examined to determine if it is a male (Berner, 1986). In some cases, the animal may need to be placed on a microscope slide before examining (for guidance on how to do this, refer to section 13.6.16.4 of "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms", USEPA, 2002b). At the end of the test, if 50% or more of the surviving organisms in a block are identified as males, the entire block must be excluded from data analysis for the reproductive endpoint. For blocks having fewer than 50% of surviving organisms identified as males, the males (not the entire block) must be excluded from the analysis of reproduction. Determinations regarding test acceptability criteria for survival and reproduction must be made prior to the exclusion of any blocks. In addition to these test acceptability criteria, if fewer than eight replicates in the control remain after excluding males and blocks with 50% or more of surviving organisms identified as males, the test is invalid and must be repeated with a newly collected sample within 30 days of the original test's end.

4.21.3.6.3 Although developmental and behavioral effects are often difficult to quantify and may not provide suitable endpoints, they might be useful for interpreting effects on survival and reproduction. Daphnids should be carefully observed during the test for abnormal behavior, such as erratic swimming. Morphological examination of organisms alive at the end of the test might be useful as well. WET Test Report Forms should include documentation of any abnormal appearance or behavior.

TABLE 4.3 CHRONIC TEST CONDITIONS FOR *C. DUBIA* STATIC RENEWAL TESTS

TABLE 4.3 CHRONIC TEST CONDITIONS FOR *C. DUBIA* STATIC RENEWAL TESTS	
1. Test type:	Static renewal
2. Test duration:	Until ≥80% C. dubia in one control have produced 3rd brood or at the end of 8 days, whichever comes first
3. Temperature (°C):	25 ± 1°C; temperatures must not deviate (i.e., maximum minus minimum) by more than 3°C during the test
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	50-100 ft-c (540-1075 lux)
6. Photoperiod:	16-h light, 8-h darkness
7. Test chamber size:	30 ml maximum
8. Test solution volume:	15 ml minimum/replicate
9. Renewal of test concentrations:	Daily
10. Age of test organisms:	< 24-h old; released within same 8-h period
11. No. organisms per test chamber:	1
12. No. replicate chambers per concentration:	Minimum 10
13. No. organisms per concentration:	Minimum 10
14. Feeding regime:	Feed only 0.1 ml each of YCT and algal suspension per test chamber daily
15. Cleaning:	Daily, when chambers are renewed
16. Aeration:	None. If DO is low in sample, sample ~~should~~ may be aerated prior to renewal (see Section 4.20.3.3)
17. Dilution water:	Receiving water or synthetic water (see Section 4.4)
18. Dilution series:	5 effluent concentrations and dual controls (minimum); If IWC ≤30%, then 100, 30, 10, 3, 1%; if IWC >30%, then 100, 75, 50, 25, 12.5%; any additional or alternate dilutions must be specified in the WPDES permit (see 4.1.2)
19. Test acceptability:	Both controls must contain ≤20% males & there must be ≤20% males over all concentrations; one control must have ≥80% survival, CV between replicates ≤40%, ≥80% surviving females w/3rd brood, and mean young/surviving female ≥15
20. Sampling requirement:	Specified in WPDES permit and Section 2

4.21.4 FATHEAD MINNOW (*PIMEPHALES PROMELAS*) SUB-CHRONIC PROCEDURES

4.21.4.1 SUMMARY OF METHOD

4.21.4.1.1 This method estimates chronic toxicity to the fathead minnow, using newly hatched larvae in a seven-day, static renewal test. Larvae <24 hours old are exposed to different concentrations of effluent and receiving water and results are based on survival and growth. For guidance on fathead minnow culturing methods, refer to "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (USEPA, 2002b).

4.21.4.2 START OF THE TEST

4.21.4.2.1 The test is started with new disposable polystyrene cups or clean 30-mL borosilicate glass beakers that are labeled and color-coded with tape. Each following day, a new set of plastic cups or clean glass beakers is prepared, labeled, and color-coded with tape similar to the original set. New solutions are placed in the new set of test chambers, and the test organisms are transferred from the original test chambers to the new ones with corresponding labels and color-codes. Each day, previously used glass beakers are cleaned for the following day, and previously used plastic cups are discarded.

4.21.4.2.2 Test solutions must be block randomized using a template or a table of random numbers. When using the randomized block design, test chambers are randomized only once, at the beginning of the test. A number of different templates should be prepared, so that the same template is not used for every test. The larvae should then be pooled and placed into each 30 mL test chamber until each chamber contains 2 larvae for a total of 20 larvae for each concentration. The test organisms should come from a pool of larvae consisting of at least three separate spawnings. The amount of water added to the

chambers when transferring the larvae to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

4.21.4.3 FEEDING

4.21.4.3.1 The fish in each test chamber should be fed a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii two times daily at 6-h intervals or three times daily at 4-h intervals. The nauplii should be rinsed with freshwater before use. The amount of food provided should be sufficient to ensure the presence of a small amount of uneaten food at the next feeding. Larvae are not fed during the final 12-h of the test.

4.21.4.4 DAILY RENEWAL

4.21.4.4.1 At the time of daily renewal the fish are transferred to a new test chamber containing a fresh test solution using a plastic Pasteur pipette, which has been trimmed at the end to create a 5mm bore diameter. Water transfer is kept to a minimum by allowing the fish to swim out of the pipette into the new test chamber. Injuries to individual fish should be noted on the test sheets.

4.21.4.5 OBSERVATIONS DURING THE TEST

4.21.4.5.1 The number of live and dead larvae in each chamber are recorded daily and the dead discarded.

4.21.4.5.2 To protect the larvae from unnecessary disturbance during the test, daily test observations and fish transfer should be done carefully. The larvae should remain immersed during the performance of the above operations.

4.21.4.5.3 Although developmental and behavioral effects are often difficult to quantify and may not provide suitable endpoints, they might be useful for interpreting effects on survival and growth. Fish should be carefully observed during the test for abnormal behavior, such as uncoordinated swimming. Morphological examination of organisms alive at the end of the test might be useful as well. WET Test Report Forms should include documentation of any abnormal appearance or behavior.

4.21.4.6 TERMINATION OF THE TEST

4.21.4.6.1 The test shall be terminated after 7 days of exposure. At termination, the surviving larvae in each chamber should be counted and recorded. For dry weight analysis, replicates are combined in pairs using a random number table, resulting in 5 replicates for weight analysis. Immediately prior to the dry weight analysis, each group should be anaesthetized and dipped in distilled water to remove food particles. Anaesthetized fish are then transferred to a tared weighing boat, and dried at 60°C for 24-h or 100°C for a minimum of 6 hours. Immediately upon removal from the drying oven, the weighing boats must be placed in a desiccator until weighed, to prevent the absorption of moisture from the air. All weights should be measured to the nearest 0.01 mg. Subtract tare weight to determine the dry weight of the larvae in each replicate. For each test chamber, divide the final dry weight by the number of original larvae in the test chamber to determine the average individual dry weight and record on the data sheet. For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria. Average weights should be expressed to the nearest 0.001 mg. If the larvae are preserved, they must be dried and weighed within 2 weeks. Preservation is not recommended, but if necessary, must be achieved by freezing.

TABLE 4.4 CHRONIC TEST CONDITIONS FOR P. PROMELAS STATIC RENEWAL TESTS	
1. Test type:	Static renewal
2. Test duration:	7 days
3. Temperature (°C):	25 ± 1°C; temperatures must not deviate (i.e., maximum minus minimum) by more than 3°C during the test
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	50-100 ft-c (540-1075 lux)
6. Photoperiod:	16-h light, 8-h darkness
7. Test chamber size:	30 ml
8. Test solution volume:	20 ml/replicate
9. Renewal of test concentrations:	Daily
10. Age of test organisms:	< 24-h old
11. No. organisms per test chamber:	2
12. No. replicate chambers per concentration:	Minimum 10
13. No. organisms per concentration:	Minimum 20
14. Feeding regime:	Feed 0.1 ml < 24-h brine shrimp nauplii 3x daily at 4-h intervals or 0.15 ml 2x daily, at 6-h intervals. Larvae are not fed during the final 12-h of the test; No additives allowed to food
15. Cleaning:	Transfer fish to new chambers daily.
16. Aeration:	None, unless DO ≤ 40% saturation. Rate should not exceed 100 bubbles/min.
17. Dilution water:	Receiving water or synthetic water (see Section 4.3)
18. Dilution series:	5 effluent concentrations and dual controls (minimum); If IWC ≤30%, then 100, 30, 10, 3, 1%; if IWC >30%, then 100, 75, 50, 25, 12.5%; any additional or alternate dilutions must be specified in the WPDES permit (see 4.1.2)
19. Test acceptability:	One control must have Survival ≥ 80% ,CV between replicates ≤ 40%, and avg. dry weight ≥ 0.25 mg
20. Sampling requirement:	Specified in WPDES permit and Section 2

4.21.5 *SELENASTRUM CAPRICORNUTUM* STATIC TEST PROCEDURES

4.21.5.1 SUMMARY OF METHOD

4.21.5.1.1 *Selenastrum capricornutum* cells are continuously exposed for 4 days to different concentrations of effluent and receiving water. Chlorophyll content or cell number at 96 hours is used to estimate an inhibition concentration where there is a significant reduction in growth in effluent concentrations when compared to a control (See Section 5.5.).

