Phytoplankton Non-diatom Taxonomic Analysis

(Modified from *Standard Methods 10200F* and *10900C*)

1. SCOPE AND APPLICATION

1.1 This method details the counting rules and procedures for identifying and counting non-diatom algae and counting diatoms in phytoplankton samples.

1.2 This method is used for quantitative or qualitative phytoplankton samples collected by SOP FS 7000, *General Biological Community Sampling* (FS 7100, *Phytoplankton Sampling*).

1.3 This method is to be used for all samples with Laboratory Information Management System (LIMS) test codes beginning with "PKD". Portions of this method (as noted in the SOP) apply to phytoplankton samples with LIMS codes "BLOOM-ID" and "ALGAL-ID".

2. SUMMARY OF METHOD

2.1 The processed PKD sample is inverted gently to mix well, and a portion is diluted to achieve a counting density of 5-10 natural algal units per grid.

2.2 A modification from SM 10200F.2, a Utermöhl counting chamber is used. Laboratory inverted microscopes vary slightly with respect to objective magnification, eyepiece magnification, and added magnification components. Counts and identifications in the wet material are performed at 400X to 450X magnifications.

2.3 For PKD samples, natural units of non-diatom algae are counted and identified to lowest practical taxonomic level moving from left to right across the counting chamber. Diatoms are not identified, but a running tally is kept of the number of diatom frustules encountered. Counting and identification continues until a target of 295-305 natural units is encountered. Once a grid is begun, all algae within the grid must be counted and/or identified. If an algal unit is lying across the edge of the grid, there are rules for when that specimen is or is not included in the count and identification.

2.4 For BLOOM-ID and ALGAL-ID samples, dilution is not required. These samples are not counted. They are viewed to determine the dominant taxon (or taxa) in the sample. Taxa are recorded on the bench sheet as being dominant, co-dominant, or present. It is also noted if the sample is not algae. This test was developed for identification only; no attempt should be made to determine whether the sample looks like it represents an algae bloom. If the customer needs information on species densities, a follow-up PKD-QN-C analysis should be performed.

3. APPARATUS AND EQUIPMENT

3.1 Inverted microscope
3.2 Plastic centrifuge tubes, 50-mL
3.3 Volumetric pipettes, 10-mL
3.4 Disposable transfer pipettes
3.5 Utermöhl counting chamber
3.6 Cover slips, 24 x 60 mm
3.7 Petri dishes, 100 x 15 mm

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3.8 Filter paper to fit Petri dish
3.9 Laboratory counter
3.10 Labeling tape
3.11 Safety equipment (see section 10)

4. REAGENTS AND CHEMICALS

4.1 Deionized (DI) water

5. SAMPLE COLLECTION AND PRESERVATION

5.1 Samples are collected, preserved, and delivered to the lab according to SOP FS 7000.

6. SAMPLE PREPARATION

6.1 Samples are prepared per SOP AB-11.

7. SAMPLE ANALYSIS PROCEDURE

7.1 Custody – Check Out

7.1.1 If the sample has been transferred to another container during sample preparation or homogenization (AB-11), do not use LIMS Custody Utility to track the sample. That utility is only used to track the original sample container. Go to step 7.2.

7.1.2 If the sample is in the original container in which it was received, logon to a computer and start the LIMS program. Open Laboratory/Receiving/Custody in LIMS. When the "DEP Central Laboratory Custody" window opens, choose Custody/Checkout (Ctrl+O) or the [Check Out] button. Using the handheld barcode reader, scan barcodes on sample containers. Click [OK]. Scan ID card and rescan ID card. Close window when finished by choosing Custody/Exit [Alt+F4]. Close LIMS and log off the computer.

Note: If the scanner is unavailable or not functioning, enter the LIMS number of the sample using the computer keyboard and hit [Enter].

Note: It is very important to log off the computer when work is complete. The community computers in the laboratory are set to time out after 15 minutes. If that happens while someone is logged in, only that person or an Administrator from Scientific Support Services can unlock the computer. This makes it very difficult for someone else to do his or her work on that computer.

7.2 Preparation for Analysis

7.2.1 The sample should be contained in a 50-mL screw top centrifuge tube (SOP AB-11).

Note: BLOOM-ID samples may be in any of a variety of container types because they are often collected during routine sampling events when a bloom is observed and are not necessarily planned for (i.e. scheduled in LIMS); or quick response is needed due to a reported event, and there isn't time to schedule and receive a kit from Tallahassee.

