Inoculation of *Pseudokirchneriella subcapitata* Toxicity Assay (formerly *Selenastrum capricornutum*)

1. SCOPE AND APPLICATION

   1.1. This method is based on *EPA-821-R-02-013, Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* Method 1003.0. It is applicable to the toxicity bioassay having the green alga, *Pseudokirchneriella subcapitata* as the test species.

2. SUMMARY OF METHOD

   2.1. Test algae are concentrated; a cell count is made on the concentrated algae. An aliquot of concentrated algae is diluted to the volume and concentration necessary to provide standardized inoculums for each test replicate.

3. EQUIPMENT AND SUPPLIES – Record any equipment repairs or maintenance in LIMS **Laboratory Logbook,** *Maintenance Log*

   3.1. *Pseudokirchneriella subcapitata* culture, 4-7 days old; cultured per SOP TA-04.08.

   3.2. *Inoculation Preparation Sheet* - contained within the *Pseudokirchneriella subcapitata Chronic Bioassay/SRT Bench Sheets* logbook (Refer to the Data Archival section of this SOP for an alternative worksheet.)

   3.3. 15 mL centrifuge tubes, 2

   3.4. Thermo Scientific Sorvall T3 Centrifuge (S/N 309020018; located in B246 A)

   3.5. 500 mL Erlenmeyer flask

   3.6. 250 mL graduated cylinder

   3.7. HandyStep® pipetter and 50 mL pipet tip

   3.8. Isoton®

   3.9. Coulter Counter® Model Z2 (S/N AB114302 and AF47542) – calibrate and use per SOP TA-06.19

   3.10. 30 mL Dilu-vials

   3.11. 1 mL sterile, disposable pipets

   3.12. 10 mL sterile, disposable pipets

4. REAGENTS AND STANDARDS

   4.1. PAAP media, prepared per SOP TA-03.03.

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5. INOCULATION OF *P. SUBCAPITATA* TOXICITY ASSAY AND CONCURRENT CALCULATIONS

5.1. Pipet 10 mL of the *P. subcapitata* alga culture into each of the 2 centrifuge tubes (see Step 5.1.2)

5.1.1. *P. subcapitata* is used for samples being tested at a salinity of <1.0 ppth.

5.1.2. If one tube of *P. subcapitata* will suffice (based on the experience of the analyst), 10 mL of DI water can be placed into the second tube, instead, for the purpose of balancing the centrifuge.

5.2. Centrifuge at 1000 rpm for 10 minutes.

5.2.1. Load the centrifuge tubes opposite each other so that the load is balanced. The centrifuge will not work properly if not balanced.

5.2.2. To increase the concentration of algae, the number of tubes can be increased as needed.

5.3. Decant the supernatant and add 10 mL of the *P. subcapitata* alga culture to each of the centrifuge tubes being used for inoculum.

5.4. Tighten the caps on the centrifuge tubes and shake vigorously to re-suspend the algae.

5.5. Centrifuge again at 1000 rpm for 10 minutes.

5.6. Decant the supernatant and add 10 mL of the *P. subcapitata* culture to each of the centrifuge tubes being used for inoculum.

5.7. Tighten the caps on the centrifuge tubes and shake the tubes vigorously to re-suspend the algae.

5.8. Place 100 mL of PAAP medium into the graduated cylinder. (Volume can be increased as needed.)

5.9. Prepare the Coulter Counter® model Z2 for cell counting per SOP TA-06.19.

5.10. Place 10 mL of Isoton® in a vial and perform 3 counts on it. Record the three counts on the *Inoculum Preparation Sheet* in the spaces labeled "**A**," "**C**," and "**E**."

5.11. Calculate the mean of the three counts and record the mean in the three spaces labeled "**G**." (This is the background count.)

5.12. Make sure algae is still in suspension. Place 0.1 mL of the solution in a 30 mL vial and add 9.9 mL of Isoton®; swirl to mix.