4.21.5.1.2 Tests may be conducted either in 125 ml or 250 ml Erlenmeyer flasks or microplates (24 or 48-wells/plate, 3 ml wells). However, microplate algal assays offer many advantages over Erlenmeyer flask tests in reducing laboratory resources (especially space and time requirements) and have been shown to dramatically improve test performance (Geis, et al, 2000). Therefore, the Department strongly encourages the use of the 24-well microplate method rather than the Erlenmeyer flask method.

4.21.5.2 DILUTION WATER AND EFFLUENT SAMPLE PREPARATION

4.21.5.2.1 In most cases, the dilution water will be the receiving water, but at times it may also be a standard synthetic (reconstituted) water, stock culture medium, or some other natural water (see Section 4.4, Dilution Water and Controls). If water other than the stock culture medium is used for dilution water, 1 mL of each stock nutrient solution should be added per liter of dilution water. Effluent and dilution water should be warmed to test temperature. To remove indigenous algae, effluent and dilution water must be filtered through a GF/A or GF/C filter, or other filter providing 0.45μm particle size retention. Glass-fiber filters generally provide more rapid filtering rates and greater filtrate volume before plugging.

4.21.5.3 TEST SOLUTIONS

4.21.5.3.1 Effluents may be toxic and/or nutrient poor. Lowered growth in an algal toxicity test, therefore, may be due to toxicity or nutrient limitation, or both. To eliminate false positive results due to low nutrient concentrations (i.e., effluent that is "too clean"), 1 ml of each stock nutrient is added per liter of effluent prior to use in preparing the test dilutions. Thus, all test treatments and controls will contain at a minimum the concentration of nutrients required for adequate growth of algae.

TABLE 4.5 NUTRIENT STOCK SOLUTIONS FOR PREPARING TEST MEDIA

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 ml TYPE 1 WATER
1. MACRONUTRIENTS	$MgCl_2$•6 H_20	6.08 g
	$CaCl_2$•2H_2O	2.20 g
	$NaNO_3$	12.75 g
	$MgSO_4$•7H_2O	7.35 g
	K_2HPO_4	0.522 g
	$NaHCO_3$	7.50 g
2. MICRONUTRIENTS	H_3BO_3	92.8 mg
	$MnCl_2$•4H_2O	208 mg
	$ZnCl_2$	1.64 mg[1]
	$FeCl_3$•6H_2O	79.9 mg
	$CoCl_2$•6H_2O	0.714 mg[2]
	Na_2MoO_4•2H_2O	3.63 mg[3]
	$CuCl_2$•2H_2O	0.006 mg[4]
	Na_2EDTA•2H_2O	150.0 mg
	Na_2SeO_4	1.196 mg[5]

1 $ZnCl_2$ - weigh out 164 mg and dilute to 100 ml. Add 1 ml of this solution to Stock 2, micronutrients.
2 $CoCl_2$•6H_2O - weigh out 71.4 mg and dilute to 100 ml. Add 1 ml of this solution to Stock 2, micronutrients.
3 Na_2MoO_4•2H_2O - weigh out 36.6 mg and dilute to 10 ml. Add 1 ml of this solution to Stock 2, micronutrients.
4 $CuCl_2$•2H_2O - weigh out 60.0 mg and dilute to 1000 ml. Take 1 ml of this solution and dilute to 10 ml. Take 1 ml of the second dilution and add to Stock 2, micronutrients.
5 Na_2SeO_4 - weigh out 119.6 mg and dilute to 100 ml. Add 1 ml of this solution to Stock 2, micronutrients.

TABLE 4.6 CONCENTRATION OF MACRONUTRIENTS & MICRONUTRIENTS IN TEST MEDIA

MACRONUTRIENTS	CONCENTRATION	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2$•6 H_20	12.2	Mg	2.90
$CaCl_2$•2H_2O	4.41	Ca	1.20
$MgSO_4$•7H_2O	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14
MICRONUTRIENTS	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.0	B	32.5
$MnCl_2$•4H_2O	416.0	Mn	115.0
$ZnCl_2$	3.27	Zn	1.57
~~$FeCl_3$•6H_2O~~ $CoCl_2$•6H_2O	1.43	Co	0.354
~~$CoCl_2$•6H_2O~~ $CuCl_2$•2H_2O	0.012	Cu	0.004
Na_2MoO_4•2H_2O	7.26	Mo	2.88
~~$CuCl_2$•2H_2O~~ $FeCl_3$•6H_2O	160.0	Fe	33.1
Na_2EDTA•2H_2O	300.0	--	---
Na_2SeO_4	2.39	Se	0.91

4.21.5.3.2 When prepared as described above, the micronutrient stock solution contains ethylenediaminetetraacetic acid (EDTA). This nutrient stock formulation containing EDTA is required for culturing and testing with *Selenastrum capricornutu*m. The use of EDTA improves test method performance by reducing the incidence of false positives and increasing test method precision. In interlaboratory testing of split samples analyzed with and without the addition of EDTA, false positive rates were 0.00% with EDTA and 33.3% without EDTA (USEPA, 2001a). Interlaboratory variability, expressed as the CV for IC_{25} values, was 34.3% with EDTA and 58.5% without EDTA (USEPA, 2001a).

4.21.5.4 PREPARATION OF INOCULUM

4.21.5.4.1 The inoculum is prepared no more than 2 to 3-h prior to the beginning of the test, using *Selenastrum capricornutum* harvested from a four- to-seven-day old stock culture. Each milliliter of inoculum must contain enough cells to provide an initial cell density of approximately 10,000 cells/ml (± 10%) in the test chambers.

4.21.5.4.2 Estimate the volume of stock culture required to prepare the inoculum. As an example, if the four-to-seven-day-old stock culture used as the source of the inoculum has a cell density of 2,000,000 cells/ml, a test employing 24 flasks, each containing 100 ml of test medium and inoculated with a total of 1,000,000 cells, would require 24,000,000 cells or 15 ml of stock solution (24,000,000/2,000,000) to provide sufficient inoculum. It is advisable to prepare a volume 20% to 50% in excess of the minimum volume required, to cover accidental loss in transfer and handling.

4.21.5.4.3 Prepare the inoculum as follows:

1. Determine the density of cells (cells/ml) in the stock culture (for this example, assume 2,000,000 per ml). Calculate the required volume of stock culture as follows:

$$\text{Volume (ml) of Stock Culture Required} = \frac{\text{\#chambers used x Volume of solutions/chamber x 10,000 cells/ml}}{\text{Cell density (cells/ml) in the stock culture}}$$

$$= \frac{\text{24 chambers x 100 ml/chamber x 10,000 cells/ml}}{\text{2,000,000 cells/ml}}$$

$$= \text{12.0 ml Stock Culture}$$

2. Dilute the cell concentrate as needed to obtain a cell density of 1,000,000 cells/ml, and check the cell density in the final inoculum, if necessary.
3. The volume of the algal inoculum should be considered in calculating the dilution of toxicant in the test flasks.

4.21.5.5 START OF THE TEST

4.21.5.5.1 A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) must have four replicates. Tests may be conducted in Erlenmeyer flasks or 24 or 48-well Falcon® microplates (or equivalent approved by the Department). However, it is strongly recommended by the Department that the tests be conducted using the 24-well microplates, due to advantages including reduction of lab resources (space, time, and cost) and improved test performance (reduced variability), as described in Geis, et al, 2000.

4.21.5.5.2 The location of each test chamber must be randomized prior to algal inoculation. Mix the inoculum well, and add required amount of test solution (50 ml if flasks are used, 2 ml or 1 ml if microplate wells are used) to each randomly arranged test chamber. "Blanks" (i.e., samples without inoculum) of all effluent concentrations and controls should be prepared to adjust fluorometer and spectrophotometer readings when taking final measurements.

4.21.5.5.3 The test begins when the algae are added to the test chambers. Add the required amount of inoculum to each chamber (25 µL if using microplates, 0.5 mL if using flasks).

4.21.5.5.4 If microplates are used, plates must be covered and placed in a sealed, clear plastic baggie in order to minimize evaporation.

4.21.5.6 LIGHT, PHOTOPERIOD, AND TEMPERATURE

4.21.5.6.1 Test chambers must be incubated under continuous illumination at 400±40 ft-c (4,300 ± 430 lux), at 25 ± 1°C, and must be shaken continuously at 100 cpm on a mechanical shaker. Flask positions must be randomly rotated and microplates rotated 90º each day to minimize possible spatial differences in illumination and temperature on growth rate. Light intensity in testing areas shall be measured by placing a light meter at the level of test chambers and recorded at the start of each test (see Section 4.16.2).

