APPLICATION OF FOURIER-TRANSFORM INFRARED TECHNOLOGY TO THE CLASSIFICATION OF HARMFUL ALGAL BLOOMS (HABS)

by

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Abstract

Cyanobacteria are Gram-negative photosynthetic bacteria capable of producing toxins responsible for morbidity and mortality in humans and domestic animals. Many are capable of forming concentrated blooms that impact the environment by limiting the growth of sub-surface plants and phytoplankton. Harmful algal blooms (HABs) are also capable of producing multiple types of toxins, creating a potential hazard to recreational water users and animals drinking water from or near a bloom. Characterization of HABs is necessary to prevent these human and animal exposures and includes classifying of the type of cyanobacteria present and whether or not they are capable of toxin production, and the exact type of cyanotoxin that is actually present in bloom. Current methods used to classify cyanobacteria and cyanotoxins include microscopy, bioassays, ELISA, PCR, HPLC, and LC/MS. All of these methods, however, have limitations that include time, labor intensity, or cost. Fourier-Transform Infrared Spectroscopy (FTIR) is another potential tool for cyanobacterial classification that is not limited by these factors. To examine the practicality of this method, library screening with default software algorithms was performed on diagnostic samples received at the Kansas State University Veterinary Diagnostic Lab, followed by PCA of samples meeting minimum quality requirements to produce cluster analyses and dendrograms. Both spectrometers and software packages used were successful at distinguishing cyanobacteria from green algae in clean samples with 89.13% agreement. PCA resulted in clear classification of cyanobacteria or green algae demonstrated by a large order of magnitude difference produced by average Euclidian distance dendrograms. While this method is only capable of differentiating cyanobacteria from green algae or other aquatic environmental constituents, its simple, rapid use and low cost make it a beneficial screening tool when coupled with toxin-detection methods to characterize HABs.

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Preface

The research for this thesis was conducted to examine the practicality and real-world efficiency of Fourier-transform infrared spectroscopy (FTIR) in the classification of cyanobacteria for harmful algal bloom (HAB) characterization. Previous research has been done showing that bacterial and cyanobacterial differentiation is capable with these methods, but until now has been done using laboratory grown samples or environmental samples collected by trained laboratory staff. Here, environmental samples collected by private land owners and county extension agents were sent to the Kansas State University Veterinary Diagnostic Laboratory for analysis. The condition of these samples provide results that are indicative of the sample quality that would be seen in the practical application of FTIR, and demonstrate its true effectiveness as a tool for use in HAB characterization.

Chapter 1 - Introduction

Cyanobacteria, or blue-green algae, have impacted the health of animals and humans alike for centuries and are the most widespread, problematic freshwater nuisance algal taxa (Paerl, Fulton et al. 2001). The multiple types of toxins produced by these Gram-negative photosynthetic bacteria have the potential to cause illness and death in humans, livestock, and other animals (van Apeldoorn, van Egmond et al. 2007, Gamboro, Barbaro et al. 2012). Historical evidence exists describing an awareness of the hazards of blooms and scums created by these cyanobacteria for at least two millennia in Europe, as well as aboriginal knowledge on the African, Australian, and North American continents (Fogg, Stewart et al. 1973, Hayman 1992, Codd, Lindsay et al. 2005). Despite an awareness of the presence of toxic cyanobacterial blooms throughout history, they remain a cause of morbidity and mortality in humans and animals today. Every year livestock and companion animals die from ingesting water from water bodies harboring toxic cyanobacterial blooms. Humans experience an array of problems from skin rashes to gastrointestinal illness when recreational exposure or ingestion of contaminated waters occurs, although death in humans is very rare. In aquatic environments, harmful algal blooms (HABs) can create simultaneous exposure to multiple classes of cyanobacterial toxins, and little is known about the additive, synergistic, potentiation, or antagonistic effects of these multiple exposures on humans or animals (Codd, Lindsay et al. 2005).

Current treatment methods vary depending on the type of exposure, as well as by the animal that has been exposed. Lavage with activated charcoal or chemical treatments such as cholestyramine have been shown to bind some cyanotoxins in the gastrointestinal system, but supportive therapy is generally the only treatment available by the time an exposure has been identified (Beasley, Dahlem et al. 1989). Educating individuals to identify and avoid

cyanobacterial blooms to protect themselves, their companion animals, and their livestock from dermal exposure or ingestion of these potentially toxic cyanobacterial blooms is the best method to prevent illness and death. Even with preventative measures exposures are inevitable, especially for livestock. When these exposures do occur, a rapid and accurate diagnosis of the cyanobacterial toxin exposure is crucial, as many cyanotoxins are capable of causing death in a short amount of time with even a very small exposure dose.

Current detection methods used to identify and classify these toxic cyanobacterial blooms range from microscopic identification of cyanobacterial species (the gold standard method), to more scientifically advanced methods such as enzyme linked immunosorbent assays (ELISA) and mass spectrometry to identify specific cyanotoxins. These methods are limited by only being able to identify the cyanobacteria or specific cyanotoxins, but not both simultaneously. Additionally, these methods all require highly trained laboratory staff and sometimes very costly equipment and reagents, as well as long preparation times.

Another technology with potential use in HAB characterization due to its simplicity, cost- and time-effectiveness, and ease of use is Fourier-transform infrared spectroscopy (FTIR). The following chapters examine the use of this technology as a practical method in the characterization of HABs. Chapter 2 discusses the current literature on cyanobacterial components of HABs, their influence on the health of both humans and animals worldwide, and the current methods used in HAB characterization. Chapter 3 describes a new analysis on the practical use of FTIR in environmental cyanobacterial classification, as well as its effectiveness and weaknesses. Chapter 4 concludes by summarizing the application of this method beyond laboratory research, and how it may be incorporated into HAB characterization.

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Chapter 2 - Review of the Current Literature on the Public Health Impacts of Cyanobacteria

Cyanobacteria

Cyanobacteria, historically known as blue-green algae, are one of the oldest species on planet earth and today remain an important part of ecosystems throughout the world. Because they contain photosynthetic pigments (chlorophyll α and accessory pigments) and appear similar to algae and higher plants, they were classified as microalgae until the mid-1900's (Briand, Jacquet et al. 2003). The bluish hue that is often seen associated with blooms of these species led to them being classified as blue-green algae, but not all blue-green algae appear blue-green in color. The name blue-green algae may be deceiving as they can also appear green, brown, or red, depending on the species. It wasn't until the 1960's that blue-green algae were discovered to be a photosynthetic bacteria, rather than a type of algae (Haselkorn 2009). They are also the only group of organisms capable of reducing nitrogen and carbon in aerobic conditions (Gault and Marler 2009).

