Inoculation of Algal Assay Tests

1. SCOPE AND APPLICATION

1.1. This method is based on *EPA-600/9-78-018, The* Selenastrum capricornutum *Printz Algal Assay Bottle Test* and *EPA 1974, Marine Algal Assay Bottle Test*. It is used to add a known concentration of algal test species into test solution. It is applicable to both freshwater and salt-water test species.

2. SUMMARY OF METHOD

2.1. Test algae are collected, concentrated, diluted to the appropriate concentration for inoculation, and then added to test solutions. Test vessels are placed in an environment with controlled temperature and lighting.

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*:** freshwater algae; formerly known as *Selenastrum capricornutum*; used for Algal Growth Potential (AGP) tests and food for *Ceriodaphnia dubia*; labeled as P. sub

2.3.2. ***Dunaliella tertiolecta*:** saltwater algae; used for marine AGP tests; labeled as Dun

3. EQUIPMENT AND SUPPLIES

3.1. 15 mL centrifuge tubes, 2 or more

3.2. Centrifuge, located in qPCR lab (B246A)

3.3. Thermo Scientific Incubator maintained at 20±2 ˚C with 24-hour fluorescent lighting (360-440 foot-candles)

3.4. Shaker racks maintained at 25 ± 3 °C with 24-hour fluorescent lighting (360-440 foot-candles) and rotating at 100 ± 10 rpm

3.5. 400 mL Beaker (or any beaker 25-50% larger than volume of inoculum)

3.6. 100 mL Graduated cylinder

3.7. Eppendorf repeater pipettor and 50 mL tip(s)

3.8. Coulter® Counter Multisizer 4e, calibrated and used per SOP TA-06.41

3.9. 30 mL Dilu-vials

3.10. 10 mL Serological disposable pipet, sterile and individually wrapped, with pump

3.11. Finnipipette 100 µL pipettor and tips

3.12. Finnipipette 1,000 µL pipettor and tips

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3.13. Finnipipette II 200-1000 µL adjustable pipettor and tips

3.14. Green and red label tape

3.15. Permanent black marker

3.16. *AGP Sample Log-in Record*

3.17. *Master Setup Sheets* Excel file, located in X:\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP's Greatest Hits

3.17.1. In the "Main" tab, enter Set-up Date and Analyst.

3.17.2. In the "Setup" tab, enter sample information. Print.

3.17.3. Print the "Background" tab.

3.17.4. In the "CtRcrd10, 12, 14" tab, change the Count Day to 10 and print. Change the Count Day to 12 and print. Change the Count Day to 14 and print.

3.17.5. If running a freshwater test, print the "Map" tab.

3.18. *Inoculation Setup Sheets* Excel file, located in X:\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP's Greatest Hits

3.18.1. Start Date, Analyst, and Background Count must be entered on the FWInoc tab. All other entries should be entered in the tab appropriate to the algae species being used. FWInoc for P. sub, SWInoc for Dun.

3.19. Computer with access to FDEP Laboratory Information Management System (LIMS)

4. REAGENTS AND STANDARDS

4.1. Alga culture, 4-8 days old (cultured per SOP TA-04.08)

4.1.1. Sample salinity <1.0 ppth: *Pseudokirchneriella subcapitata*

4.1.2. Sample salinity >7.0 ppth: *Dunaliella tertiolecta*

4.1.3. Sample salinity between 1.0 ppth and 7.0 ppth: *Dunaliella tertiolecta*

4.1.3.1. Adjust sample salinity per SOP TA-08.01 before inoculation.

(All samples should have a final salinity of either <1 ppth or ≥7.0 ppth.)

4.2. Coulter Isoton®II diluent

4.3. Autoclaved deionized (DI) water for samples at salinity <1.0 ppth

4.4. Filtered ASW basal stock solution (at room temperature) for samples at salinity ≥7.0 ppth (prepared per SOP TA-09.10)

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

5.1. See SOP FS 1000 for sample collection and preservation.

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6. CULTURE PREPARATION

6.1. Label the centrifuge tubes to be used for *P. subcapitata* with green tape. Label the centrifuge tubes to be used for *D. tertiolecta* with red tape.

