Kociolek
University of Colorado

Rev 4

Standard Operating Procedures and Protocols for Algal Taxonomic Identification. 25 February, 2021

Contents

0.0 Change Record

REVISION	DATE	DESCRIPTION OF CHANGE
1		
2		
3		
4	2/25/2021	Added detail of freeze drying, QAQC added detail, and modified calibration cycles for pipettes.

1.0 Traceability of Analysis

1.1 Taxonomic Keys And References

Used In The Identification Of Soft-Bodied Algae And Diatoms

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1.2 Experts

Between Lowe (49 years) and Kociolek (36 years), this team of experts has 85 years of experience in the enumeration and identification of freshwater algae. Kociolek is the expert for the Boulder Lab.

Patrick Kociolek, Museum of Natural Sciences and Department of Ecology and Evolutionary Biology, University of Colorado, Boulder (2008-present)
Previously held the Hanna Chair in Diatom Studies at the California Academy of Sciences, San Francisco (1989-2008)
Has published nearly 300 peer-reviewed papers and 19 books and monographs on freshwater algae, specifically freshwater diatoms.
Has taught classes on freshwater algae and/or diatoms at The University of Colorado, University of Michigan Biological Station, ATREE in India, Shanghai Normal University, China and the University of La Plata, Argentina.
Editor-in-Chief of Diatombase (www.diatombase.org)
Identification and evaluation of freshwater algae for water quality monitoring for the State of California, US E.P.A. and USGS.

1.3 Training Policy

Beyond the experts, all other personnel that will make identifications for this project will have:

*An undergraduate degree in Biology, Botany, Environmental Studies or similar discipline
*Independent research experience in Phycology with an emphasis on algal identification
*Formalized training in Phycology, either taken the class in an on-campus setting or at a biological field station.

All individuals are verified by the experts (Section 1.1) prior to making independent identifications to have the following experience and training:

*Practical experience in the identification of algae, to the specified level of taxonomy for the work they are involved in.
*Know which literature to use and how to use it for algal identification.
*Proficient use of a light microscope and digital microphotography to take high resolution digital images of soft-bodied algae and diatoms.
*Experience with specimen preparation, techniques, and safety procedures.
*Excellent record-keeping skills, organization of data, and spreadsheet, database, image capture and processing, and general computer skills.

Professional development opportunities will be encouraged related to workshops, classes and other training opportunities in formal settings (on-campus, at biological field stations) and at scientific meetings.

2.0 Procedures

2.1 Sample Receiving.

All sample receiving, processing, storage and data processing will be done at Kociolek's lab at the University of Colorado, Boulder (See Figure 1 below). Most sample analysis will also be done in Boulder, with analyses of phytoplankton to be completed at Lowe's lab in Madison, Wisconsin.

Samples received at the lab will be checked in against a shipping / chain of custody statement. Samples will be checked in with bar code readers when that technology is implemented by NEON. Received samples will be immediately registered with NEON ID and inspected. If samples have some problem (breakage, empty, etc.) we will notify NEON within two working days. Sample volumes will be measured and recorded in the data sheet (Excel spreadsheet similar to Attachment 2C) for all samples. For phytoplankton, we will then ship samples to Lowe in Madison, who will complete chain of custody forms indicating sample receipt and condition.

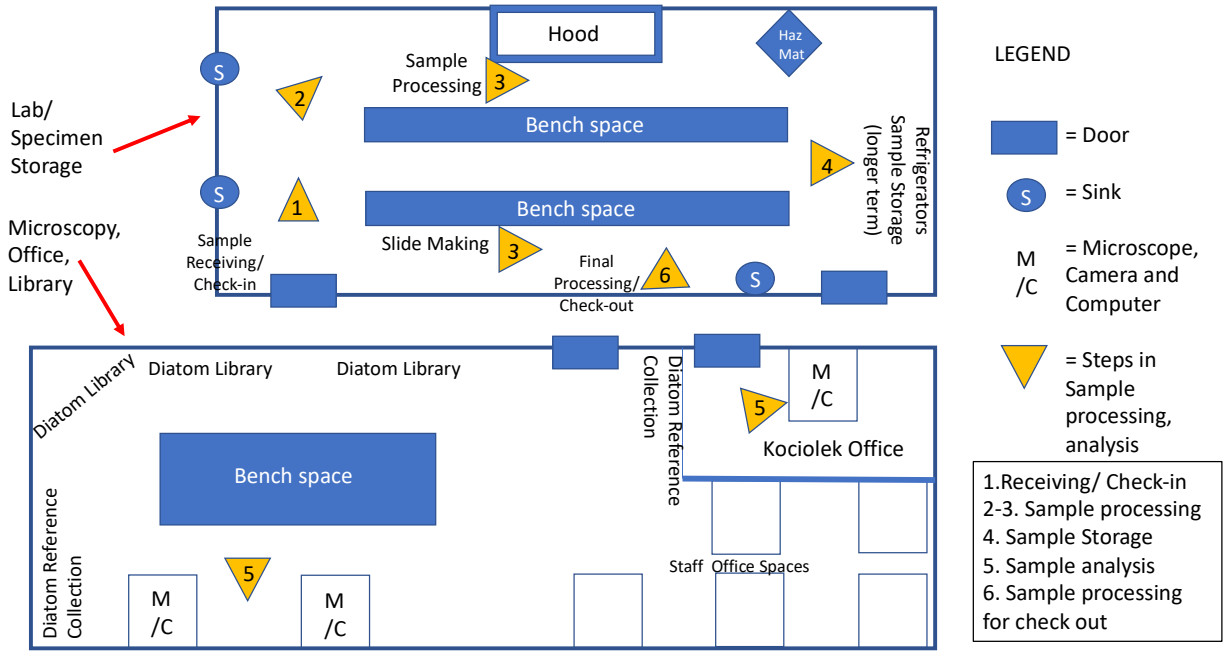

Figure 1. Configuration of Phycology Lab, and Microscopy/Office/Library spaces at the University of Colorado, Boulder.

2.2 Storage

If not processed immediately, samples will then be put into short-term storage at 4 degrees C in the lab until further processing.

