Sample Collection and Laboratory Analysis of Chlorophyll-*a* Standard Operation Procedure

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The Montana Department of Environmental Quality (DEQ) Water Quality Planning Bureau (WQPB) Standard Operating Procedures (SOPs) are adapted from published methods or developed by in-house technical and administrative experts. Their primary purpose is for WQPB internal use, although sampling and administrative SOPs may have a wider utility. Our SOPs do not supplant official published methods. DEQ may provide SOPs to other programs or partners. Distribution of these SOPs does not constitute a requirement to use a particular procedure or method unless stated in other binding communications. This document does not contain regulatory or statutory requirements unless specified.

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Although the WQPB follows this SOP in most cases, there may be situations where an alternative methodology, procedure, or process is used to meet specific project objectives. In such cases, the project manager is responsible for documenting deviations from these procedures in the Quality Assurance Project Plans (QAPPs), Sampling and Analysis Plans (SAPs), and end of project summary reports.

REVISION HISTORY

Revision No.	Date	Modified By	Sections Modified	Description of Changes
1				
2				
3	2006	M. Bostrom	Multiple	Required the use of 11-transect system for assessing wadeable stream sites; required use of hoop, template or core as base Chl*a* field methods.
4	6/05/2008	C. Shirley	Section 6	Details on Chl*a* analysis coefficients for different extraction solutes. Section added on compositing individual Chl*a* samples, by method.
5	2/14/2011	M. Suplee	Mainly 1, 2; also 3.1.3 and 6.2.4	Clarified field sampling methods. Added Appendix A, describing overall statistical confidence in average algae density (mg/m^2) derived using the SOP method. Added Appendix B (updated Aquatic Plant Tracking Form).
6	12/21/2011	M. Suplee, R. Sada	Section 1; New Section 7 & appendices C ,D,E.	Clarified wadeable stream sample frame, added large river Chl*a* sample frame. Updated Appendix B. Added visual/photo documentation method for Chl*a*. Moved aquatic plant visual assessment methods to this SOP, rather than in periphyton SOP.
7	3/25/2019	M. Suplee, R. Sada	Section 1.2.5; Section 4.1.1	**A**. Added a sampling frame for the collection of chlorophyll *a* from mid-sized rivers. **B**. Changed, from optional to required, the grinding of hoop samples (i.e., filamentous algae) to assure thorough extraction of chlorophyll *a*. Method details are provided. **C**. Minor edits throughout.
8	2/9/2021	M. Suplee, R. Sada	Sections 1.2.5; 4.0; minor changes elsewhere	Section 1.2.5: Edited reach layout section for better clarity. Section 4.0: Warm ethanol method now required for all benthic algae samples. Throughout: General clarifying edits. Appendix A: Technical memo rationalizing warm ethanol change has been included.

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ACRONYMS

ACF	Absorbance Correction Factors
AFDW	Ash-Free Dry Weight
APHA	American Public Health Association
ARM	Administrative Rules of Montana
CV	Coefficient of Variation
DEQ	Department of Environmental Quality (Montana)
EDD	Electronic Data Deliverable
EMAP	EPA Environmental Monitoring and Assessment Program
EPA	Environmental Protection Agency (US)
FERC	Federal Energy Regulation Commission
GF/F	Glass Fiber Filters
GPS	Global Positioning System
HPLC	High-performance liquid chromatography
MDEQ	Montana Department of Environmental Quality
QAPP	Quality Assurance Project Plan
SAP	Sampling and Analysis Plan
SOP	Standard Operating Procedures
STORET	EPA STOrage and RETrieval database
SVF/COC	Site Visit Form/ Chain of Custody Form
TMDL	Total Maximum Daily Load
WQPB	Water Quality Planning Bureau (DEQ)
WQX	EPA's Water Quality Exchange System

1.0 CHLOROPHYLL-*A*

Chlorophyll-*a* (Chl*a*) is measured as a means of estimating algae (periphyton or phytoplankton) biomass in a body of water. It is expressed as either mass/area for periphyton (mg/m^2), or as mass/volume for plankton species (µg/L). Heavy growths of algae generally indicate inferior water quality.

Excess algae growth may clog water filters and irrigation equipment, cause taste and odor problems in water supplies, reduce dissolved oxygen levels, interfere with fish spawning, degrade macroinvertebrate habitat, trap sediment, deflect stream flows, and impair the overall aesthetics and recreational value of a stream.

1.1 SCOPE AND APPLICABILITY

This method is developed for use in water quality assessment decision making. The sampling methods described in **Sections 1.0** through **6.0** are largely for streams and rivers. The phytoplankton sampling procedure may be used in lotic low-flow conditions, including disconnected series of pools, and in lakes and reservoirs. These sampling methods are designed to produce a quantitative measure of algae biomass by relating the total mass of Chl*a* pigment to a known area or volume. Qualitative visual assessment techniques that apply to wadeable streams are provided in **Section 7.0**.

1.2 SAMPLING DESIGN CONSIDERATIONS

1.2.1 Index period

Periphyton growth is controlled by season, nutrient concentrations, velocity of the current, days of accrual, shading, water temperature, and other factors. Because of this, sampling designs using Chl*a* must be inclusive of the times when stable flows have been achieved, as well as times when diversity and standing crop are peaking. Intensive sampling may include multiple visits to show the water body's baseline condition, period of high growth potential, and subsequent return to baseline conditions. The summer and early fall period of July 1st to September 30th is generally the time of maximum growth potential in western Montana (mountainous region). A somewhat longer sampling index period (June 16th to September 30th) is recommended for some plains ecoregions (Suplee, et al., 2008).

1.2.2 Recent conditions

Sampling events planned in advance must consider the possibility that current or recent weather patterns could influence the sampling outcome. An example of this is recent rainfall that has significantly increased the flow, scouring the substrate. If the water body has had recent significant rainfall or is currently experiencing a significant rainfall event, consider the effect of scouring and reschedule sampling event, as needed.

1.2.3 Site Locations

Selection of sampling locations depends largely on the data quality objectives (DQOs) of the water quality study. The study design necessary to satisfy these DQOs must be documented in a project plan (QAPP, SAP, or equivalent documentation). The project plan should have sufficient detail to allow minor adjustments of pre-selected sites in the field, due to unforeseen events such as site inaccessibility.

If sampling locations are to be determined in the field, field guidance should include a rationale for site selection. This is critical when different sampling crews select representative locations based on professional judgment.

1.2.4 Geo-locating site

The first measurement collected is a geo-reference for the study site. The location to be recorded is the F transect (layout of the sampling frame is discussed in **Section 1.2.5**). The "F" location will be used for geo-referencing the site for EPA's STORET/WQX database. Once the site is located in the field, use a GPS receiver and record the latitude/longitude on the Site Visit Form. Always use Datum NAD83 coordinates.

1.2.5 Sampling Frame

Sampling frames and methods vary according to a lotic waterbody's size (wadeable stream or river, medium river, large river). Sampling frame layout methods for wadeable streams and rivers as well as medium sized rivers are described in Section 10.1.5 of Makarowski (2020), and methods summarized here should be consistent with that Standard Operating Procedure.

Wadeable Streams and Rivers. A sampling frame will consist of 11 transects, with the total frame length equal to 40 times the average wetted width at the F site (**Figure 1.1**). Wadeable streams and rivers must use 40 wetted widths or a minimum of 150 meters, whichever is larger. The procedure for determining the average wetted width at the F site is: measure the wetted width at 5 places around the F site (2 upstream, 2 downstream, and 1 at the F site), average the five readings, and round to the nearest 1 m. Then, lay out the computed reach length following the contours of the stream. Since some wadeable streams and rivers can be quite wide, 40 wetted widths could entail kilometers, which is unmanageable. Therefore, if the sampling frame would exceed approximately 500 meters then it is acceptable to switch either to the medium-sized river method or to the single-transect large river method.

Samples taken at each location within the frame are *single* collections using the appropriate collection technique for the substrate encountered (listed in **Section 2** - Sample Collection Methods). The starting point right (R), left (L), or center (C) should be randomly selected at the most downstream transect (transect A). Place the remaining sampling locations progressing upstream following the R, L, C pattern. (**Note**: If a duplicate sampling event is desired, repeat the entire process but commence the duplicate's pattern at transect A at one of the two remaining transect starting points [e.g., if R was used for the first sampling, use L or C]. Follow the pattern upstream accordingly.)

The overall statistical confidence in benthic Chl*a* averages derived from an 11-replicate sampling frame as outlined above is provided in **Appendix A**. It has been found that for a typical wadeable stream benthic Chl*a* sampling event that has followed this SOP, DEQ is confident that at least 80% of the time the measured Chl*a* average will be within ± 30% of the true average.

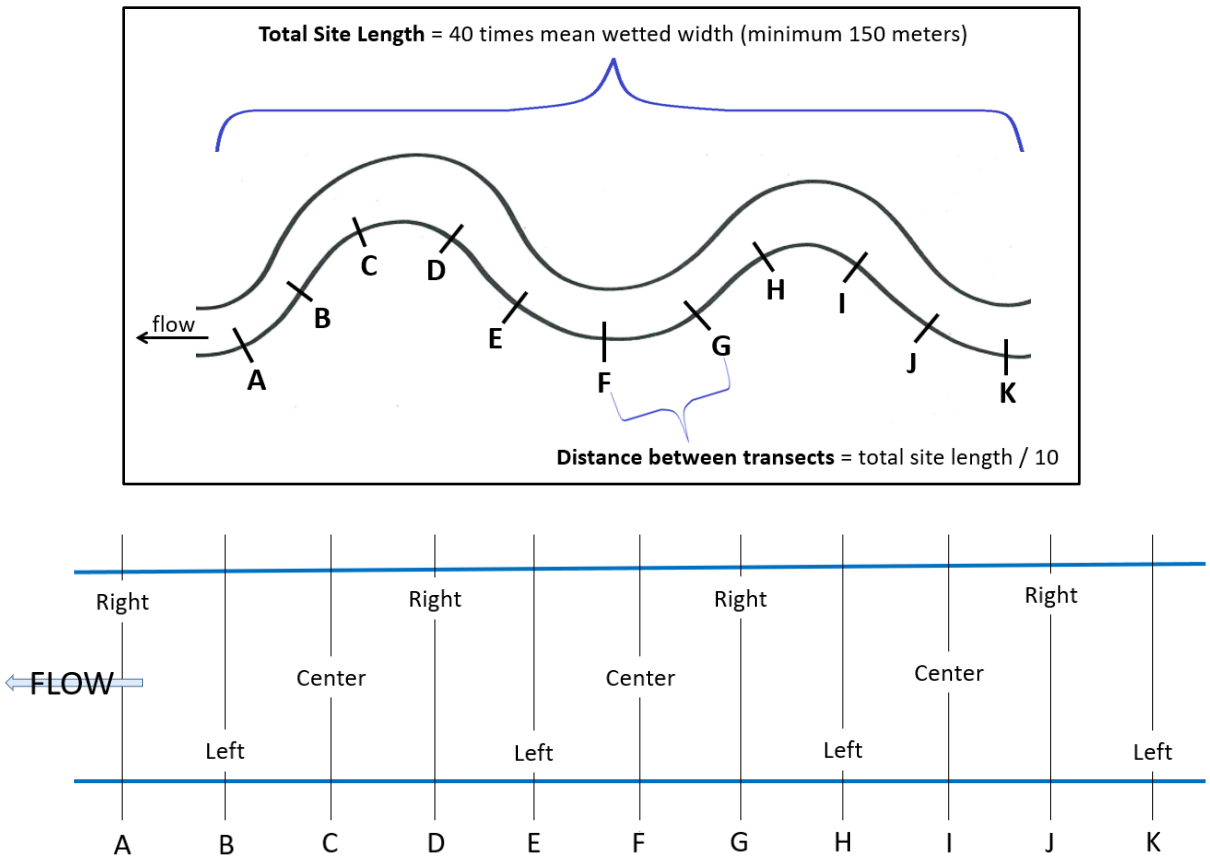

Figure 1-1. Diagram of Chl*a* sampling frame for wadeable streams & rivers.
Upper Panel. Determining total reach length and laying out the transects; the computed reach length is then laid out following the contours of the stream. **Lower Panel**. Sample collection locations at each transect. If the sampling frame would exceed about 500 m, switch to the medium-sized river method or the single-transect large river method.

Medium Rivers. The sampling frame layout for a medium river is shown in **Figure 1-2** (see also Section 10.1.5 of Makarowski (2020)). Zig-zagging in the wadeable part of the river starting from the downstream end (**Figure 1-2**), at each sampling point within the frame the field sampler will decide if a sample is to be collected using the template, hoop, or core method, depending on the dominant substrate and/or algae type present. For the template method, select (without looking) a stone near your foot or, if hoop sampling is appropriate, drop the hoop in the most representative area of where you are located. Cores are collected if the dominant substrate is fine mud with an algae film growing on it. (Cores are less likely to be encountered in medium rivers but should be included if appropriate.) Please note that acquiring data that is representative of the entire channel via this method is challenging, therefore field samplers shall take field notes describing the representativeness of the sampled zone relative to the non-sampled parts of the channel. An accompanying Aquatic Plant Visual Assessment Form (**Section 7.2**) is strongly recommended.

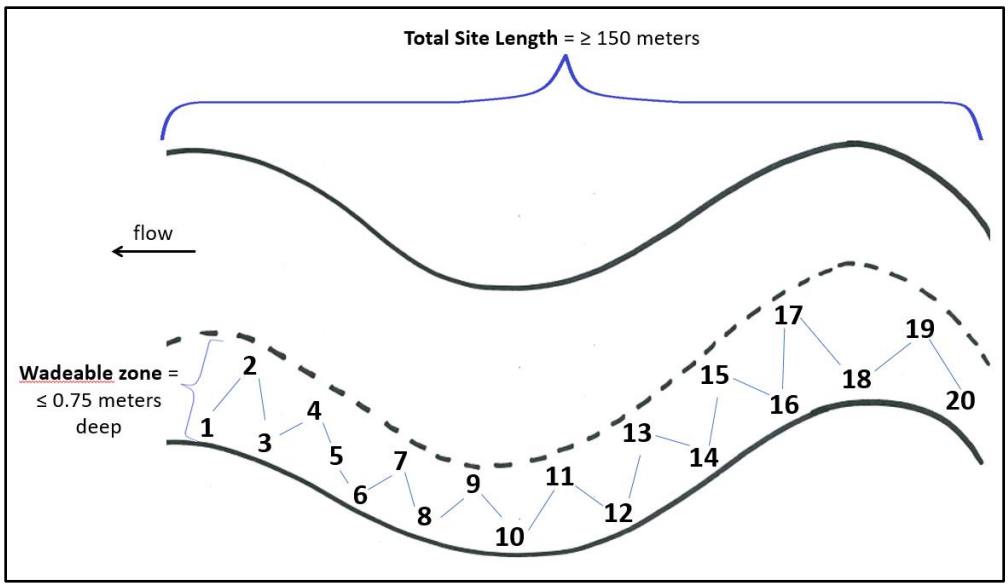

Figure 1-2. Diagram of Chl*a* sampling frame for medium sized rivers.