Advances in science in the 1900's led to cellular examination of these species to identify them as not having the cellular organelles found in eukaryotic plant cells such as algae, but rather that while they do have the cellular capacity to perform photosynthesis, their morphology is prokaryotic, as seen by the presence of peptidoglycan cell walls, no nucleus, and a lack of organelles, relating them to the domain Bacteria (Paerl, Fulton et al. 2001, Haselkorn 2009). Since these discoveries, a change in classification has occurred, moving blue-green algae to the domain Bacteria, phylum Cyanobacteria. Currently, there are 150 genera of cyanobacteria consisting of 2,654 species, of which at least 40 genera are known to produce toxins (van Apeldoorn, van Egmond et al. 2007, Koksharova 2009, Gamboro, Barbaro et al. 2012). This

recognition of cyanobacteria being prokaryotes led to further understanding of the morphological, metabolic, and ecological differences among them, as well as bacteria in general (Whitton and Carr 1982). While the classification of these organisms has changed, they remain frequently referred to as blue-green algae.

The morphology of cyanobacteria is dependent upon the particular species. There are both unicellular species (i.e. *Microcystis*), as well as filamentous species (i.e. *Anabaena*) that appear similar to green algae to the untrained eye. Filamentous species contain many individual cells that communicate via cytoplasmic channels to exchange nutrients (Haselkorn 2009). Filaments are groups of individual cells, and an individual filament should not be mistaken as being a multicellular organism. Regardless of their specific morphology, cyanobacteria are found in all depths of freshwater bodies, from the water surface to the bottom of the water body, and are always present as a normal part of aquatic phytoplankton communities.

Primarily of public health concern in freshwater systems, cyanobacteria may also be found in brackish and marine waters. Some cyanobacteria, such as *Nostoc*, are capable of surviving outside of water systems and can be found terrestrially in soil or in mutualistic symbiotic relationships with certain plant and lichen species (Paulsrud, Rikkinen et al. 1998, Haselkorn 2009). In fact, cyanobacteria have been found in more symbiotic relationships than any other microorganism, including symbiosis with green algae, diatoms, liverwort, moss, ferns, cycads, protozoa, sponges, and even shrimp and mammals (Whitton and Carr 1982). These different ecosystems influence the dynamics of the species growth, as well as their public health implications on humans and other higher trophic levels.

Cyanobacteria do not colonize, invade, or grow in human or animals hosts, but rather the public health concern with cyanobacteria comes from their production of secondary metabolites

that may be toxic to humans, animals, and the surrounding environments (Codd, Morrison et al. 2005). Toxic species have been reported in waters of over 45 countries around the world (Codd, Morrison et al. 2005). The most significant toxin-producing cyanobacteria throughout the world are from the genera *Microcystis, Anabaena, Nostoc, Aphanizomenon, Cylindrospermopsis, Oscillatoria,* and *Planktothrix*, although many other genera are capable of toxin production or are biologically and ecologically significant in other ways. Not all cyanobacteria produce toxins, however, but still play an important role by fulfilling roles of biogeochemical matter and nutrient cycling, and their involvement in the structure, maintenance, and biodiversity of environmental communities (Codd, Morrison et al. 2005, Al-Tebrineh, Gehringer et al. 2011). Many non-toxin producing cyanobacteria are used by humans as food, and many commercial markets exist for species of *Spirulina* (Whitton and Carr 1982).

Perhaps the most significant quality of the phylum Cyanobacteria is their ability to photosynthesize. Cyanobacteria were the first species to utilize sunlight for energy production; they were the first photosynthesizers in the evolution of planet earth. The evolution of these organisms throughout history gave rise to the photosynthetic capabilities seen in higher plants today. It is believed that cyanobacterial cells became endosymbionts within eukaryotes, evolving into present day chloroplasts (Whitton and Carr 1982, Raven and Allen 2003). The ability to photosynthesize light into energy provides a survival advantage to cyanobacteria that other bacteria lack, making cyanobacteria a very versatile group of organisms and clearly differentiating them from other bacterial phyla. The photosynthetic capabilities of cyanobacteria differ from many algae and higher plants because cyanobacteria utilize two photosystems for photosynthesis, and do not contain the same chloroplast organelles seen in other species. This provides cyanobacteria with the ability to switch from a bacterial photosynthesis using one

photosystem to a chloroplast type of photosynthesis involving two photosystems, depending on the environmental conditions (Whitton and Carr 1982).

Photosystem I (PSI) in cyanobacteria is similar to plant photosynthesis, and relies on chlorophyll as the dominant light-absorbing pigment (Ho and Krogmann 1982). Photosystem II (PSII) in cyanobacteria differs from the chlorophyll dependent photosystems of higher plants because it utilizes different pigments known as phycobilins, which are responsible for providing the different colors seen in many cyanobacteria (Ho and Krogmann 1982). These water-soluble, accessory pigments found in cyanobacteria are phycocyanins and phycoerythrins; phycocyanins are the pigments responsible for the blue-green hue observed in many types of cyanobacteria, while phycoerythrins are more predominant in cyanobacterial species that have a brown, orange, or red color (Cohen-Bazire and Bryant 1982). Phycoerythrins are more commonly known for their presence in red algae. While they are not as common as phycocyanins in cyanobacteria, they do play an important role in the photosynthetic activities of some cyanobacteria as well as in the characterization of certain cyanobacterial species. Cohen-Bazire and Bryant explain the differences in these phycobilin pigments by describing how "in chromatically adapting strains, growth in green light stimulates the synthesis of red-colored phycoerythrins whereas when growth occurs in red light it is the blue protein, phycocyanins, which is the dominant biliprotein" (Cohen-Bazire and Bryant 1982, p185). Additionally, these accessory pigments allow some species to carry out photosynthesis at depths that receive only green light, explaining why benthic species such as those within the genus *Oscillatoria* generally appear brownish rather than the typical blue-green (Briand, Jacquet et al. 2003). The differential expression of phycobilins within cyanobacterial species provides an advantageous photosynthetic versatility, and also can be used to differentiate types of cyanobacteria.