5.13. Perform three counts on the mixture in the vial. Record these counts on the *Inoculum Preparation Worksheet* in the spaces labeled "**I**," "**J**," and "**K**."

5.13.1. These counts are used to calculate the concentration of the algae stock solution.

5.14. Average the 3 counts from the above step. Subtract the number in Space "**G**" from the average. Multiply this number by 200. Record the result in the space labeled "**L**." This is the concentration of algal cells within the stock solution.

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5.14.1. The dilution factor is 200 because the sample to Isoton® ratio of the subsample being counted in 1:100 and the Coulter Counter® takes up a ½ mL of solution per each count.

5.15. Record the number of cells/mL required within each flask (10,000) in the space labeled "**M**."

5.16. Record the number 101 in the space labeled "**N**." (101 is the average mL of solution in each flask after inoculation with the test alga.)

5.17. Determine the number of flasks you have to inoculate, add 100 to this number and record the number in the space labeled "**O**."

5.17.1. This is the amount of inoculant being made. This number is calculated by determining the number of flasks to inoculate (1 mL of inoculant solution per flask), then adding 100 mL to ensure an adequate amount of inoculant is prepared.

5.18. Multiply the numbers entered in "**M**," "**N**," and "**O**." Record the result in the space labeled "**P**." "**P**" is the number of cells needed in the inoculum flask in "**O**" mL.

5.19. Divide "**P**" by "**L**;" place the result in "**Q**." This is the amount of stock solution required for dilution.

5.20. Pipet "**Q**" mL from the centrifuge tube containing the test algae and place into the 500 mL flask.

5.21. Place (**O – Q**) mL of PAAP medium into the flask containing **Q** mL of test algae.

5.22. Swirl and shake to mix. Pipet 1.0 mL from the flask and place into a 30 mL vial. Add 9.0 mL of Isoton® to the vial.

5.23. Perform three counts on the mixture in the vial. (Refer to SOP TA-06.19.) Record the three cell counts on the *Inoculum Preparation Sheet* in the spaces labeled "**R**," "**T**," and "**V**." Record the mean cell volume from each count in the spaces labeled "**S**," "**U**" and "**W**."

5.24. Calculate the mean of the numbers in the spaces labeled "**R**," "**T**," and "**V**." Subtract the number from the space labeled "**G**" from the mean. Multiply the result by 20. Record in the space labeled "**X**."

5.24.1. The multiplier (20) is derived from the 1 in 10 dilution of the test algae in the counting vial and from the 0.5 mL of the mixture in the vial that is processed for each count. So the total number of cells/mL (**X**) is the count multiplied by 20.

5.25. Divide the number represented by "**X**" by the number represented by "**N**" (101). Record this number in the space labeled "**Z**."

5.26. If the number recorded in the space labeled "**Z**" is within ±5% of the number of cells/mL desired for the inoculum (10,000 cells/L), then the inoculum is ready to be added to the test flasks. If the number of cells (**Z**) is not within 10% of the desired concentration, calculate the mL of the stock algae from the centrifuge tube to be

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added to the inoculum flask or the mL of DI water to be added to the inoculum flask by the following formulas:

First, calculate the number of cells in the inoculum flask by:

(**X**) cells/mL × (**O** – 1) mL = (**a**) cells

Determine the difference between the number of cells in the inoculum flask (**a**) and the total number of cells desired (**P**) by:

(**P**) cells – (**a**) cells = (**b**) cells

If (**b**) cells is < 0 (meaning that you have too many cells), then calculate the volume of inoculum to remove from the inoculum flask and the matching volume of DI water to add by:

(**b**) cells ÷ (**X**) cells/mL = (**c**) mL

Remove (**c**) mL of inoculum from the inoculum flask and add (**c**) mL of diluents to the inoculum flask. Repeat Steps 5.23.-5.26.

