Standard Operating Procedure

Preparing and processing algae samples for taxonomic analysis of diatoms

Commonwealth of Kentucky
Energy and Environment Cabinet
Department for Environmental Protection
Division of Water

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TABLE OF CONTENTS

1. TITLE PAGE AND APPROVAL PAGE

see page 1

2. REVISION CONTROL PAGE

see page 2

3. TABLE OF CONTENTS

see page 3

4. PROCEDURES

4a. Scope and Applicability

This SOP describes the method for preparing periphyton (attached algae) or phytoplankton (suspended algae) samples for taxonomic identification of diatoms. Procedures are performed by the Monitoring Section of the Water Quality Branch (WQB). Most samples come from bioassessment programs in WQB, and have been collected using the methods outlined in the SOP "Collection methods for benthic algae in wadeable streams"[2], however, the procedures listed below are applicable to samples collected using any collection method. Routine samples are usually from periphyton and are not quantitative (i.e. there is not a known sample area to which sample results will be extrapolated), but the procedures outlined here can be used for quantitative periphyton samples and for phytoplankton samples using extra steps identified for those purposes.

This SOP pertains to samples that have been received in the Division of Water (DOW) Biological Laboratory and logged into the WQB sample tracking system (see SOP "EDAS data entry and biological data management"[3]), and placed in the Algae Lab refrigerator for temporary storage. This SOP covers sample handing after samples have been received in the Algae Lab, through preparation of permanent diatom mounts that are ready to be identified by a taxonomic analysts.

Procedures involved in performing diatom taxonomic identifications are discussed in the SOP "Diatom sample taxonomic identifications"[4].

Analyses of non-diatom algae are not routinely performed, but procedures for preparing and identifying samples for taxonomic identification of non-diatom algae can be found in the SOP "Methods for Assessing Biological Integrity of Surface Waters in Kentucky, February 2008, Revision 3" [1], Section 6A.2.4.2.1.

4b. Summary of Method

A subsample is taken from the collected sample and treated with strong acid to oxidize organic material, leaving behind the siliceous diatom frustules. An aliquot of this cleaned diatom material is dried onto a coverglass which is then mounted onto a microscope slide with a high refractive index resin.

For quantitative periphyton samples, the total sample volume is recorded before the subsample is taken, and the subsample amount is carefully measured. For phytoplankton samples, the sample is settled to concentrate it prior to subsampling as with quantitative samples.

The oxidation procedure follows methods described in the manual "Rapid bioassessment protocols for use in streams and wadeable rivers, Chapter 6"[5].

4c. Definitions and Acronyms

Acronyms:
DEP Department for Environmental Protection
DOW Division of Water
MSDS Material Safety Data Sheets
WQB Water Quality Branch

Definitions:
periphyton – material adhering to benthic substrates; here, it refers to the algal component.
qualitative sample – a sample collected from composited aliquots from multiple or single habitats with no defined sample area (see SOP "Collection methods for benthic algae").
quantitative sample – a sample collected from a known area of substrate, and for which the total cell (or unit) density per unit area will need to be calculated. The processing of quantitative samples requires tracking of sample, subsample, and aliquot volumes.
phytoplankton sample – a sample of a defined volume of the water column; these samples typically have low cell densities and so must be concentrated or settled prior to processing and identification; if the water column is sampled with a plankton net, the sample can be treated like a periphyton sample.
cleaned diatom material – the subsample that has been through the digestion process and is ready to be mounted onto a microscope slide.

4d. Health & Safety

Procedures involve hazardous chemicals, and hot glassware and equipment. Do not perform these procedures without fully understanding hazards and appropriate personal protection equipment. Inattention to safety procedures could result in burns to skin, eyes, and respiratory system, damage to internal organs, contact with known carcinogens and teratogens, and fire. Material Safety Data Sheets (MSDS) for all chemicals involved in these procedures are kept in the DOW biological laboratory for reference. These procedures must be performed in a laboratory equipped with a chemical fume hood, an eyewash station, and a safety shower. All accidents must be reported to the Monitoring Section Supervisor. For life threatening emergencies, call 9-911 from any phone in the DOW laboratory. For release or spills of hazardous materials, evacuate the area and immediately contact the following the Environmental Response Coordinator (ERT Supervisor) at 564-2380 (4-2380).

Disposal of wastes generated from these procedures must follow DEP's chemical hygiene plan or instructions from the Monitoring Section Supervisor.

Nitric acid: Nitric acid is used in the diatom subsample digestion procedure, and has the potential to splash onto skin. Nitric acid is a strong acid and oxidizer, and produces hazardous fumes. Gloves and chemical splash goggles are required when transferring nitric acid to beakers, when performing water rinses of digested samples, and any other time that nitric acid is handled. All use of nitric acid must be conducted in an operating fume hood.