In addition to photosynthesis, some genera of cyanobacteria are also capable of Nitrogen fixation. Many species grow in long filaments that containing many individual cells that communicate via cytoplasmic channels to exchange nutrients (Haselkorn 2009). In the 1940's and 1950's, filamentous genera of cyanobacteria such as *Anabaena*, *Oscillatoria*, *Planktothrix,* and *Aphanizomenon* were found to have the ability to produce specialized cells known as heterocysts, leading to wide acceptance of nitrogen-fixing capabilities in cyanobacteria (Whitton and Carr 1982). Heterocysts are specialized cells in which oxygen-evolving photosystems are inactivated and all reducing power from photosynthate is used for nitrogen fixing, allowing them to provide fixed nitrogen to surrounding cells in exchange for carbohydrate from normal cell photosynthesis (Haselkorn 2009). These occur periodically between repeats of roughly 10 normal photosynthetic cyanobacterial cells in the filament (Haselkorn 2009). This specialized differentiation, like all other prokaryotic differentiation, is triggered by environmental conditions (Whitton and Carr 1982). While this would not be advantageous for an individual cell, the filamentous structure of the cells creates beneficial exchange to the filament as a whole. The heterocyst distributes usable forms of nitrogen and/or phosphorus to its neighboring cells, and this distribution trickles down to the more distal cells. The heterocysts are formed periodically throughout the filament to provide optimal exchange of usable nutrients without elimination of large quantities of photosynthetic cells. These specialized cells provide filamentous cyanobacteria with a competitive advantage over other phytoplankton that are capable of photosynthesis but are unable to fix nitrogen (Briand, Jacquet et al. 2003).

Nitrogen-fixation is a normally occurring function within the filamentous cyanobacterial genera, but environmental factors will influence the transformation of these heterocysts from the normal cyanobacterial cell makeup. Common nitrogen-fixing genera include *Anabaena,*

Aphanizomenon, and *Cylindrospermopsis* (Paerl and Huisman 2009). Through lake modeling literature researching the impacts of global warming on cyanobacteria, Elliot et. al. describe how warmer spring waters that allow for earlier phytoplankton growth and subsequent increased nutrient consumption earlier in the year have the potential to create limited nitrogen resources later in the season, creating an advantageous environment for nitrogen-fixing bacteria that can metabolize otherwise unusable forms of nitrogen (Elliot 2012). In addition to nitrogen, phosphorous has also been shown to be important to cyanobacterial growth, and support exists describing that certain nitrogen/phosphorous nutrient ratios benefit the growth of nitrogen-fixing cyanobacteria (Healey 1982). It is important to be aware that while nitrogen-fixation is highly advantageous for many cyanobacteria, not all cyanobacteria of public health concern are filamentous in nature or capable of nitrogen-fixation.

Of highest concern to public and environmental health is the influence of aquatic cyanobacteria and the toxins associated with them. These aquatic cyanobacteria have impacted trophic levels ranging from the microbial communities in which the cyanobacteria thrive, to humans, fish, birds, livestock and other mammals due to cyanotoxin release into water systems during concentrated bloom formation.

Bloom Formation

Cyanobacterial Blooms

A cyanobacterial bloom refers to a large concentration of cyanobacterial cells that occurs under optimal growth conditions (Figure 2.1). Blooms are typically seen during late summer and early fall in temperate zones, with extended bloom seasons seen in warmer subtropical climates (van Apeldoorn, van Egmond et al. 2007). They are easily observable accumulations found in

many eutrophic lakes, ponds, and rivers throughout the world, and may or may not be toxin-producing (Codd, Morrison et al. 2005, van Apeldoorn, van Egmond et al. 2007, Al-Tebrineh, Gehringer et al. 2011). Some types of cyanobacteria have gas vacuoles that allow them to be buoyant, creating visible surface scums or blooms. Surface blooms are easily identifiable, but may have different appearances based on the species within the bloom. They may look dark green or brownish and appear very similar to green algae or other surface material, but they commonly appear as a noticeably bright green or blue-green scum covering the surface of the water and are frequently described as appearing like a blue-green paint covering the surface of the water. A bright blue pigmentation of the surface bloom and surrounding water or shoreline is indicative of cyanobacteria, and occurs as the bloom begins to deteriorate (Beasley, Dahlem et al. 1989). The decline and death of a surface bloom can also create an unpleasant odor (Codd, Morrison et al. 2005, Koksharova 2009). Surface blooms, if large enough, are capable of blocking sunlight to deeper water levels, inhibiting aquatic plants and other phytoplankton from photosynthetic activities necessary to survive. This sunlight blockage to subsurface plants and phytoplankton can result in night time oxygen depletion that may cause fish deaths (Paerl and Huisman 2009). Fish deaths may also be caused by the cyanotoxins released from the bloom, by a lack of food source due to the decreased quantities of other phytoplankton, O_2 depletion caused by bacteria that are decomposing dead cyanobacteria, or a combination of these factors (Koksharova 2009).

Surface blooms are generally a cause of concern regarding human, environmental, and animal health, and are often viewed as a potential hazard, but cyanobacterial blooms are not limited to surface waters. Cyanobacterial blooms can also occur in the form of mats that lay across the bottom of a water body. These bottom mats can be formed by any type of

cyanobacteria, even those that are capable of buoyancy. *Microcystis, Anabaena, Planktothrix, Aphanizomenon, Cylindrospermopsin,* and *Nodularia* species are generally associated with surface blooms while *Oscillatoria* species are more likely to form mats or biofilms (Codd, Morrison et al. 2005).

Figure 2.1 Harmful Algal Surface Bloom – Centralia Lake, KS

It is also important to be aware that a cyanobacterial bloom does not always consist of one particular species of cyanobacteria, but rather can contain a mixture of different species. Blooms containing one or multiple species of cyanobacteria are, therefore, capable of producing

more than one type of cyanotoxin (Hisbergues, Christiansen et al. 2003, Oehrle, Southwell et al. 2010). This potential for multiple cyanotoxins to be simultaneously synthesized and released further complicates the health impact of a bloom. Algaecides such as copper sulfate are commonly used to prevent and control excessive phytoplankton growth, especially blooms in eutrophicated waters (Hadjoudja, Deluchat et al. 2010).

Factors in Bloom Formation

The environmental and public health risk associated with cyanobacterial blooms has led to a great deal of concern as to why these excessive concentrations of cyanobacterial cells occur, but the environmental parameters influencing the dynamics of these cyanobacterial communities remains poorly understood (Martins and Vasconcelos 2011). Complex combinations of nutrient concentrations, sunlight, temperature, turbidity, pH, conductivity, salinity, carbon availability, and slow-flowing/stagnant water have all been described as factors in bloom formation (van Apeldoorn, van Egmond et al. 2007).

In general, cyanobacteria grow better in warmer temperatures, making temperature the most common factor associated with cyanobacterial bloom formation (Paerl and Huisman 2009, Elliot 2012). Based on lake modeling literature, Elliot et al. describe how increasing temperatures in conjunction with decreased flushing rate and increased nutrient loads are capable of creating optimal conditions for bloom formation (Elliot 2012). Regional increase in temperature has been associated with an increase in bloom formation in aquatic environments. The heat capacity of water prevents it from increasing or decreasing as rapidly as fluctuating air temperatures, but persistent increases in air temperature over time will result in surface temperature change. This phenomenon lengthens stratification periods within water bodies and has led not only to an increase in bloom formation during the hot summer months, but also to

bloom formation both earlier and later in the year when warmer temperatures occur earlier than normal, or persist later into fall and winter (Paerl and Huisman 2009). In 2011, the Kansas Department of Health and Environment's Bureau of Environmental Health reported 26 harmful algal blooms (HABs) in Kansas public lakes and reservoirs, two of which were not lifted from advisory or warning state until early into the month of November (Bureau of Environmental Health 2012). Normal conditions for Kansas would be too cold to sustain a bloom so late into the fall, but abnormal fall temperatures provided acceptable conditions for growth.