6.1.1. More than two tubes can be used if a large quantity of inoculum is needed. The concentrated volumes of the same species can then be combined. Typically, the FDEP lab uses four centrifuge tubes.

6.2. Pour 10 mL of the appropriate alga culture into each of the marked centrifuge tubes. Replace and tighten the tube caps.

6.3. Distribute the tubes in the centrifuge so that the load is balanced. Centrifuge at 1,000 rpm for 10 minutes.

6.4. Decant and discard the supernatant.

6.5. Repeat Steps 6.1-6.4 as needed until sufficient algae are collected to prepare the inoculum, then go to Step 6.6. Regardless of the number of tubes used, there will always be three decants before the rinsing process.

6.5.1. Generally, 50 mL (or half a flask) of cultured algae is used to reach a sufficient amount of concentrated algae if two centrifuge tubes are used.

6.5.2. Generally, 70 mL (or nearly a full flask) of cultured algae is used if four centrifuge tubes are used. This is the most common practice in our lab.

6.6. If the test alga is *P. subcapitata*, add 10 mL of autoclaved DI water to the centrifuge tube. If the test alga is *D. tertiolecta*, add 10 mL of filtered ASW basal stock solution to the centrifuge tube.

6.6.1. Adding the liquid suspends and washes the algae and removes nutrients that were in the culture medium.

6.7. Tighten the caps on the centrifuge tubes and shake them vigorously to re-suspend the algae. A plastic pipet can also be used to re-suspend the algae.

6.8. Centrifuge again at 1,000 rpm for 10 minutes.

6.9. Decant and discard the supernatant.

6.10. Repeat Steps 6.6.-6.9 two more times (total of 3 rinses), then proceed to Step 6.11.

6.11. After the third rinse decant. If the test alga is *P. subcapitata*, add 10 mL of autoclaved DI water to the centrifuge tube. If the test alga is *D. tertiolecta*, add 10 mL of filtered ASW basal stock solution to the centrifuge tube. Replace the caps and shake the tubes to suspend the now concentrated algae.

6.12. Using SOP TA-06.41, prepare the Coulter® Counter model Multisizer 4e for cell counting. Save the background counts in the Background folder located in X:\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP Counts 17.

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6.13. Open the *Inoculation Setup Sheets* Excel file and fill in cells A-F with the day's background count. The mean of the three counts will be recorded in the space labeled "G."

6.13.1. Locate the most updated sheets in X:\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP's Greatest Hits. "Save As" in a new folder for the specified year and initiation date.

6.13.2. Ex: \\fldep1\Dear\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP Counts 20\9.4.20

6.13.2.1. "AGP Counts 20" is the year. A new folder is created within for initiation date.

7. DETERMINING CELL CONCENTRATION

7.1. Shake the tube to suspend the algae in the centrifuge tube. Place 0.1 mL of the appropriate algae in a 30-mL vial and add 9.9 mL of Isoton. Swirl to mix. This mixture is often referred to as the premix.

7.2. Perform three counts on the mixture in the vial. Record these counts in the *Inoculum Setup Worksheet* in the cells labeled "I," "J," and "K." Make sure the appropriate algae sheet is open—freshwater or saltwater.

7.3. The background count will be subtracted from the average of these three numbers. The resulting number multiplied by 200 is the cell concentration, which will appear in the cell labeled "L."

7.3.1. The dilution ratio for the subsample being counted to the Isoton solution is 1:100. 0.5 mL of solution is taken up by the Coulter® Counter for each count. The total dilution factor for the stock solution is then 200.

7.3.2. The concentration of the stock solution of algae is calculated by multiplying the average count minus the background by the dilution factor of 200.

7.4. Verify that the desired final concentration of cells/mL appears in the cell labeled "M." Usually, the concentrations are 1000 cells/mL for *P. subcapitata* and 400 cells/mL for *D. tertiolecta*. If a different concentration is desired, modify the set-up sheet.

7.5. Record the number 62 in the space labeled "N." This is the average mL of solution in each flask of a limiting nutrient test after inoculation with the test alga. Make SURE the Excel file is on the correct sheet.