Formalin: Samples from the field are usually preserved with formalin (~1-2%) or must be preserved upon receipt. Gloves chemical splash goggles are required when handling preserved samples. Preserving and subsampling steps must be performed in a fume hood.

Glass: Glassware, coverglass, and microscope slides should be handled with care. Broken glassware, used coverglass, and microscope slides do not go into the regular trash, but must be disposed of in a secure and sturdy container before taking to the dumpster.

Toluene: Toluene is used to prepare the Naphrax mountant. When the mountant is heated in the slide preparation process, it releases toluene fumes. All use of toluene must be conducted in the fume hood. Keep toluene away from sources of ignition and flames.

Dusts: Removal of excess mounting medium from prepared slides may create irritating dusts. Wear a dust mask while performing this task.

Hot items: Beakers and microscope slides heated on the hotplate during sample digestion and coverglass mounting will be hot. Wear hot mitts when handling beakers and handle microscope slides by unheated edge.

4e. Cautions

Handle glassware with care to avoid breakage.

Do not leave samples unattended when heating beakers on a hotplate.

Take special care to keep beaker and slide numbers with corresponding sample information during entire sample process.

With quantitative samples, take care to ensure accurate measurements of subsamples and aliquots.

4f. Interferences

Sample degradation: Improper preservation of samples may lead to decomposition of the algal cells and/or continued growth in the sample container. Check sample preservation upon sample receipt. Samples free of large clumps of macroalgae and plants are sufficiently preserved with ~2% formalin. Samples with clumps may require greater

concentrations. All samples should be refrigerated during storage to further reduce degradation.

Contamination and cross-contamination: Because algal cells are microscopic, the potential for contamination of glassware and sample storage containers is great. Glassware must be cleaned thoroughly with a high quality laboratory cleanser and hot water. Glassware and containers must be stored with lids on, in cabinets, or wrapped, to minimize settling of airborne particles. Any tools used to homogenize samples, remove subsamples, or remove aliquots must be thoroughly cleaned before use and before using on the next sample. Use deionized or distilled water for all sample dilutions, rinses, and for the final rinse when cleaning glassware. When adding acid to samples and when heating on the hotplate, keep beakers separated so possibly spills or spatter will not contaminate adjacent samples.

Uneven dispersion in diatom mounts: Excess vibrations and air movement will adversely affect the even dispersion of diatom frustules when drying onto coverglass. Uneven dispersion will reduce the accuracy of quantitative enumeration, and may interfere with the ability of the analyst to identify specimens. Minimize vibrations and air currents in the preparation room.

4g. Personnel Qualifications / Responsibilities

Procedures will be performed by personnel trained in this SOP by experienced technical staff. Personnel should have a basic understanding of laboratory safety. Personnel performing these procedures are responsible for fully understanding safety and quality assurance procedures.

4h. Equipment and Supplies

Quantitative samples
quantitative algae sample processing log
large volume graduated cylinder
large volume (5-10ml) autopipetter and disposable tips

Phytoplankton samples
phytoplankton sample processing log
1000mL glass graduated cylinder
laboratory plastic film (e.g. Parafilm™)

Diatom Sample Digestion
grease pencil
diatom processing log sheet
chemical fume hood
200 mL tall form beakers (recommended)
nitric acid
hotplate
deionized water
vacuum pump or other suction device, with small diameter fittings

20 mL glass scintillation vials
wash bottle with deionized water
litmus paper

Diatom Slide Preparation
coverglass (no. 1 thickness, 20 x 20 mm), cleaned (see below)
70% Ethanol
laboratory wipes (e.g. Kimwipes™)
metal drying plate with numbered grid
plastic transfer pipette
beaker of deionized water
glass transfer pipettes, with bulb
(or small volume autopipetter – some quantitative samples)
hotplate
insulated mitts
high quality microscope slides
Naphrax™ diatom mountant (or similar), prepared as per manufacturers instructions
flat tipped forceps
toothpicks
razor blades
dust mask
slide labels (preprinted or hand-written)
slide box

4i. Step by Step Procedure

4i1. Before beginning:

1) generate a hard copy sample processing log (qualitative diatom sample processing log, quanitative diatom sample processing log, or phytoplankton diatom sample processing log; Appendix A) using the WQB sample tracking system, or by transcribing from other sample log or sample submittal form
2) review processing log sheet for completeness and compare entries to sample container labels; resolve any discrepancies before starting and note on processing log
3) verify that samples have been properly preserved and sample containers are intact; inspect samples and sample tracking log for proper preservation; note any cases of improper preservation or leaking containers on processing log sheet
4) assign batch sequence numbers to the set of samples to be processed, and record on processing log sheet; these will be used to label beakers, drying plate grids, and slides during processing

4i2. Preliminary steps for quantitative periphyton samples only

1) transfer entire sample to a graduated cylinder and record total volume on processing log sheet
2) return sample to original sample container or transfer to other clean container

3) fit a large volume (5-10 ml) autopipetter with a clean tip
4) thoroughly mix sample, immediately draw an aliquot and transfer to beaker labeled with batch sequence number; mix sample again before drawing additional aliquots as necessary for the subsample volume desired (Use gloves to handle preserved samples!)
5) record total subsample volume on processing log
6) proceed to Step 3 under "General procedure for all samples", 4i4.

4i3. Preliminary steps for phytoplankton samples only

1) transfer entire sample to a 1000mL glass graduated cylinder and record total volume on processing log sheet (Use gloves to handle preserved samples)
2) Cover top of cylinder with plastic film. Allow sample to settle undisturbed for 2 days.
3) Using suction device fitted with small diameter tip, draw off excess water to desired total volume. Record final volume on processing log.
4) Subsample as with quantitative algae samples (above, 4i2., step 4 and 5), adding aliquots to a beaker labeled with the batch sequence number. It may be necessary to use the entire sample if cell density is low.
5) Record subsample amount on processing log
6) proceed to Step 3 under "General procedure for all samples", 4i4.

4i4. General procedure for all samples – nitric acid digestion

1) shake sample well to dislodge cells from debris that may be in the sample bottle; immediately pour off 20-40 mL of sample into a labeled beaker (Use gloves to handle preserved samples)
2) record the beaker number on the processing log, next to the sample location
3) place beakers containing subsamples on an unheated hotplate under the fume hood
4) Wear gloves and chemical splash goggles for the next steps, and work inside the fume hood!
5) Transfer an appropriate volume of nitric acid to a clean beaker which can be used to treat a set of several samples.
6) Add nitric acid slowly to each sample beaker in an approximately 1:1 ratio of acid to sample.
7) Gently heat beakers up to one hour. Note: samples that are relatively free of large particles and organic sediments may not require heating. In these cases, skip heating and allow beakers to sit overnight before proceeding.
8) Remove beaker from the hotplate and set aside, but still within the fumehood. Fill beakers with deionized water. Allow to settle undisturbed.
9) After settling ~7hr (or at least 1 hr per cm of height), suction off the water column, taking care to not disturb the settled material. Refill with deionized water. Repeat these rinsing steps until the sample is circumneautral, as measure with a clean litmus paper.
10) Label a set of glass scintillation vials. Label the lids using a permanent marker, with the following information: Station Number, Stream Name, Collection Date. Affix preprinted vial labels to each, or handwrite a label with the following

minimum information: StationID, Stream Name, Major River Basin, County, Collection Date, Replicate Number, Sample Type.

11) Suction off water column once more, but do not refill. Transfer clean diatom material to a labeled scintillation vial. If the sample is quantitative (including phytoplankton), then wash any material adhering to the beaker walls into the scintillation vial with deionized water from a wash bottle. Bring the total volume of the scintillation vial to 20 mL with deionized water.

4i5. General procedure for all samples – drying and slide mounting

1) Position the drying plate in an area free of vibrations and strong air currents; ensure that grid labels are clear.
2) Clean coverglasses by soaking in 70% ethanol; wipe dry with laboratory wipes; place coverglasses on drying plate, each in a numbered square on the grid.
3) Fill a clean beaker with deionized water; using a disposable plastic transfer pipette, flood a coverglass with water
4) Thoroughly mix the vial of cleaned diatom material by inverting; using a clean glass transfer pipette and bulb, quickly remove a small aliquot of sample and add to appropriate coverglass, gently mixing by drawing the water-sample mixture in and out of the pipette. The exact amount will vary depending on cell density and sediment in the sample. Note: for quantitative samples where densities will be calculated from the diatom slide only, use a small volume autopipetter to measure aliquots and pay special attention to mixing). Repeat for each sample to be dried.
5) Allow coverglasses to air dry overnight. Position a lamp over the plate to aid drying if necessary.
6) Position small hotplate in the fume hood and turn on; place drying plate on hotplate and leave for 15-20min to drive off any remaining moisture from coverglasses. Remove drying plate from hotplate with insulated mitts.
7) Using grease pencil, label a set of clean microscope slides with the batch sequence numbers. Spread slides out under the fume hood. Using a plastic disposable transfer pipette, spread a drop of Naphrax™ on each slide. Invert each coverglass onto the Naphrax™ on the appropriate labeled slide.
8) Place slides, a few at a time, on the hot plate, leaving an edge of each slide off the hotplate. The Naphrax™ will vigorously bubble and release toluene.
9) As the Naphrax on each slide ceases to bubble (30-40 sec), remove it to an insulated surface using flat tipped forceps, and quickly seal the coverglass to the slide using two toothpicks. Allow slides to cool, 5-10 minutes.
10) Carefully remove excess mountant from tops and edges of coverglasses by scraping with a flat single edge razor blade. Wear a dust mask to avoid inhaling irritating dusts. If any portion of the coverglasses lifts, reseal by gently reheating.
11) Affix slide labels. Use preprinted labels, or handwrite labels with the following minimum information: StationID, Stream Name, Major River Basin, County, Collection Date, Replicate Number, Sample Type. Store slides in a slide box to protect from damage.
12) After desired slides are produced from the cleaned material it can be preserved for archiving by adding a small amount of ethanol.
13) Enter processing dates and methods into the WQB electronic algae sample tracking log. Also note on the tracking log any problems or deviations from procedures, and any sample labeling issues