The second major factor in bloom formation is the nutrient load in an aquatic system. The understanding of this aspect of bloom formation is confounded by the diversity of biological and chemical compositions of environmental waters, and the difficulty of clearly defining those components (Rudi, Larsen et al. 1998). Water quality is determined by the availability of nutrients necessary for primary production, so for any type of phytoplankton to thrive, proper nutrient composition in the aquatic environment is necessary (Stehfest, Toepel et al. 2005). While cyanobacteria are capable of providing carbohydrates for themselves through photosynthesis and nitrogen fixation in certain species, the need for proper nutrient loads in the environment still exists. Paerl et. al. have described that "human-induced environmental changes, most notably nutrient over-enrichment associated with urban, agricultural and industrial development, have led to accelerated rates of primary production, or eutrophication" (Paerl and Huisman 2009, p27).

Of particular concern regarding harmful algal blooms is an excess of Nitrogen and Phosphorus released into the environment via industrial and agricultural runoff. These nutrients can be utilized not only by cyanobacteria, but also by aquatic plants and other phytoplankton within the aquatic system. Eutrophication with phosphorus has been shown to increase the

development of both nitrogen-fixing and non-nitrogen-fixing cyanobacteria (Paerl, Fulton et al. 2001). Dominance of nitrogen-fixing species is dependent on total nitrogen availability, as well as the ratios of available nitrogen to phosphorus (Paerl, Fulton et al. 2001). The "nutrient pollution" of phosphorus and nitrogen creates eutrophic environments that favor growth of all phytoplankton as well as higher forms of plant life, but in combination with other bloom-formation factors discussed here, creates a favorable environment more specific to cyanobacteria (Briand, Jacquet et al. 2003).

Limitation of nutrients is associated with the formation of heterocysts at the early onset of nitrogen deficiency (in nitrogen-fixing species), and the formation of tapered hairs in whole sections of filament at the onset of phosphate deficiency (Whitton and Carr 1982). Nitrogen-fixation in certain cyanobacteria prevents them from becoming nitrogen deficient, but does not prevent deficiencies in other important nutrients such as phosphorus (Healey 1982). Species capable of Nitrogen-fixation, however, are at an advantage by being able to utilize forms of these nutrients that are unusable by other plant and phytoplankton species. The ability to uptake and metabolize these elements allows Nitrogen-fixing cyanobacteria to thrive in nutrient poor environments, as well as those in which there is an excess of nutrients usable by all phytoplankton species.

Other dynamic factors such as turbidity, in and out flow, and wind also influence bloom formation in freshwater environments. Turbidity, or water clarity, influences the amount of sunlight that can penetrate into differing depths of the water body. While cyanobacterial surface blooms themselves can greatly increase turbidity and block subsurface phytoplankton and plants from receiving adequate sunlight for photosynthesis, the turbidity of the water prior to a bloom being present can influence its formation. Cyanobacteria themselves have strict light

requirements, with too little causing insufficient energy production, and too much inhibiting the cells by decreasing photosynthetic efficiency and possibly causing damage due to bleaching (Tamulonis, Postma et al. 2011). Turbidity can be caused by many normal factors in aquatic environments; soil erosion, waste discharge, urban runoff, eroding stream banks, bottom feeders such as carp stirring up bottom sediments, as well as more physical surface obstructions such as debris from agricultural practices or logging activity (EPA 2012). In addition to blocking sunlight necessary for photosynthesis in phytoplankton and aquatic plants, higher turbidity in water systems also increases water temperatures because the suspended particles are capable of absorbing more heat than clear water, therefore, decreasing the amount of dissolved oxygen available to fish and other aquatic animals (EPA 2012).

The flow of water both into and out of water systems is another important factor influencing the ability of cyanobacteria to form blooms. This is especially important for water reservoirs that are manmade through the damming of rivers, where the outflow of water is monitored and controlled through spillways. The flow of water into a pond, lake, or reservoir can act as a vector, carrying sediment and nutrients, as well as chemical runoff from agricultural shorelines and wastewater from countless industries into the water body. The resulting increased nutrient loads within catchments and reservoirs can cause changes in phytoplankton responses, leading to excessive growth (Elliot 2012). This nutrient loading within catchments and reservoirs is further amplified when discharge rates are not sufficient enough to counterbalance a large inflow of nutrient-rich water.

Temperature, turbidity, and wind all influence the stratification of fresh water bodies and results in seasonal turnovers of these stratified layers (Lake Access). This mixing of water temperatures, pH, and nutrients also has impacts on phytoplankton communities. Additionally,

wind can impact the presence of blooms in other ways. Calm winds coupled with water currents can act in a way that pushes surface blooms into coves or downwind areas of the water body, leading to concentrated blooms near the water's edge. Higher wind conditions, however, can create rough surface waters that can disturb surface blooms and mix buoyant cyanobacteria into the water, dispersing the bloom over the entire water column (Paerl and Huisman 2009). While there is not enough data in the current literature to provide causative evidence between warm temperatures and calm conditions being responsible for the formation of blooms, cyanobacteria are well adapted to thrive in such conditions.

Other environmental conditions, such as pH and salinity, play a role in bloom formation as well, although their influence is not as well understood. Some species of *Anabaena* and *Microcystis* are quite salt tolerant, making them more adaptable than other phytoplankton to increasing salinities (Paerl and Huisman 2009). Additionally, Paerl et. al. describe that increased water salinity influences the vertical density stratification of a water body, favoring some cyanobacterial species (Paerl and Huisman 2009).

A combination of favorable bloom-forming conditions occurred throughout the state of Kansas in the summer of 2011. Flood conditions throughout the Midwest led to significant problems for water management. Many reservoirs were rising, but to prevent further downstream flooding the spillways were unable to release larger quantities of water. This led to an increase in cyanobacterial blooms in two major ways. First, water was flooding onto agricultural land, picking up nutrients from fertilizers and other agricultural chemicals and sediment. Flood water carrying this runoff was building up in reservoirs that were not allowing high discharge as a flood prevention method. Secondly, above average temperatures were observed for region through the summer and into the autumn months. Buildup of agricultural runoff in reservoirs,

coupled with above average temperatures for much of the Midwest led to an increase in quantity and size of algal blooms throughout the state of Kansas. These conditions led to 26 HABs to be reported to KDHE for monitoring, many of which persisted into October or later (Kansas Department of Health and Environment 2012).

