EVALUATION OF A COMMERCIAL IN SITU FLUOROMETER PROBE FOR WATER QUALITY MONITORING PROGRAMS

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Abstract: Benthic Algae is often used in water quality monitoring; however, traditional methods of benthic algae assessment are relatively expensive and time consuming. The Benthotorch® is a portable fluorimeter probe used to measure *in situ* benthic chlorophyll-a ($\mu g/cm^2$) and relative abundance of cyanobacteria, green algae and diatoms ($cells/cm^2$) in about 20 seconds. During the summer of 2014, at 42 locations across Oklahoma, 119 benthic algae samples were measured with the Benthotorch®, extracted for chlorophyll a and a taxonomic assessment conducted. Medians for each method were statistically similar based on Mann Whitney tests. Regression analyses resulted in significant and positive correlations between BenthoTorch® chlorophyll *a* estimates and extracted chlorophyll *a*, although BenthoTorch® estimates underestimated extracted chlorophyll *a.* The most likely causes of discrepancies between the extracted chlorophyll *a* and the BenthoTorch® were self-shading of the algae, high biomass concentrations and sediments present in the samples. The BenthoTorch® measured an area of 1.0 cm^2 while the field samples were collected from an area of 13.1 cm^2, which is another error source. To test this, benthic algae was grown under controlled conditions in a laboratory. After a period of growth, the benthic algae was measured *in situ, ex situ/in vivo* with the BenthoTorch® and compared to extracted chlorophyll *a* measured with a spectrophotometer. The results were improved over field tests with an R^2 of 0.64 and a slope of 0.86 for the *in situ* and R^2 of 0.79 and a slope of 0.81 for the modified method. Tests for variance of sampler size concluded that the larger sampler area improved characterizing the algal variability. BenthoTorch® estimates of relative abundance were evaluated, were statistically different based on Mann Whitney tests, and were not significantly correlated in regression analyses

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CHAPTER I

INTRODUCTION

Attempts to control water pollution in the United States on a large scale began with the Refuse Act of 1899 (USEPA, 2013) and continued to be refined, revised and strengthened until the Federal Water Pollution Control Act (FWPCA) of 1972, which is more commonly referred to as the Clean Water Act (CWA). The CWA set the goal of having “fishable and swimmable” waters by 1983, as well as instituting the objective of ending the “discharge of pollutants into navigable waters” in 1985 (Poe, 1995). The CWA provided funding for States to set up and operate water quality monitoring programs. These State run programs were intended to “monitor, compile and analyze” the collected data in order to meet the goals of making the States waters compliant with the CWA (USEPA, 2003). The CWA has provided measurable improvement to numerous watersheds across the country. The CWA did not provide a way to estimate status, trends or changes in ecologic resources on a regional scale (Messer et al., 1991). The US Environmental Protection Agency (EPA) does not define how a State will achieve the CWA goals, so there is variability in state monitoring programs (USEPA, 2003).

In the late 1980’s, the US EPA devised the Environmental Monitoring and

Assessment Program (EMAP) as a way to strengthen the CWA state monitoring programs (USEPA, 2002). EMAP's goal was to provide a statistically valid and robust method to monitor current water quality and assess trends in aquatic ecosystems. This program had the goal of standardizing data and collection and storage to allow statistical comparisons and long-term trend analysis. EMAP provides guidance for monitoring. programs that includes long-term land cover, aquatic biologic indicators, as well as physical and chemical water quality parameters (USEPA, 2002). Fish, macro-invertebrates and periphyton are the biological indicators for streams, and are the most difficult to monitor because they require expense and expertise that water chemistry does not. Biologic assemblages of invertebrates, fish and periphyton all have a large spatial and temporal variability (USEPA, 1997) and require a specialist to identify and document species.

Periphyton are benthic microscopic and filamentous algae that have relatively high growth rates and reproductive cycles and respond quickly to environmental change (Stevenson et al., 1991). With the high spatial and temporal variability of periphyton in aquatic ecosystems, sampling protocols are critical to represent the aquatic ecosystem properly. Samples are currently collected by removing the periphyton from a natural or artificial substrate, processed quickly (either filtered for chlorophyll extraction or ash free dry mass, or preserved for taxonomy), and finally analyzed, which can be time consuming and costly. Taxonomic assessments are expensive because significant laboratory time is required to conduct cell counts and identification. Therefore, developing a tool to monitor periphyton *in situ* may be beneficial to monitoring agencies.

The use of *in situ/in vivo* fluorimeters in oceanography began in the mid 1960's when Carl Lorenzen modified a Turner fluorimeter to sample ocean water off the coast of Baja Mexico (Lorenzen, 1966). A commercial fluorimeter probe was developed by BBE

Moldaenke that was designed to measuring periphyton *in vivo* and *in situ*. The BenthoTorch® uses three spectral bands (470, 525 and 610 nm) to induce fluorescence in the periphyton and one at 700 nm to adjust for light scattering (BBE, 2014). The BenthoTorch® measures the fluorescence signal activated at these wavelengths, which is used to estimate total benthic biomass and the relative abundance of cyanobacteria, green algae and diatoms in either µg chlorophyll *a*/cm^2 or cells/cm^2. Although there are several researchers using the BenthoTorch®, only two studies have been published (Kahlert and McKie 2014, Harris and Graham 2015) that directly compare the BenthoTorch® to traditional periphyton sampling methods. Therefore, there is a need to examine the utility of the BenthoTorch® under different stream conditions and to compare the results to traditional sampling methods.

Periphyton

Algae consist of predominantly photoautotrophic organisms that are very diverse, although they all contain chlorophyll *a* and have unicellular reproductive structures (Stevenson et al., 1996). Benthic algae can be attached to a surface or in and among loose substrate. Phytoplankton by contrast are free moving in the water. As a result, benthic algae are representative of local conditions over time; whereas planktonic algae are representative of conditions in the water column at the time of sampling. Benthic algae can be divided into macro and micro, the latter is referred to as periphyton (Wetzel, 2001). *Cladophora* is a macro-filamentous chlorophyte that may not meet the strict definition of periphyton according to Wetzel (2001); however, it is often included in periphyton surveys for water quality (Dodds and Grubber, 1992; Suplee et al. 2009). In this study, periphyton will include macro-filamentous algae to distinguish it from other macrophytic algae (e.g. *Chara, Nitella*) that would be included in Rapid Habitat Assessments (USEPA, 1999; OCC,

2014). It takes time for periphyton to colonize, thus periphyton represent the local conditions and can be an indicator of environmental quality. Periphyton have three main morphological growth forms: unicellular, filamentous and colonial (Stevenson et al., 1996). The majority of benthic algae are Cyanobacteria, Chlorophyta (green algae), Bacillariophyta (diatoms) and Rhodophyta (red algae) (Stevenson et al., 1996). BenthoTorch® estimates the first three taxa, so they are most relevant to this study.

Relevant Phylum

Cyanobacteria

Cyanobacteria are a very diverse group of bacteria that contain species important to primary production in aquatic habitats as well as species that produce substances that are toxic to humans and wildlife (Graham et al., 2009). Cyanobacteria are prokaryotic and unicellular; colonial or filamentous (Graham et al., 2009). In general, cyanobacteria utilize chlorophyll *a* and supplementary pigments including carotenoids and phycobilins (Graham et al., 2009). Chlorophyll *b* is present in a few cyanobacteria but it is not as common (Graham et al., 2009).

Green Algae/Chlorophyta

Chlorophyta are another very large and diverse group found in all aquatic environments, including desert, glacial and hot spring environments (Graham et al., 2009). Chlorophyta include the genus *Cladophora,* which is ubiquitous worldwide and can dominate the benthos (Dodds and Gudder, 1992). *Cladophora* is also a large component of the algal mats that are viewed as a nuisance in water quality surveys (Suplee et al., 2009). All Chlorophyta contain chlorophyll *a* and *b* complimented by lutein and beta-carotene (Graham et al., 2009). Importantly for this study, Chlorophyta do not contain phycobilins that are typical of Cyanobacteria (Graham et al., 2009).

Diatoms/Bacillariophyta

Diatoms, which uniquely among the three major periphyton classes possess a silica frustule, occur as unicellular or colonial forms and are common across many aquatic habitats (Graham et al., 2009). Diatom indices are often included in water quality monitoring programs (Hill et al., 2000; Blanco et al., 2012). Diatoms contain chlorophyll *a* and *c* as well as the supplementary pigment fucoxanthin (Graham et al., 2009).

Periphyton Ecology

Many interrelated factors can influence the development of periphyton communities. The relatively short reproductive and life cycles of periphyton means that they respond very quickly to alterations in their environment and thus should be part of a comprehensive monitoring program (Stevenson and Bahls, 1999). Light, temperature, and the nutrients phosphorus and nitrogen are the primary control of biomass gain. Grazing and flow velocity are the primary regulators of biomass loss (Biggs, 1995).

Considerable research has been evaluated how the location of a stream affects the biotic community and health of the aquatic ecosystem. Whittier et al. (1988) found that ecoregion association could account for many habitat and biological indicators, but had less influence on periphyton populations. Diatoms are often used as indicators of environmental indicators in lakes and rivers (Dixit et al., 1992). In a study of diatom assemblages across ten Level I Omernik ecoregions, Potapova and Charles (2002) found that ecoregion is a predictor of diatom species. Patterns have been identified between ecoregion and periphyton assemblages in isolated studies, although environmental factors have been shown to improve periphyton community predictions. For example, Johnson et al. (2009) found that nutrient limitation was inversely correlated with percent agriculture and urban land use.

Public Perception, Impairment and Nutrients

Dodds et al. (1998) suggested that eutrophic levels for streams should be set at >10µg/cm^2 of benthic chlorophyll *a*. Ten µg/cm^2 benthic chlorophyll *a* is correlated with around 20 percent cover according to the literature (Thomas, 1978; Welch et al., 1988; Biggs, 1996; Bothwell, 1989; Dodds et al., 1997; Dodds, 2006; Dodds and Oak, 2004; Smith et al., 2003). Suplee (2009) found public perception of observed periphyton density in streams was positively correlated with benthic chlorophyll *α*. Forty to 50 percent cover was the threshold for impaired water in public surveys of Suplee et al. (2009). The 40-50 percent cover corresponds with 10-15ug/cm^2 benthic, which could be considered impaired under the Clean Water Act "fishable and swimmable, and tribal designated uses (Suplee et al., 2009; Dodds et al., 1998).

Problem Statement

Effective environmental management decisions require relevant information. Abundant useful data enable managers to better assess the environmental conditions and make decisions that are more informed. Water quality monitoring has gained importance over the last 45 years as the CWA has evolved into a more comprehensive and systematic mandate. The CWA led to the development by the US EPA of EMAP, which provides states, territories and tribal agencies information on monitoring program design, data collection and analysis, archiving and use of these data for decision-making. In part, EMAP involves gathering of biotic data used as ecological indicators, including periphyton time to colonize. Periphyton sampling is very labor-intensive fieldwork, and requires extensive lab work with expensive equipment and/or very specialized knowledge. These factors make periphyton-monitoring cost prohibitive for many agencies and researchers to gather these

data on a regular basis. A rapid assessment tool for periphyton would be a great benefit to watershed managers and policy makers.

The BenthoTorch® fluorimeter probe claims to provide a rapid, *in situ* assessment of periphyton that includes a relative abundance of the three main classes of periphyton (green algae, cyanobacteria and diatoms) as well as total biomass. Use of the BenthoTorch® would eliminate the use of artificial substrates that require multiple trips to the site or collecting/scraping periphyton from natural substrates that both require time consuming and expensive lab work and taxonomic expertise. Fluorimeters are commonly used in the lab and have been used in marine and freshwater phytoplankton studies. Despite higher biomass that improves instrument sensitivity, periphyton poses some problems that are not encountered in suspend algae. Periphyton grows on surfaces that are often irregular in texture, shape and color. Some minerals in rock and sediments have different light absorbing or reflecting properties that may cause issues when using instruments such as the BenthoTorch®. In addition, algal density on the substrate exhibits wide spatial variability. In addition, sediments can cover the algae and inhibit or reduce the signal for optical measurements. Watershed condition vary widely in low to high order streams, slope, substrate, riparian vegetation and land use, which affect the benthic algae.

Research Objectives, Hypothesis and Research Questions

The overall objective of this research was to assess the BenthoTorch® compared to traditional methods of benthic algae measurements in estimating total benthic algal biomass across major stream types and conditions throughout Oklahoma.

Hypothesis

Ho: BenthoTorch® measurements for total concentration of benthic chlorophyll-a based on fluorescence and relative abundance of the three major periphyton divisions are

equal to the traditional methods of periphyton sampling using extracted chlorophyll-*a* for total concentration and taxonomic counts for relative abundance.

Objectives and Research Questions

Article 1

Objective

1. Determine if the BenthoTorch® readings are correlated with extracted chlorophyll-*a* and taxonomic assessment using data collected from different streams across Oklahoma.

Research Questions

1. Can the factor(s) that cause differences between the BenthoTorch® and traditional measurements be identified?

Article 2

Objective

1. Develop and test a BenthoTorch® sampling method that accounts for the factors identified in Article
2. Evaluate the utility of the BenthoTorch® as a quantitative assessment tool for rapid assessment of benthic algal biomass.

Research Questions

1. What BenthoTorch® precision and accuracy are required to match traditional benthic algae sampling methods?
2. What are the costs and benefits of using the BenthoTorch® and traditional benthic algal sampling for biological assessment of streams?

CHAPTER II

LITERATURE REVIEW

Environmental Monitoring

The US EPA outlined the direction of the agency for the 1990's (USEPA, 1988). The report identified the US EPA as a regulatory agency charged with cleaning up existing pollution but suggested that the US EPA should identify methods to reduce pollution before it is generated and adopt ten recommendations by the Science Advisory Board. Recommendations 4 and 5, in particular, have influenced environmental monitoring in the years since. Recommendation 4 stated that the US EPA should "explicitly develop and use monitoring systems that help the agency anticipate future environmental conditions" (USEPA, 1988). Recommendation 5 called for the US EPA to establish the Environmental Research Institute to "conduct a core ecological research program" (USEPA, 1988). This change in philosophy for the US EPA required the development of a strategy for environmental monitoring to collect data representing ecologic conditions.

The Environmental Monitoring and Assessment Program (EMAP) was a result of the change in strategy of the US EPA in the late 1980's (USEPA, 1997). The goal of EMAP was to provide an integrated scientific monitoring system to assess the current

ecological health of the country, monitor trends, and predict future conditions by region with a known statistical confidence (Messer et al., 1991). The use of ecological indicators and an integrated data storage network are required to achieve these goals (USEPA, 1997). EMAP outlined ten elements of a state water monitoring and assessment program (USEPA, 2003):

1. Monitoring program strategy
2. Monitoring objectives
3. Monitoring design
4. Core and supplemental water quality indicators
5. Quality assurance protocols
6. Data management protocols
7. Data analysis and assessment methods
8. Reporting protocols
9. Programmatic evaluation
10. General support and infrastructure planning.

The EMAP monitoring objectives were to be consistent with the goals of the CWA as well as those of the States' water-quality management objectives (USEPA, 2003). The objectives of an EMAP monitoring program should include quantifying the water quality of the State, determining temporal variability of water quality, identify regions with water quality issues and regions that may need protection, and developing a way to measure the program effectiveness (USEPA, 2003).

The design of the EMAP monitoring program must remain pliable and responsive to the unexpected. In order to quantify the quality of water resources and identify waters that need protection, data must be collected to assess current conditions. Integrating probabilistic statistical models into the monitoring design requires regional and local sampling locations, the number of samples needed and what parameters to measure (USEPA, 2003). EMAP required the design to provide pollutant estimates within ± 10% at a 90% confidence level across regions within the States (USEPA, 2003).

It is important to consider the scale up and scale down paradigms, and the scale up paradigm with embedded scale-down components when designing a monitoring program (Root and Schneider, 1995). Root and Schneider (1995) recommended the scale up or the bottom up approach, i.e. measurements are taken at a small scale and then used to determine "possible mechanistic associations" that can be used to make predictions across larger scales. The benefits of "scale up" are fewer field trips, lower costs, and fewer samples to be statistically valid. In periphyton studies, a pattern may be seen at a few local sites that can be explained based on local conditions. For example, some diatoms have a high tolerance for acid mine drainage (Smucker et al., 2014), and thus the presence of these algae species is an indicator of water quality impairment that can be identified from the scale up approach.

Figure 1 illustrates the interrelationships between different factors from the cellular to regional scales. Whether to use a scale up, scale down or scale down embedded in scale up design depends on the question. The response of periphyton to the environment can be examined at smaller scales (within a stream reach); however, the function within the ecosystem must also be examined at lager scales (full stream reach or watershed level). Proper monitoring design for the scale transition is key to

keeping the monitoring relevant to the current environmental conditions. For example, if monitoring identifies acid mine drainage in a small isolated watershed with a dry climate and granite geology, it would pose a different risk than the same contamination in larger, more connected watershed with a wet climate and limestone geology.

Root and Schneider (1995) recommend a "strategic cyclical scaling paradigm" where the "large scale associations are used to focus small scale investigations to ensure that tested causal relationships are generating the large-scale relations" (Figure 2). The EMAP program recommends a top down approach, looking at the ecologic system as a whole and monitoring the components that are diagnostic to each region (Davis and Simon, 1995). In a state such as Oklahoma, a land cover assessment could target areas that may have been negatively impacted by land cover change. For example, loss of riparian vegetation coupled with high intensity agriculture would signal a need for more small scale monitoring in those areas. Small-scale studies may include periphyton and water chemistry monitoring to document if land cover changes have affected the ecosystem. The question is how far down the scale do you need to go for these data to be relevant?