4j. Data and Records Management

One of the following processing logs will be completed for a batch or set of samples:

- qualitative diatom processing log (used for most routine samples)
- phytoplankton sample processing log
- quantitative algae sample processing log

These logs will be generated by the WQB Monitoring Section sample tracking system, or will be filled in by hand from sample and visit logs, using the forms in Appendix A. Sample processing logs are retained in WQB Monitoring Section files indefinitely.

Samples are retained for 2 years after collection date, or until taxonomic identifications are complete. Cleaned diatom material vials and slides are archived in the DOW biology laboratory, by sampling year.

5. QUALITY CONTROL AND QUALITY ASSURANCE

The table below summarizes data quality verification steps involved in this procedure. These steps ensure that the sample is appropriate for processing and that the sample's identity is tracked throughout the process. Problems and issues must be documented on processing logs and unresolved issues must be entered into the sample tracking system. In addition to verification steps, all cautions and interferences listed in sections 4e and 4f of this SOP should be observed in order to ensure quality products.

When	Inputs	Element Verified	Verification Records
before beginning (4i1-2)	algae samples, algae sample tracking log	completeness of log, entries match sample container labels	processing log, sample tracking log
before beginning (4i1-3)	algae samples, algae sample tracking log	proper preservation	processing log, sample tracking log
before bottling cleaned material (4i4-10)	processing log, bottle labels	completeness of labels, label information matches log entries	none; correct label errors before proceding
before lableing slides (4i5-11)	processing log, slide labels	completeness of labels, label information matches log entries	none; correct label errors before proceding

6. REFERENCES

1. Kentucky Division of Water, 2008. Methods for Assessing Biological Integrity of Surface Waters in Kentucky, February 2008, Revision 3. Energy and Environment Cabinet, Department for Environmental Protection, Division of Water. Frankfort, KY.

2. Kentucky Division of Water, 2009. Collection methods for benthic algae in wadeable streams, Revision 1.0. Energy and Environment Cabinet, Department for Environmental Protection, Division of Water. Frankfort, KY.

3. Kentucky Division of Water, 2009. EDAS data entry and biological data management, Revision 1.0. Energy and Environment Cabinet, Department for Environmental Protection, Division of Water. Frankfort, KY.

4. Kentucky Division of Water, 2009. Diatom sample taxonomic identifications, Revision 1.0. Energy and Environment Cabinet, Department for Environmental Protection, Division of Water. Frankfort, KY.

5. Barbour, M.T., J. Gerritsen, B.D. Snyder, and J. B. Stribling. 1999. Rapid bioassessment protocols for use in streams and wadeable rivers: periphyton, benthic macroinvertebrates, and fish, second edition. EPA 841-B-99-002. U.S. Environmental Protection Agency; Office of Water, Washington, D.C.

7. ATTACHMENTS/CHECKLISTS AND APPENDICES

Appendix A: diatom sample processing logs (next page)

Qualitative Diatom Sample Processing Log (QUAL)

Date log generated: _____

Nitric Acid Digestion and Permanent Slide Preparation

Batch Seq #	SampleID	Stream Name	Collection Date	Rep Number	Digested Date	Bottled Date	Slide Date	Date to Tracking Log	Processed By

Quantitative Diatom Sample Processing Log (QUANT)

Nitric Acid Digestion and Permanent Slide Preparation

Date log generated: ____________

Batch Seq #	SampleID	Stream Name	Collection Date	Rep Num	Total Sample Vol	Sub-sample Vol	Digested Date	Bottled Date	Final Bottled Vol	Slide Date	Date to Tracking Log	Processed By

Phytoplankton Diatom Sample Processing Log (PHYTO)

Nitric Acid Digestion and Permanent Slide Preparation

Date log generated: _____

Batch Seq #	SampleID	Stream Name	Collection Date	Rep Num	Total Sample Vol	Concentrated Vol	Sub-sample Vol	Digested Date	Bottled Date	Final Bottled Vol	Slide Date	Date to Tracking Log	Processed By