These procedures are part of the State of North Carolina’s response to requirements set forth by the National Pollutant Discharge Elimination System (NPDES). This document supports the Department of Environment and Natural Resources’ Administrative Code Section 15A NCAC 2H.1100. Specific laboratory facility and equipment requirements, quality assurance requirements, standard test methods/procedures, standard toxicity test reporting forms, and standard scientific reporting units pertaining to Biological Laboratory certification are described here. Procedures presented here and in subsequent versions are approved by the Director before being released to the public.

METHODS AND PROCEDURES

The following documents describing NPDES test methods and procedures are recognized as standard and shall be used to measure the reporting units listed below:


7. Any other toxicological monitoring methods approved by the Director under 15 NCAC 02H.1100 or any additional methods approved and published by the Environmental Protection Agency.


9. Any biological field survey analyses which either quantify or enumerate resident aquatic populations and used to evaluate attainment of Water Quality Standards as defined in 15 NCAC 2B.0211 or 15 NCAC 2B.0220.
LABORATORY FACILITY AND EQUIPMENT REQUIREMENTS

Laboratory facilities and equipment considered as minimum laboratory resources are as follows:

(1) **Aquatic Toxicology Laboratory**
   
   (a) A minimum of 200 square feet of laboratory space.

   (b) A minimum of 20 linear feet of laboratory bench space.

   (c) A drained sink with hot and cold running water.

   (d) Adequate control of culture environment (i.e. lighting, cooling and heating) to maintain appropriate organism requirements.

   (e) A refrigerator of adequate size which will maintain sample temperatures between 0.0°C and 6.0°C.

   (f) Current copies of the procedures documents written by EPA and North Carolina’s Division of Water Quality (see Methods section for references).

   (g) Glassware, chemicals, supplies and equipment to perform any procedures included in requested certification.

   (h) Instrumentation capable of measuring dissolved oxygen, pH, temperature, conductivity, and salinity (for saltwater tests) directly from test vessels of any procedure included in certification application. Equivalent surrogate vessels may be utilized for physical measurements if injury to test organisms may result.

   (i) Instrumentation or analytical capabilities to perform measurements of total residual chlorine to a level at least as low as 0.1 mg/l and total hardness to a level at least as low as 1 mg/l.

   (j) A dissecting microscope and a compound microscope for those laboratories maintaining either of the categories of Acute Toxicity Testing/Invertebrate or Chronic Toxicity Testing/Invertebrate. The compound microscope must have a minimum magnification of 400x and a maximum magnification of ≥1000x.

   (k) A balance capable of accurately weighing 0.0001 g and Class “S” or equivalent reference weights. A balance capable of accurately weighing fish larvae to 0.00001 g for those laboratories maintaining certification for the category Chronic Toxicity Testing/Vertebrate.

   (l) Viable reproducing laboratory cultures of any test organisms included in the certification application. Use of test organisms for regulatory purposes not maintained as a viable laboratory culture may be accepted on a case-by-case basis upon receipt of written permission from the Aquatic Toxicology Unit.

   (m) Appropriate dilution water for use in whole effluent toxicity testing with chemical characteristics such that the pH is between 6.5 and 8.5 S.U. and total hardness as calcium carbonate is between 30 and 50 ppm. Should receiving waters have characteristics outside of stated ranges then alternate pH and hardness ranges may be accepted upon demonstration that the alternate ranges are better suited to testing objectives and quality assurance standards have been met.

   (n) Appropriate Chain-of-Custody documentation forms and seals.
(2) **Aquatic Population Survey and Analysis Laboratory**

(a) A minimum of 150 square feet of laboratory space.

(b) A minimum of 8 linear feet of laboratory bench space.

(c) Binocular dissecting microscopes and compound microscopes suitable for survey type.

(d) Vials, preservatives, and space to maintain representative sample collections for at least one year after collection.

(e) Current taxonomic guides and references specified by the Division.

(f) Appropriate chain of custody documentation, laboratory records and seals are to be available.

(g) Sampling equipment to support collection of appropriate biological organisms.

(h) Settling tubes and an inverted microscope with a minimum magnification of 300x for those laboratories maintaining certification for the parameter Algae.

**QUALITY ASSURANCE REQUIREMENTS**

Emphasis is placed on good laboratory practices and proper documentation. Additional quality assurance requirements to those found in the previously cited documents are as follows:

1. All instruments used in or associated with toxicity testing are to be calibrated daily or with each use and recorded in a designated notebook (i.e., automatic sampling equipment, pH meter, D.O. meter, conductivity meter, etc.)

2. A minimum of five valid reference toxicant tests must be performed and entered on a control chart for each organism and test type for which a lab is certified. A maximum of 20 datapoints are to be entered on the control chart.