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7.2.2 Mix the contents of the sample by inverting the centrifuge tube gently 3 or 4 times. If the gentle inverting of the tube does not dislodge all settled material from the bottom of the tube, invert a little more vigorously. Be careful of shaking the sample too vigorously, however, as this tends to break up some blue-green colonies.

7.2.3 Using a transfer pipette, fill the cell of the Utermöhl counting chamber with the well-mixed sample so that liquid is barely higher than the sides of the Utermöhl counting chamber. Slide a cover slip sideways to cover the cell opening to ensure that no air bubbles are introduced.

7.2.4 Place filter paper (2 pieces) into a Petri dish and moisten. Carefully place the filled Utermöhl counting chamber onto the filter paper, and then cover the dish. Let the sample settle overnight.

Note: The Petri dish creates a moist environment while the sample is settling, thus avoiding the formation of an air bubble due to evaporation. Be sure that the filter paper is smooth and that the Petri dish is placed on a level surface during settling. Otherwise, the algal cells will tend to settle out on one side of the Utermöhl counting chamber and will not be evenly distributed.

Note: BLOOM-ID and ALGAL-ID samples do not necessarily have to be settled. As long as the algae have settled enough to allow proper identification, the sample can be identified as soon as the analyst determines it is possible in order to give quick turnaround on results.

7.2.5 After the settling period, remove the counting chamber from the Petri dish and carefully wipe away any excess moisture, particularly on the underside. Observe the settled sample under the inverted microscope at 400X magnification. Count the number of algal units in 20 grids randomly selected throughout the counting chamber. Calculate the average number of algal units per grid: Total # algal units counted ÷ number of grids

- **If the average is 5-10** algal counting units per counting grid, the sample is concentrated to an appropriate counting density. Go to step 7.2.14.
- **If the average is less than 5** units, the sample is too dilute and should be further concentrated as described in steps 7.2.11 to 7.2.13.
- **If the average is greater than 10** units, the sample is too dense and should be diluted as described in steps 7.2.6 to 7.2.10.

Note: An algal “unit” is defined as a natural counting unit such as a colony or filament. An algal cell is defined as the individual cells within a colony or filament. Unicellular algal specimens, such as diatoms or flagellates, are considered as one cell and one unit.

7.2.6 If the sample needs to be diluted as determined in step 7.2.5, mix the contents of the sample by inverting the centrifuge tube gently 3 or 4 times. If the gentle inverting of the tube does not dislodge all settled material from the bottom of the tube, invert a little more vigorously. Be careful of shaking the sample too vigorously, however, as this tends to break up some blue-green colonies.

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7.2.7 Immediately pipette 1 to 10-mL of well-mixed sample into another 50-mL plastic centrifuge tube. Affix labeling tape to this tube and record the sample number, date, and volume of subsample used for dilution on the label.

Note: The number of milliliters to pipette into the dilution tube varies with the concentration of the settled material. For very dense samples, use a smaller volume, as these samples will need to be diluted more for counting.

Note: Although BLOOM-ID and ALGAL-ID samples are not counted, dilution information can still be calculated and recorded to keep the analyst in the practice of doing so for all samples, and it gives the analyst an overall idea of how dense the bloom is.

7.2.8 Use DI water to dilute the subsample. For example, if the amount of concentrated sample in the dilution tube is 2-mL, add the DI water 2-mL at a time. Stop dilution when the density of algae looks adequate for counting, based on the perceived turbidity of the sample (see note). Record the volume of DI water added on the vial label next to the volume of sample previously recorded.

Note: The amount of dilution necessary (if any) for achieving adequate counting densities varies with the amount of debris in the sample. The dilution process requires trial and error at first; with experience, one can usually eyeball the proper dilution. Sometimes the subsamples are still too dense after settling, and the dilution process must be repeated.

7.2.9 Calculate the dilution factor by dividing the total number of milliliters in the diluted subsample by the number of milliliters of concentrated material removed from the original settled sample. Record this dilution factor on the vial label as 1x, 2x, etc., meaning the sample was not diluted (1x), diluted 1 time (2x), diluted 2 times (3x), etc.

Note: Avoid using notation such as 1:2 because different people can interpret this notation differently. Some read it as one part sample in two parts total. Some read it as one part sample to two parts DI. Depending on how it is interpreted, the 1:2 notation could be read as a dilution of 2 (1 in 2) or a dilution of 3 (1 to 2).

Note: Samples not requiring a dilution have a dilution factor of 1 (1x). It is recorded this way because of the way it is used in the density calculation cited in Standard Methods. This is also the density calculation used in the Statewide Biological Database.