2.3 Processing

There are 3 types of samples to be processed: macroalgal samples, phytoplankton samples and periphyton samples. For Phytoplankton samples, we will follow the protocol develop by the Academy of Natural Sciences of Drexel University (Acker, 2016; Procedure P-13-52), using the Palmer-Maloney counting cell method. For Macroalgal samples and Periphyton samples, we will follow the protocols as described by Stanchev et al. (2015). These procedures are described below, with slight modifications for the NEON project. The different processing of samples is outlined in Figure 2.

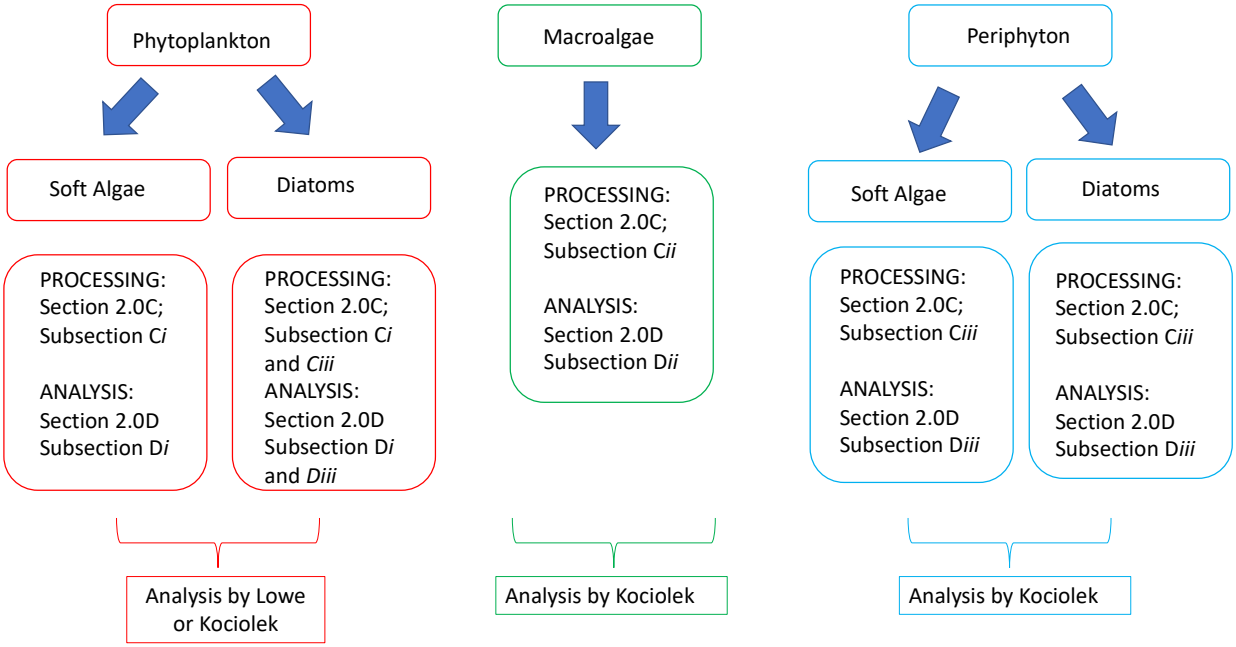

Figure 2. Overview of the different sample types and the processing protocols Analyses done by Lowe will be carried out in Madison, Wisconsin. Analyses by Kociolek will be done at the University of Colorado, Boulder, Colorado.

2.3.1 Processing of Phytoplankton Samples (adapted from Acker, 2016)

Pre-Concentrate Subsamples. The original sample will be homogenized by shaking and then split, with 250 mL sent to Lowe for concentration and analysis for soft algae, and the remainder kept at Boulder for processing of permanent diatom slides.
The original sample should be concentrated prior to adding to a counting chamber. This initial concentration should be approximately 10 times the original whole-water sample, leaving about 20 ml of concentrate for analysis. Samples are concentrated by a combination of settling in tall glass cylinders (settle for at least 24hrs) and by centrifugation (1000 g for 20 min). Determine the amount of concentration and calculate a dilution concentration factor [DCF] by dividing the final volume (this is designated as fraction volume) by the beginning volume (this was the volume of the subsample). The subsample volume, fraction volume and subsample DCF should be recorded.

We will use the Palmer-Maloney Counting Cell method. Place a rectangular cover slip (#1 thickness, 22 x 50 mm) at the top of the counting cell, covering about 1/3 of the chamber, but not across the center of the cell. Thoroughly mix the Palmer-Maloney fraction and draw it into an elongated Pasteur pipette (5.25 inch). Quickly add the fraction drop-wise into the center of the chamber. When the surface tension starts to draw the cover slip across the chamber, adjust the sides of the cover slip so that the ends of the

chamber are covered and the cover slip hangs over both sides of the ceramic portion of the counting cell. If the count is complex or if it is anticipated to take a long period of time, then add glycerin to the area where the cover slip hangs over the ceramic portion. This will seal the cover slip to the counting cell; without excess heat or vibration, the counting cell can be used for a week or more. The processing of soft algae for phytoplankton samples will be performed by Lowe in Madison, Wisconsin or in Boulder.

For the processing of phytoplankton samples for diatoms, we will follow the "nitric acid method" (page 11, below) and the "preparation of permanent diatom slides" (pages 13-14, below) protocols. This work will be performed by Lowe in Madison, Wisconsin or in Boulder.

2.3.2 Processing of Macroalgae (Taken from Stancheva et al. 2015)

Step 1: Using forceps (30 cm long), very gently pinch the material at the bottom of the tube. Search for visible macroalgal clumps, and any solid particles in the sample, such as mosses, vascular plant tissues, roots, etc.

Step 2: Gently pull up the forceps and slowly move the macroalgae and all solid particles grasped between the forceps in the solution to remove extra clinging sediment and isolate any macroalgal filaments in the sample.

Step 3: Repeat this step at least three times before proceeding to the next step.

Step 4: If macroalgal clumps are present in the sample continue onto Step 6. If no macroalgal clumps are present / visible to the naked eye, inspect the sample tube under a dissecting microscope before proceeding further.

