Set-Up and 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 determine the algal growth potential of fresh and marine waters.

2. SUMMARY OF METHOD

2.1. Prepped algal growth potential samples are poured into test flasks. 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. ***Raphidocelis subcapitata*:** freshwater algae; formerly known as *Selenastrum capricornutum*; used for freshwater Algal Growth Potential (AGP) tests and food for *Ceriodaphnia dubia*; labeled as R. sub

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

3. EQUIPMENT AND SUPPLIES

3.1. 250 mL autoclaved Erlenmeyer flasks with autoclaved foam plugs

3.2. 100 mL Graduated cylinder

3.3. 50 mL sterile pipettes

3.4. 15 mL centrifuge tubes

3.5. Centrifuge, located in PCR lab (B246B)

3.6. Thermo Scientific Incubator (Inc #7) maintained at 20±2˚C with 24-hour fluorescent lighting (360-440 foot-candles)

3.7. MRC LOM-150-LIGHT Incubator (Inc #9) with shaker rack maintained at 25 ± 3°C with 24-hour fluorescent lighting (360-440 foot-candles) and rotating at 100 ± 10 rpm

3.8. 200 mL Beaker (or any beaker larger than the volume of inoculum)

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

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

3.11. 30 mL Dilu-vials

3.12. 100 µL Finnpipette pipettor and tips

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3.13. 1,000 µL Finnpipette pipettor and tips

3.14. 200-1000 µL Finnpipette II adjustable pipettor and tips

3.15. *AGP Sample Log-in Record*

3.16. Prepared *Master Setup Sheet* Excel file, located in \\floridadep\data\DEAR\Labs\Lab5\Biology\BenchCommon\ToxCommon\AGP\AGP's Greatest Hits

3.17. 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: *Raphidocelis 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 SET UP

5.1. Prepare AGP samples per SOP TA-08.01 and lab blanks per SOP TA-08.08.

5.2. Create a run ID. Go to LIMS/Laboratory/Biology/AGP LN Manager and “Set up a new run”. Select appropriate test, algae type, analyst, and number of flasks. Set time of setup to 1500 if time is unknown or to the approximate time the samples will be inoculated. Press auto-setup. Run ID format is “AGP-yyyy-mm-dd-01”.

5.2.1. Name freshwater blanks LAB BLANK and saltwater blanks SALT BLANK. Samples are names as just their sample ID number from LIMS. Freshwater duplicates are named #######-DUP, while salinity-adjusted and saltwater duplicates are #######-SUP. (The “#” signify the Sample ID.)

5.2.1.1. Naming these correctly allows them to upload to LIMS, if names are deviated from the module will not function properly.

5.3. Arrange the autoclaved flasks in sets of 3, one set per sample. Fill out the sample “Set-up” sheet in the “Master Setup Sheet” in “AGP’s Greatest Hits” folder and save in run folder (titled with test initiation date saved in AGP\AGP Counts yy) as “Master

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Setup Sheet mm.dd.yy". Assign one lab duplicate per 10 samples and one lab blank per 20 samples, with a minimum of one duplicate and one blank per run.

5.3.1.1. A sample uses three flasks, but this is still only considered one sample for QC purposes.

5.3.2. A minimum of 360 mL of sample is needed per laboratory duplicate. Do not duplicate a field blank or field duplicate.

5.4. Invert the sample bottle to mix. Pipette 60 mL of sample or lab blank into each flask using a new pipette (or rinsing a graduated cylinder) between each new sample. Leave samples on set-up table overnight prior to inoculating. Plug each flask and store excess sample in the walk-in refrigerator.

5.4.1. The flasks and stoppers should be autoclaved the day before initiation (Thursday) and filled with 60 mL of sample after they have cooled and left to sit overnight for initiation on Friday.

6. CULTURE PREPARATION

6.1. Label the centrifuge tubes. If using both species of algae, label *R. subcapitata* with green tape and *D. tertiolecta* with red tape.

6.1.1. Typically, the FDEP lab uses four centrifuge tubes for each species of algae needed.

6.2. Pour 10 mL of the appropriate alga culture into each of the marked centrifuge tubes and recap.

6.3. Distribute the tubes in the centrifuge so that the load is balanced. Centrifuge at 1,300 rpm for 10 minutes. This is program 6 on the current centrifuge.