Qualitative assessments can be useful in demonstrating relative changes from sampling period to sampling period, but are relative changes adequate? How quantitative does the measure of periphyton need to be, and what accuracy is required? Is benthic biomass based on chlorophyll-*a* adequate to identify or corroborate a change in environmental condition, or is it also necessary to investigate relative abundance of species or higher taxa? Is relative abundance at the division level adequate, or do the algae need to be identified to genus or species level? Division level relative abundance

has not yet been used for water quality assessment. Identifying problems on a larger scale requires a higher sample number taken more frequently. However, there may be more tolerance for lower accuracy with a higher sample number. If more detail at a smaller scale is required, then the accuracy is more important. Therefore, the BenthoTorch® needs to have a similar accuracy to the traditional methods for a small scale monitoring design.

Monitoring Periphyton

Because periphyton integrate conditions over time compared to chemical monitoring at a single point in time, it has been used in water quality monitoring programs for at least the last century (Stevenson, 2014). Periphyton are considered by many as the best indicator of aquatic ecosystem disturbance because the organisms are sensitive to flow, temperature and light as well as changes in water chemistry. Rosen (1995) references several studies investigating periphyton responses to nutrients, pH, metals, and in urban runoff. Monitoring periphyton can be used to assess excess nutrients (Barry and Biggs, 2000) as well as acid mine drainage (Smucker et al., 2014).

Periphyton present a different problem in sampling compared to phytoplankton. Unlike phytoplankton, algae growing in or on benthic substrate must be physically removed in order to quantify. There are many ways to analyze periphyton samples, including but not limited to chlorophyll a, accessory pigments, ash free dry weight, bio-volume, and species identification (Weitzel et al., 1979). The collection and analysis method depends on how these data will be used.

Periphyton Sampling Methods

Based on the question, periphyton can be sampled using multi-habitat or single habitat methods. Single habitat sampling consists of a composite sample from the same type of habitat (e.g. riffle, pool, and run) from a single stream reach. Multi-habitat sampling consists of one composite sample from different habitats proportionate to prevalence within a single reach. Results from single habitat sampling may reflect differences in water quality between different streams but may miss important information held in other habitats. On the other hand, multi-habitat sampling characterizes algae between reaches but may miss water quality trends between different streams.

The US EPA recommends multi-habitat sampling when species composition analysis is performed and single habitat sampling for chlorophyll-*a* biomass analysis (Stevenson and Bahls, 1999). Single habitat sampling characterizes the overall presence of periphyton in a reach by reducing variability inherent to algal populations in different habitat types. Additionally, if the algal population composition is desired, sampling different habitat types reflects the entire population since different species prefer different environments (Stevenson and Bahls, 1999)

Researchers must choose between sampling natural and artificial substrates. It is ideal in most cases to sample natural substrates to reduce time and money spent on sampling. Natural sampling requires only one trip to the site, while artificial sampling requires one trip to place the artificial substrate on site and another to collect the substrate for analysis. Natural substrate sampling poses problems because of the depth to samples, multiple types of substrate and the difficulty in quantitatively removing periphyton from the substrate without an excess of water. However, artificial substrate

sampling can be a very useful approach to sampling periphyton in a non-wadeable river or streams with little natural substrate to sample (Stevenson and Bahls, 1999). Caution should be used in the interpretation of data obtained from artificial substrates; some observations that are credited to water quality fluctuations may actually be “artifacts” of the artificial substrates (Weitzel et al., 1979). Aside from the potential easier sample collection, some studies indicate a reduction in sampling variability, but the researcher needs to be aware of possible bias from the substrate selected (Morin and Catteneo, 1992).

The type of sample analysis depends on the goals. Chlorophyll-a based on fluorescence and ash free dry weight are used as proxies for total benthic biomass, and taxonomic analysis can be used for community composition. Periphyton samples acquired in the field need to be sent off to a reputable laboratory for taxonomic identification, or a team may choose to have one of their own trained in taxonomic identification to reduce costs. It is recommended that algae be identified to at least the genus level (Stevenson and Bahls, 1999).

Mean Benthic Chlorophyll a: Confidence and Margin of Error

Trend analyses require a known confidence and margin of error (MOE) for sampling procedures. Due to spatial and temporal variance, periphyton has many unknowns associated with sampling. Quantitative sample removal from the substrate is challenging. It is difficult to know how well the sample represents a stream reach, although with enough sampling, a confidence and MOE can be defined (MDEQ, 2011). Mean benthic chlorophyll *a* and coefficient of variation (CV) were positively correlated in streams of the Laurentian region of Quebec, Canada (Cattaneo and Prairie, 1995). The relationship between mean benthic chlorophyll *a* and coefficient of variation (CV) was

used to determine how many samples are required to account for temporal variation in stream sampling. Montana Department of Environmental Quality (MDEQ, 2011) used the same method and did not find a similar pattern. Out of 2200 individual chlorophyll data and 288 sampling events collected as part of Montana's Stream Reference Project (STREFPRO), there was no significant relationship (R^2=0.07). Using the central tendency of the CV calculations, a standard deviation may be estimated for the sampling program and used to determine the required sample size using Equation 1 (MDEQ, 2011). The sample size estimation can be rearranged, allowing the user to identify the number of samples needed to reach a desired confidence and MOE if the standard deviation is known. MDEQ (2011) back calculated a predicted standard deviation in order to assess how well their number of samples was capturing the true mean benthic chlorophyll *a*, which is given as:

$$\boldsymbol{n = \left(Z_{\alpha/2}\frac{\sigma}{MOE}\right)^2} \quad \textbf{(1)}$$

where n is the number of samples, $Z_{\alpha/2}$ is the inverse cumulative probability of the standard normal distribution, α is the given significance level, σ is the estimated standard deviation and MOE is the margin of error.

Chlorophyll Fluorescence

Photosynthesis is often expressed as an oxidation-reduction reaction using light as the energy source (Falkowski and Raven, 2007). As a molecule of photopigment absorbs one photon (from light), one electron is lost. This electron passes through the electron transport chain and eventually reduces NADP to NADPH, freeing an electron and O_2 from by photolysis. The free electron returns to the photopigment and the process can start over (Falkowski and Raven, 2007). Chlorophyll *a*, which absorbs in the red (650-700) and blue (400-450) spectrum of light, is the photopigment universal to

all algae and cyanobacteria. There are several auxiliary pigments (chlorophylls b-e, phycocyanins, fucoxanthin, etc.) used for exploiting a broader range of wavelengths used in photosynthesis (Consalvey et al., 2005). There are three possible outcomes for light energy absorbed by chlorophyll molecules in algae and bacteria. The light energy is used for photosynthesis (photochemistry), lost as heat or re-emitted as light. The re-emission of light is chlorophyll fluorescence. Fluoresced light has a longer wavelength resulting from loss of energy in the photons (entropy) (Maxwell and Johnson 2000). Exposing algae to specific wavelength of light will cause a measurable amount of fluorescence. The intensity of fluorescence is used as a measure of concentration of the chlorophyll.

Chlorophyll Analysis

US EPA protocols use chlorophyll-a as a proxy measurement for periphyton biomass (Stevenson and Bahls, 1999). It is a useful tool for the comparison of productivity within a stream or between different streams (Grzenda and Brehemer, 1960). Chlorophyll-a is found in all algae, although percent by biomass is highly variable and is dependent on the division of algae as well as environmental conditions (Graham, 2009). Richards and Thompson (1952) established the now common trichromatic method of analyzing pigments. This method uses pigments extracted from algae in a solvent and measures the absorbance of light at specific wavelengths. The absorbance was directly related to the concentration of pigments at the specified absorption spectrum. The extraction of pigments requires algal samples be destroyed in the process. Lorenzen (1966) used fluorescence on live algae samples *in situ* to measure plankton biomass continuously on marine expeditions. Lorenzen (1966) used a flow through modification of a Turner model III fluorimeter on an expedition off the coast of

Baja Mexico. The study found a positive linear relationship with an R^2 of 0.96. A limitation was the temperature fluctuated between 21 and 29 ^oC resulting in a potential error rate of 13%. Strickland (1968) cautioned that the Lorenzen's (1966) results might not be consistent with a different algal community composition or environmental conditions. Strickland used filtered seawater and concluded that light is being scattered in the water by suspended sediments. When mixing water with fluorescing and non-fluorescing particles, a more linear relationship results, but when the particles are primarily fluorescing, then the relationship is a power function (Strickland, 1968). Data from i*n vivo* measurements should be analyzed with the previous variables in mind.

Periphyton can be removed and analyzed with a portable fluorimeter or measured without removal using a fluorimeter probe that directs light at the sample and measures fluorescence. Moulton et al. (2009) used this method for a comparison with dry mass. This tool is only useful for analysis of biomass based on chlorophyll a. The results showed after a calibration that a hand held unit provides an inexpensive and reliable alternative to collecting and transporting samples back to a lab for processing.

Kahlert and McKie (2014) compared the BenthoTorch® with the traditional scraping method of periphyton followed by processing the sample in the lab for chlorophyll extraction and taxonomic assessment. Samples across 24 streams in Sweden revealed a significant relationship between the BenthoTorch and the extracted chlorophyll analysis. In Kansas streams, the BenthoTorch® compared well with relative ethanol extracted chlorophyll-a but not as well with extracted chlorophyll-a concentrations (Harris and Graham, 2015). The BenthoTorch® estimates of relative Chlorophyta, Diatoms and Cyanobacteria biomass was not consistent with laboratory measured community composition.

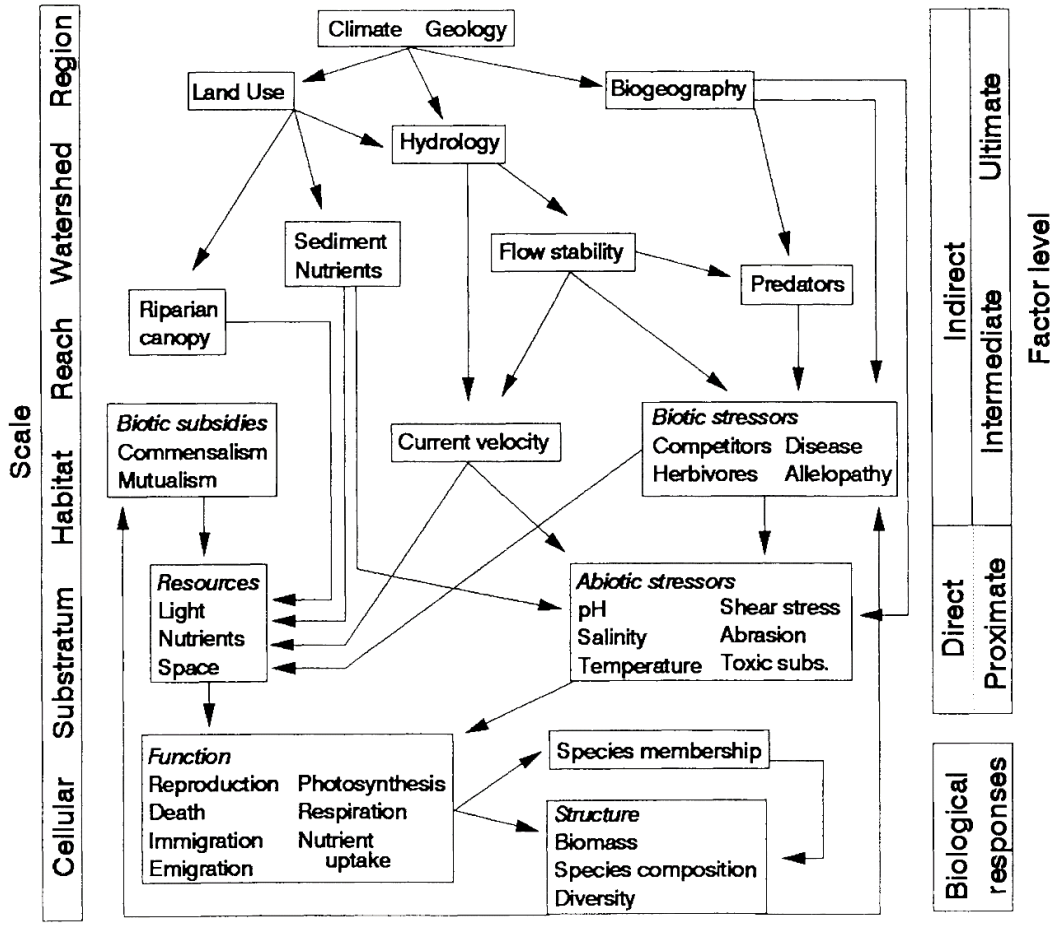

Figure 1. Hierarchical interrelationships for determining periphyton community-assemblage factors and total biomass (Stevenson, 1997).

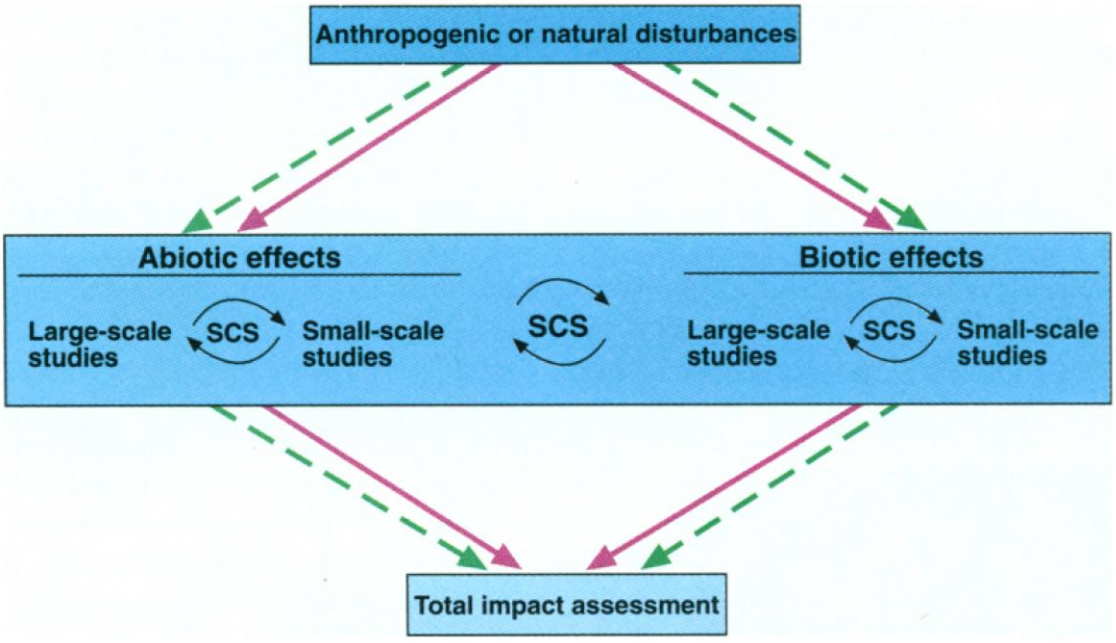

Figure 2. Components of Strategic Cyclical Scaling Paradigm (Root and Schneider, 1995).

CHAPTER III

EVALUATING THE BENTHOTORCH® FOR USE IN RAPID ASSESSMENT OF PERIPHYTON FOR VARIABLE STREAM CONDITIONS

Introduction

Periphyton growth has a complex relationship with other organisms, light, stream flow, available oxygen, temperature, available nutrients and time (Stevenson and Bahls, 1999). Periphyton requires weeks to establish and grow in a stream system and is a valuable monitoring tool for water quality and ecologic assessment (Stevenson and Bahls, 1999). Unlike water chemistry, which can fluctuate quite rapidly, e.g. dissolved oxygen, periphyton represents and integrates conditions of the aquatic community over time.

Periphyton sampling is typically conducted as a visual assessment or by removing algae from the stream substrate for a known area (Stevenson and Bahls, 1999; OCC, 2014). Visual inspection typically includes percent coverage, color and algae type and appearance. This is a very inexpensive and rapid method to assess the algal community, but these metrics are subjective and are affected by conditions at the sampling time. In comparison, the physical removal of algae from a substrate is time consuming, requires multiple steps, and can be expensive. After collection, algae

samples are analyzed by extracting chlorophyll (Stevenson and Bahls, 1999; OCC, 2014) and measuring the chlorophyll using a fluorimeter or spectrophotometer (Arar and Collins, 1997; Arar, 1997), and samples may be preserved for taxonomic analysis (Stevenson and Bahls, 1999).

There is a high potential for error in the traditional collection and analysis of periphyton, as it is difficult to know whether the entire sample has been removed and collected from the substrate. Removing the algae from the substrate disturbs the organism and can cause changes in pigment or damage the cells, and chlorophyll will degrade rapidly if not stored properly. The samples used for chlorophyll *a* extraction must be filtered within 12 hours, kept out of light, and frozen until the extraction procedure (Stevenson and Bahls, 1999). Pigments can be lost while filtering if the cells are ruptured and may not be completely removed from cells during extraction. When using a preservative, such as Lugol's Solution, it is difficult if not impossible to distinguish alga that was alive at the time of collection and those that were not. In depositional environments, there could be enough dead algal cells to skew the cell counts and biovolume estimates.

Resource constraints limit the sample number due to the extensive collection and analysis costs. The US EPA has progressed toward a more comprehensive approach to water quality assessment by monitoring long-term ecologic trends with sufficient statistical power for use in predicting future conditions (USEPA, 2003). The minimum number of samples to have reasonable predictive power may discourage monitoring agencies from using periphyton as part of their water quality assessments. Therefore, there is interest in developing a less subjective, inexpensive and rapid algal assessment method.