If (**b**) is > 0 (meaning you have insufficient cells), then calculate the mL of stock from the centrifuge tube to add to the inoculum flask by:

(**b**) cells ÷ (**L**) cells/mL = (**d**) mL

Add (**d**) mL of stock from the centrifuge tube to the inoculum flask and repeat Steps 5.23.-5.26.

5.27. Use the HandyStep® pipetter to place 1 mL of inoculum from the inoculum flask into the appropriate test flasks.

5.28. Label the *Inoculum Preparation Sheet* with the date, the test alga, analyst initials, and the samples inoculated. Save the *Inoculum Preparation Sheet* for later reference.

5.29. Calculate the Organism Code for the inoculum. For *P. subcapitata*, multiply (**Z**) × (**Y**) × (3.78×10^{-7}). Round the result to three decimals and record on the *Inoculum Preparation Sheet* as SEL.xxx, with the three numbers replacing the xxx.

5.29.1. Example: For *P. subcapitata*, where **Z** = 10,000 and **Y** = 47

($\mathbf{Z}$) × ($\mathbf{Y}$) × (3.78×10^{-7}) = $10{,}000 \times 47 \times (3.78 \times 10^{-7})$ = 0.1776600 = SEL.180

6. DATA ARCHIVAL

6.1. Use a black, waterproof pen for all bench sheets and logbooks.

6.2. To correct raw data entries, place a single line through the incorrect or illegible entry. Write the corrected entry near the error and initial the correction. Do not overwrite entries.

6.2.1. The corrected entry is written as near its appropriate space as possible or else in the comment box on the appropriate bench sheet.

6.2.2. If the correction requires an explanation, write a comment and reference the error. Initial and date the explanation.

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6.2.2.1. Use sequential alphabetical annotation to identify errors and corresponding comments (use A for first error and use A for corresponding explanatory comment; use B for second error, etc.).

6.3. Record data on *Inoculation Preparation Sheet*, which is contained within the *Pseudokirchneriella subcapitata Chronic Bioassay/SRT Bench Sheets* logbook. Alternatively, the EXCEL spreadsheet (Inoculation Setup Worksheet) located on the PC (at the Coulter Counter) can be used, printed (when complete), saved, and placed in the appropriate folders (electronic and paper).

7. QUALITY CONTROL

7.1. If a problem occurs during the sample processing or testing, the following steps are performed:

7.1.1. Lab supervisor is notified.

7.1.2. Client is notified (client makes decision for additional testing).

7.1.3. Section administrator is notified.

7.1.4. A non-conformance report (NCR) is submitted describing the problem and the corrective action.

7.1.4.1. To create NCR: open LIMS and go to Laboratory/Administrative/Non-Conformance Report.

8. SAFETY/HAZARDOUS WASTE MANAGEMENT

8.1. Proper laboratory protection, including lab hood or ventilation system, lab coat, gloves and safety glasses, is required when working with chemicals and unprocessed samples.

8.2. Refer to the FDEP Laboratory Health and Safety Manual.

8.3. Read MSDS information for reagent handling and disposal.

9. REFERENCES

9.1. EPA 821-R-02-013 Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Ed. October 2002.

9.2. DEP SOP TA-04.08, Culture of Algal-Assay Inoculation Stocks,

9.3. DEP SOP TA-06.19, Calibration and use of the Coulter Counter Model Z2

9.4. DEP SOP TA-03.03, Preparation of Preliminary Algal Assay Procedure (PAAP) Medium for Freshwater Algae Cultures.

9.5. FDEP Laboratory Quality Manual. http://www.dep.state.fl.us/labs/library/lab_sops.htm

9.6. FDEP Laboratory Health and Safety Plan, https://www.fldepnet.org/content/dear/bureau-laboratories-safety-documents

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9.7. MSDS information sheets.

10. APPENDIX OF CHANGES

12/18/2007 Added Scope and Application; Summary of Method, and Appendix of Changes.

04/29/2013 Updated format.

04/01/2014 Updated References.

02/13/2015 Formatted for 508 compliance.

03/15/2016 Updated links and rearranged references.

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