New Mexico Environment Department
Surface Water Quality Bureau

Standard Operating Procedure

for

Periphyton Sampling

Approval Signatures

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1.0 Purpose and Scope

The purpose of this document is to describe the sample collection techniques, preservation requirements, equipment, and quality control activities associated with periphyton sampling in lotic environments. This SOP does not address periphyton sampling in lakes. Procedures for these activities are addressed in SOP 12.0 *Lake Sampling*. In addition this document describes the procedures for the analysis of all chlorophyll a samples obtained by SWQB.

2.0 Responsibilities

Personnel who conduct periphyton sampling and data validation and verification activities or who supervise those who do are responsible for implementing this procedure.

3.0 Background and Precautions

Currently, the State of New Mexico has a narrative criterion to determine nutrient impairment, which states,

> *"Plant nutrients from other than natural causes shall not be present in concentrations which will produce undesirable aquatic life or result in a dominance of nuisance species in surface waters of the state"* (NMAC 2011).

This narrative criterion can be challenging to assess because the relationships between nutrient levels and impairment of designated uses are not defined, and distinguishing nutrients from "other than natural causes" is difficult.

To address this challenge, the Surface Water Quality Bureau (in cooperation with the Environmental Protection Agency and the US Geological Survey) revised New Mexico's Nutrient Assessment Protocol for Streams in 2004 using a weight-of-evidence approach. The weight-of-evidence approach uses both causal (total nitrogen and total phosphorus) and response variables (dissolved oxygen, pH, and periphyton chlorophyll *a*) for assessment purposes. Impairment threshold values are developed for each of the causal and response variables including periphyton chlorophyll *a*. These values are used to translate the current narrative nutrient criterion to quantifiable endpoints.

Site conditions or project-specific data collection objectives may necessitate the use of alternative field procedures not included in this SOP. The use of field methods other than those presented in this SOP must be approved by the Program Manager and alternative methods must be accurately documented.

4.0 Definitions

Periphyton - an assemblage of organisms that grow on underwater surfaces and includes algae, bacteria, fungi, protozoa, and other organisms.

Cobble – for this method cobble refers to any substrate particle that is large enough to sample with the delimiter, i.e. at least 2.4 cm across.

Woody Snag – any log or branch that is submersed in the water providing substrate for periphyton growth.

5.0 Equipment and Tools

- Ring or delimiter – section of 2 inch diameter PVC pipe, about 1 inch long
- toothbrush – bent back 90° and bristles trimmed to about 6 mm
- tray – to carry rocks and wood
- funnel
- 500-1000 mL sample container
- scalpel or knife
- scissors
- wash bottle
- ruler
- pruning shears or saw
- turkey baster

6.0 Step-by-step Process Description

Three different methods are used to sample periphyton depending on the dominant type of habitat in the study reach: (1) large river method, (2) targeted riffle method, and (3) reach wide multi-habitat method. If boatable EMAP monitoring is being conducted, then the large river method is used. If riffles cover at least one-fifth of the reach length (calculated as 40 times the average wetted width), a targeted riffle method is used. Otherwise, a reach wide multi-habitat method is applied. There are three parts to each of these methods. The first step is laying out the reach to determine the location where the samples will be collected. The second step is determining the type of substrate to be sampled at each of these locations. And finally, the third step is using the sampling technique appropriate for that type of substrate. When sampling a transect, each sample point must only contain a single type of substrate but a single transect can

contain multiple types of substrates. While hard substrate is preferred (cobble and wood), sand may be sampled when specific conditions and sampling criteria are met.

6.1 Substrate Sampling Methods

6.1.1 *Sampling Procedure for Non-Wadeable Large Rivers*

This sample method is used on large rivers where boatable EMAP Method is conducted (Lazorchak et al., 2000). At each of the 11 transects, collect samples from the sampling station assigned during the layout of the reach (Figure 1). Collect the substrate selected for sampling from a depth no deeper than 0.5 m. If you cannot collect a sample because the location is too deep, skip the transect. Collect one sample from each of the transects (if possible) and composite all discreet collections into one container to produce a composite sample for each stream reach.