Large Rivers. Work on large rivers (Flynn and Suplee, 2010; Flynn and Suplee, 2013) shows that an adequate number of samples needs to be collected in the wadeable region. Data collected in this manner lends itself well to computer simulation modeling (Flynn and Suplee, 2013) and other purposes. As of this SOP, it is recommended that 16 samples be collected across a large-river transect; 11 samples in the wadeable zone, and 5 samples in the non-wadeable zone (if possible). A water depth of about 1 m can be used to separate wadeable from non-wadeable zones. Wadeable samples should be equitably distributed out from the R and L banks, to the degree possible, and equally spaced. If feasible, the five non-wadeable samples can be collected via boat using an Ekman grab or similar device and should be equitably spaced (**Figure 1-3**). If infeasible, just collect the wadeable zone samples.

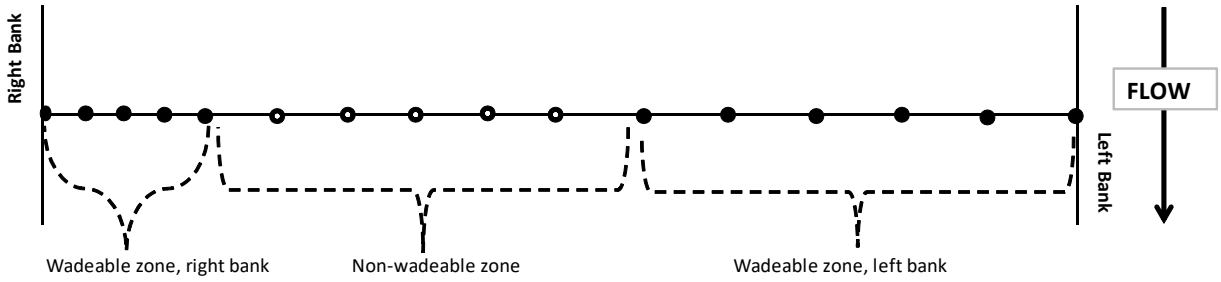

Figure 1-3. Diagram of Chl*a* sampling frame for large rivers.
Wadeable zones have a depth of about ≤ 1m.

1.2.6 Sampling Quality Control

The appropriate Quality Control samples to assess field collection activities must be designated in the project planning documents (QAPP, SAP). Because the designated sampling frame is a multi–transect sampling, information about the variability among measurements is inherent to the collection design. Therefore, duplicate samples do not *generally* need to be collected unless project DQOs require a high degree of defensibility. Documentation of the approach intended to be used to evaluate the results should be described in the quality control section of the project planning document(s).

1.2.7 Data Review and Evaluation

Data interpretation is based upon the arithmetic mean of the eleven individual results. If samples are composited according to type (core, hoop, template), a weighted average Chl*a* value (**Section 5.2.4**) is obtained based on the number of transects per collection method.

2.0 SAMPLE COLLECTION METHODS-QUANTITATIVE

Periphyton standing crop is quantified by measuring the amount of accrual on natural substrates at the study site. The sampling of artificial substrates is not recommended.

There are three methods for collecting attached algae (periphyton) from streams and rivers – the hoop, the core, and the template. A single sampling using one of the listed collection techniques is performed at each transect. The substrate and conditions encountered at the sampling locale on the transect determine the collection technique. At each of the 11 or 20 sampling locales, the algae sample collected should represent conditions prevalent in an approximately 1 m^2 area around the sampling locale on the transect. For example, if the sample is to be collected at transect D, Right (**Figure 1-1,** Lower Panel), the sampler should observe the algae conditions that prevail from the right wetted edge to 1m out along and a half a meter up- and downstream of the transect line. The sampler then selects the appropriate sampling method (hoop, core, or template) and samples the most representative point. For Center samples, observe 0.5 m on four sides of the channel center-point (upstream, downstream, towards river R, towards river L) and then sample the representative point. (These instructions are modified for the case of medium and large rivers; see details in **Section 1.2.5**.) Using the form in **Appendix B**, track the transect sampling point (R, L, C), method used, number of templates and filters used, and the dominant algae observed at the sample site. When finished, inventory the samples in the box in the upper right corner of the form in **Appendix B**.

If the water is too deep (greater than about 0.75-1.0 m) in the R, L, or C location you are sampling to properly collect a sample, adjust the sample location to the subsequent sampling locale in the pattern. Resume the normal sampling pattern (as originated at transect A) as soon as practicable. The form in **Appendix B** will aide in this.

Hoop, template, and core samples should be immediately frozen on dry ice in the field. If this is not feasible and samples are instead held temporarily on regular ice in the field, they should be frozen as soon as possible after returning from the field. Is it important to keep frozen samples frozen (prevent thawing) up to the point when they are delivered to the laboratory.

For wadeable streams and rivers, the 11 transect sampling frame (**Section 1.2.5**) is required for all collections. It has been demonstrated that this design will generally encounter one to three extreme values; thus, the relatively high coefficient of variation (73%) typically associated with the 11 samples (**Appendix A**). Analyzing each of the 11 samples separately allows the assessor to understand the patchiness of algal growth and to calculate the replicates' coefficient of variation (CV) for the sampling event. However, whether the data were each analyzed separately, or composited (discussed below), the

sampling event's average is what is typically compared to recommended or adopted Chl*a* stream-impairment endpoints[1].

Sample Compositing. Sample compositing may be used to reduce the costs associated with the 11 or 20 samples collected as part of the sampling frame described in **Section 1.2.5**. Sample compositing will, in effect, return results of each collection method as a *mean* when the composite Chl*a* concentration is calculated to the sum of the areas collected. **THE LABORATORY WILL DO THE COMPOSITING**. In the field, the sampler should keep each of the 11 or 20 samples separate but needs to check the "Composite at Lab" box on the DEQ's Site Visit Form/Chain of Custody (SVF/COC). (More on filling out this form is in **Section 3.2**.)

LABORATORY CONTRACTORS PLEASE NOTE THAT ONLY SAMPLES COLLECTED BY THE SAME FIELD TECHNIQUE CAN BE COMPOSITED! There are three sampling techniques used in this method (hoop, core, and template). Therefore, there may be up to *three* different composite samples resulting from an 11 or 20 transect sampling event.

2.1 TEMPLATE SAMPLING METHOD

The template method is used for sampling transects with substrate dominated by small boulders, cobble, and gravel *without heavy filamentous growth*.

2.1.1 Method Summary

The sampler should observe the algae density in a roughly 1 meter by 1-meter area centered on the sampling point and select a representative rock therein (or in accordance with the methods for the sampling frame as described in **Section 1.2.5**). A template with a 12.5 cm^2 area is placed on the light-facing surface of the rock with representative algae density which has been drawn from the designated locale at the transect line. The area within the template is then thoroughly scraped into a container and then filtered on site (0.70 um Glass Fiber Filters (GF/F)). At the laboratory, the algae on the filter will be processed and the resulting extract measured for Chl*a*. The template can be made, for example, from a cut-off piece of PVC pipe (Schedule 40 - 1 1/2" nominal I.D.), which results in an internal area of approximately 12.5 cm^2. Internal diameter of template should be checked and be within 3.93 to 4.05 cm (+/- 4% area error).

2.1.2 Sampling Equipment

- Waders or hip boots
- 50 cm^3 centrifuge tube or snap-cap petri dish
- 12.5 cm^2 template
- Awl or other sharp metal object to scribe template outline on the rock
- Knife and toothbrush for scraping rock
- Tap water in squirt bottle
- Shallow plastic pan to hold rock
- Hand pump vacuum with tubing

[1] It should be noted that the undesirable aquatic life impairment threshold identified in Suplee et al. (2009) is based on the quantification of stream bottom algae using the same sample collection methods (template, hoop) presented in this SOP.

- Nalgene filtering unit
- GF/F filters (0.70 um)
- Tweezers or forceps
- Cooler with ice or dry ice (preferred)
- Aluminum foil
- Large and medium Ziploc bags
- Sharpie

2.1.3 Sample Collection

The rock is placed in the shallow pan and the template placed over the upper (light-facing) surface representative of the algae on the rock. Work in a shaded area. All the growing material within the template is scraped/scrubbed and placed in the pan. In certain cases, the volume of algal material on the rock surface is small, therefore it is better to scrub the rock surface with a toothbrush and then rinse the rock surface and the toothbrush into the pan with a small volume of **tap** water (Note: Previous versions of this SOP listed de-ionized water. DO NOT USE de-ionized water as it may burst cells due to osmotic pressure differences.)

In some cases, the rocks are very small (smaller than template diameter, but still too large for core sampling). In this case, instead of using 1 representative rock, place several small representative rocks inside the template diameter, and follow the process as described in the above paragraphs, scrubbing the light-facing surfaces as best possible.

Field filtration **(MUST BE PERFORMED IN THE FIELD):** The rinse water/algae material that has been rinsed into the pan is field filtered on to a GF/F filter using a hand pump, the filter is folded in half with the sample on the inside, and then it is placed in the centrifuge tube or petri dish. Refer to **Section 2.4.3** for proper use of the Nalgene filtering unit. Minimize rinse water use to assure that all water will move through the GF/F filter.

Circumstances where rocks have very low, or high, levels of attached algae:

- Very Low Levels: In some instances, levels of attached algae or so low that scrapings from a single template will result in very little material on the GF/F filter. Little or no color will be observed on the filter after filtration. To better assure that the sample is sufficient to achieve detectable levels, up to 3 templates from the same rock (or from other representative rocks in the observation locale) can be collected and all the scraped material is then captured on the same GF/F filter. Record the number of templates aggregated on the single GF/F filter on the Aquatic Plant Tracking Form (**Appendix B**).

- High Levels: If benthic algae density from a single template is so high that the GF/F filter clogs prior to all water passing through[2], the remaining algal material/water in the upper half of the Nalgene unit may be returned to the clean pan. Make sure the pan is clean and will therefore only contain material scraped from the single template sample in question. Then, load a 2nd GF/F filter on the Nalgene unit and filter the remaining water/algae material. Both filters are

[2] This can also occur due to unusually high levels of fine sediment deposited on the rocks, intermixed with the algae.

placed in the centrifuge tube together. Record the number of GF/F filters associated with the single template on the Aquatic Plant Tracking Form (**Appendix B**).

2.1.4 Sample Handling & Labeling

Place the GF/F filter(s) into an appropriate container (50 cm^3 centrifuge tube or petri dish). Sampling location is identified on an external label with the following information:

a) Sample Type
b) Activity ID
c) Collection Date
d) Waterbody Name
e) Collector's Name

Fill out the outside label, place it on the centrifuge tube or petri dish, and cover the label completely with a strip of clear tape. Wrap the tube with aluminum foil to exclude light, write the Activity ID on the lid with a Sharpie. Place the centrifuge tube or container into a Ziplock plastic bag.

Immediately store the sample on dry ice (preferred), or ice, and away from light. Samples should be sent to the laboratory as soon as possible for Chl*a* analysis.

Record the transect number (A-K), collection position (Right, Left, Center), and the collection technique (C = Core, H = Hoop, T = Template) on the SVF/COC. If the corresponding surface area is different than the one indicated in this SOP, you must record it on the SVF/COC.

2.2 HOOP METHOD

The hoop method is designed for transects *dominated by the presence of filamentous algae*, regardless of stream substrate.

2.2.1 Method Summary

The hoop collection method is a sample from a representative area where filamentous algae dominates the sampling locale, regardless of stream substrate. Upon collection, filamentous algae is physically separated from any macrophytes present. The entire algae portion is retained and is submitted to the laboratory for extraction and analysis of Chl*a* and Ash-Free Dry Weight. The macrophyte portion may also be retained and analyzed separately for Ash-Free Dry Weight to determine macrophyte biomass (if desired). The hoop can be made by wrapping a stiff wire around the bottom of a 5-gallon bucket. Check area by measuring hoop diameter and calculating for area of a circle ($A=3.14*(D/2)^2$), adjust as necessary to arrive at an area of 710 cm^2. The diameter of the hoop is approximately 30 cm.

2.2.2 Sampling Equipment

- Waders or hip boots
- Large freezer storage bags
- Shallow plastic pan
- Aluminum Foil
- Cooler with ice or dry ice (preferred)
- Metal hoop (30 cm diameter, 710 cm^2 area)
- Scissors
- Toothbrush

- Knife for scraping rocks
- Sharpie

2.2.3 Sample Collection

Find the designated sampling location. Within the 1.0 m^2 sampling locale, locate a representative area to place the hoop. If a small number of macrophytes (< 5% by area) are present, they can be separated from the filamentous algae sample at the time of collection. If >5% macrophytes are present, collect the entire sample and perform the filamentous algae separation on the bank using a pan or bucket placed on a stable surface. Work in a shaded area.

Place the hoop over the representative area. All the algal material within the hoop is collected (i.e. filamentous and non-filamentous). Scissors or a knife may be used to detach the filamentous algae from their substrate. Filaments originating inside the hoop that are streaming beyond it in the downstream direction are to be cut off along the lower edge of the hoop, and only the parts within the hoop are retained. Similarly, filaments originating upstream of the hoop which are streaming down into the hoop (and sometimes beyond it) are to be cut along the edge(s) of the hoop, retaining only the filament parts that originally fell within the hoop. Algae attached to rocks within the hoop are scraped into the Ziplock bag. Minimize the amount of water submitted by decantation (do not decant floating algae); gently squeezing the water out of filaments works well.

2.2.4 Sample Handling & Labeling

Place all filamentous algae and other attached algae collected at the site into a large Ziploc freezer bag. Sampling location is identified on an external label with the following information:

a) Sample Type
b) Activity ID
c) Collection Date
d) Waterbody Name
e) Collector's Name

Fill out the outside label, place it on Ziploc bag, and cover the label completely with a strip of clear tape. Wrap the bag with aluminum foil leaving no space for light to enter. Place this wrapped bag into another large Ziploc bag and hand write the Activity ID on the outer bag with a Sharpie. Immediately store the sample on dry ice (preferred), or ice, and away from light. Send samples to the laboratory as soon as possible for Chl*a* analysis.

Record the transect letter (A-K), collection position (Right, Left, Center), and the collection technique (C = Core, H = Hoop, T = Template) on the SVF/COC. If the corresponding surface area is different than the one indicated in this SOP, you must record it on the SVF/COC.