The BenthoTorch®, a commercial fluorimeter probe, was developed by BBE Moldaenke to measure periphyton *in vivo* and *in situ*. It has a range of 0-15 µg chlorophyll *a*/cm^2 and a resolution of 0.01 µg chlorophyll *a*/cm^2 (BBE, 2014). The probe uses seven LED's, which include 470, 525 and 610 nm to induce fluorescence in the periphyton, and 700 nm to adjust for reflection of the substrate (BBE, 2014). The measurements are displayed on the instrument and stored for later upload. Results are provided for total benthic chlorophyll, cyanobacteria, green algae, and diatoms in µg chlorophyll *a*/cm^2 or cells/cm^2. A mathematical model is applied internally to compensate for the self-shading effect of periphyton three-dimensional structure (BBE, 2014). Internal temperature and Global Positioning System (GPS) location are also available. The included software displays fluorescence units and graphical outputs in addition to the previously mentioned data. BBE recommends the instrument be sent back to Germany for calibration every one to two years (personal communication, Tim Doyle, BenthoTorch® technical representative, 2015). The BenthoTorch® is intended to replace the traditional method of periphyton analysis without removing algae from the stream substrate, filtering the sample, extracting chlorophyll and analyzing the extraction with a spectrophotometer or fluorometer. In addition, the BenthoTorch® may replace preserving the sample and performing a taxonomic analysis. This alternative method would potentially save days or months of work and provide results with an instrument that can measure and record data in about 20 seconds.

Kahlert and McKie (2014) used the BenthoTorch® to compare conventional methods of periphyton analysis in oligotrophic streams in northern Sweden. The median BenthoTorch® measurements, spectrophotometer measurements corrected and uncorrected for phaeophytin, and microscope biovolume estimates were statistically significant across 24 sampling sites at 0.52, 0.37, 0.53 and 0.40 µg chlorophyll *a*/cm^2,

respectively. Statistical significance in a test of medians indicated the BenthoTorch® was measuring relative changes when compared to extracted chlorophyll. However, they did not specify a clear one-to-one relationship for BenthoTorch® measurements compared extracted chlorophyll *a* or biovolume estimates.

Community composition estimated by the BenthoTorch® compared poorly to traditional methods (Harris and Graham, 2015). Harris and Graham (2015) used the BenthoTorch® in streams and reservoirs in Kansas, United States. Extracted chlorophyll *a* and the BenthoTorch® correlated significantly ($p = <0.01$); however there was lower variance when chlorophyll levels were less than 4.0 µg chlorophyll *a*/cm^2 without filamentous algae ($R^2 = 0.50$) compared to chlorophyll levels greater than 4.0 µg chlorophyll *a*/cm^2 with filamentous algae ($R^2=0.27$). BBE Moldaenke stated that the BenthoTorch® was inaccurate with filamentous algae (BBE, no date), possibly due to optical thickness and shading effects. The BenthoTorch® may not be able to measure chlorophyll *a* as well as the extraction process in environments with relatively higher benthic algae levels.

This study evaluated the BenthoTorch® across 42 Oklahoma streams under variable conditions during the summer of 2014. The goal was to compare BenthoTorch® periphyton measurements with the standard US EPA method using extracted chlorophyll and taxonomic analysis (Stevenson and Bahls, 1999). In addition, these data were used to identify potential factors contributing to differences between the BenthoTorch® and traditional methods.

Methods

Site Selection

Sites were selected from an Oklahoma Conservation Commission (OCC) database of previously sampled streams. The OCC database had sites ranked on environmental quality in quintiles across each Omernik Level III ecoregion. Quality rankings were determined using previous environmental monitoring as part of the Oklahoma Non-Point Source Assessment Program (NPSAP). Sampling sites were chosen with an even distribution from high to low quality across each of Oklahoma's ecoregions. Forty-two sites (Figure 3) were evaluated during summer 2014 using traditional methods, the BenthoTorch®, and rapid habitat assessments. OCC obtained landowner access permission for the sampling sites.

Periphyton Sampling

Site Analysis

Sample locations in the stream were selected by visual inspection of the color and texture of algae and substrate type. Samples were chosen to obtain a variety of algae and substrate types. The sampling locations required a consistent surface texture. Irregular surfaces prevented the BenthoTorch® from shielding external light and risked scratching the lens. Figure 4 shows an example of a course texture substrate that both touched the lens of the BenthoTorch® and leaked dislodged periphyton under the traditional sampler. Soft sediments were also a problem because of their tendency to adhere to the BenthoTorch® (Figure 5). Because the soft sediments adhered to the cavity of the BenthoTorch®, a complete sample could not be collected for extracted chlorophyll *a* and taxonomic assessment. Therefore, soft sediment substrates were excluded from the study.

The number of samples collected was based on the diversity of the site. Samples were collected from each appropriate substrate and algae type. Filamentous algae were included because they are ubiquitous in streams and rivers in the research area. A minimum of three and a maximum of eight benthic algae samples were measured with the BenthoTorch® and collected. The BenthoTorch® battery life limited the field tests to eight samples. Sampling location selection was based on depth to algae when measured in water, substrate roughness, and the ability to use the BenthoTorch® without disturbing the periphyton. If the sample was too deep, the traditional sampler was filled with stream water resulting in an inaccurate sample (Figure 6).

BenthoTorch® Method

Chlorophyll a

The BenthoTorch® has three measurement modes: single, interval and continuous. Single mode takes one measurement and stops; interval mode takes a reading and pauses for a length of time set by the user before taking another measurement, and continuous mode records measurements every 20 seconds until stopped by the user. BenthoTorch® measurements were taken for 15 minutes in continuous mode. A stand was constructed to hold the BenthoTorch® stationary for 15 minutes (Figures 5 and 6). Fifteen-minute BenthoTorch® chlorophyll a measurements were acquired and partitioned into an initial single measurement (BT1) and the average of the last five-minutes (BT5). The average of the last five-minutes allowed the algae a ten-minute dark adjustment period to reduce variability related to removal of light. Dark adjustment period was not specified by BBE (2012) but was added after identifying measurement variance using the BenthoTorch® continuous mode during preliminary

testing. The ten-minute dark adjustment period was recommended by a BBE technical representative (Tim Doyle, personal communication).

Following each 15-minute measurement, the BenthoTorch® was cleaned to prevent contamination. Field notes and a review of digital images were used to label each sample as yes of no based on the presence of visible filamentous algae. The same procedure was used to assign a relative value (1 – low, 2- med, 3 – high), to each sample for visible sediments contained in the sample. Sediments may contain detritus or other non-alga organic material.

Taxonomy

The BenthoTorch® uses a combination of fluorescence from three spectral bands to derive the relative abundance of three divisions of algae, cyanobacteria, green algae and diatoms. Fluorometry relies on specific divisions of algae having a response at specific wavelengths (Aberle et al., 2006). The accuracy of this process depended on the relative amounts of pigments contained in the algae and the proportion of the signal it received from the fluorescing algae. This was done simultaneously with the chlorophyll *a* measurements and stored internally.

Traditional Sample Collection Method

Chlorophyll a

After each BenthoTorch® measurement, the algae under the BenthoTorch® was immediately collected using traditional methods based on Central Plains Center for Bioassessment (CPCB) (Bouchard and Anderson, 2001). A 3.8 cm diameter Schedule 40 polyvinyl chloride (PVC) pipe was used to outline the sampling area (Figure 7). A 1.27 cm thick AP Armaflex closed-cell ethylene propylene diene monomer (EPDM) foam insulation was attached to one end of the PVC pipe. The foam rubber was attached with

rubber cement, which was also used to coat the outside of the foam for additional water protection (Figure 7). Next, a cordless power drill with either a brass or plastic brush was used to dislodge the algae from the substrate, and a hand vacuum pump (Mityvac® MITMV8500) attached to a 125 ml high-density polyethylene Nalgene™ bottle was used to suction and store the sample (Figure 8). The sample area was rinsed and suctioned until the rinse water was clear. The sample collection bottle was then wrapped in foil to block all light and placed in a cooler with ice.

It is important to note the algal sample area was 13.1 cm^2, and the BenthoTorch® measures from 1.0 cm^2 in the center of the area. Care was taken to collect algae only in the 13.1 cm^2 area. However, since the BenthoTorch® only took measurements from approximately eight percent of the sampled area, additional variability was introduced.

Samples were processed in accordance with the US EPA Method 446 (Arar, 1997) within 24 hours of collection. The method was modified slightly to accommodate laboratory analysis of chlorophyll a and taxonomic analysis. The modified method, similar to Hill et al. (2000), added enough water to the sample in a graduated cylinder to rinse the sample bottle sufficiently. Next, the sample was homogenized by shaking the graduated cylinder vigorously for approximately ten seconds. Three subsamples were then collected and each filtered through an Advantec GA55-47 glass-fiber filter (0.6 μm porosity, 47-mm diameter), wrapped in aluminum foil, labeled, and frozen for at least twenty-four hours before being processed according to US EPA Method 446 guidelines for chlorophyll extraction and spectrophotometer analysis (Arar, 1997).

Taxonomy

The remainder of sample was preserved with Lugol's solution (Stevenson and Bahls, 1999) and kept in the dark until taxonomy analysis was performed by Dr. Beth

Bowles at Missouri State University in Joplin, Missouri. Biovolume estimates were conducted using the method by Hillebrand et al. (1999). Subsamples were settled in a 10 ml Utermöhl chamber and counted under a microscope at 400x magnification. Large algae were counted in half the chamber and small algae were counted along a single transect. Ten to 15 natural algae units were measured with a high variability in size. Natural units were recorded for filamentous (10 mm and under equals 1 unit), unicellular and colonies for cyanobacteria, green algae, and diatoms. Algal units smaller than 10 µm were not identified due to high error rates associated with the 400x magnification.

Quality Control

BenthoTorch®

BenthoTorch® data were uploaded and assessed for quality and completeness at the end of each sampling event. Data, including total concentration (*µg* chlorophyll *a/cm²*, cells/cm²), cyanobacteria (*µg* chlorophyll *a/cm²*, cells/cm²), green algae (*µg* chlorophyll *a/cm²*, cells/cm²), diatoms (*µg* chlorophyll *a/cm²*, cells/cm²), reflectance (unit less), and internal temperature (°C) were transferred to Microsoft Excel for analysis.

Extracted Chlorophyll a

Absorbance units measured by the spectrophotometer were transferred to Microsoft Excel and processed according to the Montana Department of Environmental Quality's Sample Collection and Laboratory Analysis procedures (MDEQ, 2008). MDEQ (2008) modified the equation from Arar (1997) to accommodate benthic samples gathered from a specific area. The original Arar (1997) equation was developed for planktonic samples gathered with specific volumes.

Supplemental Data Collection

Rapid Habitat Assessments (RHAs) (OCC, 2014; OWRB, 2006) were conducted at each site to document stream conditions. In addition, a digital camera was used to photo document the site conditions, which included the algae measurement locations before BenthoTorch® measurements and after periphyton collection, up and down steam, stream banks, and other areas of interest, such as cattle tracks or trash.

Statistical Analysis

Statistical analysis was performed using Minitab 17. Data were transformed with natural log only for taxonomic comparison to achieve normal distribution of residuals on ANOVAs. Data were not transformed for chlorophyll *a* comparison with BenthoTorch® as the residuals were normal distributed. Linear regressions were used for comparing methods of periphyton estimation and not as a predictor. BenthoTorch® was used as the response variable since it was the new method being compared to an accepted standard. Tests of ANOVA with Tukey's pairwise comparison were used to determine how periphyton estimation methods differed as well as how other factors effected the difference in methods. The difference between extracted chlorophyll *a* and BT5 and BT1 were used as the response variable and filamentous (y/n) and sediment (1, 2, and 3) were used as the treatment factor.

Results and Discussion

The purpose of the study was to compare BenthoTorch® measurements with the traditional method of removing benthic periphyton to extract chlorophyll *a* and/or conduct taxonomic algal community composition. The BenthoTorch® measurements were in the same units as the traditional method, i.e. ug/l, although the different methods may not

have equivalent results. To compare these methods, potential sources of variance were first identified and quantified.

BenthoTorch® Single and Last Five-minute Average Comparison

The BT1 and BT5 measurements were significantly different, with means of 3.3 and 4.1 µg chlorophyll *a*/cm^2, respectively (paired t-test, p=<0.01, n=106). These data were not normally distributed, and thus a Mann-Whitney nonparametric test was also conducted revealing the medians were not significantly different (p=0.62). On average, BT5 measured more chlorophyll *a* compared to BT1; however, when comparing the entire dataset of BT1 to BT5, there was not a significant difference. In practice, the user should consider how the length of measurement may affect the estimates of benthic chlorophyll *a* and adjust the sampling design accordingly. Linear regression between the BT1 and BT5 produced a 0.62 slope and an R^2 of 0.85 with a p-value<0.001 (Figure 9).

Samples with thick filamentous mats with high-density algal communities may have caused a variety of complications for *in vivo* measurements. Shading from the algae structure may have inhibited the BenthoTorch® fluorescing light signal reaching algae and physically blocked or scattered the light, preventing it from returning to the instrument. Observed sediment and detritus in the algae also attenuated the signal resulting in lower measurements. The BenthoTorch® five-minute average chlorophyll *a* measurements (BT5) may have increased over time from algae moving to allow for more light to return to the BenthoTorch®, or diatoms buried in sediments and detritus may have migrated to the surface (Consalvey et al., 2005; Perkins et al., 2002).

Laboratory Extracted Chlorophyll *a*

A one-way ANOVA with Tukey's pairwise comparison was performed on the laboratory-extracted Chlorophyll a (LEC) with the BT5 and BT1 BenthoTorch® measurements. The mean LEC of 12.7 µg chlorophyll *a*/cm^2 was significantly different from the mean BT5 and BT1 measurements of 4.2 and 3.3 µg chlorophyll *a*/cm^2, respectively ($p<0.01$, $n=106$). There was a large and significant difference between BenthoTorch® estimates and LEC at the $\alpha=0.05$ level.

A paired t-test between BT5 and BT1 showed BT5 was significantly higher compared to the BT1 ($\alpha=0.05$). Next, BenthoTorch® readings were subtracted from LEC (LEC - BT5 and LEC – BT1) to determine the variance between BT5 and BT1, and LEC. BT-LEC was used as response variable and measurement method (BT5 and BT1) was used as the treatment variable in a one-way ANOVA with a Tukey's pairwise comparison. There was not a significant difference between BT5 and BT1 at the $\alpha=0.05$ level (Table 1). Therefore, the difference between BT5 and BT1 was small compared to the difference between the LEC and the BT.

Simple linear regressions were performed using BT1 and BT5 as dependent variables and LEC as the independent variable. Results were significant at the $p<0.01$ level (Figure 10). Both BT5 and BT1 had similar intercepts and R^2, but the BT5 slopes were 200 times larger than BT1. The linear regressions were repeated with the intercept removed. The regressions were significant at $p<0.01$ and again had similar R^2. BT5 still had the higher slope compared to BT1, but only by 40 percent. The regression analysis was consistent with ANOVA's and paired t-tests. The BenthoTorch® consistently underestimated the laboratory-extracted chlorophyll *a* (LEC).

Filamentous versus Non-filamentous

Filamentous algae presented a problem for *in situ* measurements because there may be other algae growing on the filaments, the filaments may shift or move during the measurement, and the three-dimensional structure may create shading affecting the activating light penetration and strength of the fluorescence returning signal. Filamentous algae also pose a problem for obtaining homogenous sub-samples to filter for extracted chlorophyll analysis. Without a blender or cell disruptor, the filaments may clump together and/or stick to the pipette, making it difficult to obtain a representative sub-sample.

Field notes and images of the samples were reviewed and divided into groups that contained "visible" (no magnification) filamentous algae and those that did not. A one-way ANOVA with Tukey's pairwise comparison was performed to determine if there was a statically significant difference between the BenthoTorch® and LEC means when taking into account the presence of filamentous algae. The LEC-BT was the response variable and method (BT5 and BT1) was the treatment variable. There was a significant difference between the BenthoTorch® with and without visible filamentous algae at the α=0.05 level (Tables 2 and 3).

A linear regression was performed for using the BenthoTorch® as the dependent variable and LEC as the independent variable. The regressions were significant ($p < 0.01$), with the exception of filamentous BT1 with an intercept ($p = 0.06$). BT5 measurements had larger RMSE compared to BT1, which continued the trend of higher variance in the BT5 measurements. The slopes and intercepts were similar whether the intercept was included or not (Figures 11 and 12). Separating of filamentous from non-filamentous algae data reduced the influence of outliers in the regressions. While the

five-minute average and single measurements were similar, the presence of filamentous algae reduced the BenthoTorch® measurements.

Influence of Sediment on Comparison

The hypothesis that a greater amount of sediment in the samples would reduce the BenthoTorch® measurements was tested. BT5 and BT1 were each subtracted from LEC and used as the response variable, and method was used as the treatment in a one-way ANOVA with a Tukey's Pairwise comparison. Sediment levels were assigned high, medium, and low based on visual inspection in the field as well as through digital images to corroborate data after the fieldwork. The ANOVA was significant ($p<0.001$), and the Tukey's Pairwise comparison (significant at 95%) indicated that sediment had a significant effect (Table 4). There was an increasing difference between the LEC and BenthoTorch® readings. Low and Medium mean chlorophyll *a* were similar while medium and high means were similar. While sediments were contributing significantly to the discrepancy in extracted chlorophyll *a* comparisons, it was a smaller effect compared to the presence of filamentous algae.

Chlorophyll a Data ≤15 µg/cm^2

BenthoTorch® Comparison

The BenthoTorch® range was specified as 0-15 µg chlorophyll *a*/cm^2, and thus for the next analysis all LEC data greater than the 15 µg chlorophyll *a*/cm^2 were excluded. A one-way ANOVA with a Tukey's Pairwise Comparison was then conducted to determine if the mean LEC, BT5 and BT1 data were significantly different. At an $\alpha=0.05$, the comparisons were similar to the full data set with a mean LEC of 6.6 µg chlorophyll *a*/cm^2 being significantly higher than BT5 and BT1 means (3.2 and 2.7 µg

chlorophyll *a*/cm^2, respectively). Note that the means BT5 and BT1 were not statistically different (α=0.05).

Linear regression for BT1 and BT5 revealed that including the intercept in the model made little difference (Figure 13) and BT1 and BT5 were under estimating benthic chlorophyll *a* relative to LEC. Using only measurements ≤15 µg chlorophyll *a*/cm^2 made no difference on the trend for RMSE as BT5 was still larger than BT1 whether the intercept was included or not (4.9 and 3.3 µg chlorophyll *a*/cm^2, respectively).