4.21.5.7 DISSOLVED OXYGEN (DO) CONCENTRATION

4.21.5.7.1 Because of the continuous illumination of the test chambers, DO concentration should never be a problem during the test and no aeration is allowed.

4.21.5.8 TEMPERATURE

4.21.5.8.1 Temperature must be measured at the end of each 24-h exposure period in at least one test blank well or flask. At the end of the test, temperatures must be taken in at least one solution at each concentration.

4.21.5.9 TERMINATION OF THE TEST

4.21.5.9.1 The test is terminated 96-h after initiation. Test solutions may be stored at ≤ 4°C (in the dark; without freezing) for later measurement. Algal growth in each flask is then measured by one of the following methods: 1) Automatic particle counter or manual cell count, 2) fluorometrically (chlorophyll content), or 3) spectrophotometrically (light absorbance).

4.21.5.9.1.1 Cell counts

4.21.5.9.1.2 Automatic Particle Counters

4.21.5.9.1.2.1 Several types of automatic electronic and optical particle counters are available for use in the rapid determination of cell density (cells/ml) and mean cell volume (MCV) in mm^3/cell.

4.21.5.9.1.2.2 If biomass data are desired for algal growth potential measurements, a Model ZM Coulter Counter is used. However, it must be calibrated with a reference sample of particles of known volume.

4.21.5.9.1.2.3 When the Coulter Counter is used, an aliquot (usually 1 ml) of the test culture is diluted 10X to 20X with a 1% sodium chloride electrolyte solution, such as ISOTON®, to facilitate counting. The resulting dilution is counted using an aperture tube with a 100-mm diameter aperture. Each cell (particle) passing through the aperture causes a voltage drop proportional to its volume. Depending on the model, the instrument stores the information on the number of particles and the volume of each, and calculates the mean cell volume. The following procedure is used:

1. Mix the algal culture in the flask thoroughly by swirling the contents of the flask approximately three times in a clockwise direction, and then three times in the reverse direction; repeat the two-step process at least once.
2. At the end of the mixing process, stop the motion of the liquid in the flask with a strong brief reverse mixing action, and quickly remove 1 ml of cell culture from the flask with a sterile pipet. Place the aliquot in a counting beaker, and add 9 ml (or 19 ml) of electrolyte solution (such as Coulter ISOTON®).

3. Determine the cell density (and MCV, if desired).

4.21.5.9.1.3 Manual microscope counting method

4.21.5.9.1.3.1 Cell counts may be determined using a Sedgwick-Rafter, Palmer-Maloney, hemocytometer, inverted microscope, or similar methods. 400 cells per replicate should be counted to obtain ± 10% precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

4.21.5.9.1.4 Chlorophyll Content

4.21.5.9.1.4.1 Chlorophyll can also be estimated in-vivo fluorometrically. In-vivo fluorometric measurements are the recommended method because of the simplicity and sensitivity of the technique and rapidity with which the measurements can be made (Geis, et al., 2000).

4.21.5.9.1.4.2 The in-vivo chlorophyll measurements are made as follows:

1. Adjust the "blank" reading of the fluorometer using the filtrate from an equivalent dilution of effluent filtered through a 0.45µm particle retention filter.
2. Mixing:
   Flasks: Mix the contents of flasks by swirling successively in opposite directions (at least three times), and remove 1 ml of culture from the flask with a pipet.
   Microplates: To suspend all settled algae cells, a plastic pipet should be used to draw up the contents of each well and carefully squirt the sample back in the well. The tip of the pipet may be used to brush the bottom of the well while drawing up the liquid. Repeat this procedure three times per well to assure complete suspension of algal cells.
3. Place the aliquot in a small disposable vial (or place the microplate directly into the fluorometer) and record the fluorescence as soon as the reading stabilizes. (Do not allow the sample to stand in the instrument more than 1 min).
4. Discard the sample.

4.21.5.9.1.4.3 For additional information on chlorophyll measurement methods, see APHA, 1992.

4.21.5.9.1.5 Absorbance

4.21.5.9.1.5.1 A second rapid technique for growth measurement involves the use of a spectrophotometer to determine the absorbance of the cultures at a wavelength of 680 nm. Because absorbance is a complex function of the volume, size, and pigmentation of the algae, a calibration curve must be constructed to establish the relationship between absorbance and cell density.

4.21.5.9.1.5.2 The algal growth measurements are made as follows:

1. A blank is prepared as described for the fluorometric analysis.
2. The sample is thoroughly mixed as described above.
3. Sufficient sample is withdrawn from the chamber with a sterile pipet & transferred to a 1-5 cm cuvette.
4. The absorbance is read at 680 nm and divided by the light path length of the cuvette, to obtain an "absorbance-per-centimeter" value.
5. The 1-cm absorbance values are used in the same manner as the cell counts.

4.21.5.10 SUMMARY OF *Selenastrum capricornutum* CHRONIC STATIC TEST CONDITIONS

4.21.5.10.1 A summary of test conditions and test acceptability criteria is presented in Table 4.7.

TABLE 4.7 CHRONIC TEST CONDITIONS FOR *S. CAPRICORNUTUM* STATIC TESTS	
1. Test type:	Static
2. Test duration:	96-h
3. Temperature (°C):	25 ± 1°C; temperatures must not deviate (i.e., maximum minus minimum) by more than 3°C during the test
4. Light intensity:	400±40 ft-c or 4,300 ± 430 lux
5. Photoperiod:	Continuous illumination
6. Test chamber size:	125/250 ml flask; 24 or 48-well microplate (3 ml wells)
7. Test solution volume:	50 ml (flasks); 2 ml (microplates)
8. Renewal of test concentrations:	None
9. Age of test organisms	4 - 7 days
10. Initial cell density in test chambers	10,000 cells/ml
11. No. replicate chambers per concentration:	Minimum 4
12. Shaking Rate	100 cpm continuous
13. Aeration:	None
14. Dilution water:	Filtered receiving water or synthetic water (see Section 4.4)
15. Dilution series:	5 effluent concentrations & dual controls (minimum); If IWC ≤30%, then 100, 30, 10, 3, 1%; if IWC >30%, then 100, 75, 50, 25, 12.5%; any additional or alternate dilutions must be specified in the WPDES permit (see 4.1.2)
16. Test acceptability:	One control must have mean cell density of ≥ $1x10^6$ cells/ml & CV between replicates ≤ 20%
17. Fluorometer settings:	450±50 nm excitation 665±20 nm emission
18. Spectrophotometer settings:	680 nm
19. Sampling requirement:	Specified in WPDES permit and Section 2

SECTION 5 - DATA ANALYSIS

5.1 DATA INTERPRETATION AND THE ROLE OF A STATISTICIAN

5.1.1 The Biomonitoring Coordinator will review reference toxicant data, QA information, and test results for all tests submitted for compliance with a WPDES permit. Guidance regarding WET data interpretation can be found in Chapter 1.5 of the "Whole Effluent Toxicity (WET) Program Guidance Document", available from the Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921 or at http://dnr.wi.gov/topic/wastewater/WET.html.

5.1.2 Use of the statistical methods referenced in this manual does not require the assistance of a statistician. However, if data appear unusual or fail to meet necessary assumptions, a statistician should be consulted. The choice of statistical method to analyze data can become problematic if there are anomalies in the data. Analysts who are not proficient in statistics are advised to seek the assistance of a statistician before selecting alternate methods or using the results.

5.2 PLOTTING THE DATA

5.2.1 The data (individual replicate data and mean values) shall be plotted as a preliminary step to help detect problems and unsuspected trends or patterns in the responses, to help detect outliers, to demonstrate concentration-response relationships (see Section 5.3.1), and as an aid in interpretation of the results (see 5.3 below and Section 6).

5.3 CONCENTRATION- (DOSE-) RESPONSE RELATIONSHIPS

5.3.1 The concept of a concentration-response (or dose-response) relationship is a fundamental one in toxicology. This concept assumes that there is a causal relationship between the concentration of toxicants in solution and a measured response. In general, more severe responses are expected at higher concentrations of toxicant, and less severe responses at lower concentrations. The concentration-response concept is the basis for the determination of point estimates in WET testing. A biological response (mortality, growth or reproductive inhibition) is measured at a range of effluent concentrations to develop a concentration-response curve.

5.3.2 A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results. Tests that exhibit unexpected concentration-response relationships may indicate a need for further investigation or testing. If a given effluent consistently produces a specific, unexpected concentration-response relationship, there is likely a physical, chemical, or biological cause which should be further investigated.

5.3.3 The concentration-response relationship generated for each test must be reviewed to ensure that calculated test results are interpreted correctly. Chapter 1.5 of the "Whole Effluent Toxicity (WET) Program Guidance Document" provides guidance on evaluating concentration-response relationships. Based on the review of the concentration-response curve several determinations may be made, including: 1) calculated effect concentrations are reliable and should be used for determining compliance, 2) calculated effect concentrations are anomalous and should be explained, or 3) the test was inconclusive and should be repeated with a newly collected sample within 30 days of the original test's end. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Permittees and lab staff should review concentration-response information, highlight any potential problems on WET Test Report Forms, and discuss any abnormalities with the Biomonitoring Coordinator. Final decisions regarding the acceptability of tests based on review of concentration-response information shall be made by the Biomonitoring Coordinator.