2.3 CORE METHOD

Method for transects dominated by silt-clay substrate without heavy filamentous growth. These substrate types are often dominated by varying thicknesses of microalgal mats, which can have Chl*a* levels comparable to those measured in templates and hoops.

2.3.1 Method Summary

The core collection method is a sample from a representative area where a silt-clay substrate dominates the sampling point on the transect, and luxuriant plant growth is not present. A core sample is taken from the substrate. The top 1cm of the core is sliced off the plug and placed in a centrifuge tube. The sample is sent to the laboratory for Chl*a* extraction & analysis.

2.3.2 Sampling Equipment

- Waders or hip boots
- Cut-off 60 ml syringes (5.6cm^2)
- 50 cm^3 centrifuge tubes
- Cooler with ice or dry ice (preferred)
- Aluminum foil
- Small Ziploc bags
- Knife
- Sharpie

2.3.3 Sample Collection

A 5.6 cm^2 core sample is collected using a cut-off 60 cc syringe in a representative portion of the designated transect site location (**Figure 2-1**).

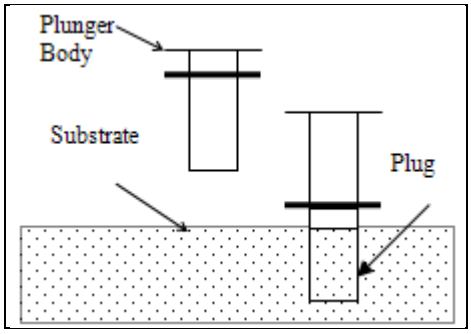

Figure 2-1. Illustration of Core Sample Collection Process.

Each core sample is taken by driving the 60-cc syringe into the substrate to a depth of 5-10 cm. The syringe plunger may have to be drawn up as the body of the syringe is pushed into the substrate, as the plunger may have too much friction within the barrel to rise up on its own. The plug may be comprised of loose sediment that will fall out of the syringe. To minimize loss of a loose plug, the sampler can place his/her fingers over the end of the syringe as it is pulled out of the hole and up through the water column.

Immediately invert the syringe containing the plug to prevent the plug from sliding out of the barrel. Extrude the core so the upper 1 cm of the core remains in the syringe (**Figure 2-2**). Slice off and discard the lower portion. Place the 1 cm portion in a 60 ml centrifuge tube.

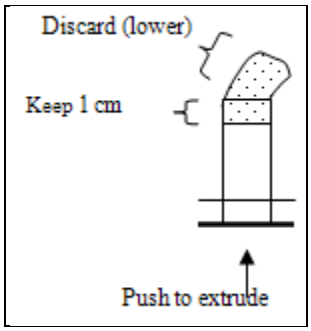

Figure 2-2. Extruding a Core Sample.

Important: Only the **upper 1 cm** of each core sample is placed in a centrifuge tube. Assure that all the material adhering to the rubber surface of the plunger-end is carefully collected, as most of the chlorophyll is located there. Carry out this work in a shaded area.

2.3.4 Sample Handling & Labeling

Sampling location is identified on an external label with the following information:

a) Sample Type
b) Activity ID
c) Collection Date
d) Waterbody Name
e) Collector's Name

Fill out the outside label, place it on the centrifuge tube, and cover the label completely with a strip of clear tape. Wrap it with aluminum foil to exclude light, write the Activity ID on the lid with a Sharpie. Place the centrifuge tube or container into a self-sealing plastic bag.

Immediately store the sample on dry ice (preferred), or ice, and away from light. Samples should be sent to the laboratory as soon as possible for Chl*a* analysis. Record the transect letter (A-K), collection position (Right, Left, Center), and the collection technique (C = Core, H = Hoop, T = Template) on the SVF/COC. If the corresponding surface area is different than the one indicated in this SOP, you must record it on the SVF/COC.

2.4 PHYTOPLANKTON METHOD (CHL*A* IN WATER)

The phytoplankton method is the sampling method for determining Chl*a* in the water column. It is used for transects dominated by pools with green color (light green to dark green), and in lakes/reservoirs.

2.4.1 Method Summary

This method uses a filtration apparatus to collect a sample. Since Chl*a* breaks down readily in sunlight, the use of a **dark Nalgene bottle is required** to minimize the exposure of the sample to sunlight[3]. The filter apparatus should be set up prior to sample collection to minimize time between sampling and filtration. The volume of water filtered must be recorded!

[3] Sampling in lakes and large rivers often involves collecting various water samples at depth, which are then placed together in a single carboy. In such cases, assure that the carboy (which is usually made of plastic) is kept covered with a canvas tarp, or similar cover, to exclude light throughout the sampling process.

2.4.2 Sampling Equipment

- Snap-shut petri dish or 50 cm^3 centrifuge tube
- 1 - hand pump vacuum with tubing
- Nalgene filtering unit
- GF/F filters (0.70 um)
- Tweezers or forceps
- Graduated cylinder (100-250 ml)
- Tap water in squeeze bottle
- 1 L (Dark) Nalgene Bottle
- Sharpie

2.4.3 Sample Collection

Filter apparatus setup

Using clean forceps, place a glass fiber filter (GF/F nominal pore size 0.7 um) on the filter holder. Use a small amount of tap water from a wash bottle to settle the filter. Rinse the sides of the filter funnel and filter with a small volume of tap water.

Sample collection and processing

Rinse a 1L dark Nalgene bottle 3 times with stream or lake water before collecting the sample.

Grab a water sample from an undisturbed location using the 1L Nalgene bottle. Cap the bottle and invert the bottle 3 times to mix thoroughly. Rinse a 100-250 ml graduated cylinder three times with *tap water*. Measure 20 ml or more of sample water in the graduated cylinder and pour into the filter funnel, place the cap loosely on the filter funnel. Draw the sample through the filter using the vacuum hand pump. ***Note: To avoid rupture of fragile algal cells, do not exceed 9.0 inches Hg on the vacuum gauge.***

Keep track of the volume of sample water filtered! The volume of sample filtered may vary from 5 ml to 1000 ml or more. When filtration slows *and* the filter has developed a distinct green (or green-brown) color, sufficient sample has been filtered. Do not allow the filter to clog. If a filter completely clogs while water remains in the upper half of the apparatus, discard the filter and start again, using less water volume. (Note that this differs from the allowable approach for benthic algae templates presented in **Section 2.1.3.**)

After filtration is complete, unplug the hand pump, remove the filter funnel from the filter holder, and remove the filter with clean forceps. Avoid touching the colored portion of the filter. Fold the filter in half, with the colored side folded on itself. Place the folded filter paper inside the petri dish and snap it shut (or place the folded filter inside centrifuge tube).

2.4.4 Sample Handling & Labeling

Sampling location is identified on an external label with the following information:

a) Sample Type
b) Activity ID
c) Collection Date
d) Waterbody Name
e) Collector's Name

Fill out the outside label, place it on the petri dish/centrifuge tube, and cover the label completely with a strip of clear tape. Wrap it with aluminum foil to exclude light, write the Activity ID on the foil with a **SHARPIE**. Place the petri dish/tube into a self-sealing plastic bag.

Immediately store the sample on dry ice. Always use dry ice for phytoplankton samples unless no other option is available; in such circumstances, regular ice may be used. Samples should be sent to the laboratory as soon as possible for Chl*a* analysis. Store the sample away from light.

3.0 RECORDING THE CHL*A* SAMPLING EVENT

The Chl*a* sampling event must be recorded including information on the location, collection methods used, and the area/volume collected. DEQ uses a SVF/COC to record this information in the field for later entry into the MT-eWQX database for submission into EPA's STORET database.

3.1 GEO-LOCATION

As described in **Section 1.2.4** of this SOP, the F transect is used to geo-reference the sample site. Other locations may be recorded to document the extent of the sampling frame, however, these must be clearly distinguished so that the F middle transect is easily recognized. Regardless of how many locations are recorded, in the database only the F middle transect will be used to geo-locate the site.

3.2 RECORDING THE SAMPLING EVENT - DEQ SITE VISIT FORM/CHAIN-OF-CUSTODY (SVF/COC)

DEQ established a site visit form for recording monitoring sampling event metadata. This form was designed to geo-locate a sampling event to the single site in multi-parameter sampling events. Therefore, when using the DEQ site visit form, it is imperative that the geo-location of the F transect for a Chl*a* sampling event is distinguished from the latitude and longitude of the other measurements.

3.2.1 Filling Out the Form

Indicate a Chl*a* sample was taken by checking the "Chlorophyll a" box on the SVF/COC. Note the *Sample ID* on the form. Indicate the sample collection procedure and location used at each transect on the Site Visit Form using the following abbreviations. The first letter represents the sample type and the second letter represents the location on the transect. (Note: forms for medium and large rivers may differ somewhat from this.)

First letter (Technique/Type)

- Template = T
- Hoop = H
- Core = C

Second letter (Position)

- Right = R
- Left = L
- Center = C

For example: Transect: A_C-R_, B_H-C__, C_C-L__, D_T-R__, etc...

Record the geo-location of the F transect under Site Visit Comments. Record the mean wetted width from the F site (**Section 1.2.5** Sampling Frame) under Site Visit Comments. Include each sample on a separate line and include the area or volume collected so the laboratory may complete the calculations to area or volume. Phytoplankton samples must include documentation of volume filtered. ***The laboratory must not accept samples until the field crew provides these documents.***

3.2.2 Compositing

If compositing is desired, THIS FACT MUST BE NOTED ON THE SVF/COC by checking the box "Composite at Lab." Because Chl*a* readily breaks down in sunlight, samples must be composited in subdued light at the laboratory prior to processing. Samples collected by different techniques CANNOT be composited. However, method-specific composites may be made (i.e. Core Composite, Hoop Composite, and/or Template composite).

The sampler is responsible for providing the area, type (core, hoop, template), and the number of each type of each individual sample collected and recording this on the SVF/COC. The laboratory is responsible for multiplying the number of composites by the area of each sample for determining the denominator of the final result.

3.2.3 Ash-Free Dry Weight

Ash-free dry weight (AFDW) must be determined for all benthic algae samples (Suplee and Sada de Suplee, 2016) and is essential for proper computation of Chl*a* from hoop samples. AFDW collected from natural stream-sediment surfaces is a useful measurement for estimating algal biomass. It provides an additional means of assessing accumulated algal biomass independent of Chl*a.* Chl*a* levels tend to be highest during peak growth, and then decline later as the Chl*a* molecules degrade as the algae senesce (Stevenson, et al., 1996).

AFDW is to be determined using Standard Methods 10300 C (APHA, 1998). AFDW can be determined from the same sample in a subsequent analysis that follows the Chl*a* analysis. AFDW can be determined from individual replicates, or as a weighted average. **AFDW results from core samples should not be included in determining a site's average AFDW**. This is because the method measures organic material from the entire core sample, not just the surface where the algae are growing, and will therefore over-report AFDW.

4.0 CHL*A* SAMPLE EXTRACTION

Sample extraction and spectrophotometric (or HPLC) determination of Chl*a* are to be performed in an analytical laboratory by a qualified laboratory technician or chemist. Sample extraction and determinative techniques described herein are *modified* from the procedure described in EPA 446.0 (Arar, 1997). These modifications are:

- Use of the monochromatic equation for phaeopigment-corrected Chl*a* with the extraction solvent ethanol (Suplee, et al., 2006),
- Option for using high-performance liquid chromatography (HPLC), and
- Use of the warm ethanol method (derived mainly from Sartory and Grobbelaar (1984)) for benthic algae samples.

The solvent purity and grade used for extraction can greatly influence the outcome of the analysis. Therefore, this procedure limits extraction solvent options to those listed in **Table 5-1**. If HPLC will be used, strict adherence to solvents and instrument conditions described in Standard Methods 10200H (5) must be maintained.

Caution: ALL CHLOROPHYLL WORK MUST BE PERFORMED IN SUBDUED LIGHT.

If processing must be delayed, hold solid and filter samples at -20^{o}C and protect them from exposure to light. Solid and filter samples taken from water having a pH 7 or higher may be placed in airtight plastic freezer bags and stored frozen for 3 weeks. Samples from acidic water must be processed promptly to prevent Chl*a* degradation.

The four different sampling techniques result in different types of media which are submitted to the laboratory, and extractions to accommodate each media follow.

4.1 CHL*A* EXTRACTION FOR BENTHIC ALGAE SAMPLES

All benthic algae samples (templates, hoops, and cores) must be processed, and Chl*a* extracted, using the warm ethanol method provided here. Please see **Appendix F** for details as to why this method is required for benthic algae samples.

Method sources. Sartory and Grobbelaar (1984), Laboratory Protocols of the University of Montana Watershed Health Clinic (Dr. Vicki Watson), and EPA Method 447.0 (Arar, 1997).

Warm Ethanol Procedure:

1) If frozen, remove samples from freezer and allow to thaw in subdued light at room temperature. Remove any excess water by straining or decanting.

2) Once thawed, proceed as follows. **If** it is a hoop sample, examine the sample for uniformity; if it is not uniform, chop/scissor and mix the filaments to create as uniform a mass as possible. Then, collect a subsample of about a tablespoon's worth (15 ml) of the homogenous algae material and grind the subsample in a mortar and pestle with a measured volume of 95% ethanol (EtOH) for one minute. Use just enough solvent to achieve a light green color. The solvent and sample are then placed in a 50-cc centrifuge tube and placed in the dark. **If** the sample is on a GF/F filter (template sample) or if it is a mud surface sample (core sample), grind the entire sample in a mortar and pestle with a measured volume of 95% ethanol (EtOH) for one minute. For templates, use just enough solvent to achieve a light green color; for core samples, the mud may obscure the color so please see additional instructions in **Section 4.1.2.1** below. The solvent and sample are then placed in a 50-cc centrifuge tube and placed in the dark.
   a) **However**: if the sample is on a GF/F filter and if it has little algae on it, adding enough solvent to grind it may result in too much dilution. So, instead, each filter is placed directly in a 50-cc centrifuge tube with just enough solvent to allow for the sample volume required for analysis – and then the filter is beaten with a glass stirring rod for ≤ one minute.

3) Once all samples are ground (or beaten), the centrifuge tubes with sample are warmed in a water bath to 78 degrees C and held there for 5 minutes. The samples are then allowed to sit in the dark or subdued light for 1 hour at room temperature.

4) The chlorophyll extracts in their centrifuge tubes are then centrifuged (and/or filtered) to clarity (recommended centrifuge times for the slurry are 675 g for 15 min or at 1000 g for 5 min).

5) For all sample types (hoop, template, core), analyze Chl*a* in the clarified extract from step 4. **Chl*a* extracts may be held overnight in a freezer and analyzed the next day if it helps with sample processing workflow.**

6) Determine the AFDW for each hoop and template sample, but do not determine AFDW for core samples.

   a) For each filamentous algae (hoop) sample, take all the remaining algal material and determine its AFDW. Make sure to label this material to match it to its corresponding subsample to document they are from the same original sample; you will need to relate the two in step 7a below.