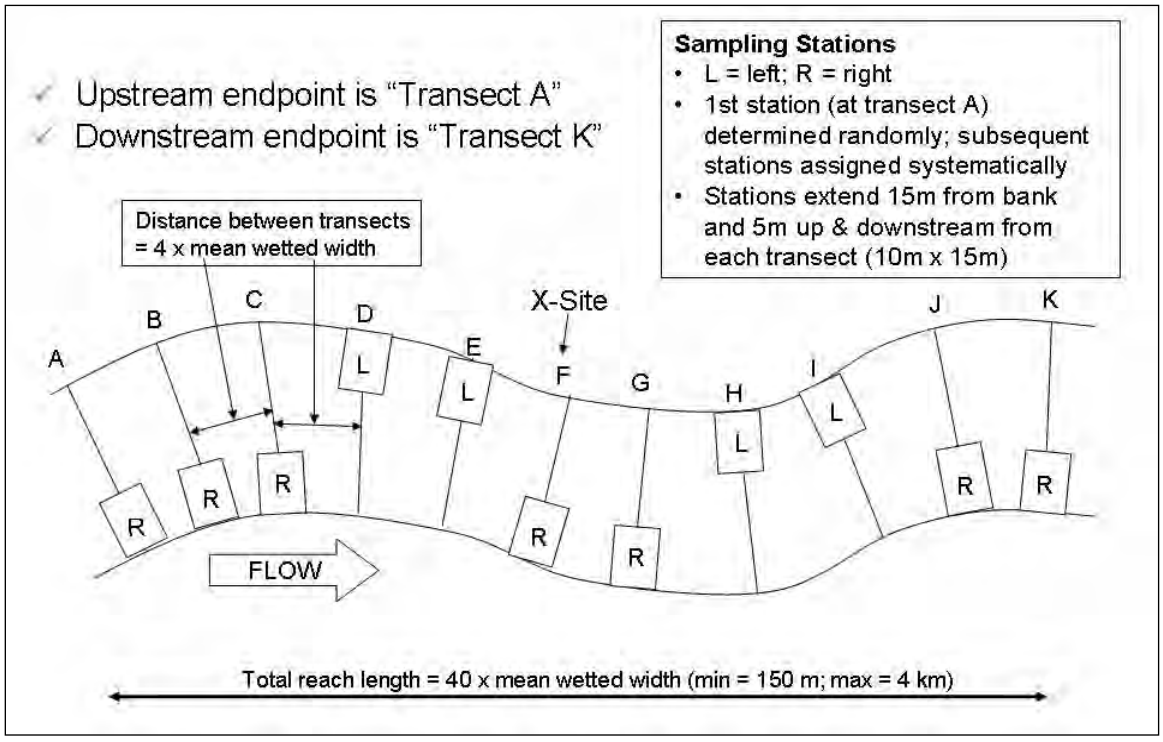

Figure 1. Substrate sampling design for reach-wide periphyton collection in large rivers

Starting with Transect "A" (i.e., the most upstream transect), collect a single substrate sample from the assigned sampling station using the procedure below.

1. Collect a sample of hard substrate (rock or wood) that is small enough (< 15 cm diameter) and can be easily removed from the river and collect a periphyton sample with the appropriate technique described in section 6.2.

2. If hard substrate is not present at the Transect, use the area delimiter to confine an area of soft sediments and sample with the *Suction Method* described in Section 6.2.3.

3. Place the sample collected at each transect into a single sample container to produce the composite sample. Keep the sample container in a cooler on ice while you travel between transects and collect the subsequent samples. The samples need to be kept cool and dark because a chlorophyll sample will be filtered from the composite.

4. Repeat Steps 1-3 for Transects B through K. Record sample collection information on the *Field Form.* The information to be recorded includes the substrate and habitat type as well as the dimensions of any woody snags sampled. Tables 1 and 2 define the different substrate and habitat types.

Table 1. Sampling habitat definitions

HABITAT	CHARACTERISTICS
Pool	Still water; low velocity; smooth, glassy surface; usually deep compared to other parts of the channel
GLide	Water moving slowly, with smooth, unbroken surface; low turbulence
RIffle	Water moving, with small ripples, waves, and eddies; waves not breaking and surface tension is not broken; “babbling” or “gurgling” sound
RApid	Water movement is rapid and turbulent; surface with intermittent “white water” with breaking waves; continuous rushing sound

Table 2. Substrate types

SUBSTRATE	CHARACTERISTICS
Fine/sand	Non-gritty to gritty particles (silt/clay/muck up to ladybug sized;<2 mm diameter)
Gravel	Fine to coarse gravel (ladybug to tennis ball sized; 2 mm to 64 mm diameter)
Coarse	Cobble to boulder (tennis ball to car sized; 64 mm to 4000 mm diameter)
Other	Bedrock (larger than car sized; > 4000 mm diameter); hardpan (firm, consolidated fine substrate); wood of any size; aquatic vegetation, etc. Note the type of “other” substrate in comments section of field form.