6.4. Decant and discard the supernatant.

6.5. Combine the four tubes of algae down to two tubes by pipetting out the algae pellet and adding it to another tube.

6.6. Repeat steps 6.2.-6.4.

6.7. Combine the two tubes down to one.

6.7.1. There should always be an even number of tubes in the centrifuge for balancing purposes. When an uneven number of tubes are in use, fill an empty tube with an equivalent amount of water and use to balance the centrifuge.

6.8. Repeat steps 6.2.–6.4.

6.9. Rinse the sample by adding 10 mL of autoclaved DI water (for *R. subcapitata*) or 10 mL of filtered ASW basal stock solution (for *D. tertiolecta)* to each algae filled centrifuge tube.

6.10. Tighten the caps and resuspend by shaking vigorously or using a plastic pipette.

6.11. Centrifuge at 1,300 rpm for 10 minutes. Program 6.

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6.12. Decant and discard the supernatant.

6.13. Repeat Steps 6.9.-6.12 two more times.

6.14. Add 10 mL autoclaved DI water (for *R. subcapitata*) or 10 mL filtered ASW basal stock solution (for *D. tertiolecta*). Shake the tubes to resuspend. This will be your working vial of algae for inoculating.

6.15. 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 \\floridadep\data\DEAR\Labs\Lab5\Biology\BenchCommon\ToxCommon\AGP\AGP Counts [YY]\Background.

6.15.1. A new folder is made each year.

6.16. Prepare the *Master Setup Sheet* Excel file, located in \\floridadep\data\DEAR\Labs\Lab5\Biology\BenchCommon\ToxCommon\AGP\AGP's Greatest Hits.

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

6.16.2. In the "Setup" tab, enter sample information.

6.16.3. Fill in the Inoculation Setup Tabs (FWInoc or SWInoc)

6.16.3.1. 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 R. sub, SWInoc for Dun.

6.16.3.2. Fill in cells A-F with the day's background count. The mean of the three counts is recorded in "G".

6.16.4. Save the file as "Master Setup Sheet MM.DD.YY" in in the current run folder located at \\floridadep\data\DEAR\Labs\Lab5\Biology\BenchCommon\ToxCommon\AGP\AGP Counts [YY]\[MM.DD.YY].

7. DETERMINING CELL CONCENTRATION

7.1. Shake the tube to suspend the algae. Place 0.1 mL of algae in a 30 mL Dilu-vial labeled "premix" and add 9.9 mL of Isoton. Swirl to mix. This mixture is the "premix".

7.2. Perform three counts on the mixture in the vial. Record these counts on the *Inoculum Setup* tabs in cells "I," "J," and "K" using the appropriate tab from the "Master Setup Sheet", freshwater or saltwater.

7.3. The background count is subtracted from the average, and this is multiplied by 200 to give the cell concentration, "L".

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7.4. The desired final concentration of cells/mL appears in cell “M”. The default concentrations are 1000 cells/mL for *R. subcapitata* and 400 cells/mL for *D. tertiolecta*.

7.5. Enter “61" in cell “N” (60 mL sample + 1 mL inoculum).

7.6. Determine the number of flasks that must be inoculated. Add 100 to this number and enter it in cell “O”. This is the amount of inoculum to be prepared.

7.6.1. For example: 3 blank flasks, 3 sample flasks, 3 duplicate flasks = 9 flasks + 100 = 109 flasks in “O”.

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 into the 100 mL graduated cylinder. Bring up to 100 mL with the appropriate water (autoclaved DI or filtered ASW basal stock solution).

8.2. Pour into the beaker and swirl to mix. Pipet 1.0 mL from the beaker into a 30 mL Dilu-vial labeled “Inoc”. Add 9.0 mL of Isoton to the vial.

8.3. Perform three counts on the mixture in the vial. Record counts on the *Inoculum Setup* tabs in the cells labeled “R,” “T,” and “V”. Record the mean cell volume from each count in the cells labeled “S,” “U,” and “W”.

8.4. The background count is subtracted from the average of the numbers “R,” “T,” and “V.” The resulting number multiplied by 20 is the inoculum concentration, which appears in cell “X”.

8.5. The number in “X” is divided by the number in “N”. This is the concentration of algal cells in each flask if inoculated with the prepared inoculum. This number auto-populates in the space labeled “Z”.

8.6. If the number in “Z” is within 10% of the desired concentration, 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 counts.

8.6.1. In the Inoculum Count Check MCV box (below “Y”) the words “Good Concentration” or “Adjust Concentration” will appear. If it says “Good Concentration” the inoculum is ready to use, move on to step 8.7. If the

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words “Adjust Concentration” appear, adjust the inoculum by following the instructions in the Master Setup Sheet.

8.6.1.1. In the “If Z < 900 or Z > 1100” box, find the value of “**b**”.

8.6.1.2. In the boxes below that, if “**b”** < 0 remove “**c**” mL from the inoculum and replace it with “**c**” mL of appropriate water. If “**b**” > 0 then remove “**d**” from the inoculum and add “**d**” mL working algae from the vial.

8.6.1.3. Run the concentration on the Multisizer again and repeat steps 8.3 to 8.6 until you get a “Good Concentration”.

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

8.8. Save the Master Setup Sheet.

9. INCUBATION

9.1. 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 the shaker rack in Inc #9 maintained at 25 ± 3°C with 24-hour fluorescent lighting (360-440 foot-candles) and rotating at 100 ± 10 rpm. If there is an excess of flasks and the room temperature is within range, freshwater samples can be placed on the shaker racks in the culture room maintained at 25 ± 3°C with 24-hour fluorescent lighting (360-440 foot-candles) and rotating at 100 ± 10 rpm. If the room is out of temperature, the freshwater tests are placed in the appropriate stationary incubator (usually Inc #8), separate from the saltwater tests, at a temperature of 25 ± 3°C with 24-hour fluorescent lighting (360-440 foot-candles). These samples will need to be placed on the shaker racks for an hour twice a day rotating at 100 ± 10 rpm or hand swirled twice a day. Saltwater tests are placed in an incubator maintained at 20 ± 2˚C with 24-hour fluorescent lighting (360-440 foot-candles) (Inc #7) and hand swirled at least once a day.

10. DATA ARCHIVAL

10.1. *Master Setup Sheet* Excel file, located in \\floridadep\data\DEAR\Labs\Lab5\Biology\BenchCommon\ToxCommon\AGP\AGP's Greatest Hits

10.2. *AGP Sample Log-in Record*

10.3. FDEP Laboratory Information Management System (LIMS)

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

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10.5. 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.

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

10.5.2. 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.

10.5.3. Use sequential alphabetical annotation to identify errors and corresponding comments.

11. QUALITY CONTROL

11.1. AGP samples are prepared, filtered, and stored in the walk-in refrigerator per SOP TA-08.01. If a sample's initial pH was <6.0 or >10.0, re-check the pH and adjust the sample prior to set-up per SOP TA-08.06.

11.2. Lab blanks are prepared per SOP TA-08.08.

11.3. AGP tests require 3 flasks per sample, 3 flasks per laboratory blank, and 3 flasks per laboratory duplicate.

11.4. Foam plugs should be replaced semi-annually or when they crumble or change color, or after use in a saltwater/salt adjust test.

11.5. Glassware is washed, autoclaved, and stored per DEP SOP BG-05.

11.6. Aseptic techniques are used to prevent contamination of samples and cultures.

11.7. Incubator and culture room temperatures are monitored continuously by an electronic data logger system.

11.8. If the Culture Lab or incubator temperature is out of tolerance, notify the toxicity group supervisor.

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

11.10. The rotation of the shaker racks is verified to be 100 ± 10 rpm. The rpm is adjusted as needed by turning the speed adjustment knob and counting the rack rotations.

11.11. Light intensity is verified monthly and recorded in the *Light Intensity Measurement Log*.

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

11.12.1. Lab supervisor is notified.

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

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11.12.3. Section administrator is notified.

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

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

11.13. Refer to FS1000 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 bodily injury. The shaker racks are operated by a timer system that activates the shakers twice daily for a 60-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 Laboratory Quality Assurance Manual for sample disposal.

12.5. Refer to the Laboratory Health and Safety Manual.

12.6. Read MSDS information for reagent handling and disposal.

13. REFERENCES

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

13.2. EPA 1974, Marine Algal Assay Procedure Bottle Test

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

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

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

13.6. DEP SOP TA-08.06, pH Adjustment of Samples: Algal Assay and Toxicity Bioassays

13.7. DEP SOP TA-08.08, AGP Lab Blank Analysis and Blank Data Review

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

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

13.10. FS1000, General Sampling

13.11. DEP Laboratory Quality Assurance Manual

13.12. DEP Laboratory Health and Safety Manual

13.13. 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.

01/28/2015 Formatted for 508 compliance.

09/07/2023 Combined TA-08.02 & TA-08.03.

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