7) Computation of Chl*a* to unit area for hoop, template, and core samples is shown below.

   a) *For filamentous algae (hoop) samples*: Chl*a* for the entire original filamentous-algae sample is calculated as:
   [(mg Chl*a*/L) X L solvent] = mg Chl*a* in subsample; then
   [(mg Chl$a_{subsample}$) X (AFDW$_{subsample}$ +AFDW$_{remaining\ sample}$)] ÷ AFDW$_{subsample}$ = mg Chl$a_{\ entire\ sample}$
      i) A single standard hoop has an area of 0.071 m^2. Dividing the mg Chl$a_{\ entire\ sample}$ from above by this area provides Chl*a* density in **mg chlorophyll *a*/m^2**. Please see **Section 5.2** for additional details.

   b) *For template samples:*
      i) [(mg Chl*a*/L) X L solvent] = mg Chl*a* in the sample; then
      ii) A single standard template has an area of 0.00125 m^2. Dividing the mg Chl*a* in the sample by this area provides Chl*a* density in **mg chlorophyll *a*/m^2**. Please see **Section 5.2** for additional details.

   c) *For core samples:*
      i) [(mg Chl*a*/L) X L solvent] = mg Chl*a* in the sample; then
      ii) A single standard core has an area of 0.00056 m^2. Dividing the mg Chl*a* in the sample by this area provides Chl*a* density in **mg chlorophyll *a*/m^2**. Please see **Section 5.2** for additional details.

A few additional details pertinent to each method are provided below.

4.1.1 HOOP SAMPLES

Samples are shipped to the laboratory in a Ziploc bag covered with aluminum foil, and often with a second (outer) ziploc bag protecting the aluminum foil. Samples should arrive frozen and remain frozen until ready for extraction.

4.1.2 CORE SAMPLES

The sample will be returned from the field in a 50 cm^3 centrifuge tube wrapped in aluminum foil. This foil wrapped tube should be in a protective (outer) Ziploc bag. Samples should arrive frozen and remain frozen until ready for extraction.

4.1.2.1 Sample Extraction

- When ready to extract, remove sample from freezer and allow it to thaw.
- Add enough solvent to cover the sample. Record volume added. A minimum of 13 ml of solvent will probably be needed for analysis. Additional solvent may be added, however the more solvent added, the greater the dilution of pigments – *don't dilute into a non-detect.*

4.1.3 TEMPLATE SAMPLES

Field crews normally have filtration equipment and return samples on filters in centrifuge tubes. Regardless, the template area must be included on the SVF/COC prior to proceeding with the analysis because results are reported as mass/area. Filtered samples should be returned to the laboratory frozen and remain frozen until analysis. Template samples must arrive at the laboratory as a frozen filter sample. This extraction must be performed in subdued light to minimize the degradation of Chl*a* pigment. In the unlikely event that samples are not field-filtered, the laboratory must perform filtration per **Section 4.1.3.1** below.

4.1.3.1 Sample Extraction (*Note: these steps normally have been done in field*)

- Assemble filtration apparatus and quantitatively transfer and filter entire sample through a 47 mm glass fiber filter with a nominal pore size of 0.7 um (Whatman GF/F filters).
- Volume filtered is irrelevant because results will be related to template area.
- Place filter into a labeled centrifuge tube.

4.2 CHL*A* EXTRACTION FOR PHYTOPLANKTON SAMPLES

This extraction must be performed in subdued light to minimize the degradation of Chl*a* pigment. Phytoplankton samples should arrive frozen at the laboratory as a filter in an aluminum-foil wrapped petri dish or similarly covered 50 cm^3 centrifuge tube.

4.2.1 Filter Extraction

- When ready to extract, remove sample from freezer and allow it to thaw.
- Add enough solvent to the centrifuge tube (or sample beaker if sample was in a petri dish) to cover the sample. Record volume added. Generally, a minimum of 13 ml of solvent is needed for analysis. Additional solvent may be added, however the more solvent added, the greater the dilution of pigments – *don't dilute into a non-detect*.
- It may be necessary to mix the solvent and sample using a mechanical shaker.
- Keep in the dark overnight.
- The next day, proceed with spectrophotometric analysis (**Section 5**).

5.0 SPECTROPHOTOMETRIC DETERMINATION OF CHL*A*

The Spectrophotometric determination of Chl*a* is to be performed according to EPA Method 446.0 (Arar E.,1997) or Standard Methods 10200 H (APHA, 1998). Both of these methods include the monochromatic calculation required for spectrophotometric analysis with phaeophytin-*a* correction.

Each laboratory must have current standard operating procedures (SOPs) that describe their instruments, reagents, interferences, standards, instrument setup, calibration procedures, analytical procedures, quality control requirements, calculations, and reporting protocols. Except as provided below, these SOPs must describe a method in general accordance with the reference methods EPA 446.0 or Standard. Methods 10200H.

A reference sample must be run with each analysis to determine method bias at a 10% frequency. The reference may be purchased from Sigma Aldrich or any certified vendor. Acceptance limits are +/- 20% of True Value. Control charting of reference sample performance is suggested to better control method performance.

Chl*a* methods in general list Absorbance Correction Factors (ACF) or give values for k and A. It is preferred that the laboratory calculate the values of k and A from average values obtained by analyzing 20-30 aliquots of a reference material. This will allow the calculation of a method ACF specific to the laboratory's instrument and purity/grade of reagents used. If reference values (**Table 5-1**) from literature will be used rather than calculating its own ACF, the laboratory must demonstrate acceptable method performance in an initial method validation and re-establish this acceptable performance annually or as changes in instrument conditions or reagents require. Above all, recognize the potential for high or low bias to exist in this method and do not blindly follow published or literary values for the ACF without verification.

$$\text{Absorbance Correction Factor} = k \times A$$

$$k = \frac{(664b/665a)}{(664b/665a)-1}$$

$$A = \frac{l\,(cm) \times \text{Concentration (mg/l)}}{\text{Absorbance 664b}}$$

5.1 CORRECTION FOR PHAEOPHYTIN-A

Both reference methods (EPA 446.0 and Standard Methods 10200H) provide calculations for obtaining monochromatic (Chl*a* corrected for presence of phaeophytin-a) and trichromatic (Chl*a*,*b*,*c*) results. Montana law (ARM 17.30.602[4]) requires that Chl*a* water quality measurements be corrected for phaeophytin [pheophytin].

Refer to Standard Methods 10200H(2) (APHA, 1998) or EPA Method 446.0 (Arar E.,1997), for instrument requirements, sample analysis requirements (calibrations, reagents, wavelengths, and calculations).

The calculations presented in the reference methods are for a phytoplankton (water) sample and can be applied directly for those samples.
Chl*a* mg/m^3 = [(Absorbance Correction)((664b-750b)-(665a-750a))*V1/(V2*L)]

Where: V1 = Volume of extract (L)
V2 = Volume of sample (m^3)
a = after acidification
b = before acidification
L = Light path or width of cuvette, cm

The calculation for periphyton replaces area for volume.

Chl*a* mg/m^2 = [(Absorbance Correction)((664b-750b)-(665a-750a))*V1/(A1*L)]

Where: V1 = Volume of extract (L)
A1 = Sample collection area (m^2)
a = after acidification
b = before acidification
L = Light path or width of cuvette, cm

The allowed solvents in its purity form are listed in **Table 5-1**.

Table 5-1. Approved Solvents and Absorbance Correction Factors.

Solvent Purity	Solvent	Absorption Peak Ratio(APR)	Specific Absorption Coefficients (E_{1cm})	A	K	Absorbance Correction = (A x K)
90%	Acetone (Ace)	1.7 note 1	89.0 L/(g*cm) note 2	11.0 note 1	2.43 note 1	26.7 note 1,4
95%	Ethanol (EtOH)	1.72 note 2	83.4 L/(g*cm) note 2	11.99 note 3	2.39 note 3	28.6 note 3

1. APHA, 1998
2. Values listed by Sartory (Sartory D.P. and Grobbelaar J.U.,1984)
3. Calculated from values listed by Sartory (Sartory D.P. and Grobbelaar J.U.,1984)
4. Significant figure error. Error carried forward.

5.2 CALCULATION TO AREA

In order to determine the density of periphyton algae by measuring Chl*a*, results obtained from the instrument and determined for the sample (in mg) must be related to the area sampled rather than a volume of water. Thus, mg Chl*a* ÷ sample method area (m^2) = mg Chl*a*/m^2. The area obtained from the three collection techniques varies. If the area information is not readily available on the SVF/COC, the laboratory must not begin the extraction and analysis until it is provided. Also, if compositing is used, the number of composites and total area sampled must be submitted on the SVF/COC.

5.2.1 Area of Hoops

A hoop has a standard area of 710 cm^2. *Confirm area of hoop prior to use*. If compositing is used, the number of hoop samples composited must be confirmed from the SVF/COC. Calculate as follows:

$$\frac{710\ cm^2 \times "number of hoops"}{10{,}000\ cm^2/m^2} = X\ m^2$$

For example, for one hoop: $\frac{710\ cm^2 \times 1}{10{,}000\ cm^2/m^2} = 0.0710\ m^2$

5.2.2 Area of Cores

A 60 ml plastic syringe results in a core sample with a standard area of 5.6 cm^2. *Confirm area of syringe prior to use*. If compositing is used, the number of cores composited must be confirmed from the SVF/COC. Calculate as follows:

$$\frac{5.6\ cm^2 \times "number of cores"}{10{,}000\ cm^2/m^2} = X\ m^2$$

For example, for one core: $\frac{5.6\ cm^2 \times 1}{10{,}000\ cm^2/m^2} = 0.00056\ m^2$

5.2.3 Area of Templates

Templates may vary from the 12.5 cm^2 size standard. Confirm area of template prior to use. All template samples must list the size of the area scraped on the SVF/COC.

$$\frac{12.5\ cm^2 \times "number of templates"}{10{,}000\ cm^2/m^2} = X\ m^2$$

For example, for one template: $\frac{12.5\ cm^2 \times 1}{10{,}000\ cm^2/m^2} = 0.00125\ m^2$

5.2.4 Reach Wide Chl*a* Composite Calculation

Each type of sample will contribute more or less to the sample as a whole depending upon the number of like-kind samples composited. A weighted average of Chl*a* is determined from the following equation.

$$\sum [(Rc*Nc)+(Rt*Nt)+(Rh*Nh)]/[(Nc+Nt+Nh)]$$

Where: R(c,t,h) = Chlorophyll-a lab result from a core, template, and or hoop
N(c,t,h) = Number of each type

For example. Assume: 3 cores were taken (Chloropyll-a = 60.0 mg/m^2
6 templates (Chloropyll-a = 360.0 mg/m^2)
2 hoops (Chloropyll-a = 160.0 mg/m^2)

	# of Type	R
Cores:	3	60.0
Templates:	6	360.0
Hoops:	2	160.0

Therefore: $\sum [(3X60)+(6X360)+(2X160)]/11$

Reach Weighted Average for Chloropyll-a = 241.8 mg/m^2

6.0 DETERMINATION OF CHL*A* BY HPLC

Standard Methods 10200 H(5) or EPA Method 447.0 may be used if the laboratory confirms the data generated by HPLC compares to the spectrophotometric method. All method specific QA/QC protocols must be followed. For calculation under SM 10200 H(5) use the data from **Table 5-1** of this document.

7.0 SAMPLE COLLECTION METHODS-QUALITATIVE

Provided here are two different visual assessment methods. **Section 7.1** describes methods that may be used to document benthic algal Chl*a* in cases where algal growth levels are uniformly low. **Section 7.2** describes a visual aquatic-plant survey that is not restricted to algae, and which should be completed during each Chl*a* sampling event (be it quantitative or visual).

7.1 VISUAL ESTIMATION OF BENTHIC ALGAL CHL*A*

Field personnel may decide that, based on visual assessment, benthic algal Chl*a* is low (<50 mg/m^2) at all transects of a stream site. Review the photos in **Appendix C** to see what this level of algal growth looks like. For purposes of stream assessment, benthic algal Chl*a* levels this low do not require quantification.

For each stream site, **EITHER** quantitative samples are collected at all 11 or 20 transects (per methods in **Sections 1.0** through **6.0**), **OR** photos are taken to document that Chl*a* is <50mg/m^2 at all 11 or 20 transects. **A mixture of photos (i.e., no sample taken) and quantitative Chl*a* samples from a site is not**

permitted. If you are not confident that algae levels at all transects are equal to or lower than the photos in **Appendix C**, then proceed with quantitative Chl*a* sampling.

If all transects appear to be <50mg/m^2, take at least one digital photo per transect (A→K). Each photo should represent a close-up aerial view of the channel substrate at the transect. Use a polarized lens to reduce glare from the sun and water's surface to enhance photo quality. Record the photo number and a brief description of each photo on the Photograph Locations and Description Form. If conditions do not allow for substrate photos through the water column and the bottom is rocky, some representative rock samples should be taken to the bank and photographed for each transect. A polarized filter should be used.

7.2 AQUATIC PLANT VISUAL ASSESSMENT FORM

The general composition, amount, color, and condition of aquatic plants are visually assessed in the field using the Aquatic Plant Visual Assessment Form (**Appendix D**)[4]. This information helps describe the health and productivity of the aquatic ecosystem, records nuisance aquatic plant problems, documents changes in the plant community over time, and can be used to help corroborate quantitative Chl*a* results.

The Aquatic Plant Visual Assessment Form should be filled out while collecting quantitative measurements of stream bottom Chl*a* or when taking photos to document visual estimates of Chl*a* <50 mg/m^2. It may also be used when collecting samples for periphyton standing crop, composition, and community structure per the periphyton SOP, WQPBWQM-010. If the stream being assessed does not entail laying out a longitudinal reach (this is uncommon but might occur for certain projects), use only the "F" labeled form in **Appendix D**. For this latter scenario, use one "F" site form per stream site.

At each transect (A, B, etc.), the assessor will evaluate the entire wetted stream bottom as it appears 5 m above and 5 m below the transect line (i.e., an evaluation zone comprising 10 linear m of stream bottom, with 5 m of stream bottom downstream of the transect line and 5 m upstream).

Actual Cover in Channel: First part of the form. Refers to the area coverage of the stream bottom by the plant type in question, within the evaluation zone. Circle the percent coverage category that most closely fits what you see.

Predominant Color: The colors of aquatic plants are clues to their identity, state of growth, and health of the aquatic ecosystem. Record the predominant color of the plants or algae from the pick list, using the letter codes. Be sure to lift up your sunglasses to record accurate color categories. See **Appendix E (Section E1.0)** for photo examples. **Note:** Color reference is to the actual colors observed, not the types of algae the assessor may identify.

