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 used to add a known concentration of algal test species into test solution. It is applicable to the toxicity bioassay having the green alga *Pseudokirchneriella subcapitata* as the test species. This SOP is used for samples with test-IDs BSY-CS-FSC and BSY-CD-FSC.

2. SUMMARY OF METHOD

   2.1. Test algae are collected and concentrated. The concentrated algae are counted, then diluted to the appropriate concentration for inoculation. A known concentration is necessary to provide standardized inoculums for each test replicate.

   2.2. Interferences include contaminated glassware, airborne particulates (including dust and microscopic organisms), and improperly prepared samples.

   2.3. Definitions

      2.3.1. ***Pseudokirchneriella subcapitata***— (formerly *Selenastrum capricornutum*); a non-motile, unicellular, freshwater green alga

3. EQUIPMENT AND SUPPLIES--Record any equipment repairs or maintenance on the form located in the *Dilution Water Parameters* logbook

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

   3.2. 15 mL centrifuge tubes, minimum of 2

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

   3.4. 1 Beaker, ≥100 mL

   3.5. 100 mL graduated cylinder

   3.6. 30 mL Dilu-vials

   3.7. Disposable transfer pipets

   3.8. 100 µm fixed pipet, with tips

   3.9. 1000 mL fixed pipet, with tips

   3.10. 10 mL sterile, glass serological disposable pipets, with pump

   3.11. HandyStep® pipetter with 50 mL pipet tip

   3.12. Computer with access to DEAR (X:) share drive

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4. REAGENTS AND STANDARDS

4.1. *Pseudokirchneriella subcapitata* culture, 7-10 days old; cultured per SOP TA-04.08.

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

4.3. Isoton®

5. PREPARATION OF *P. SUBCAPITATA*

5.1. For samples with a salinity of <1.0 ppth, add 10 mL of the *P. subcapitata* alga culture to each of 2 centrifuge tubes.

5.1.1. Analyst experience can determine if one tube of *P. subcapitata* will suffice.

5.1.2. To increase the final concentration of algae, the number of tubes can be increased and then combined as needed.

5.2. Place the tubes in the centrifuge so the load is balanced. Centrifuge at 1000 rpm for 10 minutes.

5.2.1. The centrifuge manual should be referred to for proper use.

5.3. Decant the supernatant.

5.4. Add another 10 mL of the *P. subcapitata* alga culture to each of the concentrated algae pellets. Tighten the caps on the centrifuge tubes and shake vigorously or use a transfer pipet to re-suspend the algae. Place the tubes in the centrifuge so the load is balanced. Centrifuge at 1000 rpm for 10 minutes.

5.5. Decant the supernatant. Using a transfer pipet, remove one algae pellet and add it to the second tube. Add another 10 mL of the *P. subcapitata* alga culture to the tube containing the algae pellets. Tighten the cap and shake vigorously or use a transfer pipet to re-suspend the algae.

6. PREPARATION OF INOCULUM (Record the following data in *Sel cap SRT Inoc* worksheet; refer to Section 7 for calculations.)

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

6.2. Open the *Sel cap SRT Inoc* Excel worksheet found in X:\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP's Greatest Hits. Select the **FWInoc** tab. Fill in the Start Date and Analyst.

6.3. Record the daily Background Counts in cells labeled **A**, **C**, and **E**. Record their corresponding MCVs in **B**, **D**, and **F**. The average background count will automatically be calculated, and the result will populate all cells labeled **G**.

6.4. In a Dilu-vial labeled “Premix,” pipet 0.1 mL of the suspended concentrated algae and 9.9 mL of Isoton®. Swirl to mix.

6.5. Perform three counts on the premix. Record the counts in cells labeled **I**, **J**, and **K**. Cells labeled **L** will automatically be calculated.

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6.6. Cell **M** is the concentration of algae required in each flask. This should be 10,000 cells/mL.

6.7. Cell **N** is the volume of solution in each flask after inoculation with the test alga. This should be 101 mL.

6.8. Cell **O: # of Flasks** is the number of flasks to be inoculated. This is dependent on the sample load and it determines the amount of inoculum needed. (1 mL inoculum per flask.)

6.8.1. The number in Cell **O: Inoculant Vol** can be rounded up to ensure an adequate amount of inoculum is prepared. This number is typically 100.

6.9. Cells labeled **P** are automatically populated based on cells **M**, **N**, and **O**.

6.10. Add **Q** amount of suspended concentrated algae to a graduated cylinder. Use PAAP to bring up to the volume entered in **O: Inoculant Vol**. Pour into a beaker and swirl to mix.