7.2.10 Repeat steps 7.2.2 to 7.2.5.

7.2.11 If the sample needs to be further concentrated as determined in step 7.2.5, repeat the settling process described in SOP AB-11 using a 100-mL graduated cylinder for settling.

Retain the 50-mL centrifuge tube. After it has been rinsed well with DI water, the newly concentrated sample aliquot can be poured back into this tube. A new Algal Sample Bottle Label should be printed to place on the graduated cylinder.

Record the initial and final sample volumes from this settling step on the Algal Sample Bottle Label that is affixed to the graduated cylinder. When the settled sample is poured back into the 50-mL centrifuge tube, transcribe the new initial and final sample volumes to the Algal Sample Bottle Label that is affixed to the centrifuge tube. These new volumes

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can be written underneath the original volumes recorded and noted to be from a second concentration step.

Note: The decision of how much more to settle the sample will be based on how close to the 5-10 algal unit per grid target the sample is in step 7.2.5. With experience, the proper concentration can usually be determined by judging the perceived turbidity of the sample.

7.2.12 If the sample was further concentrated to achieve the appropriate counting density, calculate the new final volume to be used for density calculation as follows:

a. Determine the initial concentration factor by dividing the initial sample volume by the initial settled sample volume. Record this concentration factor on the Algal Sample Bottle Label affixed to the 50-mL centrifuge tube next to the initial volumes established per SOP AB-11.

b. Determine the secondary concentration factor by dividing the second starting sample volume by the second settled sample volume. Record this concentration factor on the Algal Sample Bottle Label next to the second set of volumes established in step 7.2.11.

c. Multiply the concentration factors together to get a final (total) concentration factor.

d. Divide the initial sample volume by the final concentration factor. This number is your final sample volume to be recorded on the Phytoplankton Bench Sheet and used in algal density calculation.

Example:

a. Initial sample volume=1000-mL; settled sample volume = 200-mL; concentration factor = 5
b. Initial sample volume (aliquot reserved for analysis) = 50-mL; settled sample volume = 10-mL; concentration factor = 5
c. Final concentration factor = 5 × 5 = 25
d. Final sample volume= 1000-mL/25 = 40 mL

7.2.13 Repeat steps 7.2.2 to 7.2.5.

7.2.14 When the appropriate counting density is reached, calculate the final dilution factor by multiplying the dilution factors of each serial dilution. Samples not requiring a dilution have a dilution factor of 1 (1x). Record the final dilution factor on the final dilution tube.

7.2.15 Record the following pre-analysis information the Phytoplankton Bench Sheet:

Record:	Note:
Initial sample volume	For quantitative phytoplankton samples. The initial sample volume is the original volume of the sample when it was first checked in.
Final sample volume	The final sample volume is the volume after all sample preparation is complete.
Final dilution factor	Failure to record dilution factor prevents accurate determination of algal spatial density.
Length of the Whipple grid in mm	Length is based on magnification and microscope calibration (SOP AB-01).
Counting chamber #	Located on the side of the Utermöhl counting chamber

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Record:	Note:
Depth of counting chamber in mm	Although the cell depth is not needed for analysis of qualitative samples, it is good practice to record it on the bench sheet. This helps to stay in the habit, reducing the probability of forgetting to record it for a quantitative sample.
Preservative	This is a precaution to make sure the analyst is aware of the substance they are working with, even though the sample and preservative have been diluted at this point in the analysis.
Settling time	-

Note: For BLOOM-ID and ALGAL-ID samples, this information is not needed, but it is a good idea to record it to keep the analyst in the habit of recording the information.

7.3 Custody – Check In

7.3.1 If the sample is not in the original container, go to step 7.4. This likely applies to most PKD samples, except BLOOM-ID and ALGAL-ID samples.

7.3.2 If the sample is in the original container in which it was received, log onto LIMS. Open Laboratory\Receiving\Custody. When the "DEP Central Laboratory Custody" window opens, choose Custody\Check In [Ctrl+I] or the [Check In] button in the "Custody" window. Scan barcodes of sample labels on bottles. (Follow prompts) Scan ID card and rescan ID card. Choose File\Exit. Log out of LIMS and log off the computer.

Note: If the scanner is unavailable or not functioning, enter the LIMS number of the sample using the computer keyboard and hit [Enter].

7.4 Sample Analysis

7.4.1 Natural unit counts (cells, colonies, or filaments) should be used rather than individual cell counts. Those units that extend into the grid a distance of at least 1/4 of the grid width (or length) should be counted. Of the algae which extend into the grid a distance of less than 1/4 of the grid width, those crossing the top and left borders of the field are counted as “in”. Those crossing the bottom and right boundaries of the field are not counted. Use the laboratory counter to keep a running total of the number of fields observed.