Condition: Aquatic plants go through seasonal cycles of growth, maturity, and decay. The condition of a plant or algae will indicate the approximate stage of this seasonal cycle. It can also help explain cases

[4] Variations of this form have been developed for project-specific purposes, for example for medium rivers where visual assessment can only occur from one bank. Please check with the Monitoring and Assessment Section of the Water Quality Division to determine if modified forms are in use; the forms will be documented in project-specific Sampling and Analysis Plans (SAPs).

where, for example, Ash-Free Dry Weight (AFDW) to chlorophyll *a* (Chl*a*) ratios are found to be unusually high. Growing plants and algae show new growth and bright colors. Mature plants and algae are larger but have more subdued colors because of age, epiphytes, and sediment deposits. Decaying plant and algae display a loss of both pigmentation and physical integrity. Record conditions as Growing, Mature, or Decaying on the form using the letter codes. See **Appendix E (Section E2.0)** for photo examples.

Thickness Category for Microalgae: Non-filamentous microalgae can be present on stones and fine sediment surfaces and can develop a fairly wide array of Chl*a* levels depending upon the mat thickness. The categories (Thin, Medium, Thick) will help corroborate Chl*a* and AFDW measurements collected and also show the progression of algal growth at a site. Use a mm-scale ruler to measure the mat thickness. See **Appendix E (Section E3.0)** for photo examples.

Length Category for Filamentous Algae: Increasing length of filamentous algae has been associated with recreation impacts (Biggs, 2000; Suplee, et al., 2009). Highly enriched waters tend to grow long filaments, 1-2 meters or more in length at times. Record filamentous algae filament lengths as Short or Long on the form. When filaments are >2 cm in length, record their approximate lengths in the comments section. **Appendix E (Section E4.0)** has photo examples.

Finally, **Section 5.0 of Appendix E** shows a few photos of other aquatic plants found in Montana streams, but is by no means complete. It is recommended that a good aquatic plant identification guide (e.g., (DiTomaso and Healy, 2003) be taken to the field and consulted when filling out the form.

8.0 Reporting Results

Results of Chl*a* analyses must be reported in conformance with MT-eWQX specific format. Data providers are required to populate the data in a MT-eWQX EDD. Detailed guidance for populating the MT-eWQX EDD can be found in the MT-eWQX Guidance Manual located on the Web at http://deq.mt.gov/Water/SurfaceWater/SubmitData.

9.0 References

APHA. 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association.

Arar E. 1997. EPA Method 446.0 - *In Vitro* Determination of Chlorophylls a, B, C_1 + C_2 and Pheopigments in Marine And Freshwater Algae by Visible Spectrophotometry, Revision 1.2. Washington, D.C.: U.S. Environmental Protection Agency.

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APPENDIX A – STATISTICAL CONFIDENCE IN DEQ'S BENTHIC ALGAE CHL*A* SAMPLING

Definitions:

- **Sample Frame**: Within an assessment reach, a "site", which is a short longitudinal stretch of stream, or a perpendicular transect of a stream/river. For wadeable streams, a site's longitudinal length is defined as 40 times the average wetted width at its midpoint, or 150 m minimum[5]. Sites can have multiple sampling events (i.e., across-time sampling).

- **Population**: The algae growing on or across the surface of the stream bottom within the defined longitudinal area of the site, or across the perpendicular transect.

- **Sampling Unit**: An individual benthic algae sample collected via the template, core, or hoop method, which is subsequently measured for chlorophyll *a* (Chl*a*) and/or ash free dry weight.

Suplee et al. (Suplee, et al., 2009) demonstrate that average benthic algae levels up to about 150 mg Chl*a*/m^2 are considered acceptable by the Montana public majority, whereas average levels of 200 mg Chl*a*/m^2 or more are very undesirable. This algae level threshold is an important component in the assessment of beneficial use attainment, particularly for the western and mountainous regions of the state. As such, it is important to understand the variability and confidence levels associated with DEQ's Standard Operating Procedure (SOP) for sampling benthic algae. This section describes the datasets used, methods, and confidence intervals estimated for DEQ's algal sampling protocols. The purpose of the analysis outlined here was to answer the following question:

1. What is the accuracy and confidence in the average Chla value determined for a sampling event? (The above question is posed given that DEQ's Chla SOP calls for the collection of 11 replicates from a site.)

A.1. Datasets, Averages, Coefficients of Variation

All available datasets were collated for the purpose of understanding variability and confidence in DEQ benthic algal sampling methods (**Table A-1**). Data from the Stream Reference Project (STREFPRO) were restricted to sites in western Montana ecoregions, or transitional ecoregions, and did not include algae collected from eastern Montana prairie streams. This is because prairie streams are substantially different ecologically (e.g., they grow considerably more algae naturally, often have fine sediment bottoms, and are commonly intermittent), and can be better assessed for nutrient impairment by examining other factors like dissolved oxygen. The other datasets were from streams or rivers that typically have gravel/cobble substrates and good flow. The University of Montana's Clark Fork River

[5] 40 times the mean wetted width (150 m minimum) was derived from USEPA's method to asses wadeable streams (Lazorchak, et al., 1998). Similarly, the USGS NAWQA program uses 20X the wetted width, and Simonson *et al.* (1994) specify 30-35 times the wetted width. These stream lengths have been shown to be sufficiently long to encapsulate key stream characteristics (e.g. depth, substrate type, cover, shading, bank height, etc.) which strongly influence algae and aquatic plant growth. Simonson et al. (1994) find that the aforementioned stream variables could be estimated within ± 5% of the true mean 81-89% of the time using 11 transects spaced along a reach that is 30-35 times the mean wetted width. This longitudinal length was also found to encompass at least three riffle-pool sequences (Leopold, et al., 1964).

dataset and the PP&L FERC 401 certification dataset were helpful in that many sampling events straddled or exceeded the nuisance threshold value of 150 mg Chl*a*/m^2 identified in Suplee et al. (Suplee, et al., 2009).

The term *sampling event* is used here to describe the sampling of algae (***n*** = 10 or 11 replicates) on a given day, as opposed to *site*, since a site may have been sampled repeatedly over time (e.g., monthly during the summer) and we wanted to include these temporal sampling events. Conditions for a sampling event to be used in the analysis were (a) the total number of replicates collected and analyzed for the sampling event was 10 or 11, and (b) the average benthic Chl*a* calculated from a sampling event's replicates was >19 mg Chl*a*/m^2. These conditions were established so that the amount of effort expended for any given sampling event was uniform (i.e., replicate ***n*** was nearly identical), and so that replicates whose result were non-detects (ND) would not be a substantial part of the dataset. (Sampling events with average algae >19 mg Chl*a*/m^2 usually had none, or at most 1or 2 replicates, as NDs, whereas sampling events whose average algae was <19 mg Chl*a*/m^2 often had several or more NDs.) Also, 19 mg Chl*a*/m^2 is the average algal density we have so far determined for western MT reference streams (*see*: http://deq.mt.gov/wqinfo/nutrientworkgroup/agendasMinutes/2009/july09/NWG_july16-09.pdf), and since we are most interested in variability of algal growth closer to the nuisance threshold, 19 mg Chl*a* /m^2 seemed liked a natural low-end cutoff.

Table A-1. List of Datasets Available for Use in this Analysis.

Dataset	Source	No. Sampling Events in Dataset	Number of Sampling Events Used	Notes
Stream Reference Project (STREFPRO; 2004-2008)	DEQ	72	21	Since these were reference sites, many Chl*a* means <19 mg/m^2
Outstanding Fisheries Project (2006-2007)	DEQ	65	32	
Monitoring – Reassessment (2006)	DEQ	15	0	Data not used. All sampling event averages <19 mg Chl*a*/m^2, or n <<10.
Tabacco & Flint Watershed TMDLs (2007)	DEQ	19	14	
Yellowstone River QUAL2K Model (2007)	DEQ	10	0	Data not used. Sampling event averages <19 mg Chl*a*/m^2, or <<10
Upper Gallatin R. TMDL (2005-2008)	DEQ	?	0	Data not used. 11 transect replicates were composited (no replicates)
PP&L Madison/Missouri Sampling (FERC 401 cert. Compliance)	PP&L	54	47	
Clark Fork River Algae Sampling ('87-'05)	University of MT	328	105	Only used sampling events for which n=10 or 11

The average, standard deviation (**s**), and coefficient of variation (CV; [**s**/average]·100) was calculated from the replicate Chl*a* measurements for each sampling event. The CV is very useful as it allows comparison of any given sampling event's standard deviation to all other sampling event's standard deviations, regardless of whether the sampling event's average algae level was low or high (or in between). After screening the sampling events with the two conditions above (sample size, mean algae >19 mg Chl*a*/m^2), a total of 218 individual sampling events were collated. These 218 sampling events, each comprising 10 or 11 replicate Chl*a* samples each, represent more than 2,200 individual measurements of stream bottom Chl*a*. Template, hoop, and core samples are all represented. They also represent a wide array of stream types and conditions such as rivers with varying amounts of eutrophication (Clark Fork, Madison, and Missouri rivers), low and mid Strahler-order streams with little or no human impacts (STREFPRO), mid-order streams with various degrees of human impacts (TMDL datasets), etc.

A correlation was run between the sampling events' average benthic algal Chl*a* values and their associated CVs (**Fig. A-1**)(Cattaneo and Prairie, 1995). This was carried out to ascertain if there was any clear relationship between the two (e.g., lower algae levels are closely associated with higher replicate CVs, or visa-versa), which could influence subsequent analyses. No clear pattern was noted; the correlation coefficient (r^2) between the two variables was very low, only 0.07. Low average Chl*a* values were associated with about eight unusually high CVs (**Figure A-1**, outside of gray box), but the vast majority of low-Chl*a* averages had CVs typical of the entire range.

A histogram of CVs for the complete dataset was generated (**Figure A-2**). **Figure A-2** shows a clearly defined central tendency for the replicates' CVs (average = 69%). Thus, across all datasets, a typical Chl*a* sampling event comprised of 10 or 11 replicates typically had a replicate CV of 69%.

There were significant differences in CV patterns between the five different datasets (Analysis of Variance; $p < 0.05$). But each dataset contributed information to the whole that could not be ascertained individually. For example, the Clark Fork River dataset had a lower mean CV than did the other datasets, but provided much information on replicate variability for Chl*a* samples bracketing the nuisance threshold (150 mg Chl*a*/m^2).

The central tendency of the CVs provides a mechanism to estimate an overall confidence and interval width for the SOP algae sampling method (more on this, **Section A.2** below). Consideration must first, however, be given to the fact that the datasets' individual CV patterns differ. Among the 5 datasets, the most variable dataset was the Outstanding Fisheries Project (average replicate CV = 88%), the least variable dataset was the Clark Fork River (CV = 58%), and the dataset falling exactly midway was the Tobacco & Flint TMDL dataset (average replicate CV = 73%). The latter is a DEQ dataset collected following this SOP's methods. Since the central tendency of all datasets is a CV of 69%, and since a DEQ dataset with a mean CV of 73% falls exactly midway among the datasets, 73% is a good estimate of the *typical* replicate variability one would encounter in a *typical* DEQ benthic Chl*a* sampling event. This CV value was used to estimate the overall confidence level associated with the method.

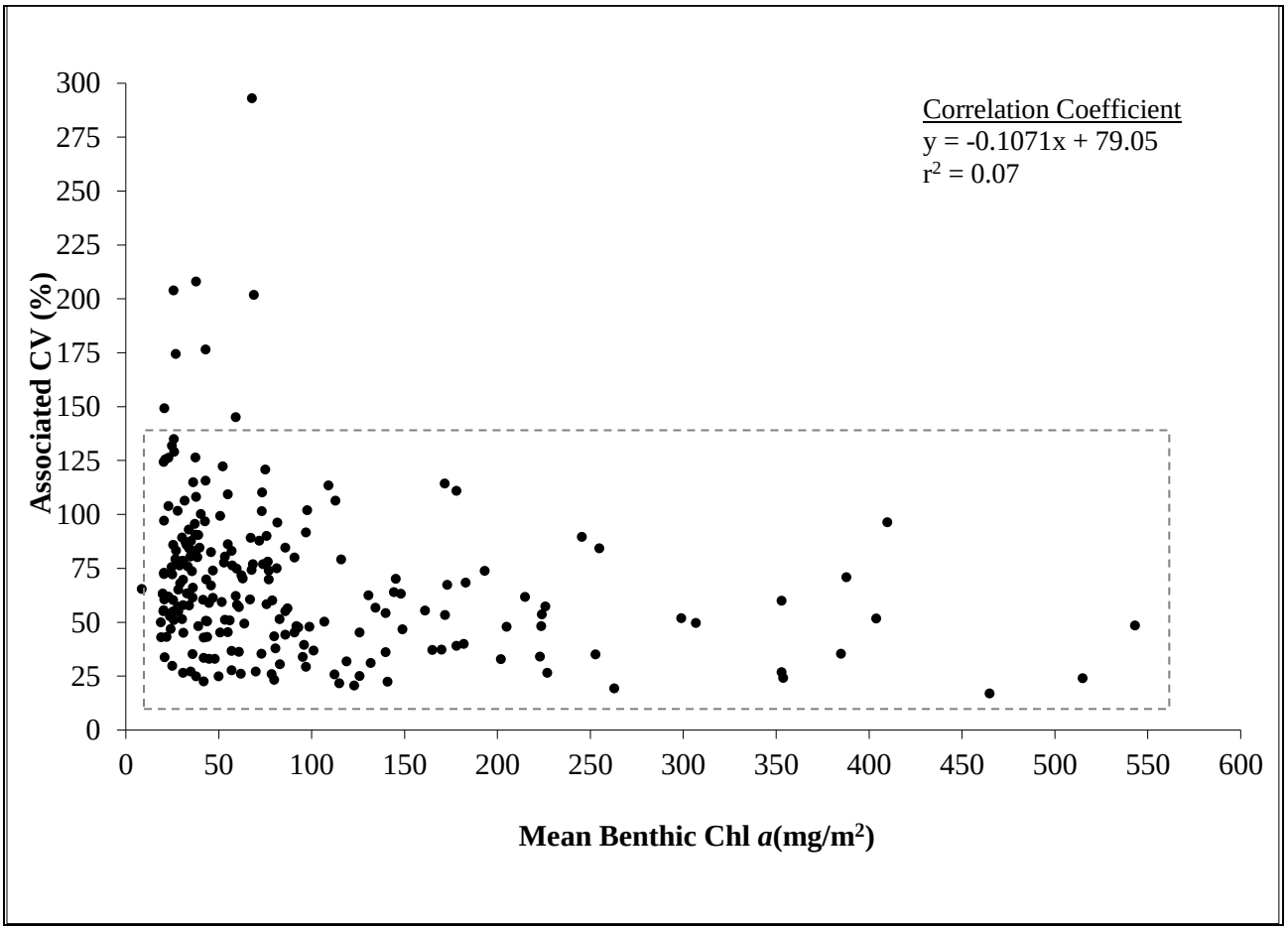


Figure A-1. Scatterplot between mean benthic Chl*a* and Corresponding CVs.
Gray outlined box show that the vast majority of CVs, over a large Chl*a* range, range from about 25 to 120. Regression correlation (R^2=0.07) shows that there is no relationship between the two variables.