6.1.2 *Sampling procedure for Wadeable Streams – Targeted Riffle Collections*

Use this method if riffles cover at least one-fifth of the reach length (calculated as 40 times the average wetted width). Select a representative riffle and use the procedure described below:

1. Starting at the bottom (downstream section) of the selected riffle, visually determine a diagonal line running continuously through the riffle feature area. Select a landmark on

the far bank that corresponds to the end of this diagonal transect running through the riffle feature. The diagonal line should capture as much of the targeted feature as possible. Collect one substrate sample at each of nine equidistant points along this diagonal, starting with a point near one bank and ending at a point near the opposite bank. Sample points may be measured or estimated by pace steps depending on conditions. If the stream width is less than 3 feet, the samples may be collected from 2 or 3 separate diagonal cross sections by collecting 3 to 5 samples from each diagonal. If you cannot safely wade across the entire width, go as far as possible, then turn back and continue to collect samples on a diagonal back to the bank at the opposite end of the riffle from where you started.

2. Within in each sample location visually estimate the halfway point between the present sampling location and the next sampling location. Confine present sample collection to an area equal to the radius of the halfway point (the sample area). To collect substrate for periphyton sampling, bend down to lightly touch the bed sediments at the sample point without directly looking at the bed substrate. Pick up the first stone that you touch. If it is too big to retrieve, then repeat attempting to collect a stone from other locations in the same sample area. If you touch a silty, sandy, or gravely patch among the cobbles, take the nearest stone that can be picked up within the sample area. If no cobbles are present in the sample area, sample a woody snag (Section 6.2.2) that is within the distance to your next sample point in any direction. However, if you must go further than your next equidistant point to find a suitable cobble or woody snag among finer sediment, use the *Suction Method* to sample the fine substrate at that location (Section 6.2.3).

3. Cobble and portions of snags may be placed in a tray and transported to the bank for periphyton sampling. If other than 9 cobble samples are collected, record sample collection information on the *Habitat & Biota Worksheet* including the number of each non-cobble sample collected (i.e., woody snag or fines) as well as the diameter and length of any woody snags sampled. *Combine each sample into a single composite sample and process as described in Section 6.2.*

6.1.3 *Sampling Procedure for Wadeable Streams – Reach-wide, Multi-habitat Collections*

If riffles cover less than one-fifth of the reach, a reach wide, multi-habitat composite will be collected using a modified EMAP method (Peck et al., 2006).

1. Establish 5 equidistant transects following protocols in Section 6.1.3.1 *Reach Layout* of SOP 5.0 - Physical Habitat Measurements. Start sampling on the LEFT side of Transect A (left when looking downstream) and follow the pattern of LEFT-CENTER-RIGHT as you move upstream to each consecutive sampling point (Figure 2). That is to say, collect a sample at 25% of the wetted width (LEFT) along Transect A, at 50% of the wetted width (CENTER) along Transect A.5, and at 75% of the wetted width (RIGHT) along Transect B. Repeat this pattern for the remaining 6 transects (B.5 – E). If you cannot collect a sample at the designated point because of deep water or unsafe conditions, relocate to another random point on the same transect. Record sample collection information on the *Field Form.*

2. Use the appropriate sampling method for each substrate type encountered (*refer to Section 6.2*). Record sample collection information on the *Habitat & Biota Worksheet* including the Transect – Substrate/Habitat Table*. Combine each sample into a single composite sample and process as described in Section 6.3.*

RECORD the habitat and dominant substrate type at each sample location.