5.4 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

5.4.1 A critical assumption in the statistical analysis of toxicity data is independence among observations. Statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one observation would provide no information about the error in any other observation. One of the best ways to insure independence is to follow randomization procedures. The purpose of randomization is to avoid situations where test organisms are placed serially, by level of concentration, into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

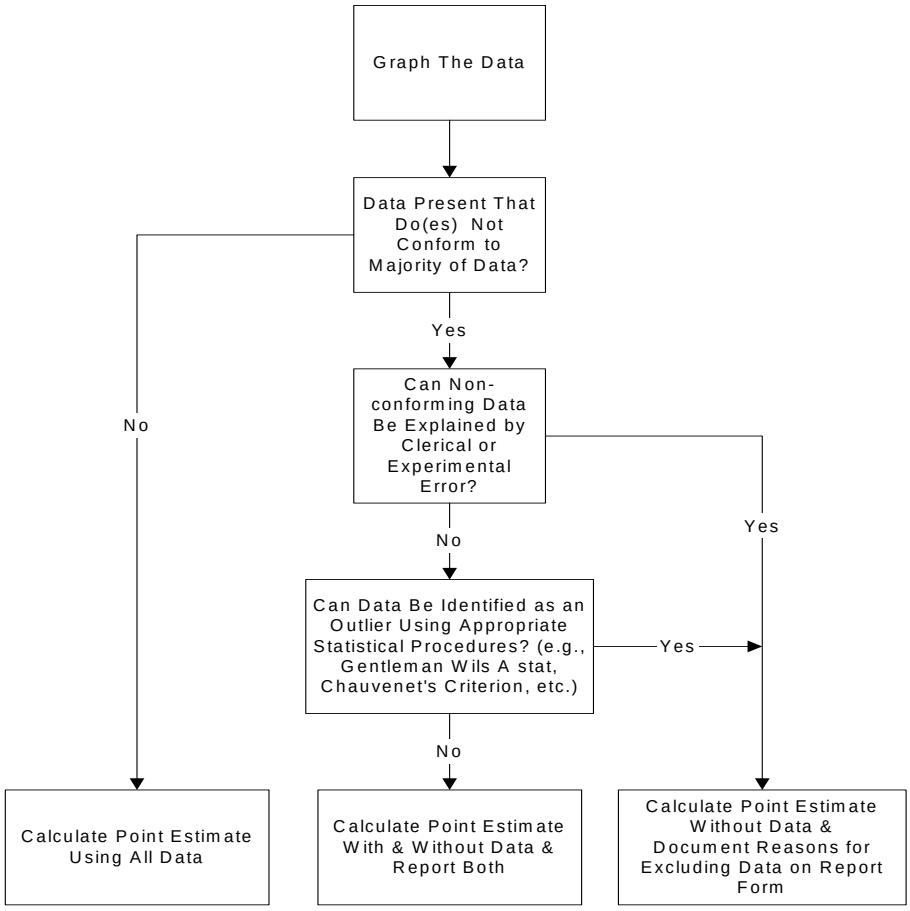

Figure 5.1 Process For Identifying Outliers

5.4.2 Data that do not appear to be in conformance with the substantial majority are often referred to as outliers, and might be due to random variation or to clerical or experimental errors. Statistical outlier detection procedures are screening procedures that indicate whether a datum is extreme enough to be considered, not due just to random variation. Outliers may be detected by plotting the data, by an analysis of the residuals, or by using an appropriate statistical procedure (Gentleman Wilk's A statistic, Dixon's test, Chauvenet's Criterion, etc.). Barnett and Lewis (1978) describe many outlier detection procedures, and Feder and Collins (1982) illustrate the use of several outlier detection procedures with aquatic toxicological data. An explanation must be sought for any questionable data points. Outliers may be discarded only if an acceptable explanation can be given. If outliers can be shown to be due to clerical or experimental error, they should be either corrected or deleted from the data prior to analysis. If one wishes to argue that a data point is an outlier, statistical analyses must be performed with and without the outlier and the results of both analyses reported along with an explanation. **Figure 5.1 illustrates the process that must be used to demonstrate that a data point is an outlier. The detection and treatment of outliers should not be based upon whether the results put a discharge in or out of compliance but must**

be applied equally for all suspect data.

5.5 STATISTICAL ANALYSIS AND CONFIDENCE INTERVALS

5.5.1 For determination of the appropriate acute endpoint, the flowchart given below (Figure 5.2) guides the analyst to the proper choice of statistical methods based on assumptions and determinations that can be made from the data. In acute and chronic tests, the proper statistical method should be performed using USEPA (see http://www.epa.gov/nerleerd/stat2.htm) or commercially available software to derive the desired effect concentration. For point estimation techniques the statistical methods generally produce an effect concentration with associated 95% confidence intervals. However, under certain circumstances confidence intervals are not produced or are unreliable (e.g., if test data do not meet specific assumptions required by the statistical methods, if point estimates are outside of the test concentration range, or if specific limitations of statistical software are encountered). Currently, confidence intervals are not used in determining compliance, but must be reported (when available) and may be used as supplemental information when interpreting test results.

5.6 ACUTE TOXICITY TEST DATA ANALYSIS

5.6.1 The required acute toxicity test consists of at least 2 controls and 5 concentrations of effluent in which the endpoint is an estimate of the effluent concentration which is lethal to 50% of the test organisms in the time period prescribed by the test, expressed as the LC_{50}. Theoretically, it is a calculated test concentration which would cause mortality to 50% of the test population.

5.6.2 ENDPOINTS

5.6.2.1 Point estimates such as the Lethal Concentration are derived from a mathematical model that assumes a continuous concentration-response relationship. By definition, any LC value is an estimate of some amount of adverse effect. Thus the assessment of a "safe" concentration must be made from a biological standpoint rather than with a statistical test. In this instance, the biologist must determine some amount of adverse effect that is deemed to be "safe", in the sense that from a practical biological viewpoint it will not affect the normal propagation of fish and other aquatic life in receiving waters. For the acute test methods in this manual, the LC_{50} has been chosen as the level of effect which represents unacceptable toxicity which has the potential to impact the aquatic life community. Therefore, the objective of these acute toxicity tests is to estimate an effluent concentration which causes lethality to half of a test population after a species specific exposure period.

5.6.2.2 The LC_{50} is determined by the Graphical, Spearman-Karber, Trimmed Spearman-Karber, or Probit Method as described in USEPA 2002a (p72-108). Figure 5.2 will assist in choosing which statistical method to use. Test endpoints must be reported as an LC_{50} and in Toxic Units (TU_a). 95% confidence intervals must also be given (when available), as an estimate of the precision around the reported LC_{50}.

- When a Zone of Initial Dilution (ZID) is not allowed, the Acute Toxic Unit (TU_a) shall equal $100/LC_{50}$. A passing test shall always be reported as 1.0 TU_a (LC_{50} >100% = 1.0 TU_a).

- When a ZID is allowed, the *Relative* Acute Toxic Unit (rTU_a) shall equal $ZID\%/LC_{50}$; where the ZID% is 3.3 times the percent dilution determined through application of the zone of initial dilution, according to s. NR 106.06(3)(c). A passing test shall always be reported as 1.0 TU_a (LC_{50} > ZID% = 1.0 TU_a).

- For more guidance regarding the correct application of a ZID, TU, and LC_{50}, see Chapter 2.4 of the "Whole Effluent Toxicity (WET) Program Guidance Document", available at http://dnr.wi.gov/topic/wastewater/WET.html or from the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

5.6.2.3 If, in the judgment of the Department, the methods used to determine positive test results are not

deemed appropriate for a specific data set, empirical interpretation methods may be used.