3. A reference toxicant test should be performed every two weeks for each organism used in acute whole effluent toxicity testing, or alternatively, acute reference toxicant tests may be performed such that NC NPDES acute tests are performed within one week of an acute reference toxicant test for the organism in question. In the case of the latter, to maintain acute certification for an organism, acute reference toxicant tests must be performed, at minimum, on a quarterly frequency. For organisms obtained from outside suppliers, a reference test must be performed with each batch of organisms received from the supplier.

4. A reference toxicant test should be performed once per month for each organism used in chronic whole effluent toxicity testing, or alternatively, tests may be performed such that NC NPDES chronic tests are performed within two weeks of a chronic reference toxicant test for the organism in question. In the case of the latter, to maintain chronic certification for an organism, chronic reference toxicant tests must be performed, at minimum, on a quarterly frequency. For organisms obtained from outside suppliers, a reference test must be performed with each batch of organisms received from the supplier.

5. The endpoint for chronic reference toxicant tests will be the IC25 as determined by the linear interpolation method described in *EPA-821-R-02-013* and *EPA-821-R-02-014*, or subsequent versions.
(6) Acceptable alternative culture media utilized to culture the algae *Selenastrum capricornutum* for use as *Ceriodaphnia* food are:


   (b) Additional nutrients may be used in the preparation of algae medium described in Section 13.6.15 of EPA-821-R-02-013 and Appendix A1, Section 3.10.3 of EPA-821-R-02-012. Specifically, the volume of nutrient stock solutions found in Table 1 on page 147 of EPA-821-R-02-013 or p 133 of EPA-821-R-02-012 may be adjusted so that solutions 1.A, 1.D, and 2 are added at a rate of 2 ml/L and solutions 1.B and 1.C are added at a rate of 6 ml/L.

(7) A representative of each test organism cultured shall be taxonomically identified to the species level at a minimum frequency of once per quarter. Test organisms obtained from an outside supplier shall be taxonomically identified to the species level at a minimum yearly. All specimens shall be preserved and held for a minimum of one year.

(8) If closed incubators are utilized for toxicity testing and/or test organism culturing purposes, culturing and testing activities may not be contained within the same incubator.

(9) Effluent samples collected for chronic tests are to be first used within 36 hours of collection and not more than 72 hours after first use of the sample for test renewal. The beginning of this period is defined as the time of the collection of a grab sample or the time of collection of the last subsample of a composite sample to the time that the organisms are introduced to the test solution.

(10) A single sample log will be maintained for all samples entering the laboratory which lists, at a minimum, sample identity including any sample number(s), sample temperature at receipt, time and date of receipt, name of person received from, and name of person receiving sample.

**PROCEDURE MODIFICATIONS**

Modifications from test protocols from the cited EPA documents follow. These modifications are in addition to those specified in individual procedures documents. References to the EPA manuals are given to provide context to the modification being made to the EPA method.

(1) For freshwater tests, the pH of the control solution at test initiation and subsequent test solution renewals must fall in the range of 6.5-8.5 standard units. Total hardness must measure between 30 and 50 mg/l CaCO₃.

(2) All acute and chronic toxicity tests will be conducted at 25.0 ± 1.0°C, except chronic tests for *Mysisopsis bahia* will be conducted at 26.0 ± 1.0°C. Variances may be requested for species which may require alternate temperatures. (Ref. EPA-821-R-02-012, p 46, 9.12.1; EPA-821-R-02-013, pp 75-76, Table 1; pp 164-165, Table 3; EPA-821-R-02-014, pp 78-79, Table 3; pp 178-179, Table 3; pp 241-242, Table 3).

(3) Organisms used in acute toxicity tests will have food made available for a minimum of two hours prior to initiation of testing. For cladoceran species, this feeding amount can be greater than but not less than 0.05 ml of YCT and 0.05 ml of a solution of the algae *Selenastrum capricornutum* (with a cell concentration of 1.71 X 10⁷ cells/ml) per 15 milliliters of culture solution. (Ref. EPA-821-R-02-012, p 46, 9.11.1)

(4) For each sample used in a toxicity test, pH, specific conductance or salinity (for saltwater tests), and total residual chlorine will be measured and recorded from an undiluted aliquot on the day the sample is first used. Temperature, dissolved oxygen, pH and salinity (for salt water tests) will be measured in the control and the highest toxicant concentration tested at the beginning of the test, prior to and following each renewal, and at the termination of the test. (Ref.
(5) *Ceriodaphnia dubia* used in toxicity tests will be obtained from individual cultures, from third or subsequent broods of adults not being more than 14 days in age, containing eight or more neonates, with an average adult mortality not exceeding 20% per culture board (*Ref. EPA-821-R-02-012. p 131, Appendix A1, 3.7.6.*).

(6) Chronic *Ceriodaphnia dubia* analyses will have an additional test acceptability criterion of complete third brood neonate production by at least 80% of the surviving control organisms. (*Ref. EPA-821-R-02-013. p 165, Table 3.*)

(7) *Ceriodaphnia dubia* neonate reproduction totals from chronic tests shall include only organisms produced in the first through third broods.

