DIVISION OF WATER QUALITY

NORTH CAROLINA DEPARTMENT OF
ENVIRONMENT AND NATURAL RESOURCES

NORTH CAROLINA PHASE II CHRONIC WHOLE EFFLUENT TOXICITY TEST PROCEDURE

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Version 3.0

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North Carolina Phase II
Chronic Whole Effluent Toxicity Test Procedure
(*Ceriodaphnia* multi-concentration or full-range toxicity test)

This procedure has been established as a modification of Method 1002.0 described in the U.S. Environmental Protection Agency document entitled "Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms," Fourth Edition (EPA-821-R-02-013). Appendix 2 outlines a further modification of the methodology described here which utilizes a single effluent concentration and a control treatment to determine compliance.

This test procedure has been approved by the Director of the North Carolina Division of Water Quality under the Certification Criteria/Procedures document described in the Biological Laboratory Certification Rules (15A.NCAC.2H.1100) as a standard procedure for evaluation of the effects of toxic substances to sensitive aquatic species. It shall be considered acceptable proof effluents are not causing chronic impacts to aquatic life in receiving streams due to toxic substances. It does not directly address mutagens, carcinogens, teratogens, or disease-causing agents and may be superseded by other water quality regulations. Depending on the designated use and specialized concerns of a particular water body (or effluent discharge), additional monitoring and/or restrictions (either chemical or biological) may be required. These monitoring requirements may include, but are not limited to, additional toxicity testing using alternate test organisms, unmodified EPA protocols, or increased sampling or test solution renewal frequencies.

The test organism used for this test is *Ceriodaphnia dubia*, a small cladoceran common in lakes and larger rivers frequently used as an aquatic toxicity test organism. The organism has a rapid life cycle at 25°C, potentially producing numerous offspring during a seven-day period.

The measures of effect used in this test are number of offspring produced and mortality within the test period. This document will outline approved variations from the EPA procedure. Only those modifications outlined here, in the North Carolina Biological Laboratory Certification/Criteria Procedures Document, or approved by written exception made by NC DWQ may be made to the EPA guidelines. This document is organized into six sections which include:

1) Effluent sampling and handling,
2) Test procedure,
3) Interpretation of results,
4) An outline of daily activities to be performed prior to and during the test period (Appendix 1),
5) A summary of the mini-chronic procedure, which outlines methodology to determine permit toxicity limit compliance utilizing one effluent concentration and a control treatment (Appendix 2), and
6) Quality Assurance Checklist (Appendix 3).

EFFLUENT SAMPLING AND HANDLING

All effluent samples collected for this procedure must be 24-hour composites unless grab samples or other alternate sampling regimes are specifically allowed by the facility's permit or monitoring requirement. Sampling should be performed below the last waste treatment process, including disinfection. There may be no removal of chlorine or any other effluent constituent from the sample by either chemical or physical methods prior to testing with the exception of allowable filtration of the effluent through 60 µm nylon screen or plankton netting and reduction of excess dissolved oxygen to the saturation level, as per EPA methods.

Sample collection materials may be tempered glass, polyethylene, perfluorocarbon plastics including Teflon®, 304 or 316 stainless steel, polypropylene, polyvinylchloride, Tygon®, or silicone. All non-perfluorocarbon plastics should be discarded after use. It is the responsibility of the collector to assure that contamination is not influencing test results. There may be no chemical residue present which will affect effluent toxicity. Care should be taken that sufficient sample volume is collected in order to perform the test.

Effluent samples must be immediately preserved on ice such that they achieve and maintain a temperature between 0.0°C and 6.0°C, inclusive, from collection, in the case of grab samples, or initiation of collection through the use of an iced or refrigerated sampler, in the case of composite samples. The single allowable exception to this protocol is the situation in which a grab sample arrives at the performing laboratory within three hours of collection. In that circumstance, the sample container must be completely covered in ice in its shipping container immediately after collection and arrive at the laboratory in that same condition. All other samples must be received by the certified biological laboratory at a temperature between 0.0°C and 6.0°C, inclusive, or the resulting data will be considered unacceptable for submittal for compliance purposes. The sampling container must be completely filled, with no air pockets, to minimize loss of volatiles. Aliquots only should be drawn from the original sample for warming and subsequent use in tests.

Each effluent sample collected for this procedure must follow certain timing/scheduling constraints. By definition of this method, each composite sampling must be performed over two calendar days (Day One through Day Two, and Day Three through Day Four, as defined in Appendix 1). For purposes of defining the month in which the test is indicative of compliance, the start date of the first sample for any given test will be considered the month (and quarter) in which the test was performed. The sampling schedule is intended to be performed on Monday through Tuesday and Wednesday through Thursday. Shifting the sampling days is acceptable, assuming that the relative chronology and sequence of sampling and testing activities remains constant and the certified biological laboratory is capable of meeting such a schedule. Effluent samples for chronic tests are to be first used within 36 hours of collection and not more than 72 hours after first use for test renewal. Sample holding time begins at the time of collection of a grab sample or the time of collection of the last subsample of a composite sample, and ends when the organisms are introduced or transferred to the test solution. First use of the sample is defined as the time the last organism is introduced to the test solution when initiating/renewing a test. Samples must be stored at 0.0-6.0°C, with minimum head space. For example, a composite sample initiated on Monday at 10:00 AM and terminated at 10:00 AM on Tuesday must be used for the first time by 10:00 PM on Wednesday. Likewise, the second sample, initiated at 10:00 AM on Wednesday and terminated at 10:00 AM on Thursday, must be used for the test renewal by 10:00 PM on Friday. The second sample must be used for the final test renewal not more than 72 hours after first use. As such, careful coordination should take place between sampling personnel and the certified biological laboratory so that sampling schedules can be accommodated within protocol constraints of the testing method.

Preparation of split samples must be performed carefully to insure that each laboratory receives and analyzes similar samples. This similarity should take into account possible variables including, but not limited to, sample mixing, sample containers, lack of air space in sample containers, sample temperature, pH, conductance, and total residual chlorine. Additionally, if concurrent analyses are sought on split samples, performing laboratories should coordinate analytical times and dates. Analyses of split samples performed at significantly different times or on different dates will be considered as independent analyses.

TEST PROCEDURE

The test shall be performed as a minimum of six treatments exposing 10 test organisms to each. The first treatment shall be considered the control population and shall be exposed at 0% effluent and 100% dilution water. One of the minimum of five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the facility's instream waste concentration (IWC). The IWC is calculated as follows:

$$\%\ \boldsymbol{Effluent}\ (\boldsymbol{IWC}) = \frac{\text{Permitted Discharge Volume} * 100}{\text{Permitted Discharge Volume} + 7Q10^{\boxtimes}}$$

$^{\boxtimes}$ *Where 7Q10 is defined as the lowest average 7 day flow in the receiving stream which has a probability of reoccurrence every ten years. All terms must have equivalent units.*

At least two of the effluent test treatments must be of a lesser effluent concentration than the IWC, with one being one-half the concentration of the IWC. No concentration should be greater than two times that of the next lower concentration or less than one half of the next higher concentration. The following are possible test concentrations for a facility with an IWC of 45%:

22.5%$^{\oplus}$
35.0%$^{\oplus}$
45.0%$^{\oplus}$
70.0%
90.0%

$^{\oplus}$ *Indicates required concentrations for this example, i.e. IWC and two lower concentrations.*

Dilution water must be the culture water used to maintain the test population or be suitable for that purpose. The pH of this water at test initiation and initiation of 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 $CaCO_3$.

Ten test organisms will be exposed to each treatment in individual test chambers. The test will run until at least 80% of the surviving control organisms produce three broods of young, not to exceed a seven day +2 hours exposure, using the chronology specified in Appendix 1. Termination should be contingent upon whether the control reproduction mean has reached the minimum acceptable average value of 15.0 young per surviving female.

The objective of this test is to determine the effluent's No-Observed-Effect Concentration (NOEC), Lowest-Observed-Effect Concentration (LOEC), and Chronic Value (ChV). The NOEC and LOEC are determined by identifying which effluent concentrations tested have significant detrimental impact upon reproduction and/or survival as compared to the control population. The lowest effluent concentration tested which displays significant impact upon survival or reproduction is the LOEC. The highest effluent concentration tested which does not display significant impact to either survival or reproduction as compared to the control population is the NOEC. The ChV is defined as the geometric mean of the NOEC and LOEC.

After effluent collection on Days One through Two, the test treatments will be prepared and the test initiated on Day Three (Appendix 1). An aliquot of the first composite sample is brought to room

temperature and utilized to mix test solutions which are then distributed to the test vessels. The specific conductivity, pH, and total residual chlorine of the undiluted effluent sample must be measured and recorded. Effluent samples are to be refrigerated at a temperature between 0.0° and 6.0°C except for those aliquots drawn for mixing test solutions. The pH, dissolved oxygen and temperature of the control and highest effluent testing concentration must be checked and recorded. At all times temperature of the test solutions must be 25.0°C (±1.0°C) and dissolved oxygen must be equal to or greater than 5.0 mg/l.