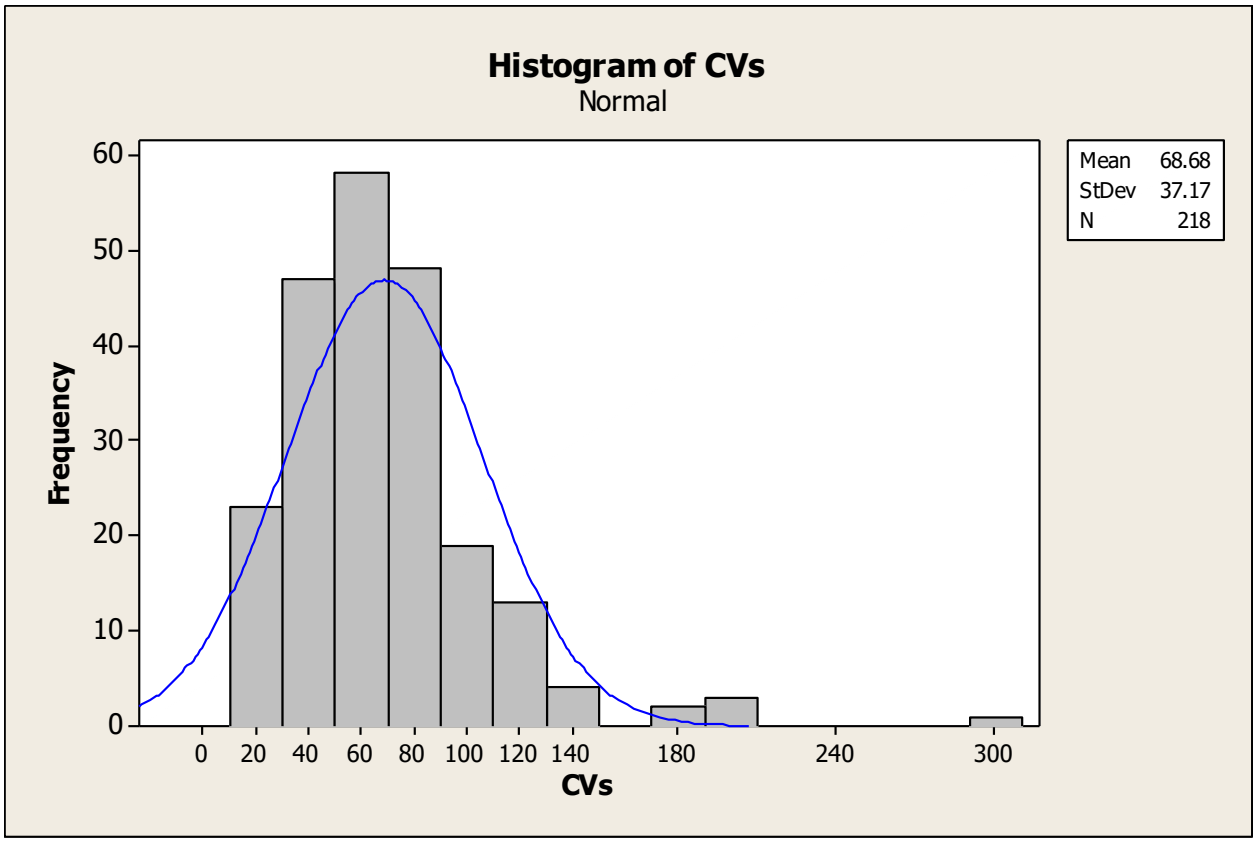


Figure A-2. Histogram of CVs for the 218 benthic Chl*a* sampling events.

A.2. Estimation of the Confidence Level and Interval Width for DEQ's Benthic Algae Sampling Protocol

The next objective was to estimate the interval width (i.e., the range across which the true population average for a sampling event is likely to fall) and the confidence in that interval. The goal is to be able to make a statement like "with the sampling protocol used, I am usually within ± X of the true benthic algae average Y% of the time".

Two statistical equations (Ott, 1993) and a third statistical approach (bootstrapping; Manly, 2001) were used together to develop these estimates. The two equations used are:

$$\mathbf{n} = \frac{\mathbf{t}^2\mathbf{s}^2}{\mathbf{d}^2} \qquad (1)$$

Where **n** is the number of sample replicates collected, **t** is the two-tailed critical value of the Student's **t** distribution, **s** is the standard deviation of a sampling event's replicates, and **d** is the pre-selected half width of the desired interval width of the sample mean.

$$\mathbf{x} \pm \mathbf{Z}_{\alpha/2}\,\mathbf{S}_x \qquad (2)$$

Where **x** is the sampling event average Chl*a*, $\mathbf{Z}_{\alpha/2}$ is the Z-table value having a tail area of $\alpha/2$ to its right, and $\mathbf{S}_x$ is equal to the standard deviation of the sampling event divided by the square root of **n** (**n** =11).

The bootstrapping approach will be discussed momentarily.

Equation 1 is solved iteratively (Thornton, et al., 1982), and is commonly used to determine an appropriate sample size for future sampling events, given the variability of a test sample's replicates and the desired interval width. In our case, sample size is about 11, since that is the SOP for Chl*a*. The standard deviation, **s**, needs to be determined for a given sampling event, and **d** needs to be determined based on what degree of precision around the true average is wanted.

Again, our objective was to determine the confidence and accuracy with which our current protocol estimates an average benthic algae level. The most reasonable standard deviation (**s**) to use in equation 1, therefore, is the one associated with the central tendency for all types of streams sampled via these methods (**Figure A-2**). Per our rationale in **Section A.1**, a CV of 73% was used to back-calculate **s**. This is easily done, as **s** = [CV· average]/100. (For example, an 11-replicate sampling event with an average algae density of 114 mg Chl*a*/m^2 will have, at a CV of 73%, an **s** of 83.) The desired interval width also needed to be determined; we believed a precision of ± 20-30% of the mean was reasonable. This is because, at a threshold of 150 mg Chl*a*/m^2 (nuisance threshold), the upper confidence bound for the measured average should fall short of the unacceptable (i.e., 200 mg Chl*a*/m^2) algae level, given a reasonable level of confidence.

Equation 1 was solved iteratively (Thornton et al., 1982) with a fixed **n** = 11, varying confidence levels from the Student's t distribution, and varying values of **d** between 20 and 30% of the average. We found that, with a typical replicate CV of 73%, we are 80% confident that the average benthic Chl*a* measured during a sampling event will be within ± 30% of the true population average. **Stated differently: for a typical benthic Chl*a* sampling event that has followed the SOP, DEQ is confident that 80% of the time the measured Chl*a* average will be within ± 30% of the true population average**.

It should be noted that if the replicates from a sampling event produce a much lower CV, then greater confidence in the interval width is possible (e.g., 90 or 95% confidence), the interval width can be narrowed, or both. Conversely, sampling events with much higher CVs will estimate the mean Chl*a* with less confidence, or with a wider interval width, or both. The confidence limits estimated in the above paragraph are, therefore, for *typical* Chl*a* sampling events.

Bootstrapping was used to cross-check these results. Bootstrapping is a method that, in the absence of any other knowledge about a population, considers the distribution of values found in a random sample of size **n** from a population to be the best guide to the distribution in the population itself (Manly, 2001). To carry out the bootstrap, test datasets comprised of 11 replicates which produced a particular average algae level (e.g., 150 mg Chl*a*/m^2) were created, each test dataset having a replicate CV of 73%. For any given algae level (say, 150 mg Chl*a*/m^2), the bootstrap program sampled (with replacement) the original 11 replicates, and then generated an average for the re-sampled observations. This was repeated 200 times, i.e., creating 200 generated datasets and 200 corresponding averages. A confidence interval and upper and lower bound for the generated average Chl*a* levels were then calculated. In all cases the results of the bootstrap closely matched the output from equation 2, for both the confidence level and the interval width.

Finally, we examined the consistency of averages calculated from field duplication efforts. These involved the collection of 11 replicates along a site, labeled "Duplicate 1", followed immediately by the collection of another 11 replicates, labeled "Duplicate 2". The Duplicate 2 samples were collected either

1 m up- or downstream of the Duplicate 1 collection points, or were collected via one of the site's alternative longitudinal sampling patterns (see **Section 1.2.5** and **Figure 1-0**, this SOP). There is no way to know which (if either) of the two duplicate values is the "true" Chl*a* average, therefore the average of all the replicates (n=22) at a site was assumed to be the true site average. The % difference between the collective average and each duplicate's average was then evaluated (i.e., did duplicate 1 and duplicate 2 each fall within 30% of the all-replicates average). Results are shown in **Table A-2**.

Table A-2. Field Duplicated Sampling Events for Benthic Algal Chl*a*.

Values are the average benthic Chl*a* calculated separately for two sampling events (N = 11 reps each), carried out at the same stream site, same day.

Site	Benthic Algal Chl*a* (mg/m^2)		CVs (%)		Duplicates' average Chl*a* within expected range*?
	1st Duplicate	2nd Duplicate	1st Duplicate	2nd Duplicate	
Rock Creek (Y02ROCKC01)	**21**	**21**	126	149	Yes
Beaver Creek (M09BEVRC05)	**51**	**67**	99	61	Yes
Little Thompson River (C13LTTPR40)	**32**	**23**	106	104	Yes
Swamp Creek (C13SWPCR20)	**12**	**11**	62	61	Yes
E. Gallatin River (site EG10)	**136**	**130**	n/a	n/a	Yes
E. Gallatin River (site EG13)	**54**	**161**	n/a	n/a	No
Armells Creek (M31ARMLC07)	**58**	**65**	70	89	Yes
Middle Fork Judith River (M22JUDSF01)	**31**	**36**	80	96	Yes
Moose Creek (M03MOOSEC04)	**26**	**27**	112	132	Yes
Eagle Creek (M10EAGLC01)	**79**	**96**	83	79	Yes
Box Elder Creek (Y26BOXEC08)	**22**	**31**	74	78	Yes

*Compared to ± 30% of the whole dataset average, i.e., all replicates collected for the 1st duplicate and the 2nd duplicate. Note: In some sites the templates, hoops or cores were composited and so a CV could not be calculated. Shown as n/a in the 'CVs (%)' columns.

Overall, the duplicates' averages all fell within ± 30% of the corresponding overall average, with one exception. Nine of eleven successful duplications is 91% success; this is in fact superior to the calculated statistical confidence level (80%) of the method. These data therefore show that actual field duplication will produce results equal to or better than our statistically determined expectations derived from replicate variability.

APPENDIX B – AQUATIC PLANT TRACKING FORM

Aquatic Plant Tracking Form

Total # of samples per method	
Templates:	
Cores:	
Hoops:	

Site Visit Code: _____

Waterbody: _____

Date: _____

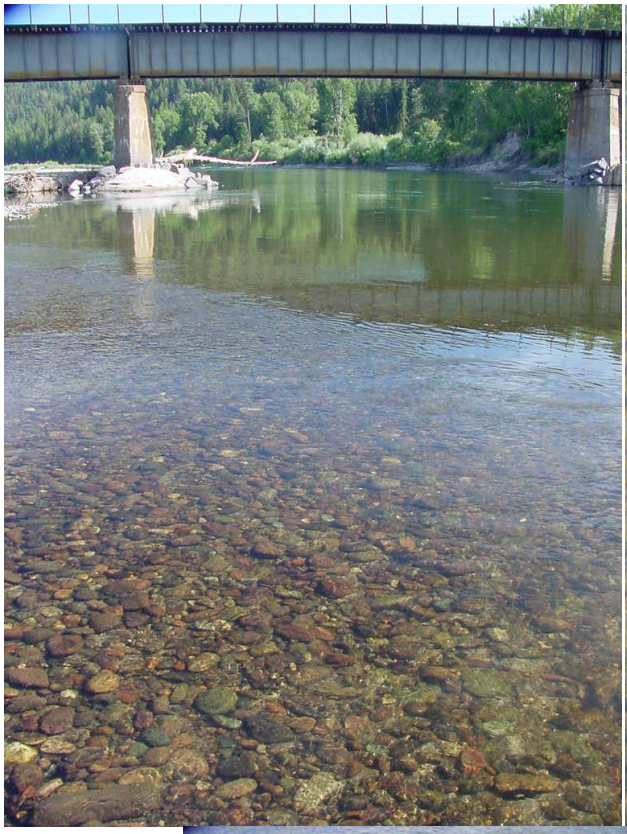
Visit No.: _____

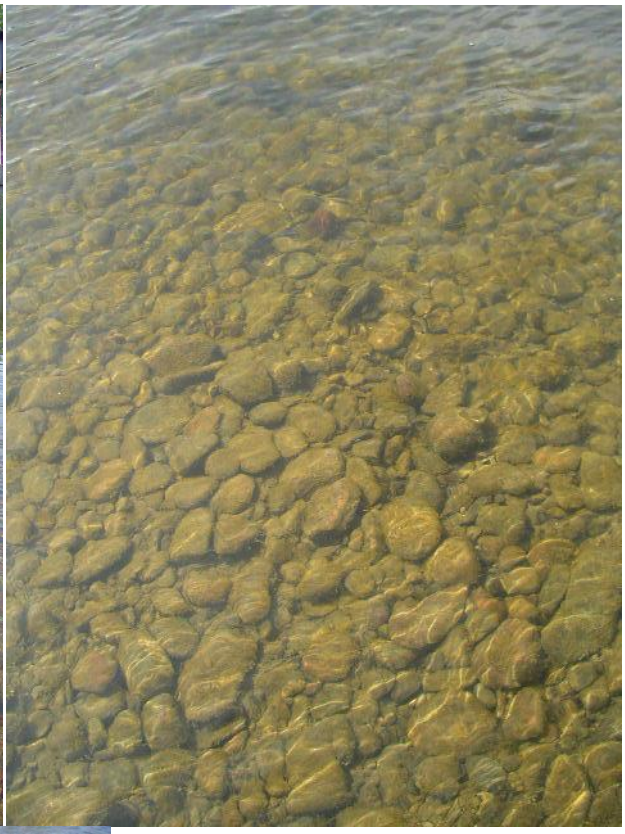
Transect	Position on stream (R, L, C)	Sampling Method*	*For templates*: Number of templates collected	*For templates*: Number of GF/F filters on which the sample has been collected	Dominant Algae†	Notes
A						
B						
C						
D						
E						
F						
G						
H						
I						
J						
K						

* H - Hoop; C - Core; T - Template; N - None collected (e.g., transect dry/inaccesible); H/N - Hoop-appropriate transect, but no sample retained (~100% macrophytes or moss)

† F - filamentous; M - algal film on a mud surface; R - non-filamentous algae on rock surfaces; n/a - no algae present

APPENDIX C – EXAMPLE PHOTOS DEPICTING BENTHIC ALGAE LEVELS OF 50 MG CHL*A*/M^2 OR LESS.