Step 5: Using forceps, remove the macroalgae from sample very gently, squeeze it to remove as much liquid as possible and then place it into the tube with 10 mL DI water. Continue until no macroalgae remain.

2.3.3 Processing of Periphyton Samples (Adopted from Stancheva et al. 2015)

Step 1: Obtain the 60 mL centrifuge tube containing the periphyton quantitative sample. Homogenize the microalgal fraction of the periphyton quantitative sample by gently but thoroughly inverting the centrifuge tube several times. The sample must be well homogenized prior to sub-sampling (Step 2).

Step 2: Pipette 5 mL of homogenized microalgae fraction into a 50 mL centrifuge tube labeled with the sample information.

Step 3: Fill the centrifuge tube with DI water to the 50 mL mark. Let the sample settle for a minimum of 12 hours.

Step 4: Once the sample has thoroughly settled, gently remove the supernatant layer down to a volume of 5 mL by using a pipette. Avoid disturbing the algal material on the bottom of the tube.

Step 5: Label a 15 mL graduated centrifuge tube with the following information:
NEON sample ID

Collection Date (MM/DD/YYYY)
"Soft algae" on the label.

Step 6: Transfer the 5 mL of sample material from the 50 mL centrifuge tube to the labeled 15 mL graduated centrifuge tube.

Step 7: Rinse down the sides of the 50 mL centrifuge tube several times with DI water to capture any remaining algae clinging to the sides. Transfer the rinse liquid to the labeled 15 mL graduated centrifuge tube.

Step 8: Fill the labeled 15 mL graduated centrifuge tube with DI water to the 15 mL mark.

Step 9: Centrifuge the sample for 5 min at 4000 RPM on a table-top centrifuge.

Step 10: Remove the supernatant layer until 1 mL sample is left by using a pipette. Avoid disturbing the algal material on the bottom of the tube.

This procedure concentrates the microalgal fraction 5 times while removing most of the glutaraldehyde before microscopic examination. From this 1 mL sample, a semi-permanent slide is prepared for analysis. If a small amount of material (sediment and algae) is present, centrifuge for 5 min at 4000 RPM on a table-top centrifuge and concentrate the sample to 0.5 mL. Record any additional dilution or concentration performed on the sample and the final sample volume used for slide preparation in the ID Datasheet.

Pipette-mix the sample and subsample with pipette from the center of the well-mixed material. Place 1 drop (0.05 mL) of sample on a standard microscope slide and cover with a cover slip. Proper preparation of the slides is vital to performing identifications. The following should be noted while preparing slides:

*Ensure that the volume of the drop is not so large that it creates the formation of bubbles or causes the cover slip to float.

*Avoid having too much or too little material on the slide. Too much material results in layers of cells, specimen overlap and a non-flat cover slip which interferes with accurate identification. Too little material increases the amount of time required to complete analysis and may not be adequate for proper identification and enumeration.

*Small thick clumps of spreading filaments intermixed with colonial algae can sometimes occur in the microalgal fraction. Clumping of material not only interferes with accurate identification and enumeration, but can circumvent the assumption of random distribution of specimens on the sides. These clumps usually contain several different species, so they should to be dispersed before proceeding with analysis. Gentle tapping on the cover slip or spreading the clump apart with a pair of dissecting needles will reduce clumping.

Inspect the semi-permanent microalgal slide at lower magnification (200x) using a compound microscope to confirm that microalgae are evenly distributed. Gently adjust the cover slip if algal clumps are present. Cover slip may be sealed with nail polish to prevent evaporation. This semi-permanent microalgal mount is good for analysis for at least two hours.

2.3.4 Preparation of Permanent Diatom Slides

Two methods are available for diatom cleaning: the nitric acid method (American Public Health Association, 1981) and the hydrogen peroxide and potassium dichromate method (Van der Werff 1955). Nitric acid processing provides the "cleanest" (less organic material remaining), but can destroy lightly silicified species. In cases where taxa with lightly silicified valves are identified from the preserved samples, we will use the hydrogen peroxide method. Diatoms will be cleaned by these methods from both Phytoplankton and Periphyton samples.

2.3.4.1 Cleaning of Diatom Samples: Nitric Acid Method

Concentrated nitric acid is extremely hazardous, and therefore specific handling and disposal procedures must be in place. Staff should consult the appropriate MSDS provided by the supplier. Nitric acid should always be handled in a positive-draw fume hood by trained staff wearing safety goggles, rubber gloves, and lab coats. Diatom quantitative samples are preserved in glutaraldehyde, so they must be handled carefully.

Step 1: Shake the sample vial vigorously and pour 20 mL (30 mL for sparse samples) of homogenized sample into the beaker labeled with the sample information.

Step 2: Add a small amount of nitric acid to the beaker to test if a violent or exothermic reaction occurs. If violent reaction does occur, or if carbonates are abundant in the sample, the sample can be pre-treated by adding 10% HCl.

Step 3: When it has been determined that no violent reaction will occur, slowly add the remaining volume of nitric acid to the beaker. In all, a volume of nitric acid approximately equal to the volume of sample processed is added to the beaker.

Step 4: Place the beaker on a hot plate under a positive-draw fume hood. Boil the sample and nitric acid mixture until the organic content turns white. This white material is the siliceous cell walls of the diatoms. This step typically takes 30 minutes to 1 hour, during which the volume of the material will be reduced to about ½ (see Note below).

Step 5: Once the boiling step is complete, allow the sample to cool. Transfer the sample to a 15 mL centrifuge tube labeled with the NEON sample ID and centrifuge at 3500 rpm for 8 minutes.

Pour the supernatant off into an appropriately designated waste container. Add DI water to the centrifuge tube containing the diatom sample and centrifuge at 3500 rpm for 8 minutes.

Step 6: Repeat the cycle of decantation, addition of new DI water, and centrifugation 5 times, or until the pH of the water is neutral (or the same as the deionized water being used – may not be pH 7). The result should be a pellet of nearly white material at the bottom of the tube.

Step 7: Label a 15 mL graduated centrifuge tube with the following information:
*NEON sample ID
*Collection Date (MM/DD/YYYY)
*Note "diatoms" on the label to distinguish from the other samples.