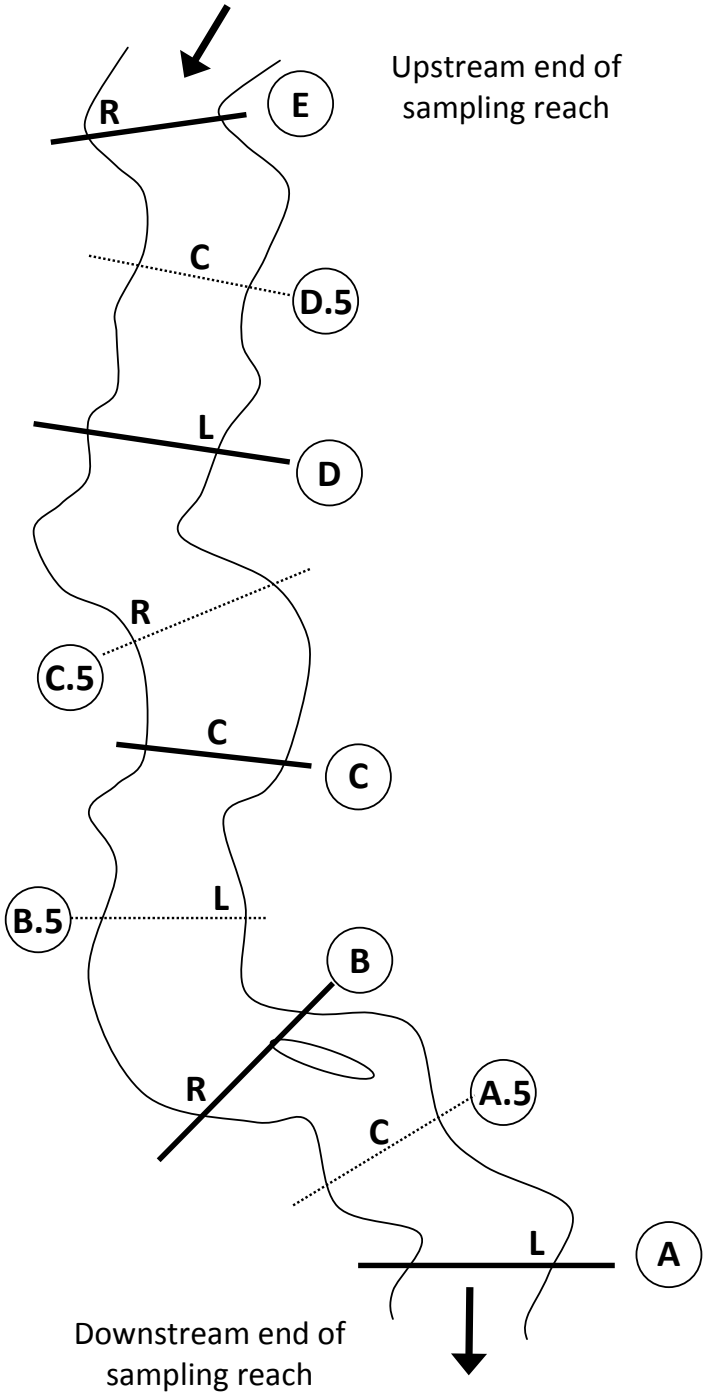

Figure 2. Substrate sampling design for reach-wide periphyton collection in wadeable streams

6.2 Periphyton Sampling Methods

6.2.1 Ring Method for Cobble and Gravel (Epilithic) Habitats

1. If a > 2 inch diameter coarse substrate is available at the sample location as described above in Section 6.1, collect one cobble sample from each location and place stones in a plastic tray, top side facing up (i.e., in the same orientation that they were found), and transport them to the bank to sample periphyton.

2. Place the delimiter or ring on top of the stone to define a circle in the center, or as near as possible to the center, of the stone. Use a knife or scalpel to mark around the inside of the ring and scrape off as much periphyton growth as possible from within the ring and place in sample container. Using a wash bottle of stream water, rinse off the scalpel into the appropriately labeled sample container.

3. Holding the ring in the center of the rock, scrub the defined area for 30 seconds with the modified toothbrush. Remove the slurry from within the circle by removing the ring, holding the rock at an angle over a funnel placed in the sample container and rinsing with a wash bottle of stream water. Thoroughly rinse the slurry from the scrubbed area and embedded in the toothbrush into the container. (Note: only use small amounts of wash water to avoid running out of space in the sample container and making homogenizing the sample more difficult).

4. If the sampling point falls over a mat of filamentous algae, a modified approach is required for sample collection. Place the ring on the center of the rock. Along the inner edge of the ring, cut any filaments passing into or out of the ring. Remove any loose filaments from outside the ring. Scrape off all of the filaments inside the ring and rinse it off the scalpel into sample container. Holding the ring in place, scrub the defined area for at least 30 seconds (or the time required to remove all visible biomass) with the toothbrush, then rinse the slurry from within the circle and embedded in the toothbrush into the sample container as described above.

5. Calculate the area sampled using the following equation:

   Sampling Area = $n\pi r^2$, where
   n = number of discrete collections (generally 9),
   π = 3.1416, and
   r = radius of the delimiter in centimeters (2.3 cm for 2-inch PVC pipe).

Note the number of delimiters sampled on the *Periphyton and Benthic Macroinvertebrate Field Form*. Add the area of all woody snags and delimiters of hard and soft substrates sampled to calculate the total area sampled and record on the *Chlorophyll a Analysis Sheet*.

6.2.2 Sampling method for Woody Snags (Epidendric Habitats)

Collecting quantitative microalgal periphyton samples from epidendric (woody snags) habitats presents a challenge because they generally have an irregular surface and are difficult to remove without loss of algal biomass (Moulton et al., 2002). If the woody snag is large enough, has a relatively smooth surface, and can be removed from the water, it can be sampled in a similar manner to epilithic habitats using the ring method (*Section 6.2.1*). Otherwise, periphyton is collected from woody snags by using the cylinder scrape method described below.