APPENDIX D – AQUATIC PLANT VISUAL ASSESSMENT FORM

Waterbody: _____ Site Visit Code: _____

Date: _____ Reach: EMAP Layout

Visit No.: _____

Transect Letter: A

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category
Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Transect Letter: B

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category
Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Transect Letter: C

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category
Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Site Visit Code: _____

Date: _____

Transect Letter: D

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category
Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Transect Letter: E

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category
Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Transect Letter: F

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category
Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Site Visit Code: _____

Date: _____

Transect Letter: G

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category
Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Transect Letter: H

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category
Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Transect Letter: I

AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long
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Microalgae	0 1 2 3 4			
Filamentous Algae	0 1 2 3 4			
Macrophytes	0 1 2 3 4			
Moss	0 1 2 3 4			

COMMENTS

Site Visit Code: _____

Date: _____

Transect Letter:	J				
AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long	
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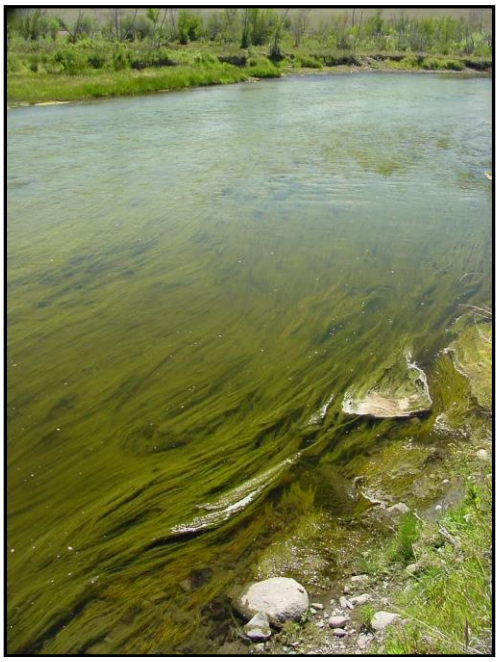
Microalgae	0 1 2 3 4				
Filamentous Algae	0 1 2 3 4				
Macrophytes	0 1 2 3 4				
Moss	0 1 2 3 4				
COMMENTS					

Transect Letter:	K				
AQUATIC PLANT VISUAL ASSESSMENT FORM	0 = Absent (0%) 1 = Sparse (< 10%) 2 = Moderate (10-40%) 3 = Heavy (40-75%) 4 = Very Heavy (>75%)	**G** = Green **GLB**=Green/light brown **LB**= Light brown **BR** = Brown/reddish **DBB** =Dark brown/black	**Gr** = Growing **M** = Mature **D** = Decaying	**Thin =** < 0.5 mm thick **Medium** = 0.5-3 mm thick **Thick** = > 3 mm thick **Short** = < 2 cm long **Long** = >2 cm long	
	Actual Cover in channel (circle one)	**Predominant Color**	**Condition**	**For microalgae & filamentous algae:** Record thickness or length category	
Microalgae	0 1 2 3 4				
Filamentous Algae	0 1 2 3 4				
Macrophytes	0 1 2 3 4				
Moss	0 1 2 3 4				
COMMENTS					

APPENDIX E – EXAMPLE ALGAE PHOTOS DEPICTING DIFFERENT COLOR AND GROWTH CONDITIONS, PER CATEGORIES IN THE AQUATIC PLANT VISUAL ASSESSMENT FORM

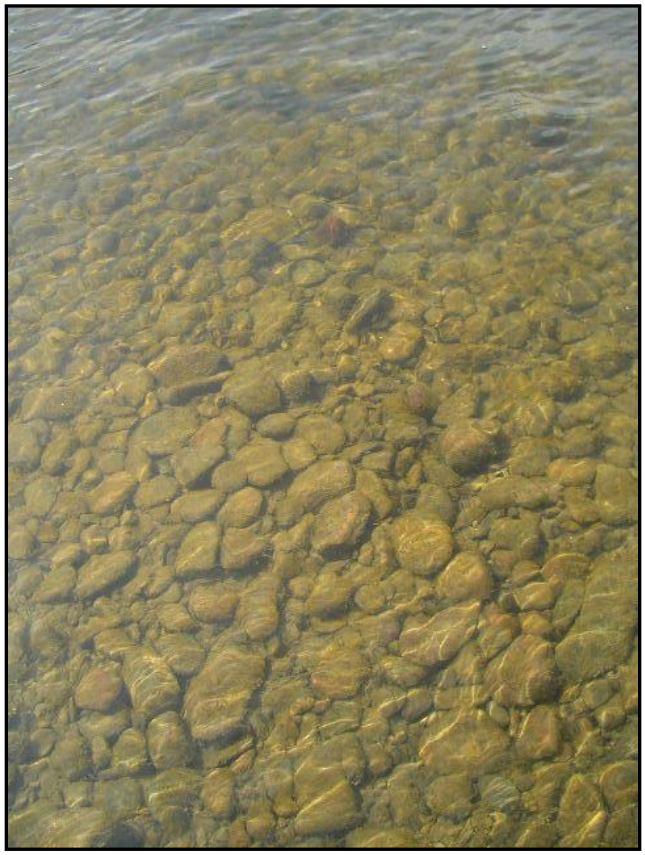
E1.0 Predominant Color

E1.1 Green



E1.2 Green/light brown

E1.3 Light brown



E1.4. Brown/reddish

E1.5 Dark brown/black - No example available

E2.0 Condition

E2.1 Growing (filamentous algae)

E2.2 Growing (Diatoms. Note the golden-brown color on rocks)

E2.3 Mature

E2.4 Decaying

E3.0 Thickness Category for Microalgae

E3.1 Microalgae Thin (note thickness on rocks that don't have filaments)

E3.2 Microalgae Medium

E3.3 Microalgae Thick

E4.0 Length Category for Filamentous Algae

E4.1 Short

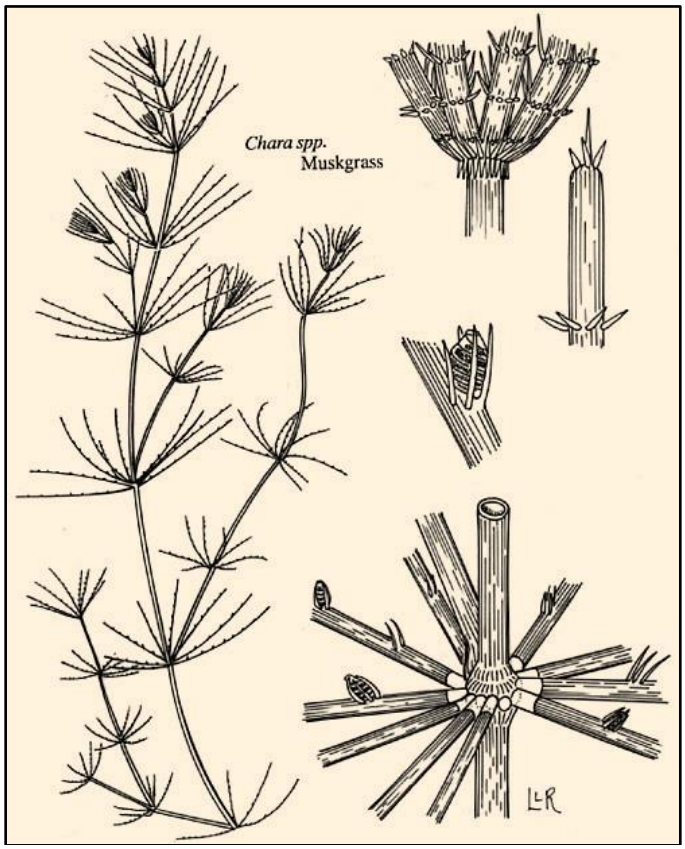
E4.2 Long

E5.0 A few examples of other aquatic plants in streams

E5.1 Macrophytes

E5.2 Moss

E5.3 Chara (branched algae, often associated with good water quality)



APPENDIX F – EVALUATION OF CHL*A* METHODS AND SOLVENT EXTRACTION TECHNIQUES FOR FILAMENTOUS ALGAE SAMPLES

DEQ
Montana Department of Environmental Quality

Memo

TO: Water Quality Planning Bureau
FROM: Michael Suplee, Ph.D. and Rosie Sada
COPY:
DATE: January 11, 2021

SUBJECT: *TECHNICAL MEMORANDUM*: Evaluation of Chlorophyll *a* Analysis Methods and Solvent Extraction Techniques for Filamentous Algae Samples

Background and Objectives. In 2019 DEQ sampled observably-heavy growths of the filamentous algae *Cladophora* as part of an ongoing study on the Smith River, but laboratory chlorophyll *a* (Chl*a*) results via High Performance Liquid Chromatography (HPLC) came back at densities we thought were very low for the levels of filamentous algae observed. Concerns over whether this could be related to Chl*a* extraction techniques or to the HPLC analytical method led to the study described here. We emphasize that this study is focused on filamentous algae.

- Objective 1: Compare the two analytical methods (HPLC *vs.* spectrophotometric) currently used by our contracted laboratories to measure algal Chl*a* in filamentous algae.

- Objective 2: Compare DEQ's standard ethanol extraction technique against a warmed ethanol extraction technique to determine if there are differences in their ability to extract Chl*a* from filamentous algae. (*NOTE: All laboratories prefer to use ethanol to extract Chla and ethanol is a DEQ-approved solvent for this purpose (DEQ, 2019); therefore, we did not examine the effect of the other DEQ-approved solvent, acetone.)*

Methods. Large quantities of filamentous algae from three locations (Smith, Clark Fork, and Yellowstone rivers) were collected and frozen in summer 2020. Each quantity of algae from each river was later thawed in subdued light, chopped, and mixed to create a uniform algal mass (no mixing of river samples occurred; each river was kept separate). From each uniform algal mass, 24 equal-mass aliquots (replicates) were measured out using a scale, wrapped in aluminum foil and numbered, and refrozen until provided to the laboratories. The laboratories were (a) Energy Lab in Helena, (b) the State DPHHS Environmental Lab, and (c) the University of Montana Watershed Health Clinic of Dr. Vicki Watson (UM Lab). To minimize any bias that may have occurred during preparation of the aliquots, the numbered aliquots associated with each river were randomly assigned to each laboratory. During preparation it was noted that the Clark Fork River samples were dark green, The Smith River samples were dull light green and the Yellowstone River samples were light yellow green.

Each laboratory was asked to analyze four aliquots from each river sample via (a) DEQ's current ethanol extraction technique described in DEQ's Standard Operating Procedure (DEQ, 2019) and (b) via a warm-ethanol extraction technique based on Sartory and Grobbelaar (1984) which was provided in Appendix A of the project's sampling and analysis plan (DEQ, 2020). Energy Lab and the State Lab both use HPLC for their Chl*a* analyses, whereas the UM Lab uses the spectrophotometric method. All samples were also analyzed for Ash Free Dry Mass (AFDM). **Figure 1** illustrates the sample preparation, submittal, and analysis process for a single laboratory.

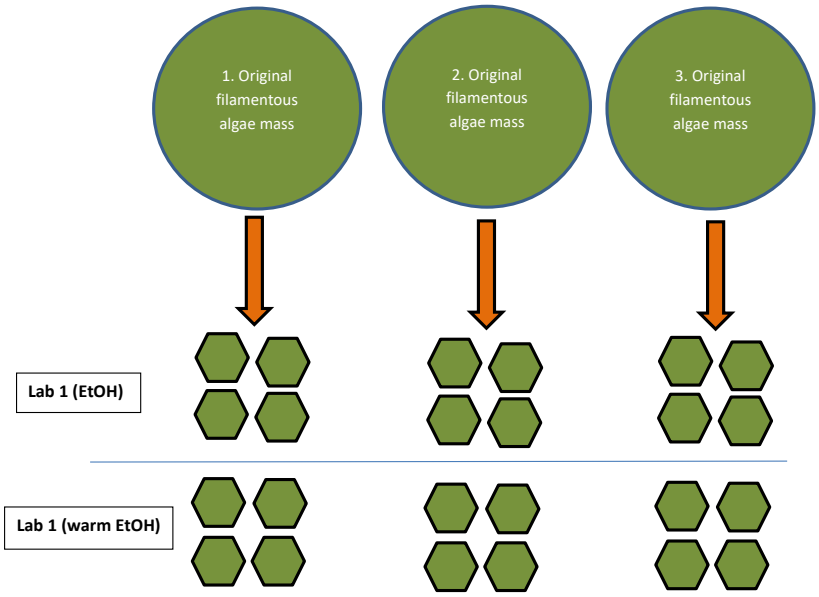


Figure 1. Illustration of Sample Preparation and Submittal to a Single Laboratory. Three uniform masses of filamentous algae from three rivers were each divided into eight equal-mass aliquots in DEQ's Watershed Lab. The aliquots were wrapped in aluminum foil, labelled, and refrozen prior to submittal to the laboratory. The laboratory was asked to extract Chl*a* from four of the aliquots using the current DEQ ethanol extraction technique, and to extract Chl*a* from the other four aliquots via a warm ethanol technique. Each aliquot was also analyzed for AFDM.

Results. One of the laboratories misunderstood the instructions and composited the replicate aliquots, therefore their results were of limited value for this study and are not presented. The other two laboratories (one using spectrophotometry, the other HPLC) correctly analyzed the aliquots as instructed and the results here are based on their data. Because the analysis was reduced to two laboratories, we used T-tests and assumed equal variance to analyze differences in analytical methods and solvent extraction techniques.

Figure 2 is a box and whisker plot of the Chl*a* aliquots organized (on the horizontal axis) by river, extraction technique, and finally analytical method. The Clark Fork River had the highest Chl*a* values by far, whereas the other two were lower and fairly similar; both laboratories using different analytical methods provided similar ranges for each river. **Figure 3** shows the data organized the same way but for AFDM instead. For AFDM all three samples had more distinct density ranges, and each laboratory's reported values fell reasonably tightly within each range. There were some distinctions between

laboratories in terms of AFDM, the laboratory using the spectrophotometric method (UM Lab) providing consistently lower AFDM by a small amount.

