Development of a Comprehensive State Monitoring and Assessment Program for Wetlands in Massachusetts

Appendix K

Assessment of Wetland Communities: Diatom Analysis

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Assessment of Wetland Communities: Diatom Analysis

Preliminary Diatom Analysis

Our goal for the preliminary diatom analysis is to get a general sense of whether diatoms as a group, or certain groups of diatoms, have the potential upon further analysis to yield indices of biological integrity (IBIs) for assessing wetland condition.

The preliminary algae analysis will focus on algae samples collected from leaf litter in the 2008 field season. We are focusing on leaf litter samples because we appear to have gotten good samples from the field work and have four subplot samples for all sites (we were not always able to get four sub-samples per site for water and substrate sampling because of the lack of standing water at some sites).

We propose analyzing leaf litter algae samples for ten sites, five sites with high IEI scores and five sites with low IEI scores. We will seek to get a good spread of sampling dates for both high and low IEI sites.

Currently we have separate leaf litter algae samples (50 ml) for each of the four subplots at each site. We are proposing to create one composite sample for each site. The procedure for compositing the samples will be conducted as follows: 1) agitate the samples to re-suspend diatoms, 2) collect 10 ml with a clean pipette (to prevent cross-contamination of samples), 3) combine the 10 ml from each subsample into a vile for a total of 40 ml. The composited samples will be sent to an outside expert for analysis. This will leave us with 40 ml of each of the original subsamples for further analysis.

The samples would be sent to Bowling Green State University for diatom community analysis. The analysis would be overseen by Rex Lowe. The samples would likely be cleaned by acid digestion to remove excessive organic material and mounted on slides with Naphrax mounting medium. The community analysis would be based on a 600 valve (individual diatom) count (see attached laboratory procedures).

The analysis would be complete within 3-4 weeks upon receipt of the samples. We would analyze the results of the diatom community analysis for any preliminary relationships to the CAPS ecological integrity metrics. This will inform whether a full analysis of the algae samples is warranted and if we should continue to sample algae in the 2009 field season.

Analysis of Leaf Litter Samples for Diatoms

Based on the results of preliminary diatom analysis it was decided to proceed with identification and analysis of leaf litter samples collected in 2008. We are proposing to create one composite sample for each site. The procedure for compositing the samples will be conducted as follows: 1) agitate the samples to re-suspend diatoms, 2) collect 10 ml with a clean pipette (to prevent cross-contamination of samples), 3) combine the 10

ml from each subsample into a vile for a total of 40 ml. The composited samples will be sent to an outside expert for analysis. This will leave us with 40 ml of each of the original subsamples for further analysis.

Analysis of Water Samples for Diatoms

One question that arose in the interpretation of leaf litter diatom samples is whether some species of diatoms may be missing from leaf litter samples because they were suspended in standing water at the time of sampling. If this was the case then leaf litter samples from sites that lacked standing water might contain species that were absent or less abundant in leaf litter samples from sites with standing water.

To gain insight into this issue we will analyze composite samples collected from standing water for 29 sites sampled in 2008 for which we have water samples from all four subplots. Sites with water samples from four subplots will be comparable with leaf litter samples because these were collected from four subplots at all sites and composited for identification.

By comparing results from water and leaf litter samples at these 29 sites we will determine to what degree these methods produce overlapping results. We will also compare combined water and leaf litter results from these sites with the results from leaf litter samples collected in 2008 from other sites. Based on the results of these analyses we will determine whether it makes sense to identify water sample diatoms from all sites sampled in 2008.

Identification

Samples will be sent to Bowling Green State University for diatom community analysis by Rex Lowe. The community analysis would be based on a 600 valve (individual diatom) count using the same laboratory procedures as for the preliminary analysis (see attached laboratory procedures).

Quality Control and Assurance

Sample and slide quality can affect the outcome of these procedures. Minor deviations that do not affect the area scanned or number of specimens observed will be described on bench sheets. Other deviations will be discussed with the Phycology Section Project Manager for inclusion in the project QA/QC notes.