A combination of accelerated nutrient loading, increasing temperatures, and enhanced stratification are likely to favor cyanobacteria and influence the formation of blooms (Paerl and Huisman 2009). The presence of blooms, however, is not always indicative of a public health risk, as not all blooms secrete cyanotoxins into the environment (Al-Tebrineh, Gehringer et al. 2011). Blooms that are found to be toxic in the environment are then classified as Harmful Algal Blooms (HABs). It is important to understand that because not all cyanobacteria produce toxins, not all blooms are hazardous or warrant concern if exposure to humans or animals occurs (Beasley, Dahlem et al. 1989). To be classified as a HAB, the bloom must be identified as potentially toxic by identifying the presence of a known toxin-producing species, or by identification of the harmful toxin itself within the bloom and surrounding water.

Cyanobacterial Toxins

At least forty genera of cyanobacteria are capable of toxin production (van Apeldoorn, van Egmond et al. 2007). This toxin production is a result of cyanobacterial secondary metabolism, but not all species of cyanobacteria have the genetic makeup to create these toxic metabolites. Environmental factors similar to those influencing bloom formation (temperature, nutrient load, pH, and light exposure) also contribute to toxin production, but are not well understood (Martins and Vasconcelos 2011). Additionally, the species capable of toxin production do not all produce the same toxins. An array of cyanotoxins is synthesized by different species, all of which have different effects on other organisms, resulting in an array of

public health impacts. Additionally, some species are capable of producing more than one type of cyanotoxin, and some species are capable of producing both non-toxic and extremely toxic variants, further complicating the impacts on the surrounding environment (Meriluoto 1997). Table 2.1 describes cyanotoxins of public health concern and the genera responsible for their synthesis.

There are three major categories of cyanotoxins that significantly impact public health; the cyclic peptides (microcystin and nodularin), alkaloids (neurotoxins anatoxin and saxitoxin, and cylindrospermopsin), and lipopolysaccharide irritants (Gamboro, Barbaro et al. 2012). The cyclic peptide microcystin is the most commonly encountered and detected cyanotoxin in freshwater environments throughout the world (Al-Tebrineh, Gehringer et al. 2011, Marie, Huet et al. 2012).

Treatment for exposure to these cyanotoxins is limited, and usually consists of supportive therapy for animals and humans until either the toxins are passed, or mortality occurs. Lavage with activated charcoal or chelating agents such as cholestyramine has been shown to bind microcystins and anatoxins in the gut and help eliminate them from the body (Beasley, Dahlem et al. 1989). These treatments are time sensitive, however, and must be administered shortly after an exposure has occurred to offer beneficial influence. Additionally, their effectiveness is also dependent upon the exposure dose and the type of cyanotoxin.

Table 2.1 Classification of cyanotoxins by type and producing genera.

Cyanotoxin	Basic Structure	Type	Genera
microcystins	cyclic peptides	hepatotoxin	*Microcystin* *Anabaena* *Oscillatoria* *Planktothrix* *Nostoc*
anatoxin-a	alkaloid	neurotoxin	*Anabaena* *Oscillatoria* *Aphanizomenon* *Cylindrospermopsis*
anatoxin-a(s)	guanidinium methyl phosphate ester	neurotoxin	*Anabaena* *Oscillatoria* *Aphanizomenon* *Cylindrospermopsis*
saxitoxin	tricyclic carbamate alkaloid	neurotoxin	*Anabaena* *Aphanizomenon* *Cylindrospermopsis*
cylindrospermopsin	tricyclic alkaloid	cytotoxin	*Aphanizomenon* *Umezaka* *Rhadiopsis*

Microcystins

The most commonly encountered and widely distributed cyanobacterial toxins in freshwater systems are a group of cyclic heptapeptides known as microcystins (Briand, Jacquet et al. 2003, Al-Tebrineh, Gehringer et al. 2011, Marie, Huet et al. 2012). They are also the most well-known and understood of all the cyanotoxins, and an abundance of information regarding their toxicity and detection is present in the literature. Microcystins are a large group of hepatotoxins named after the genus *Microcystis* from which they were first described, and over 70 variants have since been characterized (Meriluoto 1997, Codd, Morrison et al. 2005,

Haselkorn 2009, Koksharova 2009). Microcystins are not, however, limited to the genus *Microcystis* but have also been found in several other unicellular and filamentous cyanobacterial genera, including *Anabaena, Oscillatoria, Planktothrix* and *Nostoc*, and often these different genera occur together within a bloom (Hisbergues, Christiansen et al. 2003). Microcystin production is directly proportional to growth rate of the producing species, with the highest concentration occurring during the late logarithmic growth phase (van Apeldoorn, van Egmond et al. 2007).

Microcystins are hepatotoxic protein phosphatase inhibitors, tumor promoters, and possible human carcinogens. Primarily inhibiting protein phosphatase 1 (PP1) and 2A (PP2A) of hepatocytes, it has also been demonstrated that they may have some influence on kidney structure and function (Lowe, Souza-Menezes et al. 2012). Covalent bonds result in irreversible inhibition of PP1 and PP2A leads to hyperphosphorylation of cytoskeletal proteins that deforms hepatocytes and causes swelling of the liver (hepatomegaly) (MacKintosh, Dalby et al. 1995, van Apeldoorn, van Egmond et al. 2007, Lowe, Souza-Menezes et al. 2012). This loss of structure in the liver leads to blood accumulation and frequently results in death from hemorrhagic shock (Briand, Jacquet et al. 2003). Lowe et. al. also demonstrated that some alteration of the structure and function of the kidney occurs in rats exposed to sublethal doses of microcystin-LR (Lowe, Souza-Menezes et al. 2012). Microcystins have also shown the ability to pass the blood-brain barrier via anion transporting polypeptides, although this does not appear to be of significant involvement in their toxicity (Bruno, Melchiorre et al. 2009). Animals exposed to microcystins show weakness, anorexia, pallor of extremities and mucous membranes, as well as mental derangement, with death occurring within a few hours to days depending on the exposure (van Apeldoorn, van Egmond et al. 2007).

Environmental concentrations of these toxins during blooms are highly variable. This variation can be attributed to non-producing subpopulations present in the bloom, and environmental factors such as simultaneous increases in temperature and phosphorus concentrations (Martins and Vasconcelos 2011). Additionally, microcystins are mostly retained within cyanobacterial cells until lysis or death. This creates higher water concentrations when blooms are aged and declining, or when algaecides used to control blooms lyse the cells, releasing the intracellular microcystin (van Apeldoorn, van Egmond et al. 2007).

Examples of currently described microcystins include microcystin-LR (MC-LR), MC-LA, MC-YR, MC-YA, MC-YM, and MC-RR, where the first two letters denote the toxin as microcystin and the latter two letters identifying the varying L-amino acids present in the specific toxin (Beasley, Dahlem et al. 1989). The amino acid variants are labeled using standard amino acid symbols; L denoting leucine, Y denoting tyrosine, A denoting alanine, M denoting methionine, and R denoting arginine in the previous examples given. These differing amino acid variants can influence the lipophilicity and polarity of the microcystin, in turn affecting each variant's specific toxicity (WHO, 1998). Microcystin-LR is the most significant toxin in regards to public health, as well as the most studied and is frequently used in antibody and genetic testing for microcystins.

Microcystins have also been very well described genetically. The *mcy* gene cluster responsible for microcystin biosynthesis is 55kb in size and codes for 10 microcystin genes (Borner and Elke 2005). The microcystin synthetase genes are responsible for synthesis of all the differing variants of microcystin, toxic and non-toxic. This gene cluster is found in all microcystin-producing genera, although it may differ slightly in organization and sequence (Borner and Elke 2005).

Anabaena, Microcystis, and *Planktothrix* are the cyanobacterial genera known to include most of the microcystin-producing species worldwide (Hisbergues, Christiansen et al. 2003). The structure of these microcystins has also been described and is very similar in resemblance to the cyanotoxin nodularin, produced by marine and brackish water cyanobacteria of the genus *Nodularia*. Although much is known regarding their synthesis and toxicities, the significance of microcystins for the cyanobacterial cells themselves is unknown (Koksharova 2009).

Cylindrospermopsins

Another cyanotoxin that affects the liver is cylindrospermopsin. Cylindrospermopsins are produced by species of *Clyindrospermopsis, Aphanizomenon, Umezaka,* and *Rhadiopsis* (Meriluoto 1997, Shen, Lam et al. 2002, van Apeldoorn, van Egmond et al. 2007). They are tricyclic alkaloid cytotoxins that block protein synthesis, and while it is not targeted to hepatocytes like the microcystins, the liver is one of the first organs in which clinical symptoms occur, as well as the kidneys, thymus, spleen, and heart (WHO 1999, Briand, Jacquet et al. 2003, van Apeldoorn, van Egmond et al. 2007). Cylindrospermopsins cause widespread necrotic injury and are considered carcinogenic due to the induction of DNA stand breakage, with the loss of whole chromosomes from kinetochore and spindle dysfunction also being a possible cause of carcinogenesis (Shen, Lam et al. 2002, Codd, Morrison et al. 2005).

These cytotoxins have bioaccumulated in freshwater mussels, some crayfish species, and rainbow fish (van Apeldoorn, van Egmond et al. 2007). Young blooms hold a larger percentage of toxin within the cells, but the concentration of cylindrospermopsins in the water column increases with bloom age (van Apeldoorn, van Egmond et al. 2007).

Anatoxins

Anatoxins are a group of neurotoxins with two primary variants that differ both in their structure and mechanism of action. Not as frequently encountered in most regions as microcystins, anatoxins are still of significant concern to public health due to their widespread presence and toxicity in humans and animals. The most common producers of anatoxins are species of *Anabaena*, but they have also been derived from *Oscillatoria, Aphanizomenon,* and *Cylindrospermopsis* (van Apeldoorn, van Egmond et al. 2007). It has been shown that phosphorus levels have no effect on anatoxin production in blooms, but unlike the microcystins, not much is known about the environmental or genetic influences on toxin production (van Apeldoorn, van Egmond et al. 2007).

Anatoxin-a is an alkaloid toxin similar in structure to cocaine, and acts as a potent postsynaptic depolarizing agent by binding to acetylcholine receptors causing persistent stimulation (Meriluoto 1997, van Apeldoorn, van Egmond et al. 2007). Differing from anatoxin-a, anatoxin-a(s) is a guanidinium methyl phosphate ester that inhibits cholinesterase at nerve junctions (van Apeldoorn, van Egmond et al. 2007). The (s) in the name comes from the hypersalivation symptoms seen in mice studies (Briand, Jacquet et al. 2003). Due to the high potency of anatoxins, detrimental effects from exposure occur rapidly in humans and animals. Depending on the species, exposure dose, and amount of food in the stomach, ingestion of anatoxins can led to death within minutes to hours and is usually caused by asphyxia due to respiratory muscle paralysis (van Apeldoorn, van Egmond et al. 2007).

Saxitoxins

Saxitoxins and neosaxitoxins are known more commonly as paralytic shellfish toxins (PST). Primarily produced by *Anabaena* species, they have also been reported from

Aphanizomenon and *Cylindrospermopsis (WHO 1999)*. Saxitoxins and neosaxitoxins are very stable tricyclic carbamate alkaloids (van Apeldoorn, van Egmond et al. 2007). They act as neurotoxins causing muscle paralysis by blocking neurotransmission from binding to sodium channels (Briand, Jacquet et al. 2003). Exposed animals typically show neurological effects such as nervousness, jumping, ataxia, convulsions, and paralysis, with death occurring in minutes when paralysis of respiratory muscles occurs (Briand, Jacquet et al. 2003, van Apeldoorn, van Egmond et al. 2007).

Exposure to Cyanotoxins and Harmful Algal Blooms

Animals

Bloom formation with the presence of one or multiple cyanotoxins creates hazardous environmental conditions for humans and animals alike. Recreational use of public waters and ingestion of contaminated drinking waters are the most significant exposure routes for humans and animals, respectively. Domestic animal cyanotoxicosis has been reported in cattle, pigs, horses, water buffalo, sheep, dogs, cats, and poultry, with farm-raised fish deaths also being attributed to cyanobacteria (Codd, Lindsay et al. 2005).

Ingestion is the exposure route most commonly associated with mortality in animals, but thanks to prevention and education measures in most locations, human ingestion is not commonly seen. Livestock and other animals do not avoid drinking from cyanobacterial scums or their surrounding water, which is likely cause for the numerous reports of livestock mortality in the literature (Briand, Jacquet et al. 2003). Some animals, such as dogs, may even be attracted to eating cyanobacterial surface scums due to their taste or odor (Codd, Edwards et al. 1992). These exposures to animals are sometimes easily identifiable by evidence of blue or green algae

on their coats or near their mouths (Beasley, Dahlem et al. 1989). Physical contact can be a significant exposure for some aquatic animals, such as beavers and muskrats, but is generally not as significant to livestock and domestic animals.

The regional and global impact that these toxins have on wildlife, livestock, and domestic animals is much greater than the impact on humans. Despite numerous publications describing animal and livestock illness from cyanotoxin exposure, the health risks for domestic animals is still widely ignored (Briand, Jacquet et al. 2003). Most of the described cyanotoxins act quite rapidly based on the common ingestion exposure in animals, making treatment windows very small. Artificial respiration, lavage of activated charcoal or cholestyramine, and supportive therapy may allow for recovery if provided shortly after the exposure occurred (Beasley, Dahlem et al. 1989). For livestock and domestic animals, exposures are generally not observed by the owner. Consequently, treatment is generally not given until clinical signs have already developed. At the onset of symptoms, cyanotoxins have likely passed beyond the gastrointestinal system, making lavage and supportive therapy much less likely to promote recovery.

Humans

Contrary to animal exposures, dermal exposure is the most common exposure seen in humans. Physical contact with the skin or mucous membranes can result in skin rashes and itchiness at the exposure site. Accidental ingestion of contaminated water also occurs, primarily by children. Accidental ingestion is much more cause for concern and can lead to gastrointestinal discomfort as well as more serious hepatotoxic, neurotoxic, or cytotoxic symptoms, depending on the dose and specific toxin ingested. Exposures may also occur via inhalation of aerosolized water droplets from boat wake, waves, and whitecaps that contain toxins or toxin-producing cyanobacterial cells (Gamboro, Barbaro et al. 2012). While this generally results in a much lower

dose than ingestion, it can result in respiratory problems. Recreational water activities, such as water skiing, wakeboarding, and tubing can result in these accidental ingestion and inhalation exposures. Toxicosis in humans has also been reported in rare cases involving medical processes using contaminated water supplies (Azvedo, Carmichael et al. 2002)

Regarding human exposures, currently no national guidelines exist stating a maximum acceptable cyanobacteria or cyanotoxin level within recreational waters to guide the regulation of public advisories and warnings. Many state and local governments have developed programs based on their own needs to create awareness and educate recreational water users of cyanobacterial risk, which has assisted in preventing significant human health impacts from HABs. Additionally, the noticeable appearance of HABs allows recreational users with little or no scientific training to recognize that a potential hazard exists (Codd, Morrison et al. 2005). The extent of human health problems from recreational exposures is highly underreported outside of regional health authorities due to the relatively mild illness (skin and GI irritation) associated with the common human exposures (Codd, Lindsay et al. 2005). Due to the international impact of microcystins, however, the World Health Organization has created a recommended guideline of 1µg/L of microcystin-LR in drinking water supplies based on a 44 day study in pigs exposed to drinking water containing extracts of *M. aeruginosa* containing microcystis-LR (WHO 1998, Codd, Lindsay et al. 2005, Oehrle, Southwell et al. 2010). A small number of countries, including France, Italy, Poland, Australia, Canada, and Brazil, have recognized and implemented this value into regulation, but it simply remains a guideline elsewhere (Oehrle, Southwell et al. 2010).

The WHO guideline value may be beneficial concerning drinking water supplies, which can sometimes become contaminated with cyanobacteria, but does not translate well to

environmental waters used for recreation or agricultural waters for livestock. A guideline value for recreational waters has been created by WHO, but it pertains to cell counts rather than microcystin or other toxin concentration. They have determined that 20,000-100,000 cyanobacterial cells/mL results in a relatively low probability of adverse health effects, but cell counts >100,000 cells/mL have a moderate probability of effects and should be monitored (WHO 2009). The U.S. does not federally regulate drinking or recreational water to the guideline level provided by WHO, but the Environmental Protection Agency has put microcystin, anatoxin-a, and cylindrospermopsin on the CLIII (Oehrle, Southwell et al. 2010). The CLIII is a list of unregulated contaminants that is used to prioritize data collection for certain agents to determine if regulation is required. Continued data collection on the impacts of these toxins, as well as regional public and environmental health departments, recreational facilities, and water managers providing early warning to the public prior to the development of unacceptable health risks remains the best way to maintain minimal human health impacts from exposure to HABs (Codd, Lindsay et al. 2005).

Cyanobacterial Monitoring and Detection

HAB Monitoring

Many states where HABs pose a risk have developed their own methods of monitoring and testing public use recreational waters. The Kansas Department of Health and Environment's Bureau of Environmental Health (BEH) has developed specific guidelines for the monitoring and tracking of HABs throughout Kansas. Once notified of a potential HAB by the public or local governing organization, BEH verifies the presence of the HAB and collects samples to be analyzed. Based on the results of cell counts and ELISA tests, the department will issue advisories or warnings, if necessary, and relay this information the public through news releases,

postings at the water body, and electronic media (Bureau of Environmental Health 2013). Public Health Advisory status is given to public use waters when either the microcystin is 4-20µg/L, or cell counts are within 20,000-100,000 cells/mL of any toxin-producing species, with re-tests occurring within four weeks; Public Health Warnings are more serious, however, and are issued if the detected microcystin level is ≥20µg/L or >100,000 cells/mL, and are monitored weekly until the warning status is lifted (Bureau of Environmental Health 2012). Kansas uses these methods, matching the WHO cell count guidelines, because microcystin is the predominant cyanotoxin of concern for the region. Not all states use the same methods, however, but will use different monitoring methods as well as advisory and/or warning criteria based on the risks specific for that region.

While these methods have proven to be effective for prevention of HAB exposure in Kansas public waters, agricultural waters posing a threat to livestock or domestic animals remain un-monitored by state or local government organizations. Private water monitoring is generally left to the discretion of the landowner. If a HAB is suspected on private land in the state of Kansas, water samples are sent to Kansas State University's Veterinary Diagnostic Lab for diagnosis. Just as the monitoring methods and advisory criteria differ between regions, so do the laboratory detection methods used to confirm the presence of cyanobacteria and/or cyanotoxins. Based on the risks in a region and the laboratory equipment and technicians, many different detection methods are used in determining the potential threat of HABs.

Cyanobacterial and Cyanotoxin Detection Methods

An array of techniques is available to detect the presence of harmful cyanobacteria and their toxins. These methods range from light microscopy to advanced molecular techniques used to classify strain differences within the same group of cyanotoxins. While microscopy remains

the gold standard for diagnosing cyanobacterial presence, molecular methods such as HPLC, protein phosphatase inhibition assays, flouroimmunometric assays, ELISA, qPCR, and LC/MS have proved beneficial in their ability to determine strains and specific toxins (An and Carmichael 1994, Lawton, Edwards et al. 1994, Mehto, Ankelo et al. 2001, Oehrle, Southwell et al. 2010, Al-Tebrineh, Gehringer et al. 2011). All of these methods rely upon morphological classification, chemical markers, macromolecular, immunological, and/or DNA studies (Kansiz, Heraud et al. 1999).