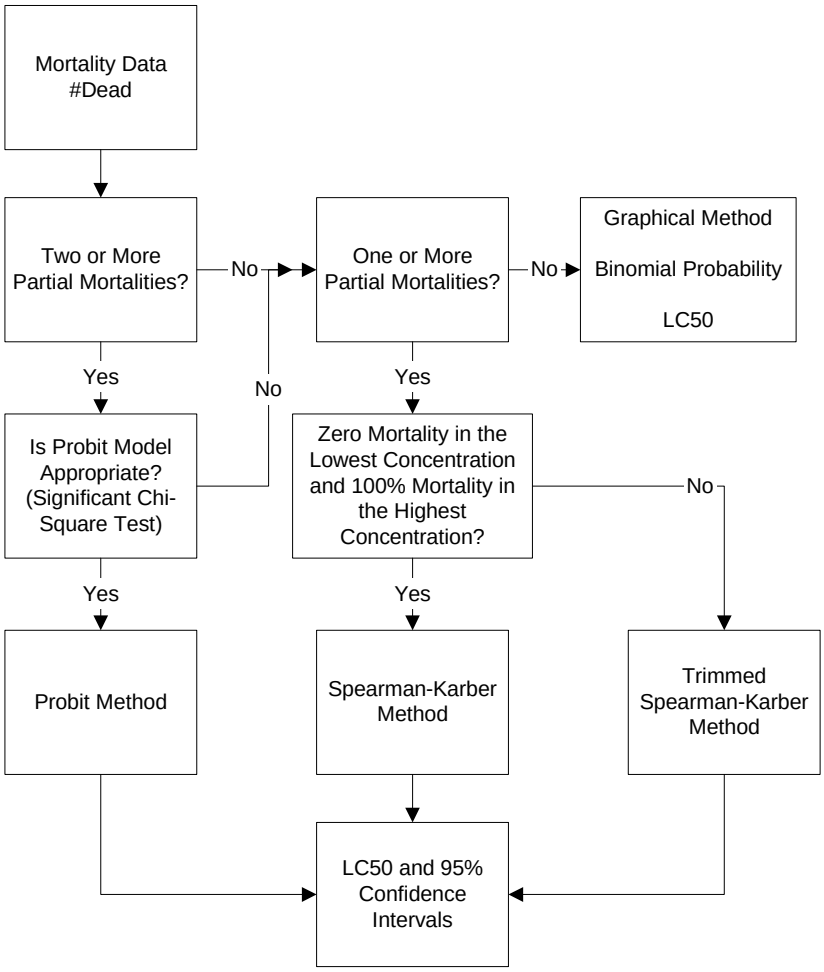

Figure 5.2 Determination of the LC_{50} From a Multi-Concentration Acute Toxicity Test

5. 7 CHRONIC TOXICITY TEST DATA ANALYSIS

5.7.1 ENDPOINTS

5.7.1.1 Point estimates such as the Inhibition Concentration (IC) are derived from a mathematical model that assumes a continuous concentration-response relationship. By definition, any IC value is an estimate of some amount of adverse effect. Thus the assessment of a "safe" concentration must be made from a biological standpoint rather than with a statistical test. In this instance, the biologist must determine some amount of adverse effect that is deemed to be "safe", in the sense that from a practical biological viewpoint it will not affect the normal propagation of fish and other aquatic life in receiving waters. For the chronic test methods in this manual, the IC_{25} (IC_{50} for *S. capricornutum*) has been chosen as the level of effect which represents unacceptable toxicity which has the potential to impact vertebrate and invertebrate communities.

5.7.1.2 The objective of a chronic test is to estimate the concentration which inhibits a characteristic of a test population at a predetermined level of significance. For example, an IC_{25} in a *C. dubia* test would be the estimated concentration of toxicant that caused a 25% reduction in mean young per female; an IC_{50} in a *S. capricornutum* test would be the estimated concentration of toxicant that caused a 50% reduction in plant growth.

5.7.1.3 The endpoints of toxicity tests using fathead minnow are based on the adverse effects on survival and growth (biomass). The total dry weight of surviving larvae in a replicate shall be divided by the number of larvae used to initiate that replicate (excluding larvae lost due to transfer or handling problems).

5.7.1.4 The endpoints of toxicity tests using *C. dubia* are based on the adverse effects on survival and reproduction. The total number of live neonates produced in all replicates in the 1st three broods (excluding those lost due to transfer or handling problems) shall be divided by the initial number of replicates (excluding any replicates containing males or those lost due to transfer or handling).

5.7.1.4.1 At the end of the test, if 50% or more of the surviving organisms in a block are identified as males, the entire block must be excluded from data analysis for the reproductive endpoint. For blocks having fewer than 50% of surviving organisms identified as males, the males (not the entire block) must be excluded from the analysis of reproduction. In addition to these test acceptability criteria, if fewer than eight replicates in the control remain after excluding males and blocks with 50% or more of surviving organisms identified as males, the test is invalid and must be repeated with a newly collected sample within 30 days of the original test's end.

5.7.1.5 The endpoints of toxicity tests using *Selenastrum capricornutum* are based on adverse effects to plant growth. Algal growth may be measured by any of the following methods: 1) Automatic particle counter or manual cell count (cells/ml), 2) fluorometrically (chlorophyll content), or 3) spectrophotometrically (light absorbance; measured at 680 nm, the optical density for *S. capricornutum*).

5.7.1.6 A linear interpolation method known as the Inhibition Concentration (IC) is the endpoint required for chronic toxicity tests. The IC is defined as a point estimate of the toxicant concentration that would cause a given percent reduction in a biological measurement such as fecundity or growth.

5.7.1.6.1 The IC analysis used to determine WPDES compliance shall be conducted according to USEPA methods (USEPA, 2002b; API, 1988). The "p" value shall be set equal to 25 (for *C. dubia* and fathead minnow) or 50 (for *S. capricornutum*) The number of bootstraps selected for the analysis shall equal 200. The Inhibition Concentration (IC_{25} or IC_{50}) value used to determine positive toxicity, shall be the bootstrap estimate generated by the computer program. Alternate statistical procedures may be used to determine the inhibition concentration, if approved by the Department prior to use.

5.7.1.6.2 Test endpoints must be reported as an IC_{25} in fathead minnow and *C. dubia* tests, and as an IC_{50} in *S. capricornutum* tests. Chronic results must also be reported in relative Toxic Units (rTU_c). 95% confidence intervals should also be reported, as an estimate of the precision around the IC value.

- The *Relative* Chronic Toxic Unit (rTU_c) shall equal IWC/IC_{25} (IC_{50} for *S. capricornutum*). A passing test shall always be reported as 1.0 rTU_c ($IC > IWC = 1.0\ TU_c$). The instream waste concentration (IWC) is an estimate of the proportion of effluent volume to total volume of effluent + receiving water.

- For more guidance regarding the correct application of the rTU_c, IWC, and IC, see Chapters 1.3 and 2.4 of the "Whole Effluent Toxicity (WET) Program Guidance Document", available at http://dnr.wi.gov/topic/wastewater/WET.html or from the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

5.7.1.6.3 Use of the Linear Interpolation Method is based on the assumptions that the responses 1) are monotonically non-increasing (the mean response for each higher concentration is less than or equal to the mean response for the previous concentration), 2) follow a piecewise linear response function, and 3) are from a random, independent, and representative sample of test data. The assumption for piecewise linear response cannot be tested statistically, and no defined statistical procedure is provided to test the

assumption for monotonicity. Where the observed means are not strictly monotonic by examination, they are adjusted by smoothing. In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. The inability to test the monotonicity and piecewise linear response assumptions for this method may make it difficult to assess when the method is, or is not, producing reliable results. If, in the judgment of the Department, the methods used to determine positive test results are not deemed appropriate for a specific data set, empirical interpretation methods may be used. Alternate statistical procedures may be used to determine the inhibition concentration, if approved by the Department prior to use.

SECTION 6 - TEST REPORT REQUIREMENTS

6.1 GENERAL INFORMATION

6.1.1 The "WHOLE EFFLUENT TOXICITY (WET) TEST REPORT FORMS" shown on Pages xxx are required to be submitted for demonstrating test completion and compliance with a WPDES permit. The original, complete, signed version of the WET Test Report Form must be sent to the Department's Biomonitoring Coordinator by the date specified in the WPDES permit. Reports must contain a description and justification of any abnormal procedures, conditions, or manipulations used in the test(s). The permittee and/or laboratory should also provide any attachments or additional information which they believe to be relevant to the test. All other test documentation (e.g. bench sheets, record books, etc.) needed to fulfill manual or QA requirements must be maintained at the laboratory for laboratory certification purposes (see Section 3.16, Record Keeping).

6.1.2 WET Test Report Forms should include observations made by the permittee or lab that may influence test results or data interpretation, such as: 1) unusual treatment conditions during sampling periods (for example, plant upsets, slug loads, weather conditions), 2) deviations from test specifications or any sample manipulation that is determined to be necessary for successful completion of a test (for example, aeration, filtration, addition of chemicals), and 3) unusual behavior or appearance of test organisms (for example, young developed in the brood pouch of the adults, but not released during the exposure period; partially or fully developed young released, but all dead at the end of the 24-h period; lethargy, hyperactivity, spots or filaments, discoloration, excessive ventilation).

6.1.3 The permittee is responsible for the timely submittal of a **complete and correctly filled out form** to the Department, by the dates specified in the WPDES permit. Contact names and signatures for the laboratory who performed the test and the permittee must appear on the form in the spaces provided. This form shall be submitted to the Biomonitoring Coordinator at the address provided on the last page of the report form. Although the permittee is responsible for the submittal of this form, both permittee and laboratory are encouraged to participate in the completion of the form.