Transfer the cleaned diatom material to the labeled 15 mL centrifuge tube. Add DI water up to the 10 mL mark. This is the material from which the slides will be made.

Note: The surfaces of hot plates can get hot enough to cause boiling over or explosive conditions, especially with samples containing high amounts of organics or carbonates. Alternative methods such as

using a heating block with 26 mm diameter glass test tubes (the lower portion of each tube stay hot while the upper portion stays cool, creating a reflux action, minimizing the risk of over-boiling or drying the sample) or microwave apparatus (Acker et al., 2002) may be used to clean the diatoms.

2.3.4.2 Cleaning of Diatom Samples: Hydrogen Peroxide Method

The principal reagents used in this method, hydrogen peroxide and potassium dichromate, are extremely hazardous. 30% hydrogen peroxide is a strong oxidizer, and may require special handling and storage. Specific handling and disposal procedures must be in place for the handling these materials. Staff should consult the appropriate MSDS provided by the supplier. Both chemicals must always be handled in a positive-draw fume hood, and staff should wear safety goggles, nitrile gloves and lab coats.

Step 1: Shake the sample vial vigorously and pour 20 mL (30 mL for sparse samples) of homogenized sample into the beaker labeled with the sample information.

Step 2: Add approximately 20-30 mL of 30% hydrogen peroxide to the sample.

Step 3: Place the beaker on a hot plate under a positive-draw fume hood. Bring to boiling.

Step 4: Remove from heat and immediately add a small amount (several crystals) of potassium dichromate to the mixture using a microspatula. The addition of the potassium dichromate will catalyze a strong exothermic reaction therefore, the potassium dichromate should be added slowly. Have a squirt bottle of DI water at the ready in case the reaction begins to boil over the top of the beaker. The reaction takes approximately 5 to 10 minutes to complete. Completion of the reaction is indicated by solution changing in color from dark purple to orange.

Step 5: Add DI water up to 200 mL.

Step 6: Allow the diatom material to settle for at least 8 hours.

Step 7: Slowly and gently, to avoid disturbing the diatom material on the bottom of the beaker, decant the liquid into an appropriately designated waste container.

Step 8: Refill beaker with the diatom material on the bottom with DI water to 200 mL.

Step 9: Repeat steps 6-8 several times (approximately 3 to 5) until the cleaned diatom material is mostly colorless.

Step 10: Let the cleaned diatom material settle overnight and decant the supernatant as low as possible.

Step 11: Label a 15 mL graduated centrifuge tube with the following information:
*NEON sample ID
*Collection date (MM/DD/YYYY)
*Note “diatoms” on the label to distinguish from the other samples.
This material will ultimately be placed into glass vials for final storage with the NEON ID etched onto the vial with a diamond pencil.

Permanent slide preparation is the same, regardless of which cleaning method is chosen.

2.3.4.3 Preparation of Permanent Diatom Slides

Step 1: Drip an amount of DI water onto the cover slip with a glass pipette. The amount should be sufficient to form a thin layer of water over the entire cover slip when the diatom suspension is added. If the clean diatom suspension is very sparse, skip this step.

Step 2: Obtain the 15 mL centrifuge tube with cleaned diatom material. Agitate the vial containing the cleaned diatom suspension and quickly withdraw material from near the central portion of the sample using the glass pipette.

Step 3: Place one or two drops of diatom suspension smoothly and carefully into the layer of DI water on the cover slip (see Note 4). If the clean diatom suspension is very sparse place three to five drops directly onto a cover slip without DI water. If the cover slip overflows, discard it, clean the area, and prepare a new cover slip with diatoms.

Step 4: Air dry the material or gently dry the material on a warm hot plate. The temperature of the hot plate must not exceed 40ºC. Higher temperatures cause the water to circulate or bubble, resulting in a non-random distribution or loss of diatom valves. Avoid any procedure that rapidly evaporates the suspension. Rapid evaporation could produce strong patterns of diatoms settling on the cover slip.

Step 5: When the cover slips have visibly dried, place them on a hot plate at an elevated temperature to drive off any remaining moisture.

Step 6: Confirm the prepared cover slip contains a random distribution of diatoms sufficiently dense for conducting identification and enumeration procedures. On average, 15 to 30 diatom valves should be visible in a single field of view. Confirm density and random distribution in 5 fields of view. If clumps of diatom valves are on the slide to the point where individual specimens cannot be viewed prepare another cover slip.

Step 7: Add a small amount of mounting medium (Naphrax) to a cleaned microscope slide and put the cover slip (diatoms down) on the mounting medium with forceps.

Step 8: Put the microscope slide with the cover slip on a hot plate preheated to 120 to 150ºC. Leave on hot plate until bubbles stop forming under the cover slip, indicating that all the solvent from the mounting material has been driven out of the medium.

Step 9: Use forceps to safely remove the slide from the hot plate. Gently tap down on the cover slip to remove any air bubbles and to even the distribution of diatoms.

Step 10: Once the slide cools, scrape any excess mounting medium that remains outside the cover slip with a single-edged razor.

Step 11: Scribe the NEON sample ID and slide number (six digits, the first three corresponding to the slide number, the last 3 corresponding to the position in the slide box, from 001 to 100) on the back of the slide with a diamond pencil. Attach adhesive labels to the completed slides and include the following information:
*NEON sample ID
*Collection date (MM/DD/YYYY)

2.4 Analysis

Data will be recorded in Excel Spreadsheets organized as indicated in NEON Attachment 2C. There will be four types of analysis conducted: 1)Phytoplankton, 2)Soft Algae in Periphyton samples, 3)Diatoms in Periphyton samples, and 4)Macroalgal samples.

Count data: For Phytoplankton samples, we will count and identify 300 natural algal units and will count and identify 600 diatom valves. For periphyton samples we will count and identify 300 natural algal units. For permanent diatom slides from periphyton samples we will count and identify 600 diatom valves. For macroalgal samples, we will estimate the relative percentages of each macroalgal taxon present in a sample.