If diatoms account for more than 10% of the phytoplankton community, duplicate diatom slides will be made for QA purposes. Simpson's Similarity Index (SIMI) will be used to compare QA counts. A 60% similarity threshold is used. If samples are less than 60% similar the sample will be recounted and a remark added to the database.

Data Analysis

The overarching goal of the data analysis is to determine whether CAPS IEI and the component ecological integrity metrics (e.g., habitat loss, connectedness, etc.) are related to observed ecological conditions, and to further quantify the magnitude and nature of those relationships. To accomplish this goal, we will use a variety of statistical methods including principally quantile regression (Cade et al. 1999) and a custom analytical method based on the method of indicator species analysis (Dufrene and Legendre 1997). The data input for both analytical methods will be a list of the sample points and the corresponding values for each of the CAPS metrics and a suite of variables representing the presence or standardized abundance of each species or group of species and/or one or more derived biotic indices (e.g., Simpson's diversity index). For more information on data analysis see section 2.4 Analytical Method in the QAPP.

Protocol for Phytoplankton Analysis, Algal Ecology Laboratory,Bowling Green State University

Contents

1. Log-in Procedures for Planktonic Algal Samples

PURPOSE

This protocol describes procedures for logging samples in when they are received by my laboratory at BGSU. These procedures cover the steps from the receipt of samples to the submission of data.

OVERVIEW

There are three main steps in the log-in procedure:

1. Unpacking and examining samples to confirm that all are intact and that the containers are not damaged or leaking. Check that the number of samples received equals the number expected.
2. Logging samples into a project database employing an Excel spread sheet with all sample data that are provided by the project manager.
3. Assign each sample a unique character short name that identifies the habitat and date.

5. Diatom Identification

Preparation of Slides for Diatom Identification

To identify and enumerate diatoms accurately at the species and variety levels, it is necessary to remove most extracellular and intracellular organic matter from the siliceous frustules of diatoms and other material in the sample. Removing the organic matter is necessary so that all details of diatom structures necessary for taxonomic identification are clearly visible. This protocol describes two methods for removing organic material from a plankton sample. The first method (incineration) is preferred if the sample does not contain excessive quantities of particulate organic matter and will be applicable to most phytoplankton samples. The second method is used only when

necessary to prepare samples rich in particulate organic matter by digesting the sample with nitric acid.

METHODS

To produce a diatom slide most organic materials must be removed from the sample so that diatom frustules can be clearly seen and identified. This requires a preliminary examination of the raw sample with a microscope to determine the proper amount to process. Diatom preparation data (Beaker #, and notes) are recorded on the appropriate "Diatom Slide Preparation Form" placed into the "Diatom Analysis" folder at the time of sub sampling.

Burned mounts. A portion of the diatom sub sample (########-D) is pipetted onto a clean, dry 22mm coverslip placed on a slide-warming tray. The sub sample is allowed to dry on the coverslip (normally 24 hours). The coverslip with dried diatom sub sample is transferred to a hot plate and incinerated using the "high" setting on the hot plate. One to two hours of this treatment will convert the organic matter on the coverslip to ash. The ashed sub sample is then removed from the hot plate and inverted onto a clean microscope slide onto which a drop of Naphrax® mounting medium has been placed. The margin of the slide is labeled (diamond-tipped pen directly on the glass) with the sample number (########-D) and the slide is placed back onto the warming tray for a minimum of 30 minutes to allow the mounting medium to penetrated the cleaned diatom cells. The diatom slide is then transferred back to the hot plate.

Acid digestion. In some instances it may be necessary to prepare diatom slides using acid digestion. This technique is used when there is excessive organic matter in the sample such that a burned mount contains too much ash to allow an unobstructed view of the diatoms. The entire diatom sub sample (########-D) is poured into a 1000-ml beaker and placed into the fume hood. The diatoms are allowed to settle for a minimum of 12 hours after which the supernatant is carefully poured off and replaced with distilled water to eliminate excess preservative in the sample. After 12 hours the sub sample is decanted again and an equal volume of concentrated nitric acid is added to the beaker and the suspension is allowed to sit in the fume hood for 48 hours.