Note: For BLOOM-ID and ALGAL-ID samples, there is no count performed. Simply scan the sample to determine the dominant taxon (or taxa). Taxa are recorded on the bench sheet as being dominant, co-dominant, or present. It is also noted if the sample is not algae.

7.4.2 Remove the counting chamber from the Petri dish and carefully wipe away any excess moisture, particularly on the underside. Place counting chamber on the stage of the inverted microscope.

Note: Be careful not to tip the counting chamber or bump it as this will cause the algal cells to dislodge from the bottom and float, making accurate identification and counts difficult.

7.4.3 Position the stage so that the left edge of the counting chamber is in view. Move a short distance to the right and observe all algae within the Whipple grid. Always move from left

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to right across the cell during the progression to a new field. This eliminates the possibility of accidentally changing directions and counting fields more than once.

7.4.4 At 400X magnification, identify non-diatom algae to the lowest possible taxonomic level. Only algae that appear to have been alive at the time of preservation should be counted. Algal cells/colonies/filaments without chloroplasts should NOT be counted and/or identified. Diatoms that are too lightly silicified to survive the clearing process should be identified to the lowest possible taxonomic level. All other diatoms should be counted as one group.

Note: At the magnification generally used for wet phytoplankton (400X), non-diatom taxa are usually identified to the generic level.

Note: The Leica and Zeiss microscopes have differing objectives, magnifiers and eyepieces. For example, the Leica microscopes have a 1.5X magnifier while the Zeiss have a 1.6X magnifier. The Leica eyepieces have 15X magnification while the Zeiss have a 10X magnification. The 20X objective must be used for the Leica and the 40X objective for the Zeiss to reach the 400X-450X range.

Note: Diatoms can be complexed into chains or groups. The individual frustules should be counted, not the group.

Note: On occasion certain taxa (e.g. *Dinobryon* sp., a *Chrysophyte*) may appear to be lacking a chloroplast, but upon further investigation of the sample as a whole, zoospores are observed in some cells and outside of some cells. In cases such as this, if it's apparent that the cells had chloroplasts or zoospores that may have been dislodged during sample preservation and processing, these taxa can be counted. However, decisions to count or not count taxa in situations such as this should always be discussed as a group by all taxonomists so that everyone is identifying and counting in the same way.

Note: Some examples of diatoms that do not survive the clearing process (SOP AB-02.1) are *Rhizosolenia* sp., *Leptocylindrus* sp., *Skeletonema* sp., *Odontella* sp., *Attheya* sp., *and Chaetocerus* sp. This has been determined in previously identified samples. These diatoms tend to be very lightly silicified and too fragile for the boiling process. Do not include these diatoms in the total Bacillariophyta number; include them as taxa for the wet algal taxa count.

7.4.5 While counting and identifying the algae, keep a running tally of number of fields counted, number of total units counted, and number of diatom units counted using a laboratory counter. Record each taxon, along with the number counted, on the appropriate Phytoplankton Bench Sheet.

7.4.6 Continue counting until 300 algal units have been counted and identified. If the unit count tally reaches 295 units or more and the grid being counted has been completely counted, counting may be stopped. If less than 295 units have been counted, proceed to the next grid. Once a grid is started, everything in that grid must be counted and identified. Therefore, the actual number of units counted should range from 295 to around 305 if the counting density was set up at 5-10 units per grid in the initial dilution/concentration phase. See note for time limits.

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Note: In some cases, algal density in the original sample may be so sparse that even with maximum concentration it is difficult to find 300 units in a reasonable amount of time. No more than three hours should be spent identifying any one sample.

- For samples that do not contain much sand or detritus, if 300 units cannot be found within three hours or if 20 minutes pass without encountering an algal specimen while scanning through the sample, write a comment on the bench sheet to this effect and terminate the analysis.
- For samples that contain a lot of sand and detritus, which interferes with the scanning process, and algal density is low, dilute the sample as needed then count up to 300 grids or 300 algal units, whichever comes first. Write a comment on the bench sheet noting the sand or detritus and low algal density and that counting was stopped at 300 grids.