6.1.4 Modifications to the WET Test Report Form, alternate forms, or other report types (including electronic reporting) may be acceptable for reporting WET test results, but must be approved by the Department prior to use. Modifications of the report form given in this section may also be used to submit information for non-standard tests required by the permit (e.g. toxicity identification studies) or for tests not required by the permit. All information required in this form and applicable to these non-standard tests should be submitted in the modified form.

6.1.5 **The results of all tests started for compliance with a WPDES permit, valid and invalid, shall be reported to the Department with an explanation of the tests performed and results. Tests which are started and not completed must be reported to the Department, with an explanation of reasons for not completing the test. If a permittee monitors the effluent more frequently than required by the permit, the results of that monitoring shall be recorded and reported in accordance with this manual.** The permittee shall furnish the Department, within a reasonable time, any information which the Department may request to determine whether cause exists for modifying, revoking or reissuing the permit or to determine compliance with the permit. The permittee shall also furnish the Department, upon request, copies of records required to be kept by the permittee.

6.1.6 **Leave no empty blanks on this form.** If an item is not applicable to the test, indicate this by placing an "NA" in the appropriate space and/or by explaining the blank space elsewhere on the form (for example, if replicate data is left blank so that inappropriate values do not appear in electronic graphs; the reason for the blank space should be explained in the comment area immediately below).

6.1.7 The following information must be included at the bottom of each page of WET forms submitted for WPDES compliance: facility name, WPDES permit number, and date test initiated.

6.1.8 The Department has developed electronic (Excel spreadsheet) versions of WET Report Forms, which perform some of the required steps (such as graphing of replicate data and calculation of coefficient of variation percentages), in an attempt to make reporting easier. Electronic copies of these forms (an acute, a chronic, and an acute/chronic version) may be requested by

sending a formatted 3.5" diskette (not floppy), recordable CD, or email to the Biomonitoring Coordinator. The most up-to-date contact information for the Biomonitoring Coordinator can be found on the Department's website (http://dnr.wi.gov/topic/wastewater/WET.html).

6.1.9 Guidance regarding WET data interpretation can be found in Chapter 1.5 of the "WET Program Guidance Document", available at http://dnr.wi.gov/topic/wastewater/WET.html or from the Department's Biomonitoring Coordinator at: Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI 53707-7921.

6.2 INSTRUCTIONS FOR COMPLETING THE WET TEST REPORT FORM

6.2.1 GENERAL INFORMATION

6.2.1.1 Facility: the name of the facility from which effluent samples were collected and tested should be entered here. The facility name should be the same as that shown on the WPDES permit. If the facility name has changed since the last reissuance, the new and old name should be included on the report form.

6.2.1.2 WPDES Permit No.: the WPDES permit number for the facility from which effluent samples were collected and tested should be entered here.

6.2.1.3 Outfall No.: indicate which outfall is being tested here. If there is more than one outfall being tested as a combined effluent then each outfall should be listed along with the percent effluent composition.

6.2.1.4 Laboratory Name: the laboratory that conducted the test should be identified here.

6.2.1.5 Receiving water: indicate the waterbody required by the WPDES permit and used in the test.

6.2.2 SAMPLE INFORMATION

6.2.2.1 Sample Collection: Information concerning type and time period of collection of effluent and receiving water samples used in the test are included in this area.

6.2.2.1.1 Sample type: in this space, please indicate whether the sample was a composite, grab, effluent or receiving water, etc. by using the following abbreviations:

EFF = effluent	C = composite
RW = receiving water	G = grab

(Example - if effluent sample is 24-h composite, indicate this on the form with EFF-24C)

6.2.2.1.2 Date & Time of Collection: Enter the date and time of sample collection in these boxes. Dates should be recorded as month/day/year. (Example - a sample taken on April 5, 2004 should be recorded as 04/05/04). Times should be recorded using a 24-h clock. (Example - a sample taken beginning at 1:30 p.m. would be recorded as 13:30).

6.2.2.3 Sample Temperature: Note the temperature (in °C) at time of collection and upon arrival at the lab.

6.2.2.4 pH: Enter the pH of each effluent and receiving water sample at time of collection and upon arrival at the lab.

6.2.2.5 Hand delivery: Indicate "yes" in this space if the sample was hand delivered and if time elapsed between sample collection and delivery was $\leq$ 4 hours.

6.2.2.6 Holding Time: According to section 2.4.1.2 of this manual, maximum holding time prior to the initial use of an effluent sample for toxicity testing shall be 36-h after the completion of sample collection. Indicate here whether this holding time was met for each sample.

6.2.2.7 Sample Acceptable: Indicate here whether all sample collection requirements were met and whether samples were deemed acceptable for use in the reported tests.

6.2.2.8 Comments: extra space is allotted here for comments relative to the "General Information" section of the form (e.g., if there are multiple outfalls being tested as a single composite, one could note that here, along with the percent effluent composition of the samples) and the "Sample Information" section of the form (e.g., if samples did not meet sample collection requirements, an explanation should be given here).

6.2.3 TEST INFORMATION

6.2.3.1 Date Test Initiated: the date of initial use of an effluent sample for toxicity testing (i.e., the first day of testing) should be given here.

6.2.3.2 Tests Are For: User should give the purpose for testing. Tests may be completed to meet WPDES permit compliance, as a retest of a previous failure, as a restart of a previous test that was unacceptable because it did not meet test acceptability requirements, as part of a toxicity reduction evaluation (TRE), or for other reasons. If "other" is chosen, the reasons for testing must be specified in the space below.

6.2.3.3 Date of Initial Test: If the tests being reported are retests being done as a result of a previous failure or as a restart of a previous test that was unacceptable because it did not meet test acceptability requirements, the date of the original test must be given here.

6.2.3.4 ZID Compliance Criterion: according to s. NR 106.09(2)(e), Wis. Adm. Code, "*for dischargers with an approved zone of initial dilution (ZID), a TU_a of X may not be exceeded; where X = 100 ÷ (3.3 x Dilution Factor) and the Dilution Factor = the approved ZID Concentration.*" Enter the value equal to "3.3 x Dilution Factor" (i.e., this equals the % effluent value that the LC_{50} cannot exceed for a test to "pass") in the space provided. This value should be specified in the WPDES permit.

6.2.3.5 IWC: indicate instream waste concentration (IWC) given in the WPDES permit here.

6.2.3.6 Dilution water: indicate whether receiving or laboratory water was used as diluent for each species in acute and chronic tests.

6.2.4 QA/QC CONDITIONS

6.2.4.1 Answer each of the questions by checking the appropriate box. If the criteria for a parameter were not met, indicate actual result(s) that did not meet test conditions (i.e. specify test and parameter that was not met) in the comments space provided that follows.

6.2.4.2 Comments: extra space is allotted here for comments relative to the "Test Information" and "QA/QC Conditions" sections of the form.

6.2.5 WATER CHEMISTRY DATA (EFFLUENT & CONTROLS)

6.2.5.1 Receiving Water and Effluent Data: Use this section to report all hardness, alkalinity, pH, total ammonia, and total residual chlorine data for effluents and receiving waters. The values reported here for hardness, alkalinity, and ammonia should be from sample measurements taken upon sample receipt. The values reported here for Total Residual Chlorine and pH should be from sample measurements taken after preparation for tests (i.e., after warming) and just prior to use in tests. All data (except pH) should be reported as mg/L. These data should be reported to reflect arrival conditions for each new effluent/receiving water sample received and the mean. Concentrations less than the limit of detection shall be reported as < (less than) the value of the limit of detection (e.g., if a substance is not detected at a detection limit of 0.1 mg/L, report the concentration as < 0.1 mg/L). For the purposes of calculating an average, the user may substitute a 0 (zero) for any

concentration that is less than the limit of detection.

6.2.5.2 Lab Water: Clearly specify which lab water was used in each test reported. A description of each lab water, including the type of water (for example, "moderately hard synthetic water" or "MHSW") and where it was used (for example, "used as dilution water in fathead minnow chronic test") must be entered in the first column to the right of "Lab Water". Mean values only need to be reported for each laboratory water used for testing. Extra space, if needed, is available in the " comments" area that follows this section.

6.2.5.3 Comments: extra space is allotted here for comments relative to the "Water Chemistry" section of the form.

6.2.6 ACUTE TEST CONTROL PERFORMANCE

6.2.6.1 Indicate here whether receiving water and laboratory controls met applicable test acceptability criteria.

6.2.6.2 Comments: extra space is allotted here for comments relative to the "Acute Test Control Performance" section.

6.2.7 ACUTE TEST RESULTS

6.2.7.1 Age of Organism (fathead minnow section only): indicate age of fathead minnow used in test (must be between 4-14 days, as required in Table 4.2 of this manual).

6.2.7.2 Percent survival by replicate: fill in the survival rate for each individual replicate tested. If a lab error or accident has resulted in a lost replicate, a note clearly identifying which replicate was lost and why should be included in the comments section below the appropriate test results; do not place an "NA", "LA" or other notation in the replicate space, because it will show up as a "0" on the graph and incorrectly modify the appearance of the concentration-response curve.