WARNING. THE FOLLOWING PROCEDURE IS TO BE PERFORMED ONLY IN A POSITIVE-DRAW FUME HOOD. TECHNICIANS ARE REQUIRED TO WEAR SAFETY GLASSES AND PROTECTIVE GLOVES!

After 48 hours the supernatant is poured from the beaker taking care not to disturb the sediment containing diatoms on the bottom of the beaker. Distilled water is added to beaker to dilute the acid and the diatoms are allowed to settle to the bottom of the beaker for a minimum of four hours. This distilled water addition followed by settling and pouring is repeated at least five more times or until the pH is near neutral. The date and time of each decant is noted on the "Diatom Slide Preparation Form." The cleaned material remaining in the bottom of each beaker after the final siphoning is poured into a 20-ml glass vial, which has been previously labeled with Sub sample number. Labels are

made on the side of the vial using a diamond scribe and on the cap using an indelible marker. Using a wash bottle containing distilled water, any remaining material adhering to the beaker sides is washed into the vial, and the vial is stored with others until ready to make slides.

QUALITY ASSURANCE/QUALITY CONTROL

Diatom frustules are microscopic, generally falling in the fine silt size range; therefore, there is a possibility that samples can be contaminated. Laboratory rooms where raw or processed samples are handled should be kept as clean as possible. Lab bench surfaces should be kept clean and free of debris. Techniques similar to those used for sterile experiments (bacteriological plating, etc.) should be followed to minimize the risk of cross contamination of samples. Where feasible, disposable pipettes, stirrers, etc. should be used. Where they cannot, they should be rinsed in distilled water and stored dry.

New glassware should be washed and/or rinsed prior to use. Used glassware should be vigorously scrubbed, washed with a detergent, and rinsed at least three times with distilled water to prevent contamination. (Explanatory note: at times tap water, because of algal blooms and use of diatomaceous earth filters, may contain diatoms.) All equipment should be stored dry to prevent growth of algae or fungi.

Preparation of Diatom Slides Using Naphrax Mounting Medium

PURPOSE

Accurate identification and enumeration of diatoms requires mounting of cleaned material between a microscope slide and cover slip in a medium with a refractive index near that of glass, so that the features of diatom frustules or valves are clearly visible at high magnification. Naphrax, a commercially available toluene-based mounting medium with high refractive index, is currently used at BGSU. This protocol details the steps necessary to produce high-quality diatom mounts from cleaned diatom material. This technique produces permanent mounts, preserving the diatom specimens over many decades. Procedures described in this protocol include the dilution and dispersion of cleaned diatom suspensions onto glass cover slips, the mounting of cover slips onto glass microscope slides using Naphrax mounting medium, and the labeling of permanent mounts. Naphrax should be considered a hazardous substance because it contains toluene, an organic solvent. Toluene volatilizes readily when heated. For this reason, heating of Naphrax should only be performed under a positive-draw fume hood. Personnel should wear safety glasses and protective hand wear when working with liquid Naphrax.

6. METHODS

Estimate amount of cleaned diatom material to deposit on coverslip.

Starting with cleaned material contained within 20-ml glass vials, the volume of suspended material that will need to be deposited ("dripped") on a cover slip to produce a

slide of the appropriate cell density is estimated. The ideal density to be achieved on the final mount is somewhat subjective and is based on the amount of debris in the sample and the preferences of the slide analyst. Generally, between 5 and 20 diatom specimens should be present in a single high power microscope field (1000X). To make the estimate, the sample is shaken to ensure a homogeneous dispersion of cells within the 20-ml vial. Then the vial is immediately opened and a volume withdrawn with a pipette. The material is placed on a slide and covered with a 22 x 22 mm cover slip. The preparation is observed under a compound microscope at 50X magnification and a number of fields are observed and the density of cells examined. If cells are too thick the cleaned sub sample is diluted with distilled water. If cells are too sparse the cleaned sub sample is concentrated further gravimetrically and the procedure repeated until a satisfactory density is obtained. Since these slides are not quantitative it is not necessary to be concerned with sub sample volume. If a satisfactory slide could be made by increasing the concentration of cleaned diatom material by two to five times, then do this by using a micropipetter to remove the required amount water from the vial of material after it has been allowed to settle for at least eight hours. Record the concentration factor on the "Diatom Slide Preparation Form."