The test organisms are placed singly in test vessels each containing 15 milliliters of solution. The organisms must be less than 24 hours old, within 8 hours of the same age, from third or subsequent broods, and from broods in which the adult produced at least 8 neonates. All test organisms must be produced by "individual" cultures as defined by "Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, Fourth Edition" (EPA-821-R-02-013). Neonates are transferred using an eye dropper, such that the organism is never removed from solution. There should be as little water transferred with the organism as is reasonably practical. All *Ceriodaphnia* should be fed at this time and daily thereafter. Each daily feeding will consist of addition of 0.05 ml of yeast-Cerophyll®-trout chow (YCT) food and 0.05 ml of a solution of the algae *Selenastrum capricornutum* (with a cell concentration of 1.71 X 10^7 cells/ml) per 15 milliliters of test solution. Preparation of food supplies are described by EPA-821-R-02-013 (alternative algal media preparation methods are described in "North Carolina Biological Laboratory Certification/Procedures Document"), though feeding *rates* have been modified for this protocol. Test chambers should be incubated for temperature control with the photoperiod maintained at 16 hours of light and 8 hours of darkness.

On Days Three through Four, a second effluent sample is collected to be used for renewal of the test solutions on Days Five and Eight. On Day Five the original test organisms are transferred to new test vessels containing new control and effluent solutions. The effluent solutions are mixed from the second effluent sample collected on Day Four. The specific conductance, pH, and total residual chlorine of the undiluted effluent sample must be measured and recorded. This renewal must take place within 36 hours of the second effluent sample collection time.

Mortality must be recorded at this time. Mortality in the treatment equal to the IWC should be compared to mortality in the control using the Fisher's Exact Test at a 95% confidence level. Reproduction counts should be performed in all vessels used during the initial test period (although there are usually no offspring during this phase in the life cycle). Temperature, dissolved oxygen, and pH observations must also be made and recorded for both the old and new test solutions. The new test solutions should receive food at this time.

Days Six and Seven require only that the *Ceriodaphnia* be fed. Day Eight requires renewal of the test solutions using the second effluent sample. This renewal must take place within 72 hours of the first use of the effluent sample on Day Five. Mortality, reproduction, temperature, dissolved oxygen, and pH observations must be made and recorded. Reproduction of the initial test organisms must be observed both as total number of young produced as well as brood number of the young produced (i.e. first, second, or third brood). On Day Nine, the control organisms should be observed for production of the third brood. If 80% or more of the surviving control organisms have produced a third brood, the test may be terminated. This will also hold true for observations made on Day Eight. On Day Ten, the test is terminated after making final mortality, reproduction and temperature, dissolved oxygen, and pH observations. Fourth brood neonates will be excluded from the reproduction totals and subsequent statistical analyses. The test exposure duration will be no greater than seven days +2 hours regardless of control organism reproductive success. All entries to test bench sheets should be initialed by the person making the entry in a manner that will signify which entry was made by which analyst.

INTERPRETATION OF RESULTS

The statistical comparisons for evaluating the test results should be performed as outlined in Section 13.13 Data Analysis on pages 166-188 and in Appendices B through G in EPA-821-R-02-013. To test for normality of the reproduction data, the chi-square test for goodness of fit may be used if the Kolmogorov statistic is not available (note that the Shapiro-Wilk's test should be utilized to assess the normality of datasets with 50 or fewer datapoints). The chi-square procedure is available in most basic statistics books. Confidence levels for each statistical procedure will be those specified in EPA-821-R-02-013.

As stated earlier, the objective of this test is to determine the effluent's No-Observed-Effect Concentration (NOEC), Lowest-Observed-Effect Concentration (LOEC), and Chronic Value (ChV). An "observed-effect" will be defined as either 1) a significant decrease in survival of the treatment organisms as compared to the control organisms or 2) a twenty percent or greater decrease in treatment organism reproduction which is also determined to be statistically different from control organism reproduction.

For data analysis, mean reproduction is calculated by summing the total number of young produced per treatment until either time of death or end of the experiment and dividing by the initial number of females exposed per treatment. Note that fourth brood neonates will be excluded from the reproduction totals. Percent reduction for each treatment will be calculated by subtracting the mean number of neonates produced by the treatment organisms from the mean number of neonates produced by the control organisms, dividing that number by the mean number of neonates produced by the control organisms, and multiplying by 100% as per the following equation:

$$\boldsymbol{Percent\ Reduction} = \left(\frac{\bar{Y}_1 - \bar{Y}_i}{\bar{Y}_1}\right) * 100\%$$

Where $\overline{Y_1}$ is the control organism reproduction mean and $\overline{Y_i}$ is the treatment organism reproduction mean. A chronic value (ChV) is determined as the geometric mean of the LOEC and NOEC from the toxicity test results. If the lowest effluent concentration is the LOEC, then the ChV will be considered the geometric mean of the LOEC and one-half the LOEC. If the highest effluent concentration is the NOEC, then the ChV will be considered the NOEC.

Mortality greater than 20% in the control population will be considered abnormal, and the test must be declared invalid. Average reproduction in the control population must be greater than or equal to 15.0 offspring per surviving female, or the test is considered invalid. No more than 20% of the control organisms may be males.

The control organism reproduction coefficient of variation (CV) must be less than 40.0% for the test to be considered acceptable. The CV is calculated by dividing the standard deviation of the control organism reproduction by the mean of the control organism reproduction multiplied by 100% as per the following equation:

$$\boldsymbol{Coefficient\ of\ Variation} = \left(\frac{s_1}{\bar{Y}_1}\right) * 100\%$$

Where s_1 is the standard deviation and $\bar{Y}_1$ is the mean. Note that the mean and standard deviation are calculated using the number of female organisms initially exposed to the control solution, including any which may have died during the course of the test.

If these tests are being performed as an NPDES requirement or by Administrative Letter, then the ChV must be entered on the Effluent Discharge Monitoring Form (MR-1) for the month collection was begun for the first effluent sample using the parameter code THP3B. Additionally, DWQ Form AT-3 (original) is to be received at the following address no later than the last day of the month following the month in which the analysis occurs:

Environmental Sciences Section
Aquatic Toxicology Unit
North Carolina Division of Water Quality
1621 Mail Service Center
Raleigh, North Carolina 27699-1621

APPENDIX 1. CHRONIC WHOLE EFFLUENT TOXICITY TEST PROCEDURE

DAY ONE

Start a 24-hour composite sampling device. Sampling devices should be refrigerated or cooled by ice.

DAY TWO

The composite sample will be collected, sealed, and packaged on ice to maintain a temperature between 0.0° and 6.0° C, inclusive, and shipped to the laboratory where the toxicity test will be performed. (Alternatively, a grab sample may be collected on this day if the NPDES permit specifies such a sample.)

DAY THREE

The test treatments will be set up and test organisms introduced within 36 hours of the sample collection time on Day Two. Specific conductance, temperature, and pH will be measured and recorded. Dissolved oxygen must be ≥ 5.0 mg/l, and the temperature must be maintained at 25.0°C (±1.0°C). The specific conductance, pH, and total residual chlorine of the undiluted sample must be measured and recorded. Minimize head space of the sample and refrigerate (0.0°-6.0°C). Feed *Ceriodaphnia.* Start second 24-hour effluent composite sample.

DAY FOUR

Collect and ship second effluent sample. Feed *Ceriodaphnia*.

DAY FIVE

Ceriodaphnia must be transferred to new solutions of the second sample prior to the sample reaching 36 hours in age. Record the time at which the test organisms are transferred. Mortality and reproduction counts should be performed and recorded at this time. (There are usually no offspring during this early phase of the life cycle.) Perform temperature, dissolved oxygen, and pH monitoring. The specific conductance, pH, and total residual chlorine of the undiluted second sample must be measured and recorded. Feed *Ceriodaphnia*. Minimize head space of the second effluent sample and refrigerate (0.0°-6.0°C).

DAY SIX

Feed *Ceriodaphnia.*

DAY SEVEN

Feed *Ceriodaphnia.*

DAY EIGHT

Renew all test solutions using the second effluent sample. Test solutions must be renewed within 72 hours after initial use of the sample on Day Five. Record the time at which the test organisms are transferred. Count and record mortality and reproduction. Perform pH, dissolved oxygen, and temperature monitoring. Feed *Ceriodaphnia.*

DAY NINE

Feed *Ceriodaphnia*. (Optional: Observe stage of reproduction and terminate test if 80% or greater of surviving control organisms have produced their third broods.)

DAY TEN

Perform final mortality and reproduction counts as well as temperature, dissolved oxygen, and pH monitoring.