Influence of Visible Filamentous Algae

A one-way ANOVA was conducted to examine the effect of visible filamentous algae 15 µg ≤ chlorophyll a/cm^2. As with the full dataset, data were separated into groups where filamentous algae were visible and not visible without magnification. Chlorophyll *a* was used as the response variable and the method (LEC, BT5 and BT1) was used as the treatment. The same trend is present with or without visible filamentous algae; BT5 and BT1 estimate lower amounts of chlorophyll *a* than LEC (Table 5)

BT5 and BT1 were again subtracted from LEC to analyze the effect visible filamentous algae had on the difference between the estimation methods. Tables 6 and 7 present the results for the full dataset, with the mean difference of samples containing filamentous algae being around double that of non-filamentous algae for BT5 and BT1.

Linear regressions for BT1 and BT5 were still similar to each other and followed the trends in the full data set. BT5 still has a higher RMSE compared to BT1, and visible filamentous algae exacerbated this difference (Figures 14 and 15). Filamentous algae resulted in higher p-values when the intercept was included (p = 0.10 and 0.13 for BT5

and BT1, respectively). Slopes for the BT5 regressions remained similar with or without the presence of filamentous algae (Figure 14). However, with BT1 the slopes were lower with filamentous algae present (Figure 15).

Laboratory Estimated Biovolume Comparisons

BenthoTorch®

The comparison between the BenthoTorch® measurements, i.e. total cells/cm^2, and laboratory estimated biovolume (LBV) were significantly different at an α=0.05 level. These results were similar to Harris and Graham (2015) and Kahlert and McKie (2014). A one-way ANOVA was also performed on natural log transformed data, which also resulted in a significant difference between the LBV counts and BenthoTorch® data at an α=0.05 (Table 6).

A large and statistically significant difference was observed between ln(LBV), and the ln(BT1) and ln(BT5), which was similar to the results with extracted chlorophyll *a*. Blocking of light from the algae structure reduced the BenthoTorch measurements. Samples were dominated by diatoms with surirelloid morphology, which prefer low flow and depositional habitats encountered at the sampling sites. It was possible that the depositional habitats, which included detritus and sediment, affected our comparisons.

Community Composition

Cyanobacteria, green algae, and diatom measurements were divided by total LBV, BT5 and BT1 to obtain a percent of total algal composition for each method. Mean composition percentages were used as the response variable while the algal type and method were used as the treatment in a two-way ANOVA with Tukey's Pairwise comparison (Table 7). LBV estimates have the highest composition as diatoms at 55%, where BT5 and BT1 estimated cyanobacteria as the highest (54% and 52%,

respectively). BT5 and BT1 had the diatoms in the second grouping which matched LBV estimated for cyanobacteria. All three methods were statistically similar in terms of estimates for green algae.

Filamentous Algae Effects

BenthoTorch® data were lower with the presence of filamentous algae in the samples. To examine how this affected estimates of community composition, samples were separated into groups, one with visible filamentous algae and a second without. A one-way ANOVA was performed, which resulted in significant difference between the groups at an α=0.05 (Table 8). BT5 and BT1 data were similar whether filamentous algae were visible or not; however neither BT5 nor BT1 estimated community composition similarly to LBV (Tukey's multiple comparison 95%). For the non-filamentous samples, BT5 and BT1 estimated larger cyanobacteria compared to LBV (62, 61 and 44 %, respectively), while diatoms were highest for LBV with filamentous algae. Note that BT5 and BT1 were grouped separately from LBV for diatoms in the filamentous group. Even when separating visible filamentous algae, the BenthoTorch® overestimated cyanobacteria relative to the cell counts.

BenthoTorch® with LEC ≤ 15 µg chlorophyll a/cm^2

Since the BenthoTorch® range was 0-15 µg chlorophyll a/cm^2, taxonomy was also compared only in this range. Data were sorted, and measurements for LBC above 15 µg chlorophyll a/cm^2 were removed. Natural log transformed µg chlorophyll a/cm^2 was used as response variable, and method (BT5, BT1 and LBV) was used as the treatment. Results were unchanged as ln(LBV) was still significantly larger than ln(BT5) and ln(BT1) (Table 9).

Community composition was also similar to results for the all data analysis. A one-way ANOVA using the percentage of algal groups for BT5, BT1 and LBV was performed as described above. Again, BT5 and BT1 estimated percent cyanobacteria to be statistically similar to percent diatoms for LBV (Table 10). BT5 and BT1 percent diatom data were similar to LBV percent cyanobacteria while all three methods were similar for percent green algae (Table 10).

The BenthoTorch® data were different from the biovolume estimates for a number of reasons. Biovolume estimates use cell counts and then apply equations to approximate volumes of algae "natural units". Natural units may count a certain length of filaments as one, even when it contains several individual cells. The same is used for colonial algae; many cells can be counted as one. There are also error sources in the sampling, subsampling, transect counts as well as the assumed volumes for an observed alga. In addition, it is possible that the transect or random sub-samples in the Utermöl chamber were not representative.

Variance may be partially explained with the size discrepancy in the 13.1 cm^2 sampler versus the BenthoTorch® 1.0 cm^2measurement area. The BenthoTorch® measured approximately 7.6 percent of the sampler area used for cell counts, and periphyton exhibits a wide spatial diversity at very small scales (Morin and Catteneo, 1992; Stevenson et al., 1996). Another compounding error may be temperature; increasing temperature is known to decrease the fluorescence signal (Lorenzen, 1966).

Much of the variance can be attributed to using pigment as an indicator of the volume of a cell. This is entirely dependent on environmental conditions, and two cells of identical physical size may contain drastically different amounts of pigment. Compounding this error is the fact that pigments fluctuate based on environmental

conditions. All algae use chlorophyll *a*; however, auxiliary pigments can be a significant portion of the total pigment. For this reason, the percent composition was also analyzed to determine whether the BenthoTorch® data were similar to the taxonomist in relative abundance of algal groups. A linear regression was performed on the natural log transformed LEC and LBV to examine the relationship between the two standard methods described in Stevenson and Bahls (1999). The ln(LEC) was used as the response variable and ln(LBV) was the dependent variable (Figure 16). There was a significant and positive correlation ($p<0.001$), but an $R^2=0.50$ indicates that there was still a large amount of scatter.

Study Limitations and Recommendations

The methodology limited the ability to compare the BenthoTorch® to extracted chlorophyll *a* and cell counts on all substrates. Soft sediments could not be accurately sampled and thus at some sites non-native substrates, such as concrete and riprap, were measured. Soft samples could be used in a comparison with traditional methods if the sample could be collected effectively. This would require some modification of the BenthoTorch® to shield algae from light without disturbing the sample. Note that irregular and very rough surfaces were also excluded.

The 15-minute continuous measurement provided reliable measurements when a fixed stand was used to prevent movement during data collection. In-stream sampling was also limited by the time to setup the sampling apparatus, which was considerable at some locations. Note that the additional data collection time for 15-minute BenthoTorch® measurements were still quicker compared to traditional methods. In addition, the battery life limited sampling to around two hours of measurement time without a recharge.

Further testing should be conducted to examine the effects of shading from dense algal mats. Testing the BenthoTorch® in a laboratory setting would minimize the variance introduced by temperature, substrate type and variable water quality. Removing the periphyton from the substrate and re-suspending would also reduce the spatial variability and homogenize the sample. The BenthoTorch® could then be compared *in situ* and *ex situ* on the homogenized solution and compared to extracted chlorophyll *a*.

Testing should be done to determine whether the area of the PVC sampler had a negative effect on the results. A comparison with the BenthoTorch® using samplers with a range of areas between 13.2 cm^2 and 1 cm^2 would identify how much affect the spatial variability may have had on the comparison.

Conclusions

BenthoTorch® chlorophyll *a* measurements were significantly different and positively correlated with extracted chlorophyll *a* for both BT1 and BT5. The mean BT1 chlorophyll *a* data were lower compared to BT5 chlorophyll *a* data; however, the medians were statistically similar. Therefore, if the mean chlorophyll *a* is your selected variable, given enough samples BT1 or should accurately detect relative changes in benthic chlorophyll *a*.

For the LEC vs BenthoTorch® regressions, both BT5 and BT1 had slopes well below 1.0 and underestimate extracted chlorophyll *a* by a factor of four or six. The BenthoTorch® may be a useful tool for detecting relative chlorophyll *a* differences in some locations. However, the BenthoTorch® did not compare consistently with the standard methods recommended by the US EPA (Stevenson and Bahls, 1999). In

addition, it would be difficult to integrate new BenthoTorch® data into a dataset with historic data collected using the standard method.

Three-dimensional structure of periphyton was hypothesized to shade or block light from reaching the sensor, which could contribute to the BenthoTorch® under predicting chlorophyll compared to the laboratory-extracted method. Filamentous algae show a weaker correlation, but sediment in the samples did not have a consistent effect. In summary, it was not possible to identify and account for all of the variability with these methods.

BenthoTorch® estimates of relative abundance of algal phyla were statistically different. The magnitude of difference in biovolume estimates performed by a taxonomist varied by a factor of more than 10,000 compared to the BenthoTorch® measurements. Both BT5 and BT1 estimated cyanobacteria to be the highest percent while the taxonomist estimated diatoms the highest. This was the case whether filamentous algae were visible or not. The samples were predominantly diatoms according to cell counts. Therefore, the BenthoTorch® did not compare favorably to identification performed by a trained taxonomist.

While the BenthoTorch® may not have had a strong correlation to LEC or LBV, it should be noted that they are not strongly correlated with each other. When using any method of benthic algae estimation, it is crucial to understand what the measurements represent and how they may be used. If the BenthoTorch® were to be used in streams with low periphyton density and little to no filamentous algae, then perhaps it would be consistent enough to provide value over the standard chlorophyll *a* methods.

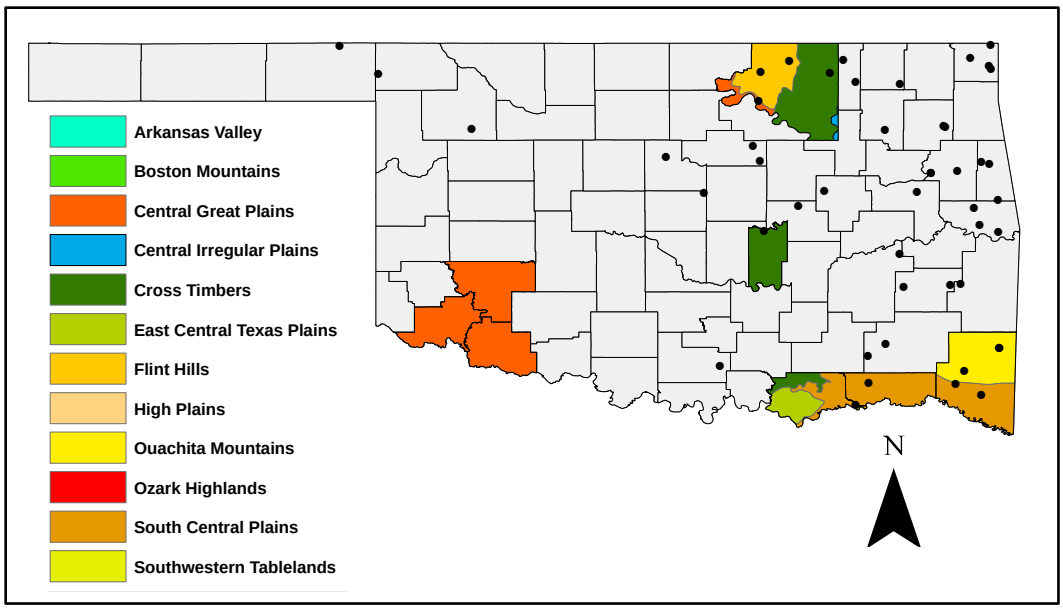

Figure 3. Oklahoma stream sampling sites with Omernik Level III ecoregions.

Figure 4. Example substrate too coarse for consistent Benthotorch® measurements and did not allow a good seal for a traditional algae sampler.

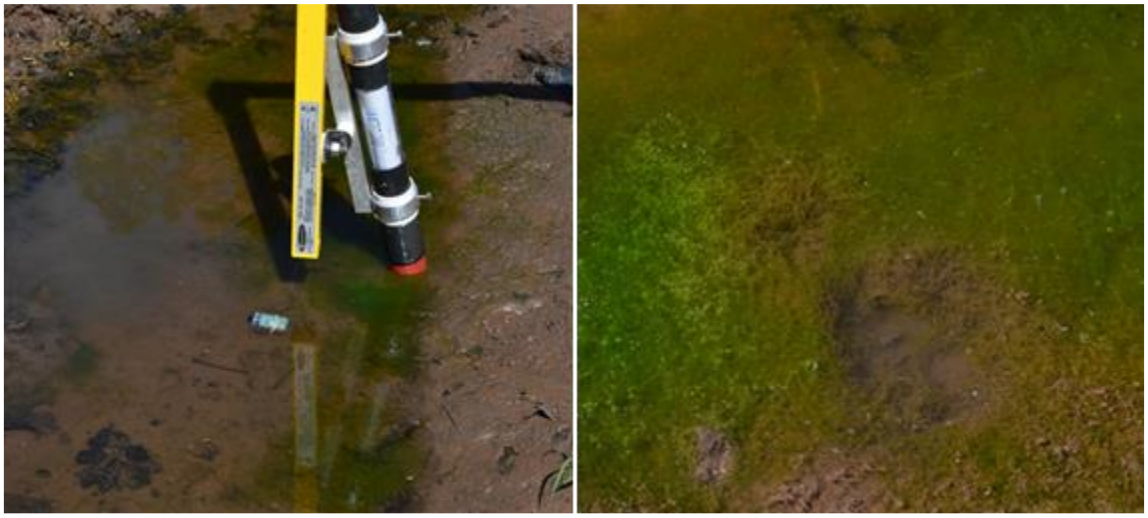

Figure 5. In situ Benthotorch® measurement on soft sediments (left) and the resulting impression (right).

Figure 6. Example of the Benthotorch® measuring chlorophyll a in situ with water too deep to collect an algae sample using standard methods.

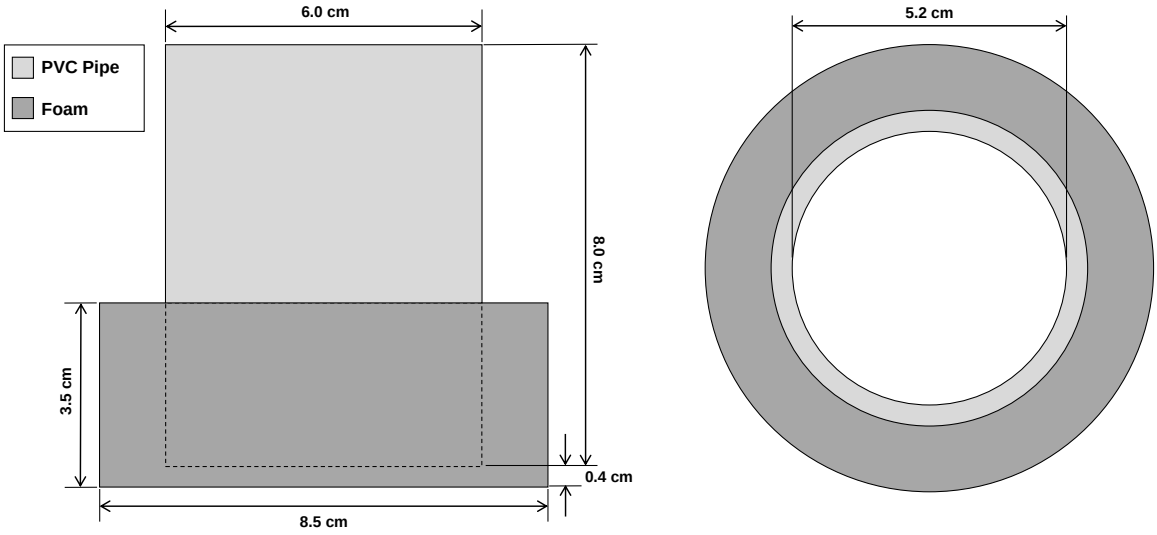

Figure 7. Schematic for the standard periphyton field sampler.