Deposit cleaned material on coverslip.

Use forceps to remove single 18 x 18-mm cover slips from the ethanol storage container, and carefully clean each by wiping with a Kimwipe. Place each cover slip on a marked space of the aluminum drying plate. Be sure the aluminum drying plate is clean and dry to avoid cross-contamination. If the intended drip count is less than 600 pl, drip a small amount of distilled water onto the cover slip with a disposable pipette, sufficient to form a thin layer of water over the entire cover slip. Agitate the sample vial to a uniform dispersion and use the adjustable pipetter to quickly withdraw the required amount from near the central portion of the sample. Eject this material smoothly and carefully onto the layer of distilled water already on the slip. By alternately drawing material up into the pipette and ejecting it, a homogeneous suspension is achieved on the cover slip. In the case where more than -600 V1 of original sample is required, the addition of distilled water is not necessary, and the sample can be ejected and mixed directly on the cover slip. In both cases, take care to ensure that the suspension covers the entire surface of the cover slip, including the extreme edges of the comers. Should the cover slip overflow, discard the cover slip, and repeat the procedure with a freshly cleaned cover slip. Discard the pipette tip when finished with each sample.

Once the aluminum drying plate is loaded with cover slip preparations, the plate should remain undisturbed until the cover slips are dry. At this point, drying of the slips can proceed at room temperature (a period of several hours will be required), or gentle heat (warm to the touch only) may be applied to hasten evaporation (a crook-neck lamp with incandescent light bulb placed 15 - 30 cm over the drying plate is one option). Once completely dry, put the aluminum plate with cover slips on the hot plate that has been preheated to 250 to 300'F. Leave for 3 to 5 minutes. This procedure ensures that nearly all water is driven from the material on the cover slips and helps assure that the diatom frustules will adhere to the surface of the glass. Remove the aluminum plate from the hotplate and inspect the cover slips. If the pattern of diatoms distributed on any of the

cover slips is not even and smooth, they should be re-dripped. If cover slip distributions seem unsatisfactory after repeated attempts, consult an algal analyst.

Mount coverslip on microscope slide.

Using a diamond scribe, etch microscope slides with Sample ID, Sub sample ID and Slide Replicate ID (e.g., GS029231 DT1 a).

THE FOLLOWING STEPS MUST BE PERFORMED IN A POSITIVE-DRAW FUME HOOD!

Using a rounded wooden splint or disposable pipette, transfer a small amount of Naphrax (volume equivalent to -2 to 4 drops of water) to the central portion of the etched side of the microscope slide. Using a rounded wooden toothpick, distribute the Naphrax over an area approximately equivalent to the size of the cover slip. Then remove the appropriate cover slip from the aluminum plate with forceps, being careful to handle the cover slip only at the extreme comers. Invert the slip and place it gently on the Covered-covered portion of the slide. Then place the slide (cover slip up) on the hotplate and apply gentle heat until the evolution of bubbles resulting from the evaporation of the toluene solvent first occurs, and then significantly diminishes. Remove the slide from the hot plate, and, using the rounded toothpicks, gently position the cover slip and press it to form a uniform, thin layer of Naphrax. Make sure that the edges of the cover slip are brought parallel to the edges of the microscope slide. Care must be taken at this stage not to press so hard as to damage or dislodge the diatoms or cause warping of the cover slip. As this procedure is taking place, the Naphrax is "setting up" (becoming hard), and the ability to move the cover slip will diminish rapidly. At this point, set aside the mount to finish cooling. Use a single-edge razor blade to carefully trim any excess Naphrax that has been squeezed out from beneath the cover slip. Great care must be taken to avoid "lifting" the cover slip by inadvertently allowing the edge of the blade to move between the cover slip and the microscope slide. Once most of the excess Naphrax has been removed and discarded, and while still working under the hood, place the mount in successive baths of acetone, and then ethanol for no more than 10 or 15 seconds each. Finally, wipe the mount clean with a Kimwipe tissue.