1. If a cobble is not available at the sample location, select the nearest woody snag.

2. If the woody snag is large enough, can be removed from the water, and has a relatively smooth surface, sample it using the ring method (*Section 6.2.1*). Otherwise, identify the part of the woody snag that will be sampled for periphyton. Carefully remove a 10 to 20 centimeter long section with pruning shears or saw and place in the tray.

3. Scrub the entire surface of each woody snag in the tray with the toothbrush. Using a wash bottle of stream water, rinse the brush and each section of woody snag into the tray. Pour the sample from the tray through a funnel into sample container. Combine each discreet sample into a single composite sample.

4. Measure the length and diameter of each cleaned woody snag section and record the on the *Field Form*. Calculate the sampling area by using the following formula (assumes a cylinder):

Sampling Area = $\Sigma \pi d_i l_i$, where
Σ indicates summing of all individual sample areas,
π = 3.1416,
d_i = diameter (cm) of each woody snag section, and
l_i = length (cm) of each woody snag section.

Note the diameter and length sampled on the *Periphyton and Benthic Macroinvertebrate Field Form for Streams* or *Periphyton and Benthic Macroinvertebrate Field Form for Large Rivers*. Add the area of all woody snags and delimiters of hard and soft substrates sampled to calculate the total area sampled. Note the total area sampled *Chlorophyll a Analysis Sheet.*

6.2.3 Suction Method for Sandy/Silty Habitat (Epipsammic/Epipelic Habitats)

1. At sampling points where depth and velocity are relatively low and that have a depositional zone consisting of sand and/or silt substrates, place the delimiter (PVC ring) onto the sediment. Push into the sediment to a depth of 1-2 cm.

2. Use a turkey baster to remove the entire top 0.5 cm of sediment and deposit into the sample container. Combine each discreet collection into the same sample container (i.e., composite the samples from the 9 sampling points). Quantitative periphyton samples are collected from the upper 5 to 7 millimeter layer of epipsammic (sand) or epipelic (silt) habitat in depositional areas (Moulton et al., 2002).

6.3 Sample Processing, Handling, and Analysis

All samples should be processed immediately after collection. If field processing (see Sections 6.3.1 and 6.3.2) must be delayed hold sample water on ice at 6°C and protect from exposure to light by using opaque bottles and storing in the dark until processing can begin or freeze samples as described below. Samples for periphyton chlorophyll a analysis must be either frozen or filtered and frozen at the end of each day, no more than 12 hours after collection. Place the labeled periphyton samples or filters on dry ice in latched coolers until transfer into a freezer. Samples or filters can be kept frozen for 28 days before analysis for chlorophyll a. Samples from acidic water (< pH 6) should be processed promptly after filtration to prevent possible chlorophyll degradation from residual acidic water on filter..

For samples where diatom taxonomic identification is needed, pour off a 45-mL sub-sample from the composite sample and preserve with 10 mL of 95% ethanol or enough Lugols solution to turn

the sample the color of black tea. Ethanol concentration in the preserved sample should be 20% by volume. Once preserved, place the sub-sample in a small cooler or other secondary containment device. Do **not** freeze this sub-sample!

6.3.1 Homogenizing the sample for chlorophyll a analysis

Most periphyton samples will contain clumps of filamentous algae or diatoms creating difficulties in sub-sampling. Representative sub-sampling is essential for accurate assessment of periphyton communities. Homogenizing the sample with a hand-held blender can significantly reduce variability in sub-sample analyses, particularly in samples dominated by filamentous algae. The blending process generally does not greatly damage cells. Certain green filamentous algae do not break apart easily (e.g., *Cladophora* sp.) during blending and tend to get wrapped around the cutting blades. The use of sharp blades on the blender, only a small volume of sample, and a slightly longer blending time usually overcomes this problem (Biggs and Kilroy, 2000), otherwise the filaments can be removed from the blades and cut with scissors.

Equipment:

- Glass or plastic beaker
- Squirt bottle with distilled water
- Kitchen or laboratory blender
- Graduated cylinder
- Small sharp scissors

1. If sample was frozen, allow sample to thaw and liquefy prior to homogenizing. Pour the contents of the sample container into a graduated cylinder. Rinse out any sample residue from the container and lid into the cylinder. Rinse off, but do not transfer, sand and gravel. Distilled or DI water is preferred for rinsing and making up the sample volume, however, tap water can be used provided the water is not heavily chlorinated. Measure and record the total sample volume on the *Chlorophyll-a Analysis Sheet*. Remember to add 45 mL to the total sample volume if that volume was removed for taxonomic identification.