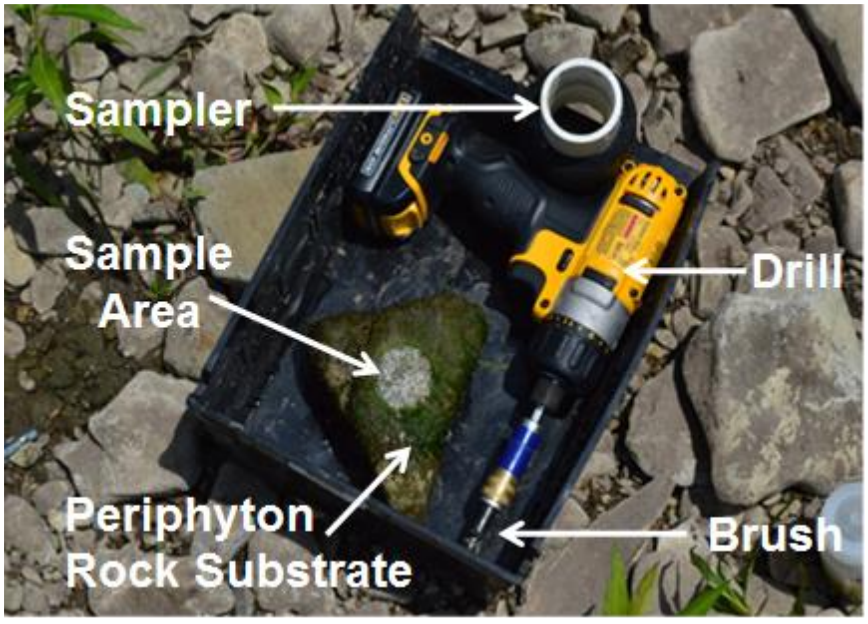

Figure 8. Periphyton field sampling equipment on a recently sampled stone: electric drill fitted with a stainless steel brush, polyvinyl chloride (PVC) sampler with ethylene propylene diene monomer (EPDM) foam collar. Algae were removed from the light colored

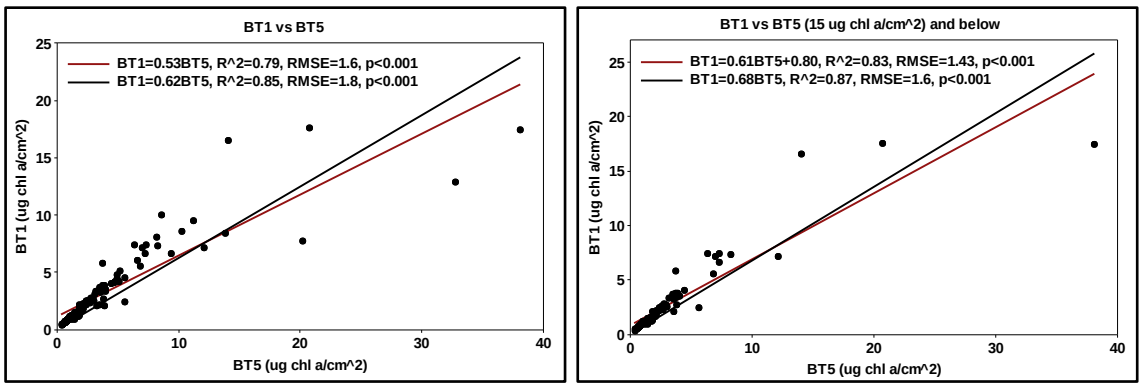

Figure 9. Single BenthoTorch® (BT1) data compared to last five-minute average data (BT5) with (left) and without an intercept (right) included in regression line.

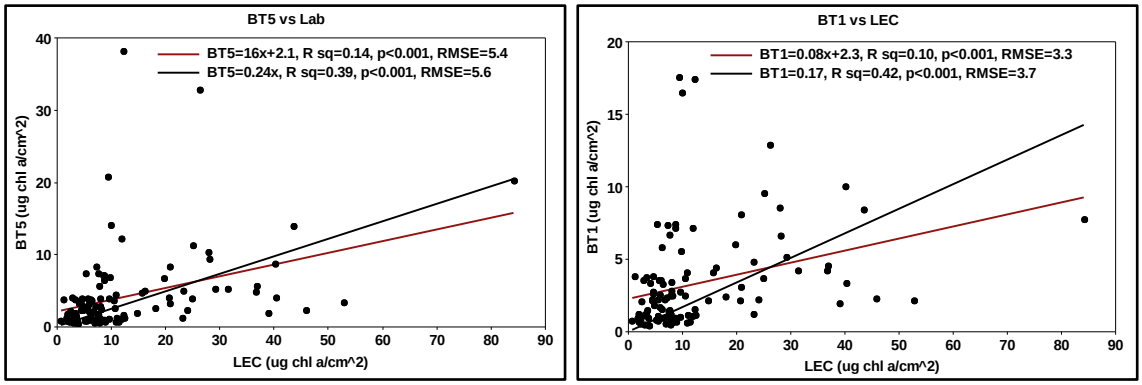

Figure 10. All data including regression lines and equations for intercept include and excluded, BenthoTorch® last five-minute average (BT5) data compared to lab extracted chlorophyll a (LEC) data (left), and single BenthoTorch® (BT1) data compared to lab extract

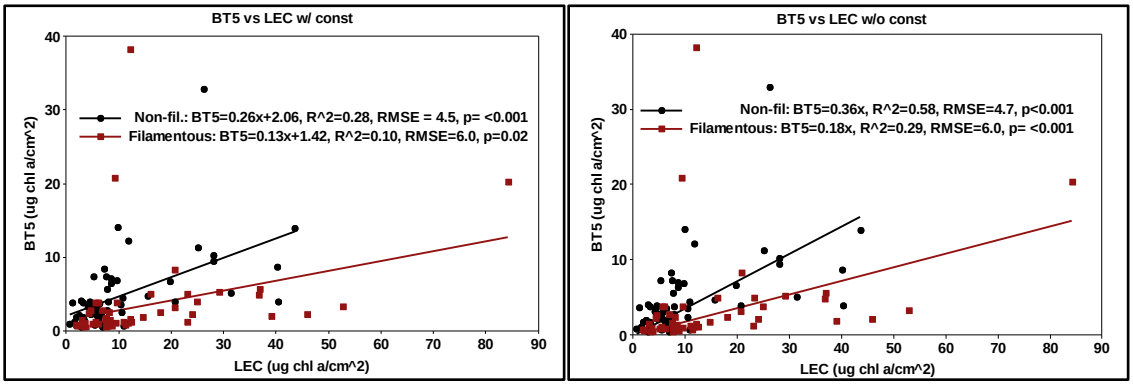

Figure 11. BenthoTorch® last five-minute average (BT5) data compared to laboratory extracted chlorophyll a (LEC) data regression lines for samples containing visible filamentous algae and no visible filamentous algae; intercept included (left), and excluded (right).

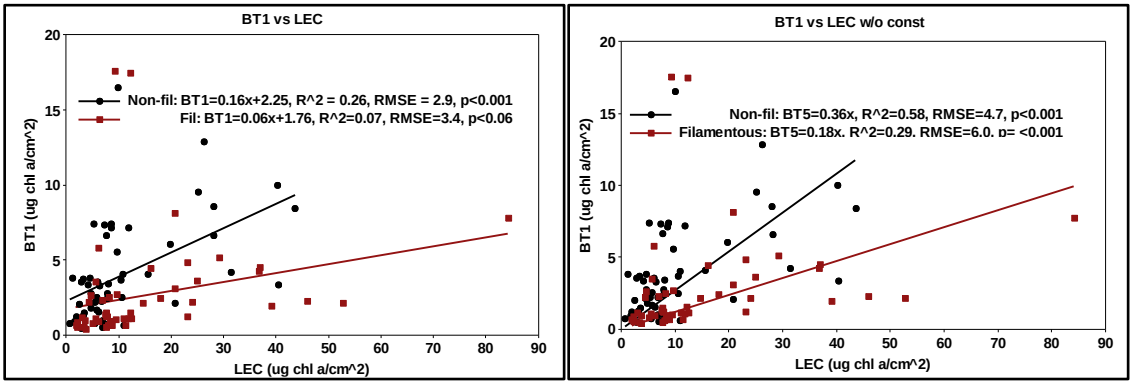

Figure 12. Single BenthoTorch® (BT1) data compared to laboratory extracted chlorophyll a (LEC) data regression lines for samples containing visible filamentous algae and no visible filamentous algae; intercept included (left), and excluded (right).

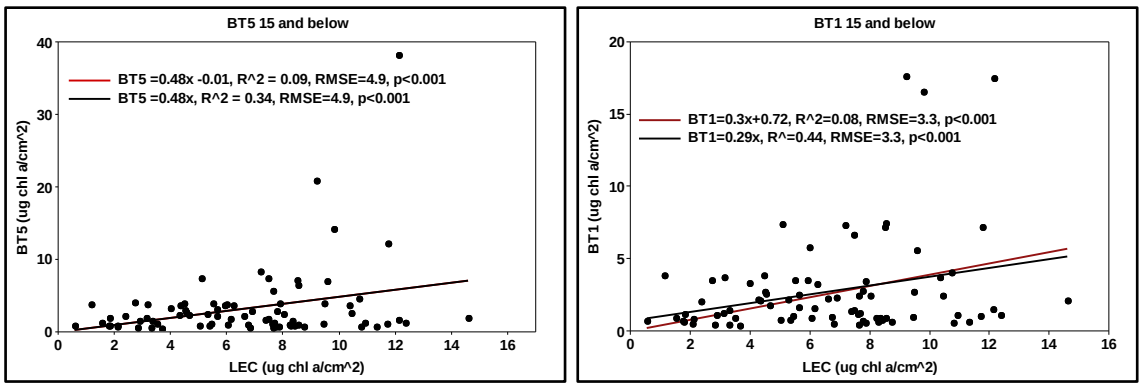

Figure 13. Data ≤15 µg chlorophyll a/cm2 including regression lines and equations for intercept include and excluded; BenthoTorch® last five-minute average (BT5) data vs laboratory extracted chlorophyll a (LEC) data (left), Single BenthoTorch® (BT1) data vs laboratory extracted chlorophyll *a* (LEC) data (right).

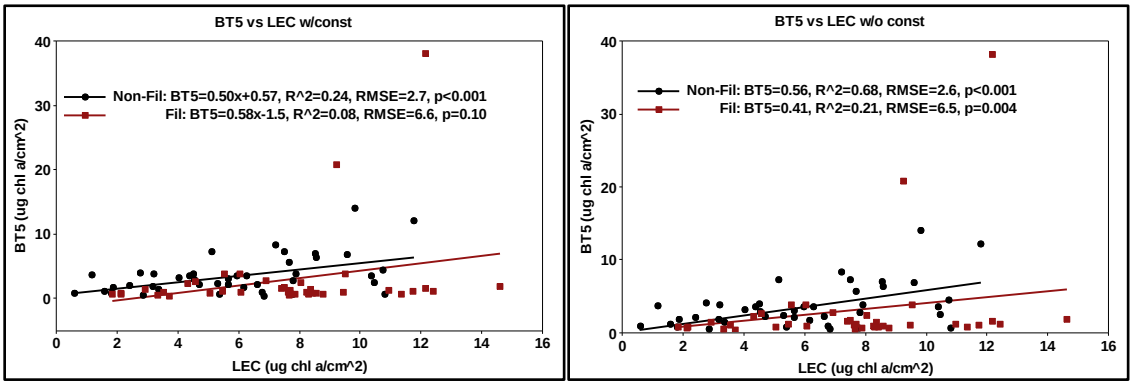

Figure 14. Data ≤15 µg chlorophyll a/cm2 with no visible filamentous algae, including regression lines and equations for intercept include and excluded, BenthoTorch® last five-minute average (BT5) data vs laboratory extracted chlorophyll a (LEC) data (left), single BenthoTorch® (BT1) data vs laboratory extracted chlorophyll a (LEC) data (right).

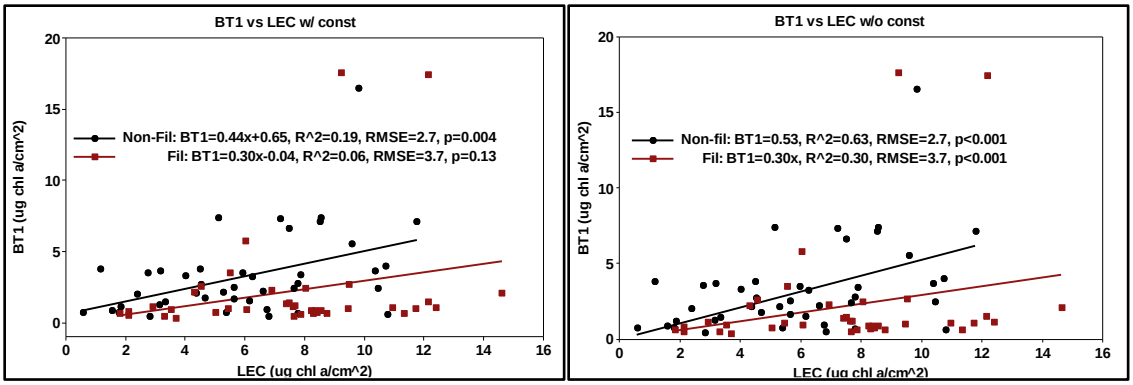

Figure 15. Data ≤15 µg chlorophyll *a*/cm^2 with visible filamentous algae, including regression lines and equations for intercept include and excluded, BenthoTorch® last five-minute average (BT5) data vs laboratory extracted chlorophyll a (LEC) data (left), single BenthoTorch® (BT1) data vs laboratory extracted chlorophyll *a* (LEC) data (right).

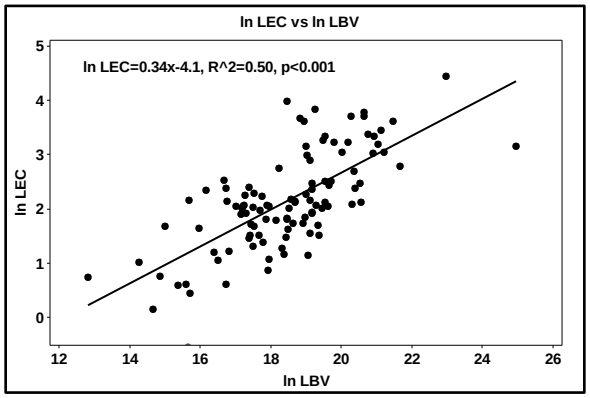

Figure 16. Linear regression line of natural log transformed laboratory extracted chlorophyll a (LEC) data vs laboratory estimated bio-volume (LBV) for data ≤15 µg chlorophyll *a*/cm^2 estimated by laboratory-extracted chlorophyll a (LEC).

Table 1. BenthoTorch® readings subtracted from laboratory extracted chlorophyll *a (LEC)* for both five-minute average (BT5) and single BenthoTorch® (BT1) compared using Tukey's Pairwise Comparison. *Means share a letter are not significantly different at α=0.05.

Treatment	Mean Chlorophyll *a* ($\mu g/cm^2$)
LEC–BT5	8.6^{a*}
LEC–BT1	9.4^{a*}

Table 2. Mean chlorophyll *a* for laboratory extracted chlorophyll *a* minus BenthoTorch® last five-minute average (LEC-BT5) compared using Tukey's Pairwise Comparison. *Means share a letter are not significantly different at α=0.05.

Treatment	Algae	Mean Chlorophyll *a* ($\mu g/cm^2$)
LEC–BT5	Non-filamentous	11^{a*}
	Filamentous	5.8^{b*}
LEC–BT1	Non-filamentous	12^{a*}
	Filamentous	6.7^{b*}

Table 3. Mean chlorophyll *a* compared by estimation methods using Tukey's Pairwise Comparison. Samples containing visible filamentous algae were analyzed separately from samples with no visible filamentous algae. *Means that share a letter are not significantly different at α = 0.05.

Method	Mean Chlorophyll *a* (µg/cm^2)	
	Non-filamentous	Filamentous
LEC	5.9^{a*}	7.4^{a*}
BT5	3.5^{b*}	2.8^{b*}
BT1	3.3^{b*}	2.2^{b*}

Table 4. Mean chlorophyll *a* from laboratory extracted chlorophyll *a minus* BenthoTorch® last five-minute average (LEC-BT5) and from laboratory-extracted chlorophyll *a minus* single BenthoTorch® (LEC-BT1) compared (separately) for effects relative amounts of sediment visible in samples using Tukey's Pairwise Comparison. *Means that share a letter are not significantly different at α=0.05.

Relative Sediment Level	Mean Chlorophyll *a* (µg/cm^2)	
	LEC-BT5	LEC-BT1
Low	1.2^{a*}	1.4^{a*}
Medium	2.7^{a,b*}	3.0^{a,b*}
High	4.9^{b,c*}	5.0^{b,c*}

Table 5. Mean chlorophyll a from laboratory extracted chlorophyll a minus BenthoTorch® last five-minute average (LEC-BT5) compared for effects of visible filamentous algae using Tukey's Pairwise Comparison. *Means share a letter are not significantly different at α=0.05.

Treatment	Algae	Mean Chlorophyll *a* (µg/cm^2)
LEC–BT5	Non-filamentous	4.6^{a*}
	Filamentous	2.4^{b*}
LEC–BT1	Non-filamentous	5.2^{a*}
	Filamentous	2.7^{b*}

Table 6. Comparison of natural log transformed mean chlorophyll a from laboratory-estimated bio-volume (LBV), single BenthoTorch® (BT1) and BenthoTorch® last five-minute average (BT5) compared in One Way ANOVA with Tukey's Pairwise Comparison. *Means share a letter are not significantly different at α=0.05.

Method	Mean Chlorophyll *a* (µg/cm^2)
ln(LBV)	18^{a*}
ln(BT5)	9.6^{b*}
ln(BT1)	9.5^{b*}

Table 7. Comparison of percent composition between periphyton estimation methods lab estimated bio-volume (LBV), single BenthoTorch® (BT1) and BenthoTorch® last five-minute average (BT5)) using Tukey's Pairwise Comparison. *Means that share a letter are not significantly different at α = 0.05.

Algae	Method	Mean Composition (%)
Cyanobacteria	BT5	54[a*]
	BT1	52[a*]
Diatom	LBV	55[a*]
	BT5	38[b*]
	BT1	35[b*]
Cyanobacteria	LBV	31[b*]
Green	LBV	17[c*]
	BT1	9.6[c*]
	BT5	8.2[c*]

Table 8. Comparison of percent composition between estimation methods (lab estimated bio-volume (LBV), single BenthoTorch® (BT1) and BenthoTorch® last five-minute average (BT5)) using Tukey's Pairwise Comparison. Samples containing visible filamentous algae w were analyzed separately from samples with no visible filamentous algae. *Means that share a letter are not significantly different at α = 0.05.

Algae	Method	Mean Composition (%)	
		Non-filamentous Algae	Filamentous Algae
Cyanobacteria	BT5	65^{a*}	$48^{b,c*}$
	BT1	62^{a*}	49^{b*}
	LBV	44^{b*}	19^{e*}
Diatom	BT5	39^{b*}	$37^{c,d*}$
	BT1	36^{b*}	34^{d*}
	LBV	40^{b*}	63^{a*}
Green	BT5	16^{c*}	14^{e*}
	BT1	2.0^{d*}	17^{e*}
	LBV	1.0^{d*}	18^{e*}

Table 9. Comparison of natural log transformed mean chlorophyll a between different estimation methods (lab extracted chlorophyll a (LEC), single BenthoTorch® (BT1) and BenthoTorch® last five minute average (BT5)) using Tukey's Pairwise Comparison for data ≤ 15 (µg chlorophyll *a*/cm^2) (estimated by lab extracted chlorophyll (LEC). *Means share a letter are not significantly different at an α=0.05.