Add paper label to slides.

Either before or after slides have been analyzed, depending on project requirements, prepare paper labels and attach them to the mounts. Labels should contain all of the critical information for identifying the sample from which they came and should be dated and initialed.

Assemble forms and transmit slides.

Put slides in plastic slide boxes; label each with name of project and subproject, Subproject ID, Box - of -, date (month/year) box prepared, and name or initials of preparer. Sign and date the "Diatom Slide Preparation Form" and the "Diatom Lab - Slide Preparation Notes" form and put them in the "Diatom Analysis" folder. Print a "Diatom

Slide Analysis Form" for use by the diatom analyst and add it to the "Diatom Analysis" folder also.

Preserve and store cleaned material.
After slides are analyzed according to the appropriate protocol, and no additional slides need to be made, process the vials containing the remaining acid-cleaned material for long-term storage. Working under a fume hood, add two - four drops of 100% buffered formalin to each vial (some contractors use alcohol as a preservative instead). Tightly cap the vials and seal them by immersing the top 1/3 of the vial in melted wax. Then transfer the vials to the appropriate storage cabinet. Be sure that the cabinet and shelves on which they are stored are properly labeled with the project information.

QUALITY ASSURANCE/QUALITY CONTROL

This procedure was originally developed in the laboratories of the ANSP and has been used for the preparation of several thousand slides. It has been modified for use in the Algae Laboratory at BGSU. Naphrax is produced under quality control conditions specifically for the purpose of high resolution slides (Northern Biological Supplies of Islip Great Britain). Naphrax mounts have proven to be stable over long periods (there are 25 plus year mounts in the ANSP Diatom Herbarium) and have been the mounting medium of choice of European investigations for over 40 years. It should be understood that, given the microscopic size and large numbers of diatoms, which are transferred from the cleaned material vials to the finished mount, there are a number of steps where contamination of the samples is possible. Laboratory rooms where raw or processed samples are handled should be kept as clean as possible. Laboratory bench surfaces should be kept clean and free of debris at all times. Techniques similar to those used for sterile experiments (bacteriological plating, etc.) should be followed to minimize the risk of cross-contamination of samples. All equipment coming into contact with sample material should be rinsed in DISTILLED WATER or RO water at least three times. Disposable pipettes should be used when possible. The distribution of specimens on the final mounted cover slips should represent the samples contained within the cleaned material vials. The degree to which this is true depends on how well the cleaned material is dispersed prior to sub-sample withdrawal, and how evenly the withdrawn material is dispersed on the cover slip. Great care should be taken to ensure that these two steps are completed properly. For certain critical applications, the project protocol may call for duplicate slide sets to test for variation in quantitative data introduced by this procedure.

Analysis of Diatoms on Microscope Slides Prepared From Samples of Planktonic Algae

SCOPE

This protocol covers the identification and enumeration of diatom taxa mounted on microscope slides.

APPARATUS/EQUIPMENT

Compound microscope:

Oil immersion objective (100x) with a numerical aperture of at least l.3;

Eyepieces of 10-15x;

DIC (differential interference contrast) or bright field condenser;

Diamond scribe mounted on microscope's objective stage

High intensity light source.

METHODS

Diatom counts.