(8) The percentage of male *Ceriodaphnia* control organisms may not exceed 20% in chronic *Ceriodaphnia* tests.

(9) The *Ceriodaphnia* control organism reproduction coefficient of variation (CV) must be less than 40% for a chronic *Ceriodaphnia* test to be considered acceptable.

(10) “Observed-effect” as referred to in NC DWQ chronic *Ceriodaphnia* procedures documents will be defined as either:

   (a) A statistically significant decrease in survival of the treatment organisms as compared to the control organisms or

   (b) A twenty percent or greater decrease in treatment organism reproduction as compared to control organism reproduction which is also determined to be statistically different from control organism reproduction.

(11) Acute tests will be terminated within one hour of their stated length.

**FORMS**

The forms for reporting whole effluent toxicity test results (see attachments) are as follows:

(1) AT-1 form, entitled Effluent Toxicity Report Form - Chronic Pass/Fail and Acute LC50, is used for reporting chronic pass/fail toxicity test results or acute LC50s.

(2) AT-2 form, entitled Effluent Aquatic Toxicity Report Form - Acute Pass/Fail, is used for reporting acute pass/fail toxicity test results.

(3) AT-3 form, entitled Effluent Aquatic Toxicity Report Form/Phase II Chronic *Ceriodaphnia*, is used for reporting Phase II chronic toxicity test results or chronic pass/fail results.

(4) AT-5 form, entitled Effluent Aquatic Toxicity Report Form/Chronic Vertebrate Toxicity Test

(5) AT-6 form, entitled Effluent Toxicity Report Form-Chronic *Mysidopsis bahia* Multi- Concentration Test
REPORTING UNITS

The list of reporting units considered as standard are defined as:

1. **LC50** - The toxicant concentration killing 50% of exposed organisms at a specific time of observation.

2. **NOEC** - (No Observed Effect Concentration) The highest or single concentration of toxicant to which organisms are exposed in a life cycle or partial life-cycle test, which causes no statistically significant adverse effect on the observed parameters (usually hatchability, survival, growth, and/or reproduction).

3. **LOEC** - (Lowest Observed Effect Concentration) The lowest concentration of toxicant to which organisms are exposed in a life cycle or partial life cycle test, which causes a statistically significant adverse effect on the observed parameters (usually hatchability, survival, growth, and/or reproduction).

4. **Chronic Value (ChV)** - A numeric value representing the geometric mean of the numeric values of concentrations analyzed as the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect (LOEC) by chronic toxicity testing. The chronic value is an estimate of the toxicant concentration that will be the actual no effect concentration based on the chronic effect tested. 
   \[ \text{ChV} = \text{Antilog} \left[ \frac{\log_{10} \text{LOEC} + \log_{10} \text{NOEC}}{2} \right] \]

5. **Biological Water Quality Rating** - A rating, ranging from Excellent to Poor, which gives an indication of water quality based on the composition of the biological community, using standardized techniques as specified by the Division of Water Quality.

6. **Total Taxa Richness** - The total number of different taxa collected, taken to the lowest practical taxonomic level.

7. **EPT Taxa Richness** - The total number of different taxa collected belonging to the orders Ephemeroptera (mayflies), Plecoptera (stoneflies), and Tricoptera (caddisflies), taken to the lowest practical taxonomic level.

8. **Diversity** - The number and abundance of taxa in a specified location summarized using a mathematical formula to allow comparisons of community structure.

9. **ET(x)** - The relative toxicity of a toxicant measured in terms of the time it takes to elicit a given response from a given percentage (x) of the exposed test organisms.

10. **TLM** - Median tolerance limit - The toxicant concentration at which 50% of test organisms survive for a specified exposure time. The term has been superseded by median lethal concentration (LC50).

11. **LC(x), EC(x)** - Lethal concentration (LC) or effective concentration (EC). A point estimate of the toxicant concentration that would adversely affect a given percent(x) of the test organisms.

12. **Maximum Acceptable Toxicant Concentration (MATC)** - Concentration to be determined within the interval bounded by the LOEC and NOEC which is used as the concentration of toxicant predicted to have no detrimental impacts on the test population.

13. **Toxic Unit Acute** - Toxic units are defined as the reciprocal of the effect concentration times 100, where the effect concentration is expressed as a percentage of whole effluent, thus 
   \[ \text{TUA} = 100 / \text{LC50} \]

14. **Toxic Unit Chronic** – Toxic units are defined as the reciprocal of the effect concentration times 100, where the effect concentration is expressed as a percentage of whole effluent, thus 
   \[ \text{TUC} = 100 / \text{ChV} \]

15. **IC(x)** - Inhibition Concentration. A point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms.