Diatom Preparation for Periphyton and Phytoplankton

1. SCOPE AND APPLICATION

1.1 This method details the process of clearing diatoms in phytoplankton or periphyton samples and preparing a fixed, mounted slide for the diatom identification process.

1.2 This method is used for quantitative or qualitative periphyton or phytoplankton samples collected by any method. Because diatoms are typically less abundant in phytoplankton, phytoplankton samples have been concentrated (SOP AB-11). An aliquot of the concentrated sample will be used in this clearing and mounting process. Periphyton samples are not concentrated since they normally contain a large number of diatoms (SOP AB-10.1). An aliquot of the well mixed periphyton sample will be used in this clearing and mounting process.

1.3 This method is to be used for all samples with Laboratory Information Management (LIMS) test codes beginning with “DTM” or “DTY”.

2. SUMMARY OF METHOD

2.1 Phytoplankton or periphyton samples to be cleared and slide mounted for diatom identification are located and a LIMS prep worksheet is created.

2.2 For clearing, test tubes are placed in a water bath. The organic material is cooked off using a combination of 2N nitric acid (HNO_3) and heat (90-100 °C).

2.3 Once the sample is cleared, the material is rinsed by filling the test tube with deionized (DI) water, centrifuging, and removing the supernatant. This process is repeated until the pH reaches between 6 and 7, indicating that the acid has been removed.

2.4 The cleared sample is pipetted onto a coverslip, dried, and affixed to a microscope slide with mounting media.

2.5 Once the mounted slide has dried, it is ready for analysis (identification).

3. APPARATUS AND EQUIPMENT

3.1 Electric hot plate (Thermo Scientific Cimarec+™)
3.2 Glass test tubes, 16 x 150mm (20 mL)
3.3 Test tube covers, size 16
3.4 Test tube rack
3.5 Beaker, 1000 mL
3.6 Masking tape and permanent marker
3.7 Plastic or glass disposable volumetric pipettes, 10 mL
3.8 Glass microscope slides, 25 x 75 mm
3.9 Coverslips, 22 mm x 22 mm
(18 mm diameter coverslips can also be used. Make sure the coverslip has sufficient area to dry enough sample that 500 diatom valves can be identified.)
3.10 Forceps (tweezers)
3.11 Disposable transfer pipettes, 3 mL
3.12 Digital thermometer, calibrated per Standard Operating Procedure (SOP) TA-06.13
3.13 pH test paper
3.14 Pencil with eraser bottom

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3.15 Centrifuge

4. REAGENTS AND CHEMICALS

4.1 Nitric Acid (HNO_3), 2N
4.2 Mounting medium with refractive index of at least 1.7.
(Examples of media that have this refractive index are Naphrax®, Hyrax®, or Zrax®. Mounting media is stored in a balsam bottle with a glass stirring rod.)
4.3 Deionized (DI) water

5. SAMPLE COLLECTION AND PRESERVATION

5.1 Samples are collected, preserved, and delivered to the lab according to FS 7000 – *General Biological Community Sampling*.

5.2 Samples are checked into the Laboratory per SOP AB-10 or AB-11.

6. SAMPLE PREPARATION PROCEDURE

6.1 For **phytoplankton** samples (DTY-), do not check out the sample using the LIMS Custody utility. The LIMS Custody utility is only used to track the original sample container. Phytoplankton samples have been prepared per SOP AB-11 and already transferred from the original sample to another container. Proceed to step 6.2.

For **periphyton** samples (DTM-), check out the sample using the LIMS Custody utility following step 6.1.1 and then proceed to step 6.2.

6.1.1 In LIMS, select Laboratory/Receiving/Custody, choose CheckOut. Using the hand-held barcode reader; scan the barcodes on sample labels on sample containers. Click [OK]. Scan the barcode on the back of the employee ID badge two times. Close window using Custody/Exit [Alt+F4].

Note: Using a computer that has a barcode reader attached makes this process quicker, but if the barcode reader is unavailable or not functioning, manually enter the LIMS number of the sample.

6.2 Using the Laboratory/Technical/Prep Worksheet Manager utility in the LIMS, create a new prep worksheet for each sample or batch of samples processed per SOP AB-02.1. Log out of LIMS and log off the computer.

6.3 Find phytoplankton and periphyton samples that are going to be used for slide preparation. Put on the appropriate safety equipment (e.g. gloves, lab coat, and protective glasses).

Note: Samples will be in 50 mL centrifuge tubes near the sink in the Algal Lab (B227A) or in the B227 walk-in refrigerator across from the Algal Lab, organized by LIMS number.

6.4 Boiling:

6.4.1 Label one 20 mL glass test tube for each sample being prepped with the sample number on a strip of masking tape. Place the label about a third of the way down from the top. Place these test tubes into a test tube rack.

Note: It is helpful to place a lid on the tube and affix the label right under the rim so it will not interfere with lid placement.

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6.4.2 For **periphyton** samples:

6.4.2.1 Shake each 50 mL periphyton sample tube vigorously, and immediately pour approximately 5 mL into the corresponding labeled 20 mL glass test tube for that sample.

6.4.2.2 Proceed to step 6.4.4.

6.4.3 For **phytoplankton** samples:

6.4.3.1 Perform the first concentration phase either through settling (6.4.3.2) or centrifuging (6.4.3.3).

6.4.3.2 Settling for 10 hours is sufficient to meet the recommended settling time of 1mm per hour of column depth, according to standard methods. If the 50 mL tube has settled undisturbed for 10 hours or more, there should be a plug of algae in the bottom. Without disturbing the plug, use a disposable pipette to remove all but 10 mL of the excess water above the plug. The excess can be discarded into a waste beaker. Waste has formalin. Dispose of it properly in the waste formalin container.. Proceed to step 6.4.3.4.

6.4.3.3 Centrifuging is necessary if the 50 mL tube hasn't had sufficient time to settle or has no plug. Place the 50 mL phytoplankton sample tubes into the centrifuge in culture room B246A.

Note: **Make sure samples are evenly spaced around the centrifuge before starting.** If you have an odd number of centrifuge tubes so that the centrifuge is unevenly balanced, fill an empty centrifuge tube with an equal amount of water and place it in to balance out the centrifuge. Different heads for the centrifuge are in the drawer labeled as centrifuge supplies beside the centrifuge in B246B.

Adjust the centrifuge to spin at 1450 rpm and set the timer for 5 minutes or use the pre-programmed #2 setting for algae samples. Start the centrifuge.

Once the centrifuge has completely stopped, gently take the samples out.

Work in a fume hood. Without disturbing the settled material, carefully pipette off the top layer of water until there is approximately 10 mL of sample left in the 50 mL tube. Rinse pipette with DI water between samples. Continue to step 6.4.3.4.

6.4.3.4 Shake or pipette each sample multiple times to get all the sample material remixed, and immediately pour approximately 10 mL into the corresponding labeled 20 mL glass test tube for that sample.

6.5 Clearing

6.5.1 Work in a fume hood. Using a disposable pipette, add 3 mL of 2N nitric acid (HNO_3) to the sample in the 20 mL glass test tube (the liquid will fill to approximately 1/3 the height of the tube).

Note: Fume hoods in the Chlorophyll Room (B205B) or Toxicity Laboratory (B246).

Note: Estimate the amount of HNO3 needed (# test tubes x 3 mL = X). Pour this amount into a small beaker and use to add to the test tubes. Dispose of any extra HNO3 by

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heavily diluting with water before pouring down the drain. Leave the water running while pouring.

6.5.2 Place a beaker (sized as needed) onto the hot plate. Fill beaker with approximately 150 mL of DI water. Remove the test tube lids (if applicable), place sample test tubes into the beaker, and, using a squirt bottle, adjust the DI water level in the beaker to equal the sample level inside the tubes.

6.5.3 Create a sample blank by filling one 20 mL glass test tube with an amount of DI water similar to the sample volumes. Insert a calibrated thermometer (per SOP TA-06.13, located in B246) into the sample blank. Temperatures measured in the blank will mimic the temperature of the samples. Place the sample blank tube in the beaker with the other samples.

6.5.4 Turn on the hot plate to 300 °C. When the thermometer in the blank reaches 90 °C, turn the hot plate down slightly to maintain temperature.

Note: A hot plate setting of approximately 230°C will hold the samples at the needed temperature.

Note: Sometimes dropping the temperature to 230°C immediately after it reaches 90°C can cause the temperature to drop below 90°C. Waiting until it reaches 95°C, or a little over 90°C before dropping the temperature can prevent this.

6.5.5 Heat samples in 90-100 °C DI water for 30 minutes.

6.5.6 Use autoclave gloves to remove the beaker of samples from the hot plate. Using autoclave gloves or forceps, transfer the test tubes from the beaker into the test tube rack. Turn off the hot plate and clean the area.

6.5.7 Using a squirt bottle, add DI water to each sample to fill each test tube to approximately half an inch from the top. The DI water dilutes the acid in the sample. Fill all the test tubes to an equal volume to balance the centrifuge. Put test tube lids on all test tubes.

6.5.8 Place samples into the centrifuge spacing them evenly. This ensures that the centrifuge runs smoothly. Centrifuge for 5 minutes at 1450 rpm (or use the pre-programmed #2 setting for algae samples).

Note: **Make sure samples are evenly spaced around the centrifuge before starting**. If you have an odd number of test tubes so that the centrifuge is unevenly balanced, fill an empty test tube with an equal amount of water and place it in to balance out the centrifuge.

Note: The glass tubes are tall. Make sure to place them so they will not come into contact with any part of the centrifuge while spinning. Typically, the middle-center spots work best to prevent the tubes from hitting the rotor arms while spinning. (Check this by tilting the buckets inwards towards the rotor. The top of the tubes should clear the rotor arms.)

6.5.9 Once the centrifuge has completely stopped, gently take the samples out. Without disturbing the settled material, aspirate the supernatant liquid with a 10 mL disposable pipette leaving approximately 5 mL of sample. Place this acidic supernatant into a waste beaker for proper disposal.

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Note: The 10 mL disposable pipette can be used to aspirate more than one sample if it is rinsed well with DI water between samples. Draw up 10 mL of DI into the pipette and dispense into a waste container several times between samples.

Note: Dispose of the waste by heavily diluting with water before pouring down the drain. Leave the water running while pouring.

6.5.10 Repeat steps 6.5.7 to 6.5.9 until the pH of the samples reaches 6 or 7 SU. Use a strip of pH test paper to check the pH. If not immediately making slides, store the samples in a fume hood.

Note: It normally takes 5 rinse/centrifuge cycles to remove the acid.

6.6 Slide Preparation:

6.6.1 Log in to LIMS and go to Laboratory/Receiving/Print Preparation Labels. Close the default options and choose Options/Algal Slide Label. Scan or enter each sample being prepared and print out the labels. Place the slide labels onto the slides by placing the barcode and sample number on the far left and folding the other half over to the back.

6.6.2 Working in a fume hood, place a coverslip onto each slide, and set the slides on the hot plate with the label portion hanging off the edge. Turn the hot plate on and set to 180°C. Safety glasses should be worn at this time.

Note: Mount 6 to 8, or fewer slides, at a time.

6.6.3 With a disposable pipette, mix the appropriate sample by drawing up sample and dispensing it back into the test tube several times. Once completely mixed, dispense the sample onto the coverslip of the correct slide on the burner. Try to completely fill the coverslip with sample, but do not overfill the coverslip so that the sample spills onto slide. If the sample spills onto the slide, remove slide from the burner and clean off the sample. Use a new coverslip and repeat. Do this for each sample.

Note: If needed, the sample can be concentrated slightly at this step by removing some liquid from the top of the tube before mixing the sample.

6.6.4 Allow the sample material to dry on the coverslip. Once dry, remove the slide from the burner.

Note: By the time the sample has been added to the last (6^{th} or 8^{th}) slide, the sample on the 1st slide should be dry.

6.6.5 Using forceps, remove the coverslip from the slide, noting which surface has the dried sample on it. With the glass rod from the balsam bottle containing the mounting medium, apply some mounting medium to the slide surface in the center where the coverslip will be placed. Using the forceps set the coverslip, sample face down, onto the mounting medium. Using the eraser side of the pencil, lightly press down on slide to affix cover slip to slide evenly distributing the mounting medium under the cover slip. Repeat for all slides.

6.6.6 Return all the slides to the burner with approximately 10 seconds time between slides. Heat the slide until the mounting medium has stopped bubbling. Remove the slide to a cool surface and apply firm, gentle pressure to or tap on the coverslip with the pencil eraser, pushing any air bubbles to the perimeter. Hold the pressure until the mounting medium dries.

Note: If the coverslip did not seal completely to the edges, remove coverslip and repeat steps 6.21 - 6.22.

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Note: If desired, the dried mounting media, which tends to form a ridge around the edge of the coverslip, can be gently scraped off with a razor blade. It is easy to remove at this point, and cleaning it away makes it easier to view the slide under the oil immersion lens. If it is not removed, the ridge often causes the lens to hang up while scanning near the edge of the coverslip.

6.6.7 Repeat steps 6.18-6.21 until all the slides have been prepared for all the samples in the batch.

6.7 Log in to LIMS and complete the prep worksheet for each sample or batch of samples using the Laboratory/ Technical/Prep Worksheet Manager utility. To complete the prep worksheet: open prep worksheet logbook, highlight the appropriate worksheet ID, open the logbook for that worksheet, highlight the samples in the list, set the correct prep person and prep start and completion dates/times, and then click Complete. Do this for all samples prepared. Log out of LIMS and log off the computer.

7. SAMPLE ANALYSIS PROCEDURE

7.1 Samples are analyzed per SOP AB-03.1 (periphyton diatom) or AB-05.1 (phytoplankton diatom).

8. QUALITY CONTROL AND DOCUMENTATION

8.1 Use a pen with black ink for all logbook and benchsheet entries.

8.2 DEP Laboratory Information Management System (LIMS)

8.3 To correct raw data entries, place a single line through the incorrect entry, write the corrected entry near the error and initial and date the correction. If the correction requires an explanation, write a comment and reference the error.

8.4 If a problem occurs during sample processing, submit a nonconformance report (NCR) describing the problem and the corrective action. To create a NCR, open LIMS and go to Laboratory/Administrative/Non-Conformance Report. For a decision tree regarding issuance of NCR's, refer to SOP LB-002.

8.5 Refer to the Laboratory Quality Manual

9. SAFETY

9.1 Lab coat, latex or vinyl gloves, and eye protection are required when working with chemicals and unprocessed samples.

9.2 Avoid breathing fumes or exposing co-workers to them. Use proper safety equipment, a lab hood or chemical ventilation system, and keep face away from open containers. Work in a well-ventilated area.

9.3 Reagent bottles must be labeled with the name and concentration of the reagent.

9.4 Refer to the Material Safety Data Sheet (MSDS) for information about reagents and chemicals in this SOP.

9.5 Refer to the DEP Laboratory Health and Safety Plan

9.6 Refer to the DEP Laboratory Quality Manual.

10. POLLUTION PREVENTION AND WASTE MANAGEMENT

10.1 Refer to the DEP Laboratory Health and Safety Plan.

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11. REFERENCES

11.1 DEP Laboratory Quality Manual, https://floridadep.gov/dear/florida-dep-laboratory/content/dep-laboratory-quality-assurance-manual-and-sops

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

11.3 DEP SOP FS-7000, *General Biological Community Sampling*

11.4 SOP AB-11, *Phytoplankton Sample Receipt and Sample Handling for Taxonomic Analysis*

11.5 SOP AB-03.1, *Periphyton Diatom Identification and Species Proportional Counts*

11.6 SOP AB-05.1, *Phytoplankton Diatom Identification and Species Proportional Counts*

11.7 SOP TA-06.13, *Thermometer Accuracy Check and Use*

11.8 SOP LB-002, *Non-conformance Reporting System*

Appendix of Changes

07/12/2010	Reformatted the layout following SOP LB-001. Clarified and re-numbered some steps.
07/10/2012	Added step 5.2.
07/02/2014	Revised Prep Worksheet Manager instructions, step 6.24.1
07/01/2015	Formatted for 508 compliance. Updated links. Created sub-sections and re-numbered steps. Reformatted References.
08/10/2017	Standardized SOP name format.
07/17/2020	Updated SOP names in references for SOP AB-03.1, SOP AB-05.1, and SOP TA-06.13.
08/24/2021	Updated links for DEP Laboratory Quality Manual and DEP Laboratory Health and Safety Plan.
08/17/2022	Additions: 3.14 material: Pencil with eraser bottom, 6.6.2 Note: Reduction from 10 to 8 slides to 8 to 6 at a time, 6.6.5, 6.6.6, Use of eraser side of pencil to affix cover slip to slide evenly distributing medium under coverslip prior to heating, 6.5.4 Note: addition: a way to prevent temperature drop in glass vials. Edits: 6.6.2 hot plate heat from 200°C to 180°C.
08/16/2023	Eliminated step **6.4.2.2**. Updated **6.4.3.3** notes: adjust the centrifuge spin speed to 1450 rpm, rinse pipette with DI water between samples. Added step **6.4.3.5**.: waste has formalin, dispose of it properly in waste formalin container. Updated **6.5.8**. Centrifuge for 5 minutes at 1450 rpm.
06/07/2024	Updated Apparatus and equipment. Updated 6.4.3.2 : all formalin waste should be disposed of properly. Added note to 6.5.8. Removed 6.5.11.

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