2.4.1 Phytoplankton (Adopted from Acker, 2016): Soft Algae

Using a high dry microscope objective (40x objective, 400x total system magnification), identify and enumerate algae in selected, random fields. Prior to enumerations, scan the counting chamber and determine the approximate number of transects or fields needed to enumerate 300 natural units (minimum of 10 and maximum of 100 random fields).

Step 1: Starting at a random starting place in the upper left-hand quadrant develop a pattern that allows for equal probability of landing in any area of the cell or chamber with the exception of the edges and the center. For Palmer- Maloney cells, a maximum pattern with 50 fields is made by having a grid of 8 x 8, subtracting 3 or 4 fields in either direction of the center.

Step 2: Using a high dry microscope objective (400x total system magnification) with a calibrated stage, identify and enumerate algae along transects, either horizontally or vertically across the chamber of the Palmer-Maloney cell.

Step 3: Without looking into the microscope, choose a location near the left edge in the upper third of the chamber (if vertical transects are analyzed, choose a location near the top edge in the left third of the chamber).

Step 4: Make a transect by moving only the horizontal stage control (or vertical control for vertical transects) a measured distance. Develop a pattern for the transects that will avoid the center and edges of the chamber. A second Palmer-Maloney cell can be used, if necessary (300 natural units should be counted with a minimum of three complete transects).

To enumerate 300 natural algal units, identify and enumerate all algal forms in the field of view:
Natural counting units are defined as one for each colony, filament, diatom cell (regardless if colonial or filamentous) or unicell. With the exception of diatoms, identify algal forms to the lowest possible taxonomic level. Enumerate diatoms as “live” if any protoplast can be observed or “dead” if the frustule is empty.

Step 5: Count the number of algal cells comprising each multicellular counting unit. Tabulate the data on the bench sheet for soft algae.

Step 6: Repeat until 300 natural algal units have been enumerated. Count only “live” diatoms as part of the required 300 natural algal units.

Step 7: Record the number of fields or total length of the transect that was observed.

Step 8: Phytoplankton Abundance is calculated as follows:

$$\text{Cells / ml.} = \frac{\text{Cell Count} * \text{field DCF} * \text{subsample DCF} * \text{fraction DCF}}{\text{volume scanned (ml)}}$$

Where:

Cell Count = number of cells enumerated

DCF = dilution concentration factor

If transects are used: volume scanned (ml) = total scan length (cm) * microscope field diameter (cm) * depth of chamber (cm)

If random fields were used: volume scanned (ml) = microscope field volume (cm3) * number of fields scanned.

The outcome of this calculation will then be converted to cellsPerBottle per the NEON data return template.

For the analysis of phytoplankton samples for diatoms from permanent slides, that is, to document 600 diatom valves in the sample, the protocol in section iii for permanent diatom slides (Page 16, below) will be followed.

When analysis is complete, Lowe will ship samples back to Boulder for storage until they are shipped to the BioArchive. Upon receipt from Lowe, Boulder will complete all chain of custody records.

2.4.2 Macroalgae (After Stancheva et al. 2015)

Thoroughly examine all the material and identify key macroalgal features needed to separate the algae by genus. These may include:
*Colonial shape, size and color in cyanobacteria (such as *Nostoc, Dichothrix, Rivularia*);
*Different life stages, heterocyst position and akinete development in cyanobacteria (such as *Anabaena, Cylindrospermum, Gloeotrichia*);
*Male and female specimens with developed reproductive structures in red and green algae (such as *Batrachospermum, Sirodotia, Oedogonium*);
*Different life stages and completely matured reproductive structures in zygnematalean algae and tribophytes (such as *Spirogyra, Zygnema, Mougeotia, Vaucheria*).

Step 1: Place each macroalgal genus identified aside.

Step 2: Prepare microscope slides for each macroalgal genus which may be presented with more than one species in the sample. The number of slides prepared depends on the need to obtain sufficient information to successfully perform species identification.

Step 3: Examine prepared slides under the compound microscope and identify macroalgae to species level. If large colonial diatoms are observed in the sample, record them.

Step 4: Take sufficient photomicrographs of all newly recorded species to support consistency of results. Take photomicrographs of previously reported species to demonstrate key aspects of vegetative morphology and reproduction used in identification.

Step 5: Record all macroalgal taxa identified; estimate relative abundance of each taxon and record this in the macroalgal datasheet based on a scale from 1-6.

2.4.3 Periphyton (After Stancheva et al. 2015): Soft Algae

The soft algae fraction is examined on a semi-permanent water mount for best observation of algal cellular morphology. Water mounts allow adjusting of the cover slip to change the position of the cells and spreading out of multilayered cell clumps for observation of critical taxonomic features.

Step 1: Using a research quality compound microscope, scan the semi-permanent slide with microalgae at magnification 200x to assess the taxonomic composition of the sample.

Step 2: Switch to a magnification of 400x (e.g., 40x objective with 10x eyepieces). At a magnification of 400x, the cover slip is composed of many horizontal optical transects.

Step 3: Identify and enumerate 300 natural algal units across a known number of horizontal optical transects. Count only intact cells with complete cell contents. Record the number of fields of view and horizontal transects traversed, each soft algal taxon identified, and the corresponding number of natural algal units enumerated in the ID Datasheet for soft algae.

Step 4: Take sufficient photomicrographs of all newly recorded species to support consistency of results. Take photomicrographs of previously reported species to demonstrate the key aspects of vegetative morphology and reproduction used in identification.

Step 5: Submit remaining microalgal fraction of the soft algal sample for archiving.

Note: The sample volume contained in one horizontal transect is determined as follows: a transect is a rectangular area of the slide in which the width is equal to the field of view and the length is equal to the length of the cover slip. With our microscope condition, at a 40x objective, the 0.55 mm width of the transect results in a cover slip (22 x 30 mm) consisting
of 40 optical horizontal transects. For microscopes where the 40x field of view differs from 0.55 mm, calculate the transect width required.

Sample volume held by one horizontal optical transect is calculated as follows: on the counting slide, 0.05 mL of subsample is placed. This subsample has been concentrated 5 times the original sample, thus 0.25 mL from the original sample is analyzed. Therefore, the original sample volume held by one horizontal optical transect is 0.00625 mL (=0.25 mL/40 horizontal transects).