2. Pour the sample into a beaker (the width of the beaker should be only slightly greater than the width of the housing holding the blades of the blender).

3. Pick out any invertebrates, pieces of gravel, leaves, moss, etc. from the sample. Non-algae solids have the potential to buffer acids concentrations. The top 0.5 cm of substrate should go into the sample container but not into the homogenized sample. The heavy debris in the sample (i.e. sand and pebbles) should be shaken for one minute and rinsed but not added to the blending container. The exception is where there is a calcium precipitate that has been removed from the substrate. This should be blended, as it frequently contains abundant cyanophytes.

4. Ensure that there is enough water to fully cover the blender-blade housing.

5. Blend for about 30 seconds or until the mixture is completely homogenized and is free of obvious clumps of material. If the sample contains filamentous algae, break the strands up by repeated cutting with a pair of sharp scissors. Every 10 seconds, or so, stop the blender and free any filaments that have become caught on the blades or blender housing and cut into lengths less than 5 mm. The end result must be a homogeneous solution suitable for sub-sampling.

6.3.2 Filtration for chlorophyll a analysis

Equipment:

- Vacuum filtration apparatus
- Hand held vacuum pump with pressure gauge
- Glass fiber filters, 47 mm Whatman GF/F
- Forceps/tweezers
- Wide aperture (~ 2 mm diameter) 5 mL pipette
- Squirt bottle with deionized or distilled water
- Aluminum foil and permanent marker

1. Set up filtering apparatus with a fresh filter.

2. Place a sub-sample in the filtering chamber. The sub-sample is made up of 3 aliquots drawn from the blended sample. The volume of the aliquots depends on the concentration of the sample and varies from 1 to 5 mL. Good color development (an obvious distinction between an unfiltered (white) and the media) on the filter is necessary, but not so much that extensive dilution will be required after extraction. To take aliquots, shake the bottle of blended sample or briefly turn on the blender and withdraw 1-5 mL with a pipette from halfway down the solution while the liquid is still agitated. Repeat this twice more to give the full sub-sample (i.e., the total sub-sample volume = 3-15 mL).

3. Apply vacuum(be careful not to have a high pressure as this will rupture cells releasing the chloroplasts; < 6 inches Hg vacuum is recommended).

4. If there is not an obvious coloring from periphyton on the filter, more aliquots should be filtered.

5. Check for any fragments of leaves, mosses, invertebrates, etc. on the filter and remove these with forceps.

6. Record the volume filtered on the *Chlorophyll-a Analysis Sheet*.

7. Remove the filter from the filtering apparatus, fold in half, and place on a sheet of aluminum foil. Fold in the sides of the aluminum foil to seal in the filter. Label the wrapped filter with RID.

8. Repeat steps 1 – 7 until the desired number of replicates of chlorophyll *a* sub-samples are filtered (generally 3).

Wrap and label (RID) filters and place in a re-sealable plastic bag. Freeze the wrapped, labeled filters. Samples should be processed as soon as possible after collection; however, the wrapped filters can be frozen for up to 28 days before analysis, if necessary. Samples from acidic water (< pH 7) should be processed promptly after filtration to prevent possible chlorophyll degradation from residual acidic water on filter. If processing must be delayed, hold sample water on ice or at 6°C and protect from exposure to light by using opaque bottles and storing in the dark.

9. Refreeze the remains of the sample for repeat analyses if needed.

Notes:

- Always check that clumps of algae or leaf fragments, etc., have not blocked the intake to the pipette.

- If the sub-samples are taking a very long time to filter, or the filter is darkly colored or covered with a thick layer of material, the sample probably needs dilution or use a smaller volume aliquot. Ensure that you record the degree of dilution and aliquot volume on the *Chlorophyll-a Analysis Sheet*, so that this can be used in the concentration calculations.

6.3.3 Extraction Method for chlorophyll a analysis

Chlorophyll is extracted in a solution of boiling 90% ethanol following the procedures described in *Spectrophotometric analysis of chlorophyll a in freshwater phytoplankton* (Sartory, 1982) and *Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis* (Sartory and Grobbelaar, 1984). The health and safety risks associated with ethanol are less than acetone and alcohols such as ethanol are superior to acetone in the extraction of photosynthetic pigments (Sartory and Grobbelaar, 1984). Ethanol is noted as a solvent in Standard Methods APHA but the procedure described in Standard Methods uses acetone and does not include any modifications for other extractants.

Equipment and reagents:

- Ethanol, diluted to 90%
- Capped 15 mL centrifuge tubes in racks
- 50 mL graduated cylinder
- Water bath
- Centrifuge
- 5 mL pipettes and tips

1. Pre-heat the water bath to 78 °C.

2. Prepare tubes by labeling, placing 10 mL of 90% ethanol in each and re-cap.

3. Remove the filter from the filtering apparatus or aluminum foil, fold in half, loosely roll up and place in tube. Make sure the filter is completely covered with ethanol. If filter was frozen, allow sample to thaw prior to placing in the tube.

4. Immerse the rack of tubes in the pre-heated bath for exactly five minutes. Make sure the tubes are held firmly in the racks. Loosen, but do not remove, the tops to prevent them popping off as the ethanol boils.

5. Remove racks of tubes from the water bath and place in a dark refrigerator overnight.

6. Using a glass rod or metal forceps, push the filter papers to the bottom of the centrifuge tubes, and cap firmly. Clarify the samples by centrifuging at 4000 rpm, approximately 2000 g, (setting 7) for 20 minutes.

6.3.4 Spectrophotometric Determination of Chlorophyll a

The chlorophyll *a* analysis is conducted using a modified method as described in 10200 H. Chlorophyll, Standard Methods for the Examination of Water and Wastewater, American Public Health Association (APHA, 1998) and summarized below. Acetone is replaced by ethanol throughout the method, e.g., for washing cuvettes, calibration, and dilutions. This modification was made because ethanol is noted as a solvent in Standard Methods and with ethanol, there is only a minor shift in the absorbance peak with acidification and interference from chlorophyll *b* is less (Sartory and Grobbelaar, 1984). The use of ethanol rather than acetone requires that the absorbance correction for chlorophyll in ethanol (28.66) as calculated from the acidification ratio

(1.72) defined by (Sartory, 1982) and the specific absorbance coefficient (SAC) of 83.4 l g^{-1} cm^{-1} for ethanol defined by (Wintermans and de Mots, 1965). This calculated ethanol value (28.66) is substituted for that of acetone (26.7) in the monochromatic equation developed by Lorenzen (1967) and used in Standard Methods to calculate chlorophyll a concentrations corrected for phaeophyton a in acetone. The absorbance peak is maintained at 665 nm for ethanol for pre and post acidification readings.

Equipment and reagents:

- Ethanol, 90%
- 0.22 N HCl Solution -- Add 9.00 mL of concentrated (37%) HCl to 250 mL deionized water and dilute to 500 mL
- 50 mL graduated cylinder
- 5 mL pipette and tips
- 10-cm path length UV quartz glass cuvettes
- spectrophotometer

1. While the sample is in centrifuge, warm the spectrophotometer by turning it ON making sure that it is set on Medium Sensitivity.

2. Press MODE for absorbance.

3. After the extraction described in previous Section is completed, withdraw the desired volume from the centrifuge tube using care to not re-suspended the pellet. The volume of the extractant used is dependent on the density of the sample, i.e. how dark green it is. Depending on density, a volume of 1 – 8 mL is diluted to 28 mL with 90% ethanol. Record the dilution factor on the *Chlorophyll a Analysis Sheet*. The dilution factor is the total volume divided by the volume of extractant.

4. Fill a cuvette with the 90% ethanol solution used for extraction and dilution. This is the blank. Place this cell in the front position of the spectrophotometer.

5. Fill a second cuvette with the sample to be analyzed and place in the back position.

6. Set the absorbance to 665 nm. Zero the blank with the INCREASE A/T dial. Read the sample's absorbance by pulling the knob. Reading should be between 0.1 and 1.0 (APHA, 1998). If the reading is greater than 1 dilute the sample and repeat the analysis. Record the value on the *Chlorophyll a Analysis Sheet* in the "665b" column.

7. Repeat Step 6, but measuring the absorbance at 750 nm. The 750 reading is subtracted from the other absorbance readings to account for the turbidity of the clarified sample. If the absorbance at 750 nm is greater than 0.005 AU, re-centrifuge the sample to further clarify the extract and repeat steps 5 – 7. Record the sample's absorbance in the "750b" column on the *Analysis Sheet*.

8. Acidify the sample and blank with 0.5 mL of 0.44 N hydrochloric acid. Plug the cuvettes and gently mix by tilting the cuvettes while keeping your thumbs on the plugs and then let stand 4 minutes. Wipe the cuvettes with a Kim wipe to remove any fingerprints. Zero the blank with the INCREASE A/T dial. Read the absorbance by pulling the knob. Record the value on the *Analysis Sheet* in the "750a" column.

9. Move the absorbance back to 665 nm. Zero the blank . Read the sample's absorbance by pulling the knob. Record the value on the *Analysis Sheet* in the "665a" column.