7.5.1. 62 mL is derived from 60 mL of sample plus 1 mL of nutrient spike plus 1 mL of inoculum, although some flasks receive up to three additional mL of nutrient spikes for a total of 64 mL.

7.5.2. If only AGP samples are being set up, then use 61 in the "N" space and other appropriate spaces.

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7.6. Determine the number of flasks you have to inoculate, add 100 to this number and enter it in the cell labeled "O." This is the amount of inoculum to be prepared.

7.7. The calculations on the sheet will produce the volume of concentrated cells needed in the inoculum "Q."

7.7.1. Cells desired in each mL of sample "**M**" times the number of mL per flask "**N**" times the number of flasks "**O**" equals the number of cells needed "**P**" in the inoculum flask in "**O**" mL.

7.7.2. The concentration needed "**P**" divided by the amount in the stock solution "**L**" gives the amount to be diluted "**Q**" in "**O**" mL.

8. INOCULATION

8.1. Pipet "Q" mL from the centrifuge tube containing the test algae and place into the 100 mL graduated cylinder.

8.2. Add the appropriate water (autoclaved DI or filtered ASW basal stock solution) to the graduated cylinder containing **Q** mL of test algae and bring up to 100 mL. (The amount of water added is **O - Q** mL.)

8.3. Pour the contents of the cylinder into the beaker and swirl to mix. Pipet 1.0 mL from the beaker and place into a 30 mL vial. Add 9.0 mL of Isoton to the vial.

8.4. Perform three counts on the mixture in the vial. Record the three cell counts on the *Inoculum Setup Worksheet* in the cells labeled "R," "T," and "V." Record the mean cell volume from each count in the cells labeled "S," "U," and "W." These counts are used to calculate the concentration of the inoculum.

8.5. The background count will be subtracted from the average of the numbers "R," "T," and "V." The resulting number multiplied by 20 is the inoculum concentration, which will appear in the cell labeled "X."

8.5.1. 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.

8.6. The number represented by "X" will be divided by the number represented by "N" (61 or 62). This gives the concentration of algal cells in each flask if inoculated with the prepared inoculum. This number auto-populates in the space labeled "Z."

8.7. If the number recorded in the space labeled "Z" is within ±10% of the number of cells/mL desired for the inoculum, 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, add more algae or DI water/ASW basal stock solution, depending on the test algae, and repeat Steps 8.4.-8.6.

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8.8. Use the Eppendorf repeater-pipettor and 50 mL tips to place 1 mL of inoculum into each test flask: *P. subcapitata* into flasks <1.0 ppth salinity, *D. tertiolecta* into flasks ≥7.0 ppth salinity. Use one sterile pipette tip per species.

8.9. Save the *Inoculation Setup Sheet* and print. The printed copy is placed in a folder that will contain all paperwork associated with an AGP/LN test (inoculation sheet, setup sheets, count sheets, etc.).

9. INCUBATION

9.1. Using a large cart, transport the flasks from the algal assay set up table to the appropriate incubators, randomizing the flasks during the process. Freshwater tests are placed on shaker racks maintained at 25 ± 3 °C with 24-hour fluorescent lighting (360-440 foot-candles) and rotating at 100 ± 10 rpm (make sure they are turned off when placing flasks in the flask holders). Saltwater tests are placed in an incubator maintained at 20 ± 2 ˚C with 24-hour fluorescent lighting (360-440 foot-candles).

9.1.1. Using an AGP/LN map sheet to prepare a map of where each flask is located is extremely helpful when it comes time for counting. Each map sheet has four lined columns. Each line is numbered 1-21. A flask code is placed above each column. The rack ID and position of the flask on the rack is recorded on the map sheet under the appropriate flask ID. The shaker holds flasks in a 5x8 matrix. The right front corner is position 1, and the left rear corner is position 40. The positions on the rear of a shaker, from right to left, would be 5, 10, 15, 20, 25, 30, 35, and 40.

9.2. If the Culture Lab temperature is out of tolerance, immediately notify the building manager (first floor, Twin Towers). If the temperature of Incubator #2 is out of tolerance, notify the toxicity group supervisor.

10. DATA ARCHIVAL

10.1. *Master Setup Sheets* Excel file, located in X:\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP's Greatest Hits

10.2. *AGP Sample Log-in Record*

10.3. *Inoculation Setup Sheets* Excel file, located in X:\Labs\Lab5\Biology\Bench Common\ToxCommon\AGP\AGP's Greatest Hits

10.4. FDEP Laboratory Information Management System (LIMS)

10.5. Algal Assay Light Measurement Log

10.6. Daily Temperature Log

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

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10.8. 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.

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

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

10.8.3. 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.).

11. QUALITY CONTROL

11.1. Glassware is washed, autoclaved, and stored in accordance with DEP SOP BG-05 to minimize the potential for contamination of the stock solution.

11.2. Aseptic techniques are used to help prevent the contamination of samples and cultures.

11.3. Incubator and culture room temperatures are monitored continuously by an electronic data logger (per SOP TA-06.28). Incubator and culture room are also manually checked daily and their temperatures recorded in the *Daily Temperature Logbook* and in the LIMS Laboratory Logbook Temperature Log.

11.4. If the Culture Lab temperature is out of tolerance, immediately notify the building manager (first floor, Twin Towers). If the incubator temperature is out of tolerance, notify the toxicity group supervisor.

11.5. Fans in the culture lab can be adjusted to assist in maintaining uniform temperatures.

11.6. The rotation of the shaker racks is verified monthly to be 100 ± 10 rpm. The rpm is adjusted as needed by turning the speed adjustment knob and counting the rack rotations. The verification is recorded in the *Algal Assay Light Measurement Log*.

11.7. Light intensity is verified monthly and recorded in the *Algal Assay Light Measurement Log*.

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

11.8.1. Lab supervisor is notified.

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

11.8.3. Section administrator is notified.

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

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

11.9. Refer to http://www.dep.state.fl.us/water/sas/sop/index.htm for sample collection and preservation, and the Laboratory Quality Manual for corrective action and handling out-of-control data.

12. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

12.2. The shaker rack can snare loose clothing, especially lab coats, and can cause serious bodily injury. BE CAREFUL! The shaker racks are operated by a timer system that activates the shakers twice daily for a 30-minute period. Always make sure the on/off switch is in the "off" position when placing flasks on and removing flasks from the shaker rack.

12.3. When replacing fluorescent lamps, make sure the shaker rack and the light switch are both turned off.

12.4. Refer to Bureau Quality Manual for sample disposal

12.5. Refer to the Laboratory Health and Safety Manual and Hazardous Waste Management

12.6. Read MSDS information for reagent handling and disposal.

13. REFERENCES

12.7. The *Selenastrum capricornutum Printz* Algal Assay Bottle Test, EPA-600/9-78=018.

12.8. *Marine Algal Assay Bottle Test*, EPA 1974.

12.9. DEP SOP FS-1000, General Sampling Procedures.

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

12.11. DEP SOP TA-08.01, Initial Algal Assay Sample Handling.

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

12.13. DEP SOP TA-06.28, Using Data Loggers to Record Temperatures.

12.14. DEP SOP TA-09.10, Artificial Seawater Basal Stock Solution for Culturing Marine Algae.

12.15. DEP SOP BG-05, Biology Wash Party Procedures.

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12.16. Laboratory Quality Manual
http://publicfiles.dep.state.fl.us/dear/labs/lab_qualitymanual_19.pdf

12.17. Laboratory Health and Safety Manual
http://publicfiles.dep.state.fl.us/DEAR/DEAR%20Safety/FDEP%20Health%20%20Safety%20Plan%20B%20-%20June%202016.pdf

12.18. MSDS Information Sheets

APPENDIX OF CHANGES

12/20/2007	Added Scope and Application; Summary of Method, and Appendix of Changes.
11/17/2008	Added Interference and Reference sections.
08/14/2013	Reformatted SOP.
01/28/2015	Formatted for 508 compliance.
07/14/2015	Updated links.
07/14/2016	Updated links.
09/13/2017	Added definitions to the Definitions section; added temperature and light intensity requirements in the Equipment and Supplies section and throughout the SOP as necessary; updated file locations throughout the SOP
08/02/2019	Updated links
09/03/2020	Update Coulter Counter and SOP used. Added to culture preparation section. Updated data archival section with universal language.

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