When additional dilutions or concentrations are applied to the initial microalgal subsample of 1 mL (see Section 4.1.3 Step 2), the sample volume per transect must be corrected by multiplying with the dilution factor (DF). Most often, the subsample of 1 mL is counted without dilutions/concentrations (DF 1), but sometimes is concentrated to 0.5 mL (DF 2), or diluted to 2 mL (DF 1/2), to 3 mL (DF 1/3), to 4 mL (DF 1/4), to 5 mL (DF 1/5).

2.4.4 Identification and Enumeration Analysis of Diatoms

Identification of diatoms requires an understanding of how the cells are put together (each cell, termed a frustule, being comprised of two valves and one to several girdle bands) and what frustular components are being identified and enumerated. The different views one may have of a frustule and/or its components is also important since attempts to key out specimens will require one to know the view in which one is seeing an individual cell. There are many fine structural elements of diatom cell walls and

knowledge of this terminology is imperative for the identification of species. Guides to this information can be found in the published literature, and commonly used books and floras, including Krammer and Lange-Bertalot (1986-1991), Round et al. (1990), and Kociolek et al. (2015 a, b). The fine structure of diatoms is the basis for the taxonomy of the group, and modern approaches to taxonomy are relying more and more on these fine structures to make effective distinctions that are not only reflected at species level, but also at the genus level.

Step 1: Position the slide on the microscope stage with its label to the right. Scan slide at medium magnification (200x or 400x) to confirm that diatoms are evenly distributed on the cover slip and to assess the taxonomic composition of the sample to be analyzed.

Step 2: Establish a horizontal transect for counting by positioning the 100x objective a short distance from the edge of slide, where valves are no longer optically distorted. Record the coordinates where the first transect begins (for QA/QC purposes, if necessary for this slide). A transect is a rectangular area of the slide in which the width is equal to the field of view and the length is equal to the length of the area studied. Record the coordinates of this starting point, if needed for future QA/QC review.

Step 3: Identify and enumerate all complete and partial valves visible in the first field of view. A partial valve is defined as having more than 50% of the valve including the central area. The valve (both complete and partial) must extend at least halfway into the transect and must include the center of the valve in the transect. Once the diatoms in the first field of view have been enumerated move on to the next field of view in the direction of the horizontal transect. If a second transect needs to be counted, move to the first field of view of the second transect. Record the coordinates where the second transect begins (for QA/QC purposes, if necessary for this slide).

Step 4: Record the first and last field of view for each counted transect and the upper right corner of the cover slip by taking the coordinates from the microscope stage. Enter the coordinates in the ID datasheet for Diatom Sample.

Step 5: Record each diatom taxon identified and the corresponding number of valves enumerated in the ID Datasheet for Diatom Sample-Heading: Diatom taxon ID; per Appendix 2C from NEON.

Step 6: Identify and enumerate 600 diatom valves across a known length of horizontal optical transects. Avoid counting valves in any disrupted areas of the mount, particularly edges that have optical aberrations. When the diatom enumeration is completed, record the last field of view counted by taking the coordinates from the microscope stage. The last field of view counted can be located at any transect point. If the sample is very sparse, continue counting for 4 hours or until 300 valves are enumerated (whichever comes first), excluding time spent learning new species.

Record the number of transects traversed and the coordinates of the last field of view counted on the bench sheet for diatom analyses.

Step 7: Take sufficient photomicrographs of all newly recorded species to support consistency of results. Take photomicrographs of previously reported species to demonstrate the key aspects of vegetative morphology and reproduction used in identification.

2.5 Digital Image Reference Collection

We will take high quality images of each algal taxon identified and counted. Image files will be stored both taxonomically (e.g. all the Blue-Green algal images in a file) and by site. This will make comparison easy and, over time, allow us to develop an algal and diatom flora for each site, facilitating counting in the future and assessing changes in the flora over the life of the NEON project.

2.6 Development of Lists of Names

We will assess names against a master list, and keep separate lists by site and taxonomy. It is not unusual for there to be both previously unreported taxa (taxa with names that are not on the list) and taxa that cannot be identified (cannot be found in the literature or may be new to science). In some parts of the United States, mostly in the western half as well as in Alaska and Hawaii, 20-40% of the taxa may not be described (Kociolek 2005, 2006). We will keep a running list of "unknowns" with photodocumentation. Any analyst who designates an unknown (such as "*Navicula* sp. 5") will need to check with an expert before that designation can be used in the analysis. Again, by keeping names by taxonomic category and by site we can develop a floristic checklist for each site, which will be a useful tool to document floristic changes over time. All images with positive determinations will also have the taxonomic reference noted that was used for the determination. We will use *AlgaeBase* (Guiry & Guiry 2019), *DiatomBase* (Kociolek et al. 2019) and *Catalogue of Diatom Names* (Fourtanier & Kociolek 2011) to check nomenclatural issues for names used. We will track all new names/designations in Boulder, and the Boulder lab will report new names to NEON for addition to the controlled taxon list, along with authorship and a URL for reference, as well as any synonyms with the old and new name, and the date of the name change.

All Chain of Custody, Data files, Images, Lists of Names and other documentation for this project will have local back up on individual computers, external hard drives associated locally for each computer, on servers for the Museum of Natural History and on the super computer at the PetaLibary run by Research Computing at the University of Colorado, Boulder.

2.7 QA/QC Review

We will perform the QA/QC protocols on 10% of all samples. All QA/QC will be done by two different taxonomists. The second taxonomist in these QA/QC reviews will always be one of the experts (even if the first taxonomist on the sample was the other expert of this team). Please see the next section (3.0) for QA/QC protocols.

2.8 Data Reporting

All data will be reported to NEON by the Boulder Lab. Data Reporting will be done using separate spreadsheets for Phytoplankton/Soft Algae; Phytoplankton/Diatoms; Macroalgae; Periphyton/Soft Algae and Periphyton/Diatoms, with fields as specified by NEON, utilizing NEON spreadsheets (Appendix 2C). In addition to the reporting of data as indicated in the NEON spreadsheets, data will also include calculations of the total number of cells per bottle for the samples provided.

Samples for which QA/QC analyses have been completed will also be documented with spreadsheets, calculating and reporting both Percent Difference in Enumeration (PDE) and Percent Community Similarity (PSc). Lowe will report data to the Boulder lab via email, and those data will be uploaded by the Boulder lab to NEON.

2.9 Archiving and Storage

All unused preserved samples (phytoplankton, periphyton, macroalgae), plus permanent diatom slides and cleaned diatom material, will be archived by CU-Boulder per directions by NEON as reference collections. Preserved samples will be kept in the bottles in which they were originally shipped. Diatom slides will be stored in typical 100-count microscope slide boxes. Cleaned diatom material will be stored in glass vials.

Freeze Drying:
All samples are frozen in a lab deep freezer, then quickly placed in a 2.5L Labconco FreeZone -50^0 benchtop Freeze Dry System. See Section 2.11.1 for a detailed procedure for the freeze dry system.

Once samples are dry they will be transferred to small scintillation vials, labelled, placed in simple 10x10 cardboard trays and be ready to be stored indefinitely under ambient conditions.

2.10 Shipment/Transport to Repository/BioArchive

When sample analysis is complete, including QA/QC analyses, and data have been uploaded to NEON, we will ship samples for long-term storage to the NEON Bioarchive by surface shipping or, given our relatively close proximity to the facility, by ourselves, following protocols supplied by NEON. If we deliver the samples to the Bioarchive ourselves, we will coordinate with the BioArchive ahead of time for the delivery of samples. We will be sending to the Bioarchive the following materials: permanent diatom slides, freeze-dried 'cleaned' diatoms, preserved soft algae and preserved macroalgae. A manifest will be prepared by the University of Colorado lab per NEON's specifications, sent to the BioArchive to accompany the shipment of materials.

2.11 Other Considerations

Instrument Inventory: All capital equipment is inventoried annually by the University of Colorado. For this project the instruments most critical for the project are the 3 Olympus BX-51 light microscopes to be used for this project, their associated digital cameras (DP-71) and image capture software and hardware. An additional Olympus BX-51 light microscope and digital camera, computer hardware and digital image capture software are also in Lowe's lab in Madison, Wisconsin. All of the Olympus microscopes have Nomarski/DIC optics, with 60X and 100X objectives with 1.42 N.A. and 1.40 N.A. objectives, respectively.

2.11.1 Freeze Drying

All samples are frozen in a lab deep freezer, then quickly placed in the freeze drier unit. The Department of Ecology and Evolutionary Biology Department has a freeze drier for our use. This freeze-drier **is a** **Thermo Savant ModulyoD** and is located in Ramaley Building, Room C335,

Diatom sample processing using the University of Colorado EBIO BioCore lyophilizer

Processing:

1. After treatment with nitric acid and subsequent DI H_2O rinse regimen, processed diatom samples are stored in DI H_2O in 14mL borosilicate vials at room temperature, awaiting lyophilization.
2. Samples are stored in the Kociolek Lab at CU Boulder until there are enough to fill the lyophilizer unit at EBIO's shared BioCore laboratory.
3. Samples are frozen at -70 degrees Celsius overnight before transport to the BioCore Lab. Samples may be frozen individually, or on racks which can be directly loaded into the lyophilizer.
4. Frozen samples are put on ice and transported from the Kociolek Lab to the BioCore Lab for immediate loading into the lyophilizer.
    a. Small sample sets (~15): Bell jars
        i. Samples may be frozen to -70 degrees Celsius and transported to lyophilizer in small batches. These will be loaded into bell jars provided at the BioCore Lab.
            1. Tips for loading bell jars
                a. Prepare one bell jar at a time.
                b. Stuff 1-2 paper towels inside each bell jar to provide a stable area for samples to lay when loading.
                c. Insert sample vials (lids removed) into bell jar, and close with rubber lid
                d. Attach loaded bell jar to available valve on lyophilizer. **Turn on before sample thaws.** Keep samples on ice until the last possible moment. If the sample thaws, it will boil when the pressure drops, and the sample(s) will be lost.
    b. Larger sample sets: lyophilizer racks
        i. ~50 sample vials may be loaded (lids removed onto a designated rack and frozen to -70 degrees Celsius prior to transport to the lyophilizer.
        ii. Samples must be transported on ice and loaded into lyophilizer immediately upon arrival at the BioCore Lab; **lyophilization should begin before the samples thaw.**
5. Diatom samples are removed from the lyophilizer after 24 hours. Samples are transported back to the Kociolek Lab and prepared for shipment to the biorepository.

Unit operating instructions for diatom processing:

1. Open drain valve to drain leftover waste from previous use. Properly dispose of liquid waste from the unit. Do not close the drain valve.
2. Push button marked "fridge" on the unit at least 30 minutes ahead of system use for diatom lyophilization.
3. If using bell jars to process smaller sample quantities, proceed to step 5.
4. If using the rack insert to dry a large quantity of samples, lower the rack insert with pre-frozen samples into the main chamber, and properly align the clear plexiglass lid. This must be done **before** turning on the pump.

5. When temperature reading reaches approximately –50 °C, turn on the main vacuum pump. Watch to make sure that the oil level in the pump drops between 0.5 to 1 cm. This means the pump has primed. If any oil droplets shoot out the top of the oil mist filter (located on top of the pump), then it is time to change the oil mist filter element. Contact BioCore manager for assistance.
6. Let the pump run for 30 seconds, then close the drain valve.
7. Pressure within the chamber will begin to drop, and you will notice that the display will eventually change from "HPr" (red lights) to somewhere between 100 and 300 μbar (green lights). The pressure display reports units as mbar until it drops below 1 mbar, at which point the numbers report pressure in μbar.
8. Load bell jars, if using, and attach to valves on the outside of the main chamber.
9. When the pressure in the main chamber has dropped and the light display is green, turn the valve connecting the bell jar to the main chamber from "vent" to "vac." Do this **slowly;** otherwise, jars may implode.
10. Connect one bell jar at a time, making sure the pressure drops back down to where the light display is green before moving on to the next bell jar. When using multiple bell jars, turn the valve halfway between "vent" and "vac" on the first bell jar before setting the valve to "vac" on the second; this maintains a low pressure in jars already connected to the system, and reduces the amount of time spent waiting for the pump to bring the pressure back down. When all jars are connected, turn all valves to "vac".
11. Depending on the amount of liquid in each sample, the lyophilization process may take several hours. The unit can be left running all day or overnight if necessary to dry all samples.