Review the "Diatom Slide Preparation Form" and the "Diatom Slide Analysis Form" contained in the "Diatom Analysis" folder and transmitted with the diatom slides from the Diatom Preparation Lab. The "Diatom Slide Analysis Form" lists sample information for each slide it accompanies, and provides space next to each listed slide to initial and date when a count is finished. It also serves as a chain-of-custody record; it must be signed by the person delivering the slides and the person receiving them. Make sure that the slides correspond with the entries on the form. Note and resolve any discrepancies. Scan slides at low to medium magnification (100x to 450x) to confirm that diatoms are evenly distributed on the coverslip, and are at a density appropriate for efficient counting. At high magnification (1000x), there should be between 5-10 diatoms per field. If there are problems with dispersion or density that would compromise the quality and accuracy of the analysis, discuss these with Diatom Preparation Lab personnel and have new slides made. Avoid counting diatoms in any disrupted areas of the mount, particularly edges that have optical aberrations. Because slides may need to be recounted for QA/QC purposes, it is very important to clearly demarcate the areas of a slide scanned during a count. After the preliminary slide examination, secure the slide in the mechanical stage and use the microscope's diamond scribe to etch a horizontal or vertical line (depending on personal preference) on the coverslip to mark the edge of the first row to be counted. Rows are narrow rectangular areas (strips) of the slide adjacent to the scribed line, with width equal to the field of view. Start rows far enough from the coverslip edge to avoid optical distortion, and end them near the opposite coverslip edge where diatoms are no longer clearly visible. Locate a starting point near one end of the etched line and make a circle with the scribe. This denotes the starting point of the count. During the count, etch a circle around the last field counted in the first row and at the beginning and end of all other rows. Always check to make sure that etching is clearly visible so that circles and lines can be located easily by others. Enumerate and identify diatoms equal in number to the number of diatoms that were recorded in the Palmer-Maloney counts. Count all partial valves that are more than 50% of the valve or that contain unique features such as recognizable central areas or distinct ends. Put initials and date on the "Diatom Slide

Analysis" form next to the entry for the slide just counted. Return it and any other related forms to the "Diatom Analysis" folder. Clean slides of immersion oil with alcohol. When finished analyzing all slides in a subproject, give the slides and "Diatom Analysis" folder to the Phycology Section Project Manager. Sample analysis may require biovolume measurements for each taxon occurring in the sample in a study unit. Biovolume measurements can be made during the routine process of counting slides or after all slides for a Subproject have been counted. It is likely that criteria for selecting specimens to measure will evolve as the number of measurements for common taxa accumulates. Biovolume measurements using standard geometric formulae are made on a minimum of five specimens/taxon and recorded on the data sheet.

Specimen documentation.

As each new species or form is encountered, specimens are photographed and stored in a digital image database. Enough photos are taken to document the full range of morphological variability in the species.

QUALITY ASSURANCE/QUALITY CONTROL

Sample and slide quality can affect the outcome of these procedures. Minor deviations that do not affect the area scanned or number of specimens observed should be described on bench sheets. Other deviations should be discussed with the Phycology Section Project Manager for inclusion in the project QA/QC notes.
If diatoms account for more than 10% of the phytoplankton community, duplicate diatom slides will be made for QA purposes. Simpson's Similarity Index (SIMI) is used to compare QA counts. A 60% similarity threshold is used. If samples are less than 60% similar the sample is recounted and a remark is added to the database.

6. Data entry

Enter data recorded on the bench sheets into the tables of the project database.

Calculation of phytoplankton abundances and biovolumes.
The calculation of phytoplankton abundance depends on the apparatus used during analysis. Biovolume values are determined by multiplying the abundance (cells/ml) by the average biovolume of each cell (cubic microns). The average biovolume of each cell is determined by averaging all values for each taxon in each group on the "Biovolume Measurements" spread sheet. If there are no records in the "Biovolume Measurements" spread sheet for the taxon, a predefined constant based on genus (for diatoms) or algae type (for non-diatoms) will be used (see table 1 for geometric shapes and volume formulae). Equations for abundance calculations are given below.

From Palmer-Maloney cell-count-data the following equation is employed to calculate cells/ml:

$$\text{cells/ ml} = \frac{(n)\,(V/v)}{}$$

S
where: n = number of cells counted, V = volume of concentrated plankton sample, v = Volume of sample enumerated and S = original sample volume in ml.

Biovolume/ml is calculated as: bv/ml = (cells/ml)(Biovolume/cell)

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