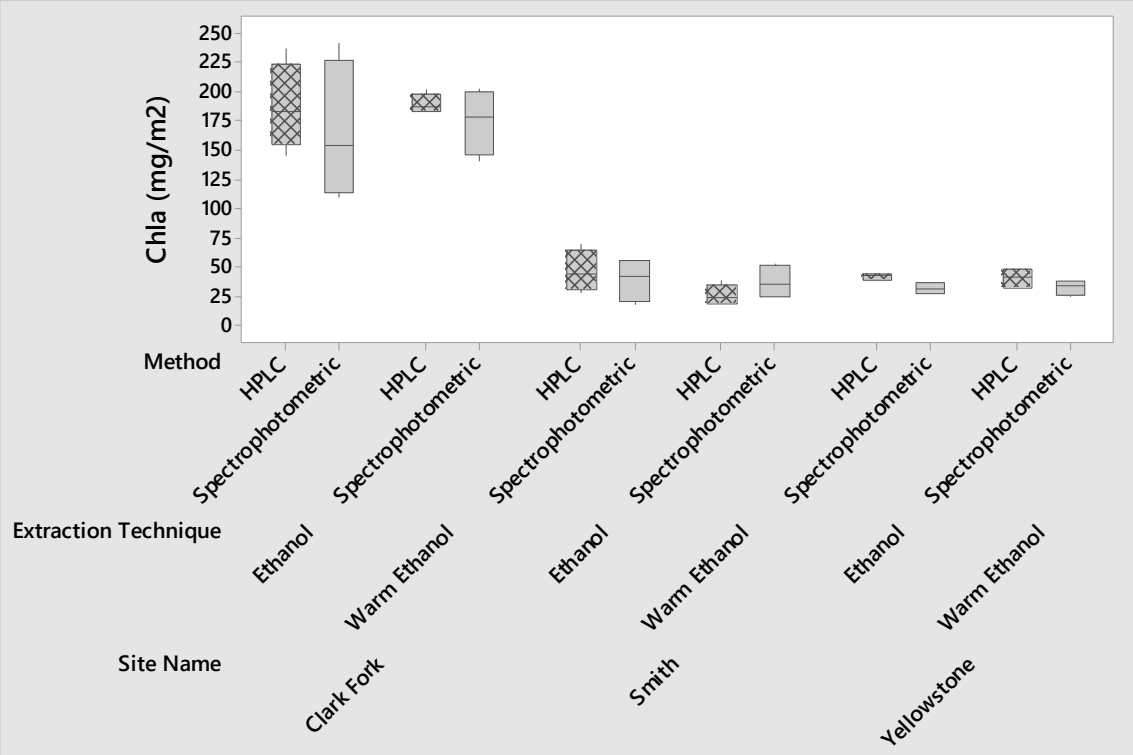


Figure 2. Box and whisker plot of Chl*a* density (mg/m^2), organized by site, extraction technique, and analytical method. Each box plot is based on the four replicate aliquots analyzed by the laboratory.

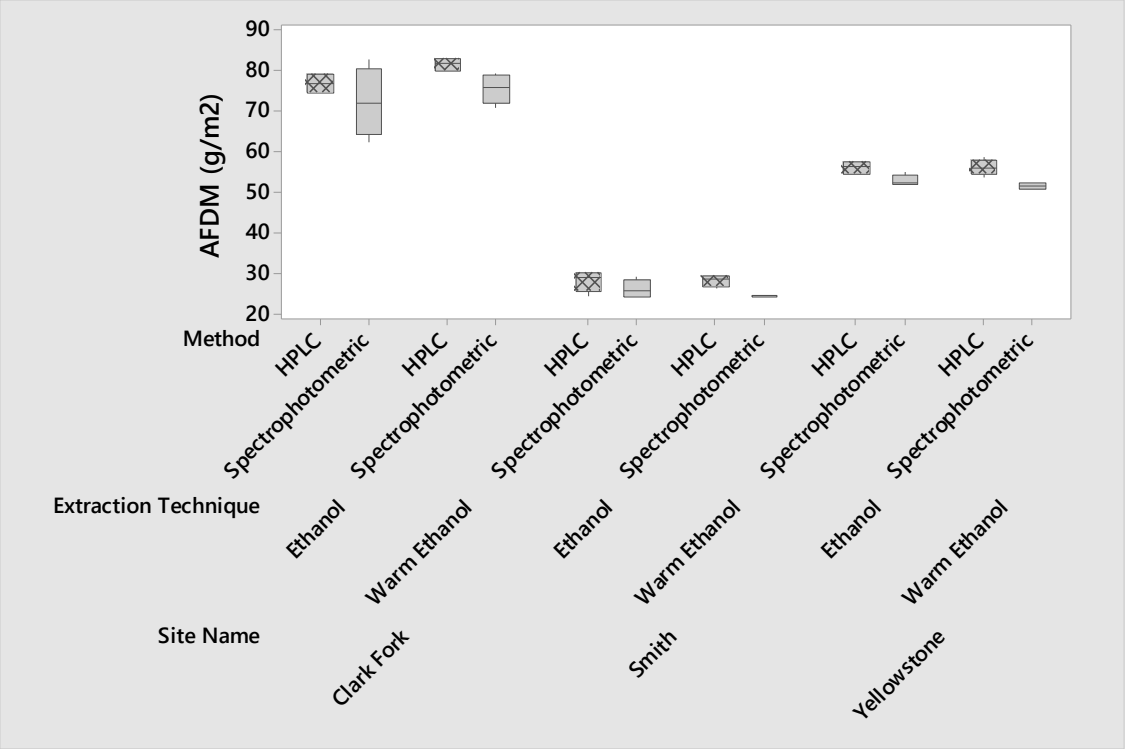


Figure 3. Box and whisker plot of AFDM density (g/m^2), organized by site, extraction technique, and analytical method. Each box plot is based on the four replicate aliquots analyzed by the laboratory.

Per objective 1 (comparing HPLC *vs*. spectrophotometry), there were no significant differences (n=6 tests) between Chl*a* extracted via HPLC compared to the spectrophotometric method except in one case, the Yellowstone River sample extracted via DEQ's current ethanol technique (**Table 1**). AFDM/Chl*a* ratio is a more rigorous parameter to examine this question because it accounts for any mass variation in the aliquots[1]; the results for AFDM/Chl*a* ratio were the same as for Chl*a* (**Table 1**, bottom row).

Table 1. T-test Results for Inter-laboratory Comparison of HPLC *vs*. Spectrophotometric for Chl*a*, AFDM, and AFDM/Chl*a* Ratio. Results for both solvent techniques are shown.

	P-values for Each Solvent Extraction Method and Sample					
	Ethanol			Warm Ethanol		
Parameter	Smith	Clark Fork	Yellowstone	Smith	Clark Fork	Yellowstone
Chl*a* (mg/m^2)	0.59	0.54	**0.01**	0.56	0.34	0.18
AFDM (g/m^2)	0.24	0.33	**0.02**	0.27	**0.02**	**0.004**
AFDM/Chla ratio	0.60	0.54	**0.02**	0.35	0.80	0.44

Note: Significant differences (≤0.05) are shown in **bold**.

[1] AFDM/Chl*a* ratio of each aliquot is the most rigorous parameter to evaluate via T-test because it accounts for variations in the original weights of each aliquot. Each aliquot was weighed on a scale, but as wet-weight; variation can be introduced at this point by the amount of residual water associated with each blob of algae.

Per objective 2 (comparing DEQ's standard ethanol extraction *vs*. warm ethanol, within the same laboratory), there were no significant differences (n=6 tests) between Chl*a* extracted via DEQ's standard ethanol extraction technique *vs*. the warm ethanol technique (**Table 2**). This was also true for AFDM/Chla ratio. Also, there was no significant different in Chla density (mg/m^2) between the two extraction techniques, by river, when the results of the two laboratories were combined.

Table 2. T-test Results for Intra-laboratory Comparison of DEQ's Current Ethanol Extraction vs. Warmed Ethanol for Chl*a*, AFDM, and AFDM/Chl*a* Ratio. Results for both analytical methods shown.

Parameter	P-values for Each Analytical Method and Sample					
	Spectrophotometric			HPLC		
	Smith	Clark Fork	Yellowstone	Smith	Clark Fork	Yellowstone
Chl*a* (mg/m^2)	0.83	0.77	0.79	0.08	0.90	0.72
AFDM (g/m^2)	0.23	0.50	0.15	0.99	**0.02**	1.00
AFDM/Chla ratio	0.75	0.71	0.77	0.07	0.85	0.57

Note: Significant differences (≤0.05) are shown in **bold**.

Since there were no significant differences in Chl*a* or AFDM/Chl*a* ratio between DEQ's current ethanol extraction technique and the warm ethanol technique, we examined the variability of these two extraction techniques to see which one is most consistent, i.e., has the lowest variability for each river (**Table 3**). The warm ethanol technique had less variability in the majority of paired cases (4/6), and it had a lower overall CV among all samples (see Grand Average CV, bottom of **Table 3**). However, for both Yellowstone River samples the CVs increased for the warm ethanol technique. The same results were observed if Chl*a*/AFDM ratio CVs were considered instead of Chl*a* CVs.

Table 3. Coefficients of Variation (CVs) Compared Between DEQ's Current Ethanol Extraction Technique and the Warm Ethanol Technique. CVs, by river, can be compared by extraction technique.

Analytical Method	Parameter	Coefficient of Variation for Each Solvent Extraction Method and Sample					
		Ethanol			Warm Ethanol		
		Smith	Clark Fork	Yellowstone	Smith	Clark Fork	Yellowstone
Spectrophotometric	Chl*a* (mg/m^2)	49%	37%	15%	41%	16%	20%
HPLC	Chl*a* (mg/m^2)	38%	20%	7%	35%	5%	22%
	Grand Average CV:			**28%**			**23%**

Finally, UM Lab completed additional analyses on their own to further understand the effects of the extraction techniques on Chl*a*. For the warm ethanol method, UM Lab measured Chl*a* and phaeophytin immediately after grinding the samples in ethanol and then again after warming the ethanol per the method. There was no significant difference in Chl*a* between the samples that were immediately measured *vs*. those that were warmed (paired T-test, one sided), however the Chl*a* was, on average, 5% higher after warming. There was a significant increase in phaeophytin after warming (paired T-test, one sided, p=0.02).

Discussion. Our findings show that there are no strong or consistent differences between Chl*a* analyzed via spectrophotometry *vs*. HPLC for filamentous algae samples. The three different river samples provided a range of Chl*a* densities, and both analytical methods provided clusters of results that were internally consistent (**Figure 2**). The only significant difference among replicate analyses was observed in the Yellowstone River sample extracted via the current DEQ ethanol technique. However, that same sample extracted using the warm ethanol technique showed no significant difference between spectrophotometry and HPLC (**Table 1**).

As seen in **Figure 2** and **Table 2**, the warm ethanol technique provides Chl*a* densities that cannot be discerned statistically from the current ethanol technique. However, we found the warm ethanol technique in most cases was less variable than DEQ's current ethanol extraction technique (**Table 3**). Moreover, UM Lab found that warming the ethanol resulted in slightly higher average Chl*a* than if the ethanol is not warmed*;* this is consistent with the intent of warming the ethanol for a short period, i.e., to achieve maximum Chl*a* extraction (see Table 4 in Sartory and Grobbelaar, 1984). Earlier analysis showed the warm ethanol technique provided the same results for diatom samples (i.e., rock scrapings) as unheated ethanol (Dr, Vicki Watson, personal communication, 1/5/2021). Further, when DEQ's core sample Chl*a* method was first tested it was found that the mud in the samples did not interfere with proper Chl*a* measurement; the extraction technique used for that study was the warm ethanol technique (Suplee et al., 2006). Taken together, these findings indicate that the warm ethanol technique is acceptable for other benthic algae samples DEQ collects as well as for filamentous algae.

We discussed the two extraction techniques with DEQ's contracted laboratories and all were supportive of the warm ethanol technique; they stated that it is about the same as the current method in terms of workload. They did note that, logistically, it works best for them if the final filtered/centrifuged Chl*a* extracts could be stored overnight in the freezer to be run the next day, because it is difficult to process the samples and run them on the instrument all on the same day. Others find that storage of Chl*a* extracts at freezing temperatures (-20^{O}C), up to 3 months, does not significantly reduce Chl*a* compared to Chl*a* samples which are measured immediately (Wasmond et al., 2006); thus, overnight storage of the Chl*a* extracts should be acceptable.

There were some systematic differences in AFDM between the two laboratories, but we have no way to determine which one is the most accurate; regardless, each laboratory provided values that clustered tightly with the other (**Figure 3**).

Recommendations. Currently the DEQ chlorophyll *a* Standard Operating Procedure (SOP) allows for spectrophotometric or HPLC analysis of algal Chl*a* (DEQ, 2019). Our findings indicate there is no systematic bias associated with filamentous algae samples using either method and, therefore, both methods can continue to be used for filamentous algal Chl*a* measurement. We recommend that the SOP (DEQ, 2019) be updated to require that the warm ethanol extraction technique be implemented for all future analysis of Chl*a* from benthic algae samples (filamentous-hoops, rock scrapings-templates, and mud surfaces-cores). As shown here, warm ethanol extraction provides more consistent results across laboratories for filamentous samples regardless of whether a spectrophotometer or HPLC is used. Since it is very common for some short filamentous algae to be included in rock scraping samples, the warm ethanol method should aide in extracting Chl*a* in those cases. The SOP should also indicate that warm ethanol extracts (sample that have already been extracted, then filtered and/or centrifuged) may be stored in a freezer overnight for analysis the next day.

References.

Montana Dept. of Environmental Quality, 2019. Sample Collection and Laboratory Analysis of Chlorophyll-*a* Standard Operation Procedure, revision 7. WQPBWQM-011. Helena, MT: Montana Dept. of Environmental Quality.

Montana Dept. of Environmental Quality, 2020. Comparison of the Effect of Two Analytical Methods and Two Extraction Techniques on the Measurement of Chlorophyll *a* in Filamentous Algae. Sampling and Analysis Plan No. WQDWQSMSAP-06. Helena, MT: Montana Dept. of Environmental Quality.

Sartory D.P. and J.U. Grobbelaar, 1984. Extraction of Chlorophyll *a* From Freshwater Phytoplankton for Spectrophotometric Analysis. The Hague: Dr. W. Junk Publishers. Hydrobiologica 114: 177-187.

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Wasmond, N., I. Topp, and D. Schories, 2006. Optimizing the Storage and Extraction of Chlorophyll Samples. Oceanologia 48: 125-144.