APPENDIX 2. NC MINI-CHRONIC *CERIODAPHNIA* WET PROCEDURE

This appendix provides a means of determining compliance by comparing a single effluent treatment to a control. The single effluent treatment procedure is described in detail in the North Carolina *Ceriodaphnia* Whole Effluent Toxicity Procedure ("Mini-chronic"), Version 3.0, revised December 2010. The option to perform this variation of the chronic procedure may only be exercised if it is included in the NPDES permit as the first test of the monitoring quarter. Discretion should be used when choosing this option. Given that the result does not produce a no-effect level, an artificial endpoint will be generated which may or may not be advantageous from a compliance standpoint. If a failure should result, at least two multiple concentration tests (one per month) must be performed by the end of the monitoring quarter.

All effluent sampling, test conditions, and test procedures are identical to those outlined in the main section of this document except for the test concentrations, number of organisms per treatment, and statistical evaluations of data. Twelve organisms will be used for each treatment. There will be only two treatments, a control and an effluent concentration equal to the IWC as defined previously. Due to the limited ability of this modification to define a chronic no-observed-effect level, the test performed using this appendix procedure may be terminated at 48 hours should the mortality in the effluent treatment significantly exceed that of the control treatment as determined by the Fisher's Exact test.

The statistical comparisons for evaluating the test results should be performed as outlined in Appendix H (entitled "Single Concentration Toxicity Test - Comparison of Control with 100% Effluent or Receiving Water") of EPA-821-R-02-013, with the exception that reproduction data are to be evaluated at a 99% confidence level. A failure will be considered as either 1) a significant decrease in survival of the treatment (effluent) organisms as compared to the control organisms or 2) a twenty percent or greater decrease in treatment (effluent) organism reproduction which is also determined to be statistically different from control organism reproduction. For compliance purposes, a "Fail" result will be averaged with other quarterly monitoring data as a chronic value (ChV) equal to the geometric mean of the IWC and one-half the IWC. In the event of a "Pass" result, the ChV will be considered to be a value greater than the IWC (">XX%"). For compliance purposes, the initial test of the monitoring quarter may be either a single or multiple concentration toxicity test if the permittee has a single concentration test specified in their permit. The NPDES permit specifies the required test type. A permittee required to perform a single concentration test may choose to perform a multiple concentration test instead. If the test result is "fail" or if a ChV lower than the limit is obtained, then at least two ChV tests (one per month) are required over the following two months. As many analyses as can be completed will be accepted. Compliance for the three month period will be determined based on the average of the follow-up ChV test results. The ChV must be entered on the Effluent Discharge Monitoring Form (MR-1) for the month during which collection was begun for the first effluent sample using the parameter code THP3B. Additionally, DWQ Form AT-3 (original) is to be sent to the same address noted above.

APPENDIX 3. QUALITY ASSURANCE CHECKLIST

The following table summarizes appropriate test conditions for any *Ceriodaphnia* chronic toxicity test performed to fulfill a North Carolina NPDES monitoring requirement. Values recorded outside of these ranges will result in an analysis being judged as "invalid" upon review by Aquatic Toxicology Unit personnel. The information should be used as a checklist for individual tests and does not cover the full range of quality control practices necessary for successful completion of this analysis.

TEST CONDITION	TEST ACCEPTABILITY CRITERION
Instream Waste Concentration(%)	By Permit, SOC, or JOC
Control Mortality	≤ 20%
Average Reproduction for Control	≥15.0 per surviving female
% Control Organisms Producing a Third Brood	≥80 % surviving controls
Maximum % Male Control Organisms	≤20%
Control Reproduction CV	<40.0 %
Initial Control Solution pH	6.5-8.5 pH units
Minimum D.O. of Control and Treatments	≥5.0 mg/l
Hardness of Dilution Water	Between 30-50 mg/l $CaCO_3$
Sample Temperature at Receipt	Between 0.0°-6.0°C
Temperature during Test	25.0 ± 1.0°C
First Use of Sample for Test Initiation and/or Solution Renewal	<36 Hours from sample collection
Subsequent Use of Sample for Test Solution Renewal	<72 hours after first use of sample

REFERENCES

North Carolina Division of Water Quality. 2010. North Carolina Biological Laboratory Certification/Criteria Procedures Document. Version 3.0. Revised December 2010.

North Carolina Division of Water Quality. 2010. North Carolina *Ceriodaphnia* Chronic Whole Effluent Toxicity Test Procedure. Version 3.0. December 1985, Revised December 2010.

United States Environmental Protection Agency. 2002. Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition. EPA-821-R-02-013, 350 pp.