Method	Mean Chlorophyll *a* (µg *a*/cm^2)
ln(LEC)	18^{a*}
ln(BT5)	9.4^{b*}
ln(BT1)	9.3^{b*}

Table 10. Comparison of percent composition for data ≤15 µg chlorophyll a/cm^2 estimated by Lab extracted chlorophyll a (LEC), between measurement methods (lab estimated chlorophyll a (LEC), single BenthoTorch® (BT1) and BenthoTorch® last five minute average (BT5)) using Tukey's Pairwise Comparison. *Means that share a letter are not significantly different at α = 0.05.

Method	Mean Composition Non-filamentous (%)	Method	Mean Composition Filamentous (%)
BT1 Cyanobacteria	62^{a*}	LBV Diatom	63^{a*}
BT5 Cyanobacteria	62^{a*}	BT1 Cyanobacteria	49^{b*}
LBV Cyanobacteria	44^{b*}	BT5 Cyanobacteria	48^{b,c*}
LBV Diatom	40^{b*}	BT5 Diatom	37^{c,d*}
BT5 Diatom	39^{b*}	BT1 Diatom	34^{d*}
BT1 Diatom	36^{b*}	LBV Cyanobacteria	18^{e*}
LBV Green	16^{c*}	LBV Green	17^{e*}
BT1 Green	2.4^{d*}	BT1 Green	17^{e*}
BT5 Green	1.2^{d*}	BT5 Green	15^{e*}

CHAPTER IV

EVALUATING BENTHOTORCH® *IN VIVO* FLUOROMETER PROBE ACCURACY FOR WATER QUALITY ASSESSMENT

Introduction

The US Environmental Protection Agency (EPA) recommended rapid bio-assessment as an efficient method to monitor long-term water quality trends in surface waters (USEPA, 1988). Physiochemical measurements assess the conditions at the time of monitoring, in contrast to bio-assessments that integrate a group or groups of organisms to assess the long-term conditions of the stream. Periphyton and filamentous macro algae are often included in bio-assessments because they are the base of the food web and serve as the biologic intermediary between substrates and the biologic community (Stevenson and Bahls, 1999). Periphyton takes time to accumulate in a stream system and responds quickly to environmental changes, making it valuable for assessing stream health. Periphyton samples are typically used to measure benthic chlorophyll *a*, which the USEPA (2000) recommended as an indirect measure of anthropogenic eutrophication. Dodds et al. (1998) suggested benthic chlorophyll *a* classify trophic levels. Although there is no universally accepted predictive relationship between benthic chlorophyll *a* and nutrients in streams, several studies have linked periphyton growth to high nutrient levels (Bourassa and Cattaneo, 1998; Clark et al., 2000; Dodds, 2003;

Dodds et al., 2002; Dodds et al., 1997; Dodds and Welch, 2000; Ice and Binkley, 2003). These attributes make benthic chlorophyll *a* an important component for assessing and monitoring aquatic ecosystems.

Periphyton is typically quantified using percent cover and/or type, sampled and measured as ash free dry mass, as extracted chlorophyll *a* and analyzed for community composition (Stevenson and Bahls 1999; OCC, 2014). Each of these methods has merits and shortcomings related to sample cost, time to obtain results, and accuracy. Percent cover is the least expensive and quickest method, and, when performed correctly, is precise but not quantitative. Ash-free dry mass represents the amount of carbon present in the benthos, but it does not distinguish between carbon from periphyton and detritus. However, chlorophyll *a* is more specific to periphyton compared ash free dry mass. Finally, community analyses require taxonomic expertise that can be time consuming and expensive.

Sampling periphyton introduces potential errors into the bio-assessment analysis since only a small sample is collected from a delineated area (Stevenson and Bahl 1999). There are different methods to delineate the area and collect the sample (Austin et al., 1981; Aloi, 1990; Bouchard and Anderson, 2001). Substrate can dictate the type and area of the sampler, and rough surfaces may reduce the sample amount collected (Aloi, 1990). In addition, periphyton has a high spatial diversity of biomass and community composition, which increases sample variability (Stevenson et al., 1996; Stevenson, 1997).

BenthoTorch® *In-Situ* Fluorometer for Periphyton Sampling

The BenthoTorch® is a Pulse Amplitude Modulated (PAM) fluorimeter probe that is specific to benthic algae (BBE, 2014). It is more specific to periphyton, i.e. micro-benthic algae, and does not measure filamentous algae adequately. An unpublished document (personal communications, Tim Doyle, Product Specialist, PP Systems and BBE Moldaenke Representative, 2016) titled "*Important notes for good BenthoTorch measurements: Procedure and comparison with standard laboratory methods*" indicated that filamentous algae, moss and lichens were not correctly measured. The BenthoTorch® measures *in situ* and *in vivo* by placing the instrument firmly against a substrate. The BenthoTorch® measurement uses seven LED's at three spectral bands at 470, 525 and 610 nm to induce fluorescence in the algae and one band at 700 nm to mitigate the effects of background reflection from the substrate (BBE, 2014). These data are recorded and saved internally in the BenthoTorch® to allow downloading to archiving data after sampling. The BenthoTorch® reports total algae, cyanobacteria, green algae and diatoms in units of either µg chlorophyll *a*/cm^2 or cells/cm^2. A single measurement takes approximately 20 seconds. The published range of the instrument is 0-15 µg/cm^2 with a 0.1 µg chlorophyll *a*/cm^2 resolution (BBE, 2014). Note that *in vivo* fluorescence and chlorophyll *a* extractions are fundamentally different; however, a strong correlation should exist, and they report the same units of benthic chlorophyll *a*. The correlation between the BenthoTorch® and chlorophyll *a* extractions is relatively high for planktonic samples, but periphyton presents many sampling challenges as noted above.

BenthoTorch® in Research and Monitoring

The BenthoTorch® has been used in monitoring and research around the world. For example, the Ontario Ministry of Environment used it to monitor the growth and volume of nuisance algae (Healthy Lake Huron, 2014). D. H. Environmental Consulting discussed BenthoTorch® use for water quality monitoring and research in South Africa (D. H. Environmental Consulting, 2014). It was also mentioned as a new method for measuring benthic algae by Furey and Liess (2015) and Hauer and Lambertii (2017). The BenthoTorch® was used to measure benthic algal biomass changes in a study looking at the effects of climate change on benthic primary producers and consumers (Fagernäs, 2014) and to measure benthic chlorophyll *a* in response to environmental variables (Anderson, 2014). Frainer (2013) used the BenthoTorch® to measure benthic algae by class in a study of ecosystem functions related to agriculture and habitat complexity. Snell et al. (2014), Piano et al. (2015) and Mrowicki et al. (2016) used the BenthoTorch® to measure benthic chlorophyll *a* for their respective studies, while Rishworth et al. (2016) used the BenthoTorch® to measure the percent composition of algal classes. Kahlert and McKie (2014), Harris and Graham (2015) and Echenique-Subiabre et al. (2016) compared the BenthoTorch® with traditional methods of periphyton analysis.

Published Comparison with Traditional Methods

A number of studies compared traditional methods of benthic chlorophyll *a* analysis to the BenthoTorch® and none have found a strong correlation with extracted benthic chlorophyll *a*. Kahlert and McKie (2014) used non-parametric tests to compare

the BenthoTorch® to traditional analysis methods for benthic chlorophyll *a* using data from oligotrophic streams in Sweden. Both methods were statistically similar for extracted benthic chlorophyll *a* but not for community composition. In streams and reservoirs in Kansas, USA, BenthoTorch® and extracted benthic chlorophyll *a* were statistically related, but higher algae concentrations and the presence of filamentous algae reduced the R^2 from 0.5 at <4.0 µg chlorophyll *a*/cm^2 to 0.27 at >4.0 µg chlorophyll *a*/cm^2. In France and New Zealand, thicker benthic algal mats greater than 2mm had a weaker correlation than mats less than 2 mm thick between BenthoTorch® and traditional methods (R^2 of 0.27 and 0.58, respectively) (Echenique-Subiabre et al., 2016). The community composition estimated by the two methods were significantly correlated when cyanobacteria was less than 50 percent of the sample ($R^2 = 0.53$, $p < 0.001$). Samples with cyanobacteria making up more than 50 percent did not correlate significantly ($R^2 = 0.03$, $p = 0.21$).

BenthoTorch® Limitations

Filamentous algae can be a problem for *in situ* fluorescence biomass estimates because high density prevents light transmission beyond the upper layers of cells. This self-shading can also occur in high-density colonial algae, where algal cells may be blocked from receiving light from the BenthoTorch® and/or the BenthoTorch® does not receive the returning fluorescence signal. In a Neotropical stream, Moulton et al. (2009) used an Aquafluor 8000 hand-held fluorometer (Turner Designs, Sunnyvale, CA, USA) to sample periphyton to compare with traditional benthic chlorophyll methods. The Aquafluor 8000 measured *in vivo* chlorophyll *a*, but required the periphyton to be

removed from the substrate. Moulton et al. (2009) found a better relationship between extracted chlorophyll *a* and the fluorometer (R^2 = 0.81) compared to Harris and Graham (2015). Moulton et al. (2009) may have had similar conditions to those encountered in lower latitudes with warmer water, longer growing seasons and higher concentrations of benthic algae observed by Harris and Graham (2015).

Periphyton Monitoring

Natural versus Artificial Substrates

Chlorophyll *a* collected from artificial substrates, e.g. glass slides, and analyzed using a spectrophotometer, has been used in periphyton monitoring (OCC, 2014) and was discussed in Stevenson and Bahls (1999). It was not recommended to compare data from artificial substrates and natural substrates. Periphyton on natural and artificial substrates are similar in principal, but the algal composition tends to differ (Weitzel, 1978; Aloi, 1990; Cattaneo and Amireault, 1992). Artificial substrates have more significant relationships with dissolved nutrients compared to natural substrates, and the effect of natural habitat is not reflected (Cattaneo and Amireault, 1992). Natural substrates can provide different habitats that favor algae that may not attach to artificial substrates (Weitzel, 1978; Aloi, 1990) and may contribute nutrients not available in the water column (Aloi, 1990).

Qualitative assessments

Qualitative percent cover data are strongly related to established ranges of benthic chlorophyll a. Suplee et al. (2009) found public perception of benthic algae to

have a strong association with benthic chlorophyll. The public perception relating benthic algae to impaired water aligned with Dodds et al. (1998), who suggested a eutrophic threshold of 10µg chlorophyll *a*/cm^2. In the images used by Suplee et al. (2009), 40-50 percent cover correlated strongly with this level across all user groups. Suplee et al. (2009) and Dodds et al. (1998) suggested 10-15 µg/cm^2 would fall into the impaired designation under the Clean Water Act "fishable and swimmable, or tribal designated uses". The BenthoTorch® could be used to add a quantitative component to the qualitative assessments. More field studies would need to be conducted to determine whether the BenthoTorch® could be used to compare current data with historic percent cover data.

Monitoring Design

Instream Data Collection

It is not possible to control natural variability, so objectively validating the accuracy of measurements is impossible (USEPA, 1999). One collection method may not be adequate for all conditions or sampling programs. Professional judgement should be used in choosing an appropriate sampling method, while statistical analyses should be used to determine the probability that a site is accurately characterized. Stevenson and Bahls (1999) outlined appropriate strategies for capturing periphyton variability at a sampling site by using a multi-habitat or single habitat design. The multi-habitat design best captured the periphyton present in the stream but was unlikely to detect subtle changes related to water quality (Stevenson and Bahls, 1999). A single habitat sampling

design would better capture small differences in periphyton, but it may not characterize the entire reach (Stevenson and Bahls, 1999).

Montana Department of Environmental Quality Confidence Analysis

In phytoplankton, coefficient of variation increases with mean chlorophyll *a*, but not in benthic communities (Cattaneo and Prairie, 1995). Using the method from Cattaneo and Prairie (1995), Montana Department of Environmental Quality (MDEQ) analyzed 2200 individual benthic chlorophyll samples from 288 Montana stream sampling events across variable conditions, Strahler stream orders, and levels of human impacts. Standard deviation and CV were calculated for each sampling event resulting in a CV of 73% to represent the variance in replicates for MDEQ sampling events. The CV was then used to estimate sample size confidence levels for their sampling program. They found that using 11 replicates for each steam reach resulted in an 80% confident level for measuring benthic chlorophyll *a* within ± 30% of the true population means.

Objectives

A number of potential factors may explain the relatively poor published relationships between BenthoTorch® and traditional methods. For example, self-shading was hypothesized to interfere with *in situ* measurements by reducing fluorescence. Another potential source of variability was the much smaller measurement area of the BenthoTorch® compared to the traditional sample area collected for chlorophyll *a* extraction. There were four objectives defining the research. The first objective was to compare *in vivo* chlorophyll *a* measurements using the BenthoTorch® fluorometer with

traditional methods of chlorophyll *a* extraction in a controlled environment. The second objective was to develop an alternative BenthoTorch® methodology to reduce variance from self-shading and irregular substrate surfaces. The third objective was to quantify the variability resulting from the discrepancy between the BenthoTorch® and traditional sampling areas. Finally, objective four was to outline a statistically valid monitoring design protocol for using the BenthoTorch® in water quality assessments.

Methods

The purpose of the experiment was to test relationships between measurements from the Benthotorch® and concurrently collected samples for extracted chlorophyll a under relatively controlled conditions. The two methods were compared in laboratory microcosms and in a single stream with low algal biomass. The methods included using the BenthoTorch® on suspended periphyton samples and measured directly on the substrate. The BenthoTorch® was compared *in situ* to the BenthoTorch® used on the *ex situ/in vivo* sample that was collected for extracted chlorophyll a in solution using the black calibration disc included with the instrument. As such, the modified method was tested using the BenthoTorch® on resuspended samples using the factory calibration disk.

***In-situ* and *Ex-situ* Comparison**

Experimental Setup

Six, 38-liter glass microcosms (50.8 cm x 27.9 cm x 33.0 cm, L x W x H) were filled with Oklahoma State University tap water treated with 5.0 ml of Tetra AquaSafe®

conditioner. Nitrogen (KNO_3) and Phosphorus ($KHPO_4$) were added to each microcosm to obtain 100 µg/L of P and 1600 µg/L of N solution to promote algal growth. The microcosms were aerated using a stone attached to an air pump. An XtraSun® 1000W Sodium grow light was suspended 60 cm above the microcosms to provide light on a 14:10 light:dark cycle to mimic light conditions during the mid-latitude growing season. Seventy-two ceramic tiles, each measuring approximately 7.6 cm x 7.6 cm, were washed with phosphate free detergent and 12 tiles were placed in each microcosm.

Periphyton used to inoculate the microcosms were collected from a local concrete culvert located adjacent to the Oklahoma State University Veterinary Medicine Teaching Hospital in Stillwater, Oklahoma; the water source was primarily from rural/agricultural runoff. Algae were removed from the culvert by placing a 13.1 cm^2 sampler directly on the concrete. The sampler was an 8.0 cm section of 3.8 cm diameter Schedule 40 polyvinyl chloride (PVC) pipe with a foam collar to create a seal between the sampler and the concrete substrate. Next, a cordless drill with a plastic brush was used to dislodge the algae, which was collected into a 125 ml high-density polyethylene Nalgene® bottle using a hand vacuum pump (Mityvac® MITMV8500). Sampling locations were selected to minimize sediments, extraneous biomass, and filamentous algae. The algae samples were returned to the laboratory where they were homogenized by shaking vigorously for at least ten seconds, decanted into a graduated cylinder to remove heavier sediments or detritus, and then pipetted in equal amounts into the six microcosms.

Sampling Procedure

Sampling of the tiles started after about a month, which allowed the algae to establish a visible biomass layer, and continued over seven irregularly spaced sampling periods between October 31, 2015 and January 13, 2016. Algae in the tanks had time to grow in between sampling and over the course of the experiment. Because the tiles accumulated more benthic algae over time, the sampling design allowed measuring benthic algae with varying densities over the course of the experiment. Tiles were selected randomly, removed from the microcosms and placed in a modified sampler mounted to a board, then measured *in situ* with the BenthoTorch®. After the BenthoTorch® *in situ* measurement, the algae sample was suctioned into a 125 ml Nalgene bottle, rinsed with water until all algae in the sampler area was removed, and brought to a final volume of 100 ml. The sample was homogenized by shaking vigorously for at least ten seconds and then subsampled. A 2.5 ml sub-sample was placed into a black calibration plate and measured with the BenthoTorch® (Figure 17). Another 20 ml sub-sample was filtered using a 0.6 µm Advantec GA55-47 glass-fiber filter, wrapped in aluminum foil and placed in a freezer. After a period of at least 24 hours, the filters were processed for extracted chlorophyll *a* according to US EPA Method 446 (Arar, 1997). Tiles were not replaced in the microcosms after sampling.