7.4.7 Add up the unit counts along the outer edge of the columns of the Phytoplankton Bench Sheet.

7.4.8 Record the following post-analysis information on the Phytoplankton Bench Sheet:

Record:	Note:
Magnification	Magnification used for taxonomic identification
# Grids	# Fields are not tracked or recorded for BLOOM-ID or ALGAL-ID samples
Total # units	Range between 295-305
# Wet algal taxa	Do not count Bacillariophyta as a taxon. This count represents a group of taxa that will be identified in the diatom portion of the analysis. Number of taxa is not recorded for BLOOM-ID or ALGAL-ID samples.
# Diatoms	-
Analyst	-
Date analysis completed	-
Analysis time in hours	-

Note: Diatom analyses are performed after the non-diatom identification is completed. Diatom slides are prepared per SOP AB-02.1, and diatom species proportional counts are performed per SOP AB-05.1.

Note: Diatom analysis is not normally performed for BLOOM-ID and ALGAL-ID samples but can be if diatoms are highly dominant and it is suspected after viewing the sample that the bloom taxon is a diatom.

7.4.9 In the appropriate logbook(s), record the necessary information for the completed sample.

7.4.10 Entry, verification, and authorization of taxonomic data in SBIO and LIMS is performed per SOPs BG-03 and BG-01 by individuals designated by the Taxonomy Supervisor

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8. DATA ARCHIVAL

8.1 Archives and slides are kept for five years, after which time they may be discarded using appropriate safety procedures. Bench sheets are filed by project and kept indefinitely.

9. QUALITY CONTROL AND DOCUMENTATION

9.1 Use a pen with black ink for all logbook and bench sheet entries.

9.2 DEP Laboratory Information Management System (LIMS) and Statewide Biological Database (SBIO).

9.3 Algal Logbook Labels printed from LIMS.

9.4 Phytoplankton Bench Sheet printed from LIMS.

9.5 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.

9.6 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.

9.7 QC of Taxonomic Identification is performed per SOP AB-14.

9.8 Refer to the Laboratory Quality Manual.

10. SAFETY

10.1 Lab coat, latex or vinyl gloves and eye protection are required when working with formalin.

10.2 Avoid breathing formalin 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 of this chemical. Work in a well-ventilated area.

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

10.4 Refer to the Laboratory Health and Safety Plan.

11. POLLUTION PREVENTION AND WASTE MANAGEMENT

11.1 Handling of waste formalin is performed per SOP IZ-10 and SOP IZ-38.

11.1.1 Formalin disposal containers are located under the sinks in the main laboratory (B205).

11.2 Refer to the Laboratory Health and Safety Plan.

12. REFERENCES

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

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12.2 DEP Laboratory Health and Safety Plan, https://www.fldepnet.org/content/dear/bureau-laboratories-safety-documents

12.3 SOP FS 7000, *General Biological Community Sampling*

12.4 SOP AB-01, *Compound and Inverted Microscope Calibration*

12.5 SOP AB-02.1, *Diatom Preparation for Periphyton and Phytoplankton*

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

12.7 SOP AB-11, *Phytoplankton Sample Receipt & Sample Handling for Taxonomic Analysis*

12.8 SOP AB-14, *Algal Identification Quality Control (QC) Procedures: Initial Demonstration of Proficiency, Ongoing QC, Monthly QC, and Quarterly QC*

12.9 SOP IZ-10, *Preparation and Proper Disposal of Buffered Formalin*

12.10 SOP IZ-38, *Formalin Assay, Recycling and Buffering*

12.11 SOP BG-01, *Data-Entry Verification*

12.12 SOP BG-03, *Taxonomic Results and Data Entry*

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

Appendix of Changes

12/09/2009 Removed sections on checking samples in and out using LIMS Custody. LIMS Custody is designed to track the original sample container, and the containers in this SOP are not the original sample containers.

11/23/2010 Reformatted the layout following SOP LB-001. Added analysis information for BLOOM-ID and reference to SOP AB-16. Re-worded text to coincide with text of AB-05. Added pre-analysis and post-analysis tables of information to record. Added section 8.

11/10/2011 Updated comment entered in LIMS for Bloom-ID. Updated name of Laboratory Safety Manual.

11/01/2012 Added Quality Control SOP numbers.

11/01/2103 Removed formalin respirator requirement. Updated formalin SOP #.

10/29/2014 Formatted for 508 compliance. Expanded note for step 7.4.6.

11/03/2015 Included the specific modification from SM 10200F.2 to the Summary of Method section. Moved much of the information previously in Data Archival section into SOP BG-03. Updated Lab Health and Safety Plan link.

11/29/2016 Updated links.

11/28/2017 Removed and revised language referring to the standard objective used in counts as 20-25X. This objective is used for Leica microscopes but 40X is used for Zeiss. Added guidance for groups of diatoms. Included the ALGAL-ID test code for sample analysis

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