10. Discard the sample and rinse thoroughly with DI water to remove all residual acid, then rinse the cuvette two times with 5 mL portions of ethanol solution.

11. Repeat Steps 5 through 10 until all of the samples have been analyzed.

12. To clean up, flush the cells with tap water and then with deionized water. Add a little ethanol to remove any residual water, fill with ethanol, cap, and place back into the storage container.

The concentration of Chlorophyll *a* (C_a) in mg/L is calculated with the following formula:

$$C_a = \frac{28.66*(665b-750b) - (665a-750a)*V_1*DF}{V_2*L}$$

28.66 = absorbance correction for chlorophyll in ethanol = K X A

K = Acid Ratio/(Acid Ratio – 1) where the acid ratio = 1.72 (Sartory, 1982)
K = 1.72/1.72-1 = 2.39

A = 1000*(1/SAC) where SAC = 83.4 $lg^{-1}cm^{-1}$ (Wintermans and de Mots, 1965)
A = 11.99

28.66 = 2.39 * 11.99

C_a = concentration (mg/L) of in chlorophyll *a*,
V_1 = extract volume = volume (L) of extract (before any dilutions)
V_2 = filtered sample volume = volume (L) of whole water sample that was filtered,
DF = any dilution factor
L = cell length, optical path length (cm) of cuvette (10 for SWQB spectrophotometer)
665b = reading at 665 nm before acidification
665a = reading at 665 nm after acidification
750b = reading at 750 nm before acidification
750a = reading at 750 nm after acidification

The DF variable was added to the Lorenzen equation to correctly calculate concentrations when a dilution factor is used. For periphyton where the final results need to be in mg/cm^2, further modification of the formula is needed. The first step is to get a “mg/sample” value by multiplying the total volume of the sample (V3) by the chlorophyll *a* concentration.

$$C_s = \frac{28.66*(665b-750b) - (665a-750a)*V_1*DF*V_3}{V_2 \text{ X } L}$$

where,

C_s = mg of chlorophyll *a* per periphyton sample
V_1 = extract volume = volume (L) of extract (before any dilutions)
V_2 = filtered sample volume = volume (L) of whole water sample that was filtered
V_3 = sample volume (L) = total volume of the sample collected
DF = any dilution factors
L = cell length, optical path length (cm) of cuvette (10 for SWQB spectrophotometer)

The "mg/sample" value (C_s) is then divided by the total area sampled to get a chlorophyll *a* value in mg/cm^2.

$$\text{Chlorophyll } a \text{ in mg/cm}^2 = C_s/\text{Total area sampled (cm}^2)$$

These calculations have been provided in the *Chlorophyll a Analysis Sheet.*

7.0 Quality Assurance/Quality Control

All staff conducting periphyton sampling will attend an annual training session on the sampling and processing of periphyton and the analysis procedures for chlorophyll *a*. Before leaving the sampling site, all field forms will be checked for completeness. There will be an initial zeroing of the spectrophotometer prior to all readings of samples and standard solutions. The spectrophotometer will be checked annually by analyzing a chlorophyll *a* calibration standard of known concentration. Additionally, the Linear Dynamic Range (LDR) will be calculated annually to determine the chlorophyll *a* concentration range over which the spectrophotometer's response is linear. Depending on study objectives, replicates and duplicates may be analyzed either in house or by an authorized laboratory which uses a similar chlorophyll *a* ethanol-based analysis method.

8.0 Forms

- *HABITAT AND BIOTA FIELD WORK COVER SHEET*
- *BUGS, HABITAT, and NUTRIENT Survey Equipment Checklist*
- *Chlorophyll a Analysis Sheet*

9.0 Revision History

Revision 3 (September 2014) – clarification regarding sample location selection and suitable substrate, optional use of ethanol or Lugols solution dependent on laboratory requirements, and minor clarifying edits to language to reduce interpretation and increase clarity.

Revision 2 (March 15, 2013) – replaced the *Field Form* with the *Habitat & Biota Field Work Cover Sheet*; for health and safety reasons, sub-samples for diatom taxonomic identification are now being preserved with 95% ethanol instead of formaldehyde.

Revision 1 (May 16, 2012) – described and justified in better detail SWQB's modified chlorophyll extraction method using 90% ethanol in place of acetone; identified appropriate setting and time for centrifuge operation; clarified procedures (e.g. absorbance peaks) and reagents for spectrophotometric determination of chlorophyll *a*; referenced formula used to calculate chlorophyll *a* concentration including absorbance correction for ethanol; enhanced the quality assurance/quality control section.

Original (July 1, 2011).

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