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 using green alga *Pseudokirchneriella subcapitata* as the test species. Tests conducted by this procedure are 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 using the "Sel cap Inoc" excel sheet. A known concentration is necessary to provide standardized inoculums for each test replicate. Test samples are then inoculated.

    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

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

    3.2. 15 mL centrifuge tubes, minimum of 4

    3.3. Coulter Counter® Model Multisizer 4e (S/N BA23044)– calibrate and use per SOP TA-06.41

    3.4. 1 Beaker (≥ 100 mL)

    3.5. 100 mL graduated cylinder

    3.6. 30 mL Dilu-vials

    3.7. Long-tipped disposable transfer pipets

    3.8. 100 µL fixed pipet, with tips

    3.9. 1000 µL fixed pipet, with tips

    3.10. 200-1000 µL adjustable pipet, with tips

    3.11. 10 mL sterile, glass serological disposable pipets, with pipet pump

    3.12. HandyStep® pipetter with 50 mL pipet tip

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3.13. Computer with access to DEAR (X:) share drive and LIMS

4. REAGENTS AND STANDARDS

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

4.1.1. Monday initiation requires either 4 or 11 day old cultures

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

4.2.1. 4 L needed during pour-up prior

4.3. Isoton®

4.4. Autoclaved DI water

5. CULTURE PREPARATION OF *P. SUBCAPITATA*

5.1. For sample collection, preservation, and handling, see SOP FS 1000.

5.2. For samples with a salinity of <1.0 ppth, add 14 mL of the *P. subcapitata* alga culture to each of 4 centrifuge tubes.

5.2.1. P. sub SRT requires more algae than an AGP. Analyst experience can determine if more than four tubes of *P. subcapitata* will suffice. The concentrated volumes of the same species are eventually combined.

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

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

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

5.4. Decant the supernatant.

5.5. Add another 14 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.

5.6. Decant the supernatant. Using a transfer pipet, remove one algae pellet and add it to the second tube. Add another 14 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.

5.7. Centrifuge at 1000 rpm for 10 min. Repeat step 5.6 and centrifuge spin until 1 centrifuge tube with algae is left.

5.7.1. Ex. With 4 centrifuge tubes, algae is added after the 1st decant to all 4 tubes. After the 2nd decant, transfers occur reducing the # of tubes down to 2. More algae is added. 3rd decant brings 2 tubes down to 1 and algae is

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added one last time. A 14 mL centrifuge tube with DI water is used to balance the centrifuge.

5.8. Perform three more spins and four rinses with autoclaved DI water. Refer to SOP TA-08.03 steps 6.6-11.

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 Multisizer 4e for cell counting per SOP TA-06.41. When creating the run ID, use code "BSY-CD-FSC." Sample ID and treatment will be the same (i.e. CTRL, 40 UG/L, 60 UG/L etc.).

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.

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. So 24 flasks would make the number 124.

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.

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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 to the dated folder for the current test within the "AGP/P. sub SRT/2023" folder.

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**):

(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**):

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(actual flask concentration **Z**) * (mean inoculum MCV **Y**) * 3.78 x 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 x 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.

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

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

8.3.1. The corrected entry is written as near as possible to the error or in the comment box on the appropriate bench sheet.

8.3.2. If the correction requires an explanation, write a comment and reference the error with a letter. Initial and date the explanation. Do not attempt to put all information (correction and comments) into a small space.

8.3.3. 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.).

8.4. FDEP Laboratory Information Management System (LIMS)

9. QUALITY CONTROL

9.1. Refer to DEP Laboratory Quality Manual for corrective action and handling out-of-control data. Refer to *EPA-821-R-02-013, Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* Method 1003.0 for test conditions and acceptability criteria.

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

9.2.1. Lab supervisor is notified.

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

9.2.3. Section administrator is notified.

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

9.3. 9.2.5 To create NCR: open LIMS and go to Laboratory/Administrative/Non-Conformance Report. Refer to https://floridadep.gov/dear/quality-assurance/content/dep-sops for sample collection and preservation, and the Laboratory Quality Manual for corrective action and handling out-of-control data. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

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

9.6. Read MSDS information for reagent handling and disposal.

10. REFERENCES

10.1. EPA 821-R02-013-2002 Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Ed. October 2002.

10.2. DEP FS 1000, General Sampling

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

10.4. DEP SOP TA-06.41, Calibration and use of the Coulter Counter Model Multisizer 4e

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

10.6. DEP SOP TA-07.10, *Pseudokirchneriella subcapitata* Chronic Toxicity Test Method

10.7. DEP SOP TA-08.03, Inoculation of Algal Assay Tests

10.8. FDEP Laboratory Quality Manual. DEP Laboratory Quality Assurance Manual and SOPs | Florida Department of Environmental Protection

10.9. FDEP Laboratory Health and Safety Plan, https://www.floridadep.net/resources/health-safety-manual

10.10. MSDS information sheets.

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11. APPENDIX OF SIGNIFICANT 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

02/01/2021 Updated equipment and links

02/02/2022 Updated culture preparation section with increased algae tubes and rinse procedure. Added SOP for reference. Updated links.

02/08/2023 Increased centrifuge tubes required. Clarified run ID. Added SOPs referenced.

01/25/2024 Checked for readability and link updates.

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