6.2.7.3 Mean percent survival: calculate an arithmetic mean of all replicates tested for each treatment.

6.2.7.4 Test endpoints: provide a 96-h LC_{50} for vertebrate test organisms and a 48-h LC_{50} for invertebrate test organisms, calculated according to the requirements in Section 5. Also report the 95% confidence intervals for the calculated LC_{50}, when available (if not available, enter an NA in the space provided).

Convert the calculated LC_{50} into Toxic Units - Acute (TU_a). NOTE: If no ZID is approved, then $TU_a = 100/LC_{50}$ if a ZID has been approved, then TU_a=ZID%/LC_{50}.

Whenever a point estimate lies above the test concentration range, the result must be reported as greater than the highest test concentration (e.g., $LC_{50} > 100\%$). A passing test shall always be reported as 1.0 TU_a ($LC_{50} > 100\% = 1.0\ TU_a$).

Whenever a point estimate lies below the test concentration range, the result must be reported as less than the lowest test concentration (e.g., $LC_{50} < 6.25\%$). $LC_{50} < 6.25\% = >16\ TU_a$.

6.2.7.5 Comments: Note any additional data relevant to the test results (e.g., abnormal effluent or lab conditions, laboratory errors or accidents, etc.) here. General observations of test organism appearance and behavior, such as erratic swimming, loss of reflex, discoloration, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, cannibalism, filamentous growth, etc. must also be noted here.

6.2.7.6 Graphs: Acute test results must be presented graphically, as well as in tabular form. Plot the replicate and mean survival data for the control and test concentrations against the concentrations. Graphs give a visual picture of the concentration-response, variability of the data, and any suspicious data or potential outliers.

6.2.8 CHRONIC TEST CONTROL PERFORMANCE

6.2.8.1 Indicate here whether receiving water and laboratory controls met applicable test acceptability criteria.

6.2.8.2 Comments: extra space is allotted here for comments relative to the "Chronic Test Control Performance" section.

6.2.9 CHRONIC TEST RESULTS

6.2.9.1 Fathead Minnow Growth and Survival Test Information:

6.2.9.1.1 Effluent Treatment: fill in the test concentrations to reflect the dilution series.

6.2.9.1.3 Mean Biomass Per Replicate: indicate the mean biomass (i.e., dry weight divided by the original number of organisms) for each of the replicate pairs tested (mg/L). (NOTE – If a lab error or lab accident has occurred resulting in a lost replicate, the lab may write a note clearly identifying which replicate was lost and why in the comments section below the appropriate test results; do not place an "LA" or other notation in the replicate space, because it will show up as a "0" on the graph and incorrectly modify the appearance of the concentration-response curve).

6.2.9.1.4 Mean Biomass (mg): calculate an arithmetic mean of all replicates tested for each treatment.

6.2.9.1.5 Calculated IC_{25}: The IC_{25}, calculated according to Section 5.5 of this manual, should be reported here. Also report the 95% confidence intervals for the calculated IC_{25}, when available (if not available, enter "NA").

Convert the calculated IC_{25} into relative Toxic Units - Chronic (rTU_c). NOTE: $rTU_C = IWC/IC_{25}$.

Whenever a point estimate lies above the test concentration range, the result must be reported as greater than the highest test concentration (e.g., $IC_{25} > 100\%$). A passing test shall be reported as 1.0 rTU_c ($IC > IWC = 1.0\ TU_c$).

Whenever a point estimate lies below the test concentration range, the result must be reported as less than the lowest test concentration (e.g., $IC_{25} < 6.25\%$). IC25 < lowest effluent concentration = > IWC/lowest effluent concentration.

6.2.9.2 *Ceriodaphnia dubia* Survival and Reproduction Test Information:

6.2.9.2.1 Treatment: fill in the test concentrations to reflect the dilution series.

6.2.9.2.2 Neonate Production by Replicate: indicate the 3 brood total of live neonates produced in each test replicate. (NOTE – If a lab error or lab accident has occurred resulting in a lost replicate, the lab may write a note clearly identifying which replicate was lost and why in the comments section below the appropriate test results; do not place an "LA" or other notation in the replicate space, because it will show up as a "0" on the graph and incorrectly modify the appearance of the concentration-response curve).

6.2.9.2.3 Mean Neonates: calculate an arithmetic mean of all replicates tested for each treatment (excluding any replicates containing males or those lost due to transfer or handling).

6.2.9.2.4 Percent Surviving Adults**:** indicate the proportion of adults that survived for the duration of the test.

6.2.9.2.5 Male Production $\leq$ 20% Over All Treatments: According to Table 4.3 of this manual, there must be $\leq$ 20% males over all concentrations in the *C. dubia* test. Indicate here whether this test acceptability criteria was met.

6.2.9.2.6 Calculated IC_{25}: The IC_{25}, calculated according to Section 5.5 of this manual, should be reported here. Also report the 95% confidence intervals for the calculated IC_{25}, when available (if not available, enter an "NA").

Convert the calculated IC_{25} into relative Toxic Units - Chronic (rTU_c). NOTE: $rTU_C = IWC/IC_{25}$.

Whenever a point estimate lies above the test concentration range, the result must be reported as greater than the highest test concentration (e.g., $IC_{25} > 100\%$). A passing test shall be reported as 1.0 rTU_c ($IC > IWC = 1.0\ TU_c$).

Whenever a point estimate lies below the test concentration range, the result must be reported as less than the lowest test concentration (e.g., $IC_{25} < 6.25\%$). IC_{25} < lowest effluent concentration = > IWC/lowest effluent concentration.

6.2.9.3 *Selenastrum capricornutum* Growth Test Information:

6.2.9.3.1 Treatment: fill in the test concentrations to reflect the dilution series.

6.2.9.3.2 Growth Measurement per Replicate: indicate the amount of growth (number of algae cells, fluorescence, or absorbance values) in each replicate. (NOTE – If a lab error or lab accident has occurred resulting in a lost replicate, the lab may write a note clearly identifying which replicate was lost and why in the comments section below the appropriate test results; do not place an "LA" or other notation in the replicate space, because it will show up as a "0" on the graph and incorrectly modify the appearance of the concentration-response curve).

6.2.9.3.3 Mean Growth: calculate an arithmetic mean of all replicates tested for each treatment.

6.2.9.3.4 Test Type: Indicate here whether tests were conducted in Erlenmeyer flasks, 24-well microplates, or other.

6.2.9.3.5 Endpoint measured: Indicate here whether cell counts were obtained/estimated via manual cell counts, spectrophotometer, or fluorometer readings.

6.2.9.3.6 Calculated IC_{50}: The IC_{50}, calculated according to Section 5.5 of this manual, should be reported here. Also report the 95% confidence intervals for the calculated IC_{50}, when available (if not available, enter an "NA").

Convert the calculated IC_{50} into relative Toxic Units - Chronic (rTU_c). NOTE: $rTU_C = IWC/IC_{50}$.

Whenever a point estimate lies above the test concentration range, the result must be reported as greater than the highest test concentration (e.g., $IC_{50} > 100\%$). A passing test shall be reported as 1.0 rTU_c ($IC > IWC = 1.0\ TU_c$).

Whenever a point estimate lies below the test concentration range, the result must be reported as less than the lowest test concentration (e.g., $IC_{50} < 6.25\%$). IC_{50} < lowest effluent concentration = > IWC/lowest effluent concentration.

6.9.2.4 Graphs:

6.2.9.3.1 Chronic test results must be presented graphically, as well as in tabular form. Plot the replicate and the mean sub-lethal (growth or reproduction) data for the control and test concentrations against the concentrations, for each species. Graphs give a visual picture of the concentration-response, variability of the data, and any suspicious data or potential outliers.

6.2.10 REPORT FORM COMPLETED BY:

6.2.10.1 Personnel responsible for filling out the form should submit it to the Department as indicated in the WPDES permit. **If the laboratory representative and the permittee responsible for the data are not given or if the form is not signed by each of these individuals, the Department will return the form as incomplete.**

6.2.10.2 Send one copy of the report form to the following address, according to the timelines specified in your WPDES permit: Biomonitoring Coordinator, Bureau of Watershed Management, Department of Natural Resources, 101 South Webster St., P.O. Box 7921, Madison, WI 53707-7921.

The Whole Effluent Toxicity (WET) Test Report Form

WET Test Report Form

WET Test Report Form

WET Test Report Form

GLOSSARY OF TERMS

Accuracy: The closeness of a measured value to its generally accepted value or an accepted reference standard.

Acute: A stimulus severe enough to rapidly induce an effect. In aquatic toxicity tests, it is usually measured as mortality or immobility observed within 96 hours or less.

Additivity: Occurs when the toxicity of chemical mixtures are greater than the exposure to each chemical individually, due to the sum of the toxic effects acting together (i.e., 1+1=2).

Aliquot: Contained an exact number of times in something; a division or part. In this manual it is used to mean a portion of the whole sample.

Ambient: The conditions of the environment into which an effluent is received, as it would be in its natural, unaltered state.