6.11. In a Dilu-vial labeled "Inoculum," pipet 1.0 mL of the inoculum and 9.0 mL of Isoton®. Swirl to mix.

6.12. Perform three counts on the inoculum. Record the counts in cells labeled **R**, **T**, and **V**. Record the MCVs in cells labeled **S**, **U**, and **W**. Cells labeled **X**, **Y**, and **Z** will automatically be calculated.

6.13. If the value in **Z** is within ±5% of the number of cells/mL desired (10,000/mL), the message "Good Concentration" will appear below the inoculum MCVs. The inoculum is ready to be added to the test flasks. If the value in **Z** is not within ±5% of the desired number of cells, the message "Adjust Concentration" will appear.

6.13.1. To adjust a high inoculum concentration, remove **c** mL of inoculum from the beaker and replace with **c** mL of PAAP. Repeat Step 6.12.

6.13.2. To adjust a low inoculum concentration, add **d** mL of suspended concentrated algae to the beaker. Repeat Step 6.12.

6.14. When the inoculum cell concentration is within the correct range, use the HandyStep® pipetter to place 1 mL of inoculum into each of the test flasks.

6.15. Save and print the *Sel cap SRT Inoc* file.

6.16. Refer to SOP TA-7.10 for test set-up and analysis.

7. CALCULATIONS

7.1. Premix concentration (**L**):

[(mean premix count) - (mean background count **G**)] * 200

7.1.1. 200 is the dilution factor. The sample to Isoton® ratio is 1:100 (0.1 mL algae:9.9 mL Isoton®). The Coulter Counter® takes up ½ mL of solution per count.

7.2. Number of cells needed for desired flask concentration (**P**):

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(number of cells/mL required per flask **M**) * (volume per flask **N**) * (number of flasks to be inoculated **O**)

7.3. Volume of suspended concentrated algae needed for inoculum (**Q**):

(number of cells needed for desired flask concentration **P**) ÷ (premix concentration **L**)

7.4. Inoculum concentration (**X**):

[(mean inoculum count) **-** (mean background count **G**)] * 20

7.4.1. 20 is the dilution factor. The sample to Isoton® ratio is 1:10 (1 mL inoculum:9 mL Isoton®). The Coulter Counter® takes up ½ mL of solution per count.

7.5. Actual flask concentration (**Z**):

(inoculum concentration **X**) ÷ (volume per flask **N**)

7.6. Calculated dry weight of *P. subcapitata* inoculum (**Organism Code**):

(actual flask concentration **Z**) * (mean inoculum MCV **Y**) * 3.78×10^{-7}

7.6.1. The organism code is SEL.xxx, where "xxx" is the first three digits after the decimal in the calculated dry weight.

7.6.2. 3.78×10^{-7} is a constant

7.7. Number of cells in inoculum (**a**):

(inoculum concentration **X**) * [(inoculum volume) – 1 mL]

7.8. **b** = (number of cells needed for desired flask concentration **P**) – (number of cells in inoculum **a**)

7.9. Volume of inoculum to remove and replace with equal volume of PAAP (**c**):

(**b** cells) ÷ (inoculum concentration **X**)

7.10. Volume of suspended concentrated algae to add to inoculum (**d**):

(**b** cells) ÷ (premix concentration **L**)

8. DATA ARCHIVAL

8.1. Record data in *Sel cap SRT Inoc* Excel file, which is found in X:\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP's Greatest Hits. Save and print.

9. QUALITY CONTROL

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

9.1.1. Lab supervisor is notified.

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

9.1.3. Section administrator is notified.

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

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

10. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

10.2. Refer to the DEP Laboratory Health and Safety Manual.

10.3. Read MSDS information for reagent handling and disposal.

11. REFERENCES

11.1. 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.

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

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

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

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

11.6. FDEP Laboratory Health and Safety Plan, https://www.fldepnet.org/content/dear Laboratory Quick Links→Health and Safety→Health and Safety Plan http://publicfiles.dep.state.fl.us/DEAR/DEAR%20Safety/EmerActionPlan2016.pdf

11.7. MSDS information sheets.

12. 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.
03/10/2017	Updated SOP to reflect no longer using paper logbook; added test IDs; defined *Pseudokirchneriella subcapitata*; updated Equipment and Supplies; updated links
02/23/2018	Updated link to Health and Safety Plan in the reference section of this SOP

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