Stopping the unit:

1. If using bell jars, slowly turn the bell jar valves from "vac" to "vent". Opening the valves too quickly causes freeze dried product to blow around and may cause loss of product. Detach bell jars and retrieve samples. If using rack insert, proceed to step 2.
2. Slowly open the drain valve. Wait 30 seconds. Turn off the pump. Turn off the freezer by pressing the "fridge" button. If using rack insert, carefully remove plexiglass lid and lift rack insert out of the main chamber to retrieve samples.
3. Close the drain valve to prevent water from draining onto the floor as the ice in the condenser melts.
4. Once the ice has melted, open the drain valve to empty the condenser. Properly dispose of liquid waste.

Additional notes/challenges:

- The rack insert does not provide a perfectly stable surface for diatom sample vials. High volume sample processing may result in sample loss due to how the rack must be loaded into the chamber. Risk of sample destruction will likely diminish with use of bell jars, but sample processing throughput will also decrease significantly.

    - For reference, the approximate maximum number of samples that can be handled by the rack insert is 75. 50 is probably safer. Bell jars may handle approx. 15 vials in total, if the unit is completely available and not being used by other labs.
    - Based on these results, we will used bell jars for processing. This has been done successfully, and while the processing rate is reduced, it provides a much lower degree of risk for the samples.
- The seals around the bottom of the main chamber and the lid must be checked when the pump is turned on. It may require some careful "jiggling" before a seal is created and the pressure begins to drop.

Once samples are dry each will be transferred to small scintillation vial, labelled, placed in simple 10x10 cardboard trays and be ready to be stored indefinitely under ambient conditions.

2.11.2 Micropipette Calibration

Calibration for each micropipette used will be done and recorded annually.

A calibration can be done in-house by using the method where the known weight of water at a specified temperature.

Tips are attached to the micropipette. The desired volume for the micropipette is set, and distilled water is drawn into the pipette. Temperature of the water is record, but must be between 20^0 and 25^0 degrees C. The set volume of micropipette is then dispensed into a beaker that has been previously weighed on a calibrated balance. This is done ten times to get a mean weight of the water dispensed. The volume of water is determined by the formula:

$$\text{Volume} = \frac{\text{Weight of the water sample}}{\text{Density of the water at the Temperature}}$$

If necessary, the micropipette will be adjusted to yield the desired volume for analyses.

The calibration will be recorded in a calibration log and include traceability information for the scale used and technician performing. Otherwise, we will pay for this service, and obtain a certificate of calibration from the lab performing the calibration.

2.11.3 pH Meter Calibration

The pH meter will be calibrated with standard buffer solution at pH 7.0 or pH 4.0 (post-cleaning solution will likely be acid). Calibration will be at least once a month, or more frequently during high-use periods. The occurrence will be logged with traceability information for the buffer solutions used and technician performing.

2.11.4 Reagents

We will purchase and note certificates of reagents in a chemical inventory specific to this project. The chemical inventory will include date purchased, and certification data for hydrogen peroxide, potassium dichromate, hydrochloric acid, nitric acid, and pH calibration solutions.

We will track use of reagents to the samples processed by batch number. For nitric acid we will monitor performance, to see if cleaning efficacy is reduced.

pH calibration solutions will be tracked to ensure they are used only before expiration dates. A monthly calibration record sheet will be used to track occurrences and traceability information.

We will document the lot numbers of the mounting media for permanent diatom slide preparation (Naphrax).

2.11.4 Laboratory Measurement Capabilities

We have our microscopes cleaned annually and assess their optical abilities by using test slides (of the diatom *Amphipleura pellucida* whose striae are at a density that is at the limits of the resolving capability of the light microscope) to demonstrate optimal resolving capabilities of the oil immersed lenses.

2.11.5 Laboratory Organization

Please see figure 1 for the physical layout of the lab and office spaces to be used for this project.

3.0 QAQC Protocols

We will perform and report both the Percent Difference in Enumeration (PDE) metric (highlighting those analyses that did not meet the QA/QC target criteria (less than or equal to 5%), and the Percent Community Similarity (PSc) metric (and highlighting and reporting those analyses not meeting the target QA/QC criterion; 85% in the data sheets). Calculations for PDE will be as follows:

$$PDE = \frac{|n_1 - n_2|}{n_1 + n_2} * 100$$

Where n_1 is the number of individuals counted by Taxonomist #1, and n_2 is the number of individuals counted by Taxonomist #2.

Percent Community Similarity (PSc) for samples going through QA/QC analysis will be calculated by the following formula:

$$PS_C = 100 - 0.5 \sum_{i=1}^{s} (|a_i - b_i|)$$

Where a_i is the percentage of species *i* in Count A (performed by Taxonomist #1) and b_i is the percentage of species *i* in Count A (performed by Taxonomist #2).

There are some recent studies related to large (regional, continental) scale algal and diatom analyses indicating that analyst bias may creep into these projects. To help minimize issues in this area, we will:

*Where possible (given the flow of samples from NEON) randomize assignments of samples for analysis.

*Leverage algae and diatom images to create floras for each site, which should help develop higher degrees of consistency and help recognize floristic changes at sites.

*We will perform QA/QC analysis on a regular basis through sample analysis, and discuss any issues related to results from these analyses.

*Facilitate communication between taxonomists involved in QA/QC analyses to make the exercise more of a learning experience.

*Set aside time during the project to discuss unknowns and/or problem taxa, or to go over floras from completed sites, to review the taxa encountered. This is to be underscored for the unknowns, since making sure all team members are consistently using designations across the study will be crucial for data analysis.

4.0 Relevant Literature

Acker, F. 2016. Identification and enumeration of algae in phytoplankton samples. Procedure No. P-13-52. 8 pages.

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