Bootstrap: A statistical method used to generate point estimates and confidence intervals for IC_{25} values. The process consists of multiple resamples of the data that is being analyzed with associated IC_{25} estimates.

Brood Board: A numbered and /or lettered grid board designed to hold *Ceriodaphnia dubia* culture vessels; this board allows tracking of the reproductive performance of individual organisms.

Certified Laboratory: A laboratory which performs tests for hire in connection with a program which requires data from a certified laboratory, and which receives certification or reciprocal recognition under ch. NR 149, Wis. Adm. Code.

Chronic: A stimulus that lingers or continues for a relatively long period of time (usually one-tenth of the life span or longer). Chronic toxicity is usually measured in terms of growth, reproduction, etc., in addition to lethality.

Cladoceran: An Order of organisms from the Family Crustacea, including such Genera as *Daphnia* and *Ceriodaphnia*, made up of small individuals (usually about the size of the head of a pin or smaller), that are commonly known as zooplankton. Found near the bottom of the food chain, they are thought to be a good indicator species for predicting the effects of toxicants on the entire aquatic community of which they are a part.

Composite sample: A combination of individual samples of equal volume taken at approximately equal intervals not exceeding one hour over a specified period of time.

Control Treatment: An exposure of the test organisms to dilution water with no effluent added; used as a standard of comparison in judging toxic effects and the validity of data.

Flow-through tests: A test system set-up that provides for continual or periodic renewal of controls and effluent treatments.

Grab sample: A single sample taken at one moment of time or a combination of several smaller samples of equal volume taken in less than a 2 minute period.

Graphical Method: A mathematical procedure which estimates an LC_{50} by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm of percent effluent concentration.

Indicator organism: A species sensitive to toxic substances, necessary for the overall health of the food chain, and representative of the indigenous population of the possible area of impact of the test material. These organisms are used to predict what is really happening in the environment when the effluent is introduced. It is assumed that knowledge of one species will give clues about the effect on a whole community.

Indigenous: Originated in and being produced, growing, living, or occurring naturally in a particular environment.

Inhibition Concentration (IC): The IC is defined as a point estimate of the toxicant concentration that would cause a given percent reduction in a biological measurement such as fecundity or growth. For example, an IC_{25} would be the estimated concentration of toxicant that would cause a 25% reduction in mean young per female or growth.

Initial Use: The point in time when organisms have been introduced into test chambers for all tests to begin the permit-required exposure period, and end the required initial holding time of the sample.

Inter-laboratory Precision: The coefficient of variation between replicates of the test endpoint (acute = LC_{50}; chronic = IC_{25}) calculated from five or more toxicity tests conducted with the reference toxicant sodium chloride (NaCl), between labs.

Intra-laboratory Precision: The coefficient of variation between replicates of the test endpoint (acute = LC_{50}; chronic = IC_{25}) calculated from five or more tests conducted with the reference toxicant sodium chloride (NaCl), within a single lab.

Instream Waste Concentration (IWC): Proportion of the volume of effluent to the total volume of water (effluent plus receiving water). The IWC is calculated as follows:

$$IWC = 100 \times \frac{Qe}{(1-f)Qe + Qs}$$

(where Qe = effluent flow; Qs = receiving water flow; f = the fraction of the Qe withdrawn from the receiving water)

Lethal Concentration (LC): The concentration of a toxic substance which is lethal to a specified percentage of exposed organisms in a given time period. For example, a LC_{50} would be the estimated concentration of toxicant that would cause 50% mortality to the test population after a 96-h exposure.

Material Safety Data Sheet: A product fact sheet which explains certain characteristics about the product such as reactivity, corrosiveness, and flammability and may contain product toxicity data.

May: Is (are) allowed to.

Must: Used to express an absolute requirement, that is, to state that the test shall be designed to satisfy the specified condition in order for the test to be acceptable.

Nauplii: Brine shrimp (*Artemia*) larva, usually in the first stage after leaving the egg.

Parentage technique: A technique allowing the performance of each female to be tracked. If a female produces one weak offspring or male, the likelihood of producing all weak offspring or all males is greater. The parentage technique enables poor performing young from a given female to be omitted from all concentrations.

Pathogenic: Causing or capable of causing disease.

Permittee: A municipality, industry, public agency, or commercial domestic establishment which is issued a permit for the discharge of pollutants issued by the Department.

Planktivorous: Organisms which feed on plankton (the passively floating or weak swimming, usually microscopic animal or plant aquatic life).

Precision: The closeness of repeated measurements on the same parameter within a sample.

Predator: Organism which exploits another for its own gain.

Primary Control: An exposure of the test organisms to dilution water with no effluent added; used as a standard of comparison in judging toxic effects and the validity of data.

Probit Method: A parametric statistical procedure for estimating the LC_{50} and the associated 95% confidence interval.

Promulgation: To put a law into action or use.

Quality Assurance (QA): A program organized and designed to provide accurate and precise results.

Quality Control (QC): Specific actions or procedures required to provide information for the QA program.

Receiving water: Water body to which effluent discharge occurs.

Reference toxicant: A chemical whose toxic qualities are known and quantifiable, which is used to demonstrate a lab's ability to obtain consistent, precise results. Labs are required to demonstrate this ability before performing toxicity tests with effluents for permit compliance reasons and on an ongoing basis.

Registered Laboratory: A laboratory which receives registration or reciprocal recognition under ch. NR 149, Wis. Adm. Code; cannot perform tests commercially for hire.

Replicate: Duplicate, repeat; usually provided because it permits easier viewing and counting of test organisms, avoids possible violations of loading limits, and ensures against the invalidation of the test which might result from accidental loss of a test vessel, where all of the test organisms for a given treatment are in a single chamber.

Required: Used to express an absolute, that is, to state that the test must be designed to satisfy the specified condition in order for the test to be acceptable.

Secondary Control: An exposure of the test organisms to laboratory water (required to be lab's culture water or standard synthetic water) with no effluent added; used as a method of judging health of the test organism population, laboratory performance, and validity of data.

Shall: Used to express an absolute requirement, that is, to state that the test must be designed to satisfy the specified condition in order for the test to be acceptable.

Should: Used to state that the specified condition is recommended and ought to be met if possible. Although a violation of one "should" is rarely a serious matter, violation of several will often render the results questionable.

Slug loads: Random doses of a chemical or compound in an influent that is not normally present at such concentrations.

Spearman-Karber Method: A nonparametric statistical procedure for estimating the LC_{50} and the associated 95% confidence interval.

Standard Operating Procedures (SOPs): A document which details the procedures and steps to be taken to successfully complete a specific task.

Static Renewal: A test system set-up that allows daily renewal of controls and effluent treatments.

Synergism: Occurs when the toxicity of chemical mixtures are greater than expected on the basis of exposure to each chemical individually (i.e., 1+1=3).

Toxicity test: A test which measures the degree of response of an exposed test organism to a specific chemical or effluent or other waters.

Trimmed Spearman-Karber Method: A modification of the Spearman-Karber Method nonparametric statistical procedure for estimating the LC_{50} and the associated 95% confidence interval, which estimates the trimmed mean of the distribution of the base 10 logarithm of the tolerance.

Whole Effluent Toxicity (WET): The aggregate toxic effect of an effluent as measured by a toxicity test.

Zone of Initial Dilution (ZID): An area surrounding the outlet of a discharge pipe which is uninhabitable by fish and other aquatic organisms, thereby allowing the mixing characteristics to be considered for purposes of effluent dilution. A ZID is often limited to a small area surrounding a discharge port where the physical turbulence of the discharge prevents organisms from swimming freely in and out of a discharge plume.

Zone of Initial Dilution (ZID) Ratio: A ZID ratio is the ratio of total water flow [stream (Q_s) + effluent (Q_e)] to effluent flow (Q_e) at the edge of the ZID, as estimated through modeling or field dispersion studies. A ZID which is approved by the Department may be used to establish alternate acute toxicity criteria pursuant to s. NR 106.09, Wis. Adm. Code.

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LIST OF COMMON ABBREVIATIONS

BOD: Biochemical Oxygen Demand

CWA: Clean Water Act

Department: Wisconsin Department of Natural Resources

DO: Dissolved Oxygen

EPA or USEPA: United States Environmental Protection Agency

IC_{25} : Inhibition Concentration of 25% of a test population

$LC_{50:}$ Lethal Concentration of 50% of a test population

NIST: National Institute of Standard Technology

NR: Wisconsin Administrative code for Natural Resources

PCB: Polychlorinated Biphenyl

PVC: Polyvinyl Chloride

QA/QC: Quality Assurance/Quality Control

Ref. Tox.: Reference Toxicant

SOP: Standard Operating Procedures

TIE: Toxicity Identification Evaluation

TDS: Total Dissolved Solids

TRE: Toxicity Reduction Evaluation

WDNR: Wisconsin Department of Natural Resources

WPDES: Wisconsin Pollution Discharge Elimination System

YCT: Yeast/Cereal Leaves/Trout chow