Data were uploaded from the BenthoTorch® after each sampling period and archived. Absorbance units from the spectrophotometer were converted to µg/cm^2 using the Lorenzen (1966) phaeopigment-corrected equation modified by the Montana

Department of Environmental Quality (MDEQ, 2011) for periphyton. The correction factor to account for turbidity introduced by solvent and phaeophytin is given as:

$$\boldsymbol{A_{cf} = k\, A_{adj}} \quad \textbf{(2)}$$

$$\boldsymbol{k = \frac{\frac{A_{664_b}}{A_{665_a}}}{\frac{A_{664_b}}{A_{665_a}} - 1}} \quad \textbf{(3)}$$

$$\boldsymbol{A_{adj} = \frac{L\, C}{A_{664b}}} \quad \textbf{(4)}$$

where A_{cf} was an absorbance correction factor, A_{adj} was the adjusted absorbance, A was absorbance with the numeric subscripts representing a specific wavelength in nm with the alphabetical subscripts *a* and *b* representing after and before acidification, respectively, *L* was length of the light path through the cuvette taken as 1.0 cm, and *C* was the concentration of Acetone in mg/l. The following equation was used to adjust phaeophytin, the periphyton sample area and volume of solution:

$$Chl\ a = A_{cf}\left(\left(A_{664_b} - A_{750_b}\right) - \left(A_{665_a} - A_{750_a}\right)\right) * \frac{V_1}{A_1 L_{path}} \quad \textbf{(5)}$$

where *Chl a* was chlorophyll *a* in mg/m^2, V_1 was the volume of extract in liters, A_1 was the sample collection area in m^2, and L_{path} was the light path or width of cuvette in cm. Next, the samples were multiplied by 0.1 to convert from mg/m^2 to µg/cm^2. Finally, the extracted chlorophyll was adjusted for the ratio of total volume to the subsample volume using:

$$V_{cf} = \frac{V_1}{V_2} \quad \textbf{(6)}$$

where V_{cf} was the volume correction factor, V_1 was the total volume of solution in ml, and V_2 was the subsample volume in ml.

Subsample Calibration Plate Adjustment

The *ex situ* method for the BenthoTorch® used a diluted sample and sub-samples. These BenthoTorch® data were adjusted to account for the difference sample volume, subsample volume and the sample area on tile to the subsample on the calibration plate. The sample area and volume were 13.1 cm^2 and 100 ml, respectively. The sub-sample volume measured *ex situ* was 2.5 ml, and the area of the calibration disc was 6.35 cm^2. The total volume of the calibration plate was 2.42 ml. Since the BenthoTorch® measured 1.0 cm^2, the volume of the calibration plate measured by the BenthoTorch® was 0.46 ml. The area adjustment factor, AC, was calculated using:

$$AC = \frac{T_1}{T_2} \quad \textbf{(7)}$$

where T_1 was total area of calibration plate cavity and T_2 was the area of Benthotorch® measurement. The calibration plate had an upper and lower section (Figure 17), with the total volume calculated using the following equation:

$$V_t = V_1 + V_2 \quad \textbf{(8)}$$

where V_1 was the volume of upper section calibration plate, and V_2 was the volume of the lower section calibration plate. The volume adjustment factor, VC, was calculated using:

$$VC = \frac{V_T}{V_3} \quad \textbf{(9)}$$

where V_3 was the volume of Benthotorch® measurement, and V_T was the total volume of the calibration plate cavity. The plate area and volume correction factor, PC, was calculated using:

$$PC = \frac{AC+VC}{2} \quad \textbf{(10)}$$

where PC was the area and volume adjustment factor for measurements on the calibration plate. The Benthotorch® calibration process (BBE, 2014) recommended only using 0.1 ml of solution, but 2.5 ml was used because the 0.1 ml volume was not representative of the sample.

Sample Area Effect on BenthoTorch® and Traditional Periphyton Sampling Method Variance

Experimental Setup

The experimental setup for the second experiment was similar to that described in the previous section. Six, 38-liter aquaria were filled with approximately 19 liters of de-chlorinated water, the same nutrients were added as described above, and 12 tiles were placed in each microcosm. Algae were gathered as described above from the same location and used to inoculate each microcosm. The algae was grown under the 1000 watt grow light with a 14:10 light:dark cycle.

Sampling Procedure

The sampling procedure was the same as described in the previous section, expect the PVC sampler was replaced with three samplers constructed by drilling a hole for the desired area and creating a seal with rubber cement. The original 13.1 cm^2 PCV

sampler design was used as the template and two more samplers were constructed with areas of 5.06 and 1.27 cm^2 (Figure 18).

The algae were given time to establish to a measureable level of biomass as described in the previous experiment. Sampling was conducted between March 5, 2016 and March 7, 2016 by randomly removing tiles from the microcosms and taking measurements using the BenthoTorch®. Each tile was also sampled three times using the three different sampler sizes. Seventy-two tiles were sampled, which corresponded to 24 tiles for each sampler size. Next, each tile was placed in a modified sampler mounted to a board and vacuumed into a 125 ml Nalgene bottle and rinsed with water until all algae in the sampler area was removed. The entire sample was filtered using a 0.6 µm Advantec GA55 47 glass-fiber filter and wrapped in foil and placed in a freezer. After a period of at least 24 hours, the filters were processed for extracted chlorophyll according to US EPA method 446 (Arar, 1997). Tiles were not replaced in the microcosms after sampling.

Statistical Methods

Data were organized, sorted, and analyzed using Microsoft Excel and Minitab 17. Linear regressions were performed using the Benthotorch® readings as response variables and the extracted chlorophyll *a* as the independent variable. One-way ANOVA's used stacked data with the chlorophyll data as the response variable and the method of chlorophyll analysis or the sampler area as the treatment. Sample size analysis was performed substituting root mean square error (RMSE) from linear

regressions performed on data collected in laboratory experiments as a predicted standard deviation.

Results and Discussion

***In-situ* and *Ex-situ* BenthoTorch® Measurements**

The purpose of this study was to compare the Benthotorch® fluorometer with the traditional method of measuring benthic algae. The study addressed three potential sources of variance identified from field studies. First, how well *in vivo* data collected from an *in situ* fluorometer compared to the traditional methods of chlorophyll *a* extraction. Second, can the sampling method be modified to minimize variance encountered in the previous *in situ* testing? Third, how much variance was introduced using a 13.1 cm^2 sample area when the BenthoTorch® only measured a 1.0 cm^2 area.

The regression between extracted chlorophyll *a* and the *in situ* BenthoTorch® measurements resulted in a 0.86 slope, an R^2 of 0.64 and a RMSE 0.62 µg chlorophyll *a*/cm^2 (Figure 19). The laboratory test regressions showed a relatively low variance, and the slope was closer to unity compared to the regression slopes and R^2 from chapter III (Figure 10), as well as Harris and Graham (2015). Harris and Graham (2015) found better correlations with lower concentrations of algal biomass (<4 µg chlorophyll *a*/cm^2, R^2=0.50). The controlled setting in the laboratory combined with relatively lower algal biomass compared to Harris and Graham (2015) and Chapter III (Figure 10), likely improved the fit with the laboratory-extracted chlorophyll a. There were a few outliers in Figure 19, which may be attributed to spatial variation in periphyton growth or issues

with measuring filamentous algae *in situ*. Even with the low biomass levels, there was still possible self-shading from the three-dimensional structure of the algae.

The second objective was to develop an *ex situ* measuring procedure to improve the correlations of BenthoTorch® measurements with extracted chlorophyll *a*. In the 2014 Oklahoma field tests (Chapter III), as well as the *in situ* laboratory tests, self-shading and spatial variance were hypothesized as the two most likely contributors to variance between the BenthoTorch® and extracted chlorophyll *a*. The *in vivo/ex situ* method was developed to minimize the effect of spatial variability and self-shading of the periphyton. A linear regression was performed between the *in vivo/ex situ* method and the extracted chlorophyll *a* resulting in a slope was 0.13, an R^2 of 0.81 and RMSE of 0.06 µg chlorophyll *a*/cm^2. The calibration plate held 2.5 ml and the standardized solution volume was 100 ml, which required an adjustment factor (Equations 7, 8, 9 and 10). Regressions using the adjusted *in vivo/ex situ* measurements increased the slope to 0.79 and the RMSE to 0.47 µg chlorophyll *a*/cm^2 (Figure 20, Table 11).

The *ex situ* method resulted in a higher R^2 and a lower RMSE (Table 13) compared to the *in situ* method. The *ex situ* method used a sub-sample from a relatively homogenous solution, whereas the *in situ* BenthoTorch® was measuring only a small fraction of the periphyton that was contained in solution. Measuring and averaging multiple sub-samples may reduce the variance, but additional testing is required. Next, a one-way ANOVA was performed using chlorophyll *a* as the response variable and extracted chlorophyll *a*, BenthoTorch® *in situ*, and BenthoTorch® *ex situ* methods as the treatment. There was not a significant difference (α=0.05) between extracted chlorophyll

a, *in situ* or *ex situ* methods. The methods compare well under a very controlled setting at low concentrations. Filamentous algae were a problem for the BenthoTorch® in field tests (Chapter III), but samples in the laboratory also contained filamentous algae. There were no control groups to determine the effect of filamentous algae.

The new method of measuring a sub-sample on the calibration plate has the potential to make the BenthoTorch® more effective for use in monitoring periphyton growth in streams in a variety of conditions. Removing and suspending the periphyton, i.e. the *ex* situ method, resulted in a better agreement between the BenthoTorch® and extracted chlorophyll a. The area of the PVC sampler was 13.1 cm^2 and the BenthoTorch® measured 1.0 cm^2, but the entire sample was contained in the BenthoTorch® calibration plate. However, due to the spatial variability of periphyton, antecedents may be very different from the solution mean of measured chlorophyll *a*.

Sampler Area Effects

Laboratory tests were conducted to analyze differences between periphyton sample sizes collected for chlorophyll *a* extraction and the area measured by the BenthoTorch®. Three sizes were used, 13.1 cm^2 (same area used for the field tests in Chapter III and the previous laboratory test), 5.06 cm^2 (roughly one third less than the original), and 1.27 cm^2 (similar to the 1.0 cm^2 area measured by the BenthoTorch®). One-way ANOVAs were conducted using measured chlorophyll as the response variable and sampler area as the treatment at $\alpha=0.05$ level. The mean BenthoTorch® measurements were not significantly different between sampler sizes. However, mean chlorophyll *a* measurements were significantly different between the three sampler sizes

(Table 14). The smallest sample area had the largest variance for both methods of chlorophyll measurement (Figure 21). There were outliers in the lab-extracted chlorophyll for the two smaller sample areas (1.27 and 5.06 cm^2) but not for the 13.1 cm^2 area sampler.

The R^2=0.76 calculated from the regression of the 13.1 cm^2 sampler was the best fit against the benthic algae measured by the BenthoTorch® (Table 14). The expectation was that the 1.27 cm^2 sampler would be the best fit. However, it was hypothesized that since periphyton can have high spatial variability at very small scales (Stevenson et al., 1996), there was a greater chance in capturing more of the variability across a larger area. It was the same statistical principal as taking samples from a population. The more samples taken, the more likely the data will be normally distributed. A smaller sampler area will increase the chance of skewing the mean of the chlorophyll *a* measurement. By averaging a larger area, the measurements become closer to the measurements taken in the 1.0 cm^2 area by the BenthoTorch®.

Proposed BenthoTorch® Method for Water Quality Monitoring

The laboratory *ex situ* method combined with the MDEQ (2011) methodology provided a starting point in designing a sampling program for the BenthoTorch® based on:

$$n = \left(Z_{\frac{\alpha}{2}} * \frac{\sigma}{MOE}\right)^2 \quad \textbf{(11)}$$

where n was the number of samples, $Z_{\alpha/2}$ was the inverse cumulative probability of the standard normal distribution, α was 1–confidence level/100, σ was the standard

deviation, and MOE was the margin of error. The standard deviation was estimated as the RMSE from regression of the BenthoTorch® and extracted chlorophyll *a.*

The MDEQ (2011) sample-size estimation method (Equation 11) based on Thornton et al. (1982), can be used to determine the required BenthoTorch® measurements to account for the discrepancy between the BenthoTorch® and extracted chlorophyll *a*. Based on professional judgement, knowledge of the system being studied and the study objectives, a specified confidence level and MOE is selected. Note that a paired t-test (p-value=0.006) indicated that the *ex situ* method was not significantly different to the extracted chlorophyll *a* in the laboratory (α=0.05). The *ex situ* method requires a robust and comprehensive test under variable field conditions for verification; however, the laboratory tests can be used as a starting point when developing a water quality monitoring program. If a new technique can be used to gather more samples with less time, then it may be possible to obtain an acceptable confidence rating and margin of error for categorizing the reach. The term 'acceptable' here is up to the monitoring agency to define. It would also be possible to establish a confidence level and MOE that will be sensitive enough to detect a meaningful difference between sampling periods and/or different reaches.

Required BenthoTorch® Measurements

Figure 22 illustrates the sample sizes required, i.e. n, to meet specific confidence levels and MOEs. A RMSE of 0.47 µg/cm^2 (Table 11) was used to estimate the standard deviation between the BenthoTorch® data and the extracted chlorophyll *a* data. These BenthoTorch® measurements would be used in place of filtering sub-samples and

processing them for extracted chlorophyll *a*. For example, if an agency wants to be 90% confident that they are capturing the mean of the sample 20% of the time, they would need to measure 17 subsamples from each collected sample. Since the BenthoTorch® can take a measurement in 20 seconds, it is likely to be a ten to fifteen-minute procedure for each sample. An 80% confidence level and 20% MOE would require 11 samples and would cut the time down by approximately five minutes. The confidence and MOE is set by a monitoring agency and should be periodically checked with a comparison to extracted chlorophyll samples. If needed, adjustments in the sampling number could be made.

Critical Effect Size and BenthoTorch® Data

Sampling Events Needed for Meaningful Change

Critical effect size (CES) is the threshold that indicates a significant and meaningful difference between two measurements (Munkittrick et al., 2009). The term significant is a statistical characterization, while meaningful refers to professional judgement of the quality of a sampling site. A biologic water-quality monitoring program should include data leading to a judgement about whether site quality is improving, staying the same or degrading. Quality is typically judged relative to a "reference site" that is considered to be relatively un-impacted (USEPA, 2002). Monitoring data are also to be used to detect long-term trends and predict future conditions for biologic communities (USEPA, 2002). Periphyton has a very high spatial and temporal variation in both community composition and quantity (Stevenson, 1996; Morin and Cattaneo,

1992). Periphyton biomass depends on normal fluctuations in environmental and ecologic conditions (Morin and Cattaneo, 1992; Stevenson, 1996). Methods outlined above should be adequate for covering the high spatial and temporal variability.

Assessing what constitutes a meaningful change between sampling periods is more difficult. Choosing an *a priori* metric for evaluating changes stream quality, e.g. mean benthic chlorophyll *a*, helps design the sampling protocol. Mean benthic chlorophyll *a* collected with a specified confidence and MOE may not be enough to quantify the benthic algae at a site. However, the standard deviation and CV could be used to identify problems that are not obvious when comparing means. It may be necessary to use a multi-metric approach to assess site quality if benthic chlorophyll *a* has an excessively high variability. For example, the fluctuations in algal levels could be compared with invertebrates and/or fish metrics. Each of these other metrics will also have a confidence and MOE associated with them, and thus these factors must be considered when choosing a confidence level and MOE for the design.

Sampling Costs

The economic value or utility of the BenthoTorch® is critical to its acceptance in a monitoring program. The proposed *ex situ* method requires the collection of samples in a solution of a known volume. It should take less than five minutes to homogenize a collected algae sample, and a subsample can be measured in about 20 seconds with the BenthoTorch®. The user can either record the reading by hand after the measurement or upload these data to a computer after the sampling is complete. This

reduces multiple steps and at least two days for processing benthic algae for extracted chlorophyll, assuming a predefined sampling site. For example, two people could collect and analyze around 12 samples in two hours, which equates to a similar number of samples to the 80% confidence with a 30% MOE used by MDEQ (2011). One person could measure about one subsample per minute with the Benthotorch and upload them too. Estimates from Figure 18 at an 80% confidence and 20% MOE would require 11 subsamples. The extracted chlorophyll method requires that collected samples be filtered. Depending on the filter apparatus and the amount of sediment in the sample, it could take several more hours to complete the filtering. If the chlorophyll is not extracted at that time, the filters must be frozen and then soaked in solvent for at least 2 but not more than 24 hours, centrifuged for 20 minutes, and run through the spectrophotometer twice with a 5-minute delay for acidification. This process adds another 5-6 hours of laboratory work including setup and cleaning and two to three days to obtain the data. For 12 samples processed for chlorophyll a, it can take up to 12 paid hours of labor. At approximately $32 an hour (total employment cost) for a state agency, or $65/hr for private, the BenthoTorch® could save 8-10 hours of labor and up to $320 for a state agency or up to $650 for a private company with a similar confidence and MOE. This includes only labor and not disposables and other equipment that would be necessary.

Conclusions

In this study, the *in situ* BenthoTorch® compared favorably in a controlled laboratory setting with relatively low concentrations of extracted chlorophyll *a* (<2.5 µg/cm^2). The results showed that under controlled conditions, measurements from the

BenthoTorch® are comparable to extracted chlorophyll *a*. Therefore, there may be locations where the BenthoTorch® would be useful in detecting relative changes in periphyton biomass within a stream reach or for comparisons with other streams. Note there is an increase in variance as the chlorophyll *a* concentration increases. This trend was also observed in the data from the field experiments in chapter III. As the level of chlorophyll *a* and biomass increase, the potential for shading and the magnitude of spatial variance also increase.

The test of an *ex situ/in vivo* method showed that it may be valuable for streams with irregular substrates, or higher benthic biomass. The relatively higher R^2 of 0.81 reflects the reduction in variance with the *ex situ/in vivo* method and the slope of 0.79 was less than the *in situ* method. The ANOVA also showed a significant difference when the traditional method was compared directly with the *ex situ/in vivo* BenthoTorch® method. This method may still prove useful in quantifying differences in periphyton between two stream reaches, temporal changes of the same stream or inclusion in benthic quality indexes. This depends on the number of samples and the required precision of the benthic chlorophyll *a* data to detect a meaningful difference. The *ex situ/in vivo* method took less time compared to the traditional method and can be completed on site. This method does not require samples to be transported back to the laboratory because the homogenizing and measurements are completed in the field.

The sampler size/area has been cited as a potential error source for algal biomass measurements (Aloi, 1990). However, studies have not quantified how much the sampling area influences variability, only that periphyton has a high variability across

small areas (Stevenson et al., 1996; Aloi, 1990). In the laboratory study, the larger sample area had a lower variance compared to a sample area similar to the BenthoTorch® measurement area. Therefore, the larger area sampler for fieldwork (Chapter III) and laboratory experiments is recommended. This also allows easier periphyton removal from the substrate and reduces the spatial variability component of the periphyton sample.

Water quality monitoring requires documenting conditions at the time of sampling, as well as long-term trends. Data quality must be comparable so that appropriate statistical comparisons are made with other sampling periods and locations. New methods must be rigorously tested and compared to established methods before being implemented. Disparate sample areas, irregular surfaces, three-dimensional structure of algal communities add potential error sources to periphyton monitoring. Even though the experiment design, collection and processing were done very carefully for this study, it is unlikely to achieve a one to one comparison with traditional methods. That being said, a new technique needs to be consistent with the established method, even if the relationship is not one to one. More testing is required to define the appropriate procedure under variable field conditions. Depending on the goals of the water-quality monitoring program, the BenthoTorch® has the potential to be an important part of a biological monitoring program. A well-designed sampling procedure using the BenthoTorch® could allow fast, inexpensive and reliable comparisons between median benthic chlorophyll a data. The procedure would have a specified confidence level and margin of error that could be incorporated into aquatic biologic assessments.

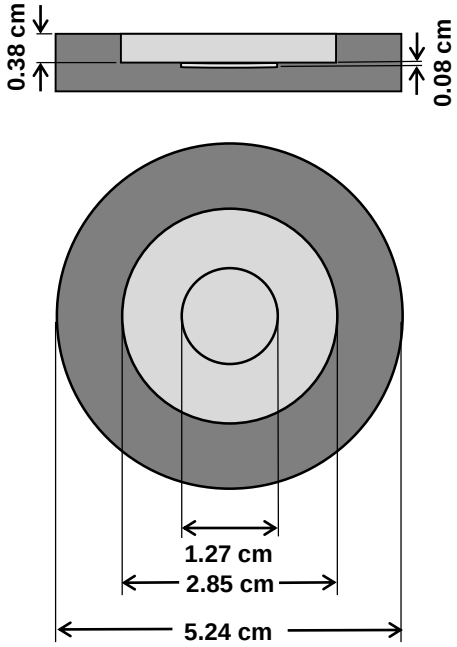

Figure 17. Benthotorch® black calibration plate provided by BBE Moldaenke.

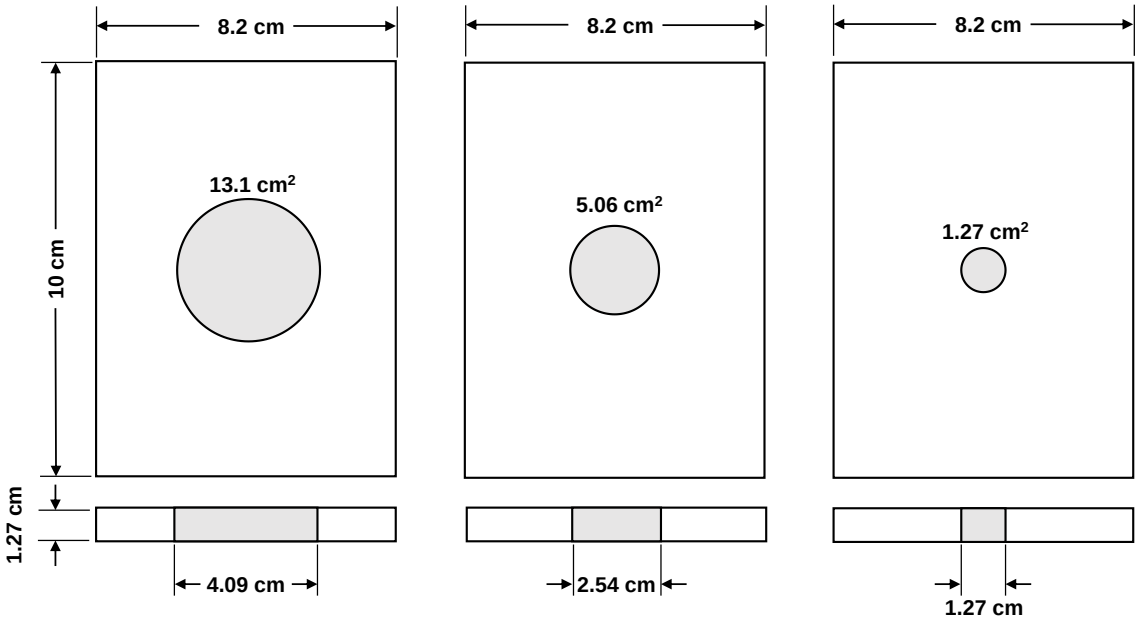

Figure 18. Sampler design to collect benthic algae from three progressively smaller areas.

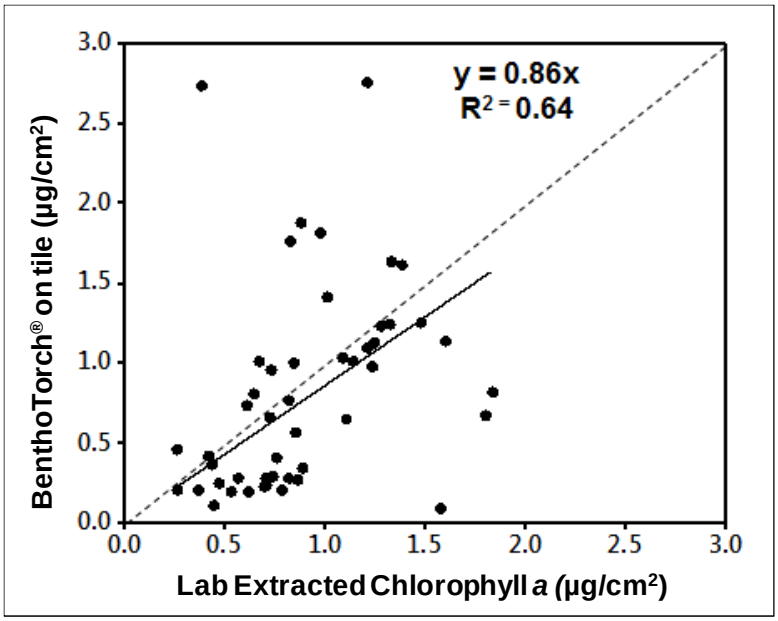

Figure 19**. Linear regression comparing the BenthoTorch® *in situ* measurements on a ceramic tile to the extracted chlorophyll *a* of the collected periphyton from the tile.**

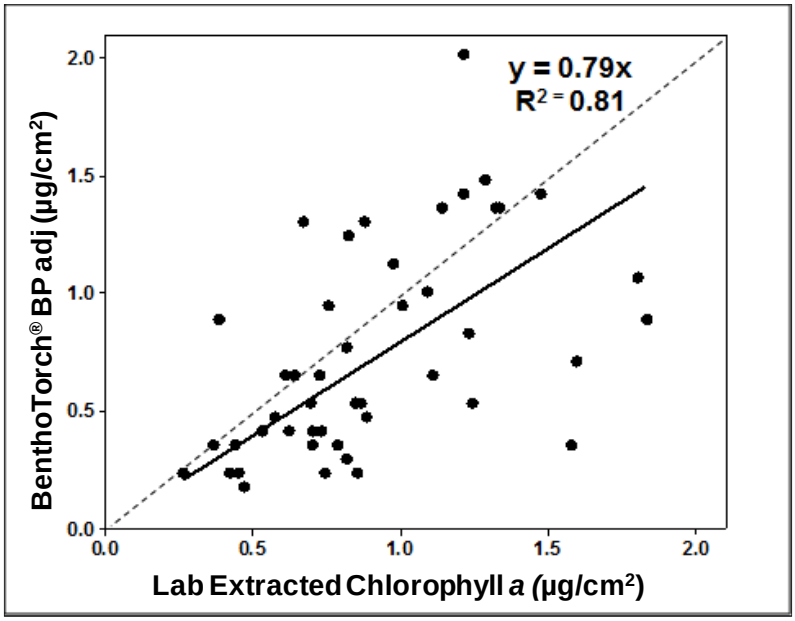

Figure 20. Linear regression comparing BenthoTorch® to extracted chlorophyll a. Calibration plate data were adjusted for the sub-sample volume/original volume area of the original periphyton and the area of the calibration plate.

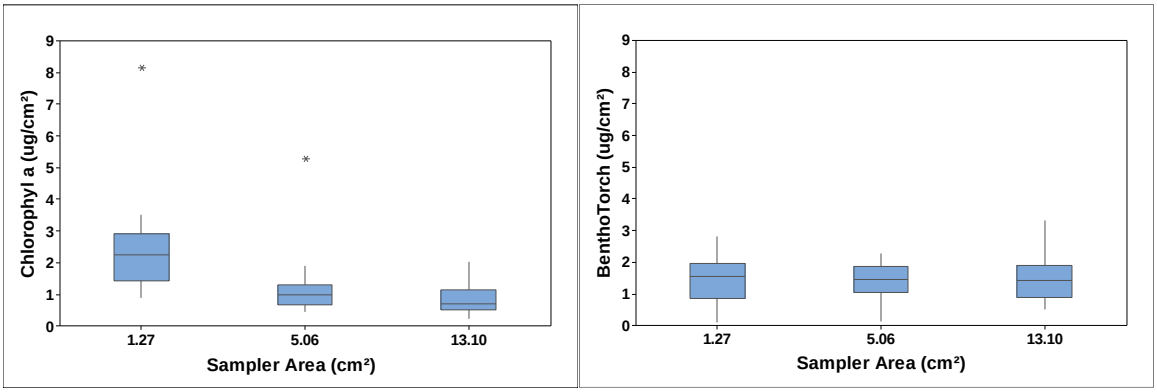

Figure 21. Boxplots of the BenthoTorch® data collected *in situ* on a ceramic tile and the corresponding extracted chlorophyll data with different sampler sizes.

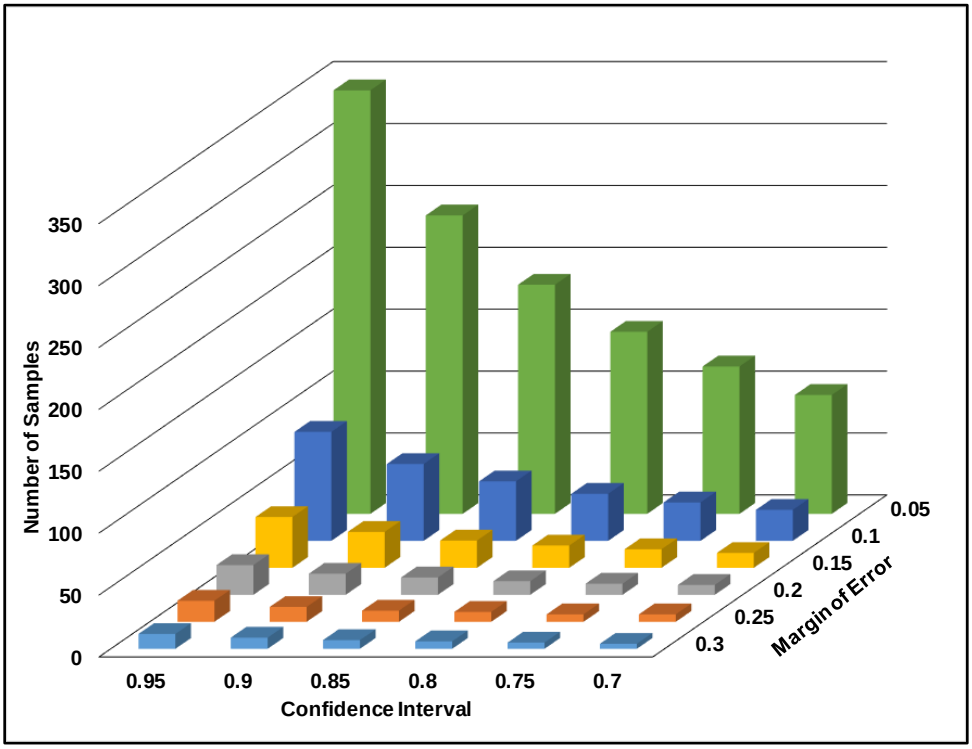

Figure 22. Number of samples required to obtain a specified confidence interval and margin of error using the root mean square error (RMSE) from *ex situ* BenthoTorch® measurements compared to extracted chlorophyll *a* in a laboratory experiment.

Table 11. Linear regression slope, R^2, and root mean square error of BenthoTorch® data vs laboratory-extracted chlorophyll a; all p-values were significant at an α=0.05.

BenthoTorch® Method	Slope	R^2	Root Mean Square Error (µg/cm^2)
In situ	0.84	0.64	0.62
ex situ	0.13	0.81	0.06
Ex situ adjusted	0.79	0.81	0.47

Table 12. ANOVA results for mean chlorophyll *a* for different methods. *Means that share a letter statistically similar at an α=0.05.

Method	Mean Chlorophyll *a* ($\mu g/cm^2$)
Laboratory extracted chlorophyll *a*	0.89^{a*}
BenthoTorch® *in situ*	0.80^{a*}
BenthoTorch® ex situ	0.73^{a*}

Table 13. ANOVA results for chlorophyll a data for three different sampler areas. *Means that do not share a letter are significantly different at α=0.05.

Sampler Area (cm^2)	Mean Chlorophyll *a* ($\mu g/cm^2$)
1.27	2.4^{a*}
5.05	1.2^{b*}
13.1	0.86^{b*}

Table 14. Linear regressions for *in situ* BenthoTorch® data from a ceramic tile vs periphyton collected from different sample areas and processed for extracted chlorophyll a.

Sample Area (cm^2)	Complete Data Set				
	n	slope	R^2	Root Mean Square Error ($\mu g/cm^2$)	p-value
1.27	4	0.47	0.65	0.99	<0.001
5.06	3	0.70	0.51	1.06	<0.001
13.1	4	1.72	0.76	0.83	<0.001

Table 15. Linear regressions for *in situ* BenthoTorch® data on a ceramic tile vs periphyton collected from different sample areas and processed for extracted chlorophyll a, excluding outliers.

Sample Area (cm^2)	Data Set Excluding Outliers				
	n	slope	R^2	Root Mean Square Error ($\mu g/cm^2$)	p-value
1.27	23	0.61	0.73	0.86	<0.001
5.06	22	1.3	0.86	0.58	<0.001
13.1	N/A	N/A	N/A	N/A	N/A

CHAPTER V

CONCLUSIONS

Field Tests

Chlorophyll a

BenthoTorch® estimated chlorophyll was statistically similar for single measurement (BT1) and a five-minute average (BT5) that included a 10-minute dark adjustment (Mann Whitney, $p = 0.05$). However, BT1 and BT5 were not statistically similar when compared to laboratory extracted chlorophyll *a* (LEC) ($\alpha = 0.05$). *In situ* testing in streams and rivers of Oklahoma did not indicate the Benthotorch® could substitute for the traditional methods of periphyton collection and analysis. The Benthotorch® did not have a consistent relationship with either extracted chlorophyll *a* or cell counts performed by a taxonomist.

Regression performed on both BT1 and BT5 had a positive slope (0.17 and 0.24 respectively) and p-values <0.001. This indicated that the Benthotorch® under measures relative to the extracted chlorophyll *a* method. An R^2 of 0.42 for single measurements and 0.39 for five-minute average indicate that the comparison is inconsistent. The presence of filamentous algae reduced the slope and R^2 while sediments present in the samples did not have a significant effect.

Cell Counts

The study did not produce statistically significant results. In measures of cells/cm^2, the Benthotorch® and the cell counts were off by a magnitude of 10,000 when compared across all samples. Percent community composition was not statistically significant either. Overall, the samples were dominated by diatoms so this may have skewed the results of community composition. Considering the presence of visible filamentous algae did not matter, the BenthoTorch® overestimated cyanobacteria relative to the cell counts with or without filamentous algae present.

Laboratory Tests

Laboratory tests under controlled conditions resulted in a closer agreement with laboratory extracted chlorophyll *a*. A lack of sediment, relatively low biomass (<3.0 µg/cm^2) and consistent substrate were the likely reason for the improved relationship between the *in situ* Benthotorch® measurements and extracted chlorophyll *a*. (slope=0.86, R^2=0.64, RMSE=0.62 µg/cm^2). The *Ex situ* method was used for a potential modification to the methods used in field tests. By removing the periphyton from the substrate and placing it in solution, the potential shading was reduced and the periphyton sample was more spatially homogenous. Regressions indicated that *ex situ* measurements compared to extracted chlorophyll *a* increased to an R^2 of 0.81. The measurement was adjusted for the difference in the area and volume of measured periphyton solution. Un-adjusted results had an R^2 of 0.13, while the adjustment increased the R^2 to 0.79. An ANOVA between the extracted chlorophyll *a*, *in situ* Benthotorch®, and *ex situ* Benthotorch® showed significant differences between the means (α=0.05).

Future Research Recommendations

The *ex situ* method is promising for inclusion in biologic monitoring programs; however, it requires additional robust testing in variable field conditions. The MDEQ (2011) methodology of using the sample size estimation in Equation 13 can be adapted to develop a standard operating procedure for the Benthotorch®. Using the RMSE of the laboratory tests as an estimate of the predicted standard deviation, there was a statistically verifiable design to begin data collection. Additional tests need to determine environmental conditions the *ex situ* method may be useful and how the procedure should be conducted. There needs to be consideration for how many samples gathered a single location within the stream as well as how many locations collected throughout the stream. A determination needs to be made about whether to composite all samples in to one for the entire stream reach, or to leave the samples separate. Whether using composite or individual samples, the Benthotorch® will require multiple measurements.

The Benthotorch® measurements *in situ* and *ex situ* should be compared to qualitative methods as well. Oklahoma Conservation Commission currently collects percent cover, and the Benthotorch® should be more precise than the qualitative method already in use.

Final Conclusions

Testing does not support using Benthotorch® measurements with data collected by traditional methods of analyzing periphyton. An *ex situ* method was tested in laboratory and showed promising results. This *ex situ* method needs to be tested in under different field conditions before a standard operating procedure can be completed.

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VITA

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