The methods used in diagnosis and monitoring vary by state and testing location, and frequently are paired to confirm both cyanobacterial presence as well as toxin hazards. As seen in the previous section, KDHE uses microscopy to perform cell counts, as well as an ELISA test to determine microcystin concentrations (Bureau of Environmental Health 2012, Bureau of Environmental Health 2013). Recent growth in the knowledge and classification of cyanobacterial toxins can largely be attributed to advances in analytical chemistry techniques. A summary of current diagnostic methods can be found in Table 2.2.

The two detection methods most commonly used in diagnostic labs are ELISA and liquid chromatography coupled with mass spectrometry (LC/MS), but many others have been developed and are used in cyanobacterial and cyanotoxin detection (Gamboro, Barbaro et al. 2012). Other methods of characterization exist (such as fluorescence in situ hybridization [FISH], spectroflourometry methods, and the use of 16s rRNA) but, due to a lack of widespread use or exploration, will not be described here.

Microscopy remains the gold standard diagnostic method for identifying the presence of cyanobacteria. Ranging from simple light microscopy to electron microscopy, these methods are used by trained technicians and professionals to visibly confirm the presence of different types of

live cyanobacterial cells, and may be able to observe the presence of dead cells which may also be indicative of harmful conditions. Microcystins are generally not secreted from cells, but rather released when the cells degrade, so the presence of degrading or dead cyanobacteria that have been known to produce microcystins may be a cause for concern. Microscopy is incapable of identifying cyanotoxins or differentiating between toxin-producing and non-toxin-producing strains based on the morphology of any observed cyanobacteria (Hisbergues, Christiansen et al. 2003). Light microscopy is the current diagnostic method used by the Kansas State University Veterinary Diagnostic Lab, where private samples, primarily from ponds used for livestock, are sent for diagnosis when a HAB is suspected due to water conditions or clinical symptoms in livestock. For these diagnostic purposes, the suspected water conditions or animal illness coupled with confirmed presence of known toxin-producing cyanobacteria is conclusive enough for land owners to assume toxic water conditions and avoid livestock use. Because cyanobacteria are always present in aquatic systems at harmless levels, microscopic examination of potential HAB samples is also used to make cell counts of cyanobacterial presence, with different states and testing facilities having minimum values defining potential hazards due to excessive growth. Some advanced microscopy techniques, such as electron microscopy, have proven to be inefficient as a diagnostic tool for large samples (Wright, Thomas et al. 1996). This is due to the complexity of the techniques and the time required for cell counts or statistical analyses (Mackey, Mackey et al. 1996).

Mouse and brine shrimp bioassays have been used to test the toxicity of materials from suspected HABs, but can typically only be used nonspecifically to determine toxicity (van Apeldoorn, van Egmond et al. 2007). These assays are non-specific and lack sensitivity, but are easy to perform and animals used in the assay can be examined post-mortem as an attempt to

determine the type of cyanotoxin(s) present (Stehfest, Toepel et al. 2005, van Apeldoorn, van Egmond et al. 2007). Bioassays are unable to characterize specific cyanobacteria or toxins, and other diagnostic methods must be used if data beyond toxicity is desired.

Table 2.2 Commonly used cyanobacterial and cyanotoxin detection methods.

Diagnostic Method	Used for:	Benefits	Limitations
1. Microscopy	Taxonomic ID of Whole Cells	Inexpensive Simple Live or dead/degrading cells Minimal sample preparation	Cannot diagnose toxicity Requires trained microscopist Time required for cell counts Not efficient for large studies
2. Bioassays	Determining Toxicity	Simple Dose-response data	Cannot determine toxin Requires live animals Can be labor and time intensive
3. Enzyme-Linked Immunosorbent Assay (ELISA)	Toxin Detection	Rapid and reliable Sensitive Commercial kits available	Availability of antibodies for many cyanotoxins Cross-reactivity depends on specific antibody used
4. Protein Phosphatase Inhibition Assay (PPIA)	Hepatotoxin Detection	Sensitive to sub-nanogram levels Rapid and inexpensive	Requires highly trained staff Some use radioactive materials Only used for microcystins & nodularins
5. PCR	ID Toxin Genes	High specificity and sensitivity Quantitative methods exist	Only shows potential toxin production Only used with genetically described cyanotoxins Complex sample prep Requires highly trained staff
6. HPLC	Detects Photosynthetic Pigments or Cyanotoxins	Very specific Ability to quantify Robust	Equipment and reagent cost Requires standards Requires highly trained staff Sample preparation and run time
7. LC/MS	Detects Photosynthetic Pigments and Cyanotoxins	More selective than HPLC alone Sensitive to ppb levels Robust Separates toxins in mixed samples	Equipment and reagent cost Requires standards Requires highly trained staff Sample preparation and run time High cost per sample

Another commonly used method to detect toxic cyanobacteria is the use of enzyme-linked immunosorbent assays (ELISA). ELISA is a useful and efficient tool in detecting the presence of certain cyanotoxins. The major limitation of this method comes from its dependency on the production and testing of fluorescently marked antibodies that bind to the target chemical/molecule (Rudi, Larsen et al. 1998). This method is commonly used in microcystin detection due to the more widely available antibodies for microcystin variants and the cross-reactivity of microcystin-LR antibodies. Currently, other antibodies for use in ELISA's are only available for certain variants of cyanotoxins, with very few reported for use on anatoxin-a (van Apeldoorn, van Egmond et al. 2007). This causes limitations because the cross-reactivity of the many microcystin variants depends on their similarity in structure to the specific variant from which the antibodies used in the assay were created, which is generally microcystin-LR (Mehto, Ankelo et al. 2001, van Apeldoorn, van Egmond et al. 2007). Cross-reactivity between strains may occur, but the toxicity of many cyanotoxin variants may be overlooked if they are incapable of binding to an antibody created from a different variant.

Because in most freshwater locations, such as Kansas, microcystins are the most significant toxin to public health, the use of ELISA for water testing is highly useful. This method is insufficient as a stand-alone test, however, due to its limitation to certain cyanotoxin variants and its significant over- or underestimation of some microcystin variant concentrations (van Apeldoorn, van Egmond et al. 2007). Flourometry immunoassays other than ELISA exist, but ELISA is by far the most commonly used for microcystin detection. To provide reliable, conclusive results, these assays must be used in conjunction with other methods, especially if non-microcystin producing strains and other cyanotoxins are to be identified.

Protein phosphatase inhibition assays (PPIA) have been developed as tests that can be used to detect toxic microcystin variants. Typically using recombinant protein phosphatase 1 in *E. coli*, these assays are sensitive to subnanogram levels and can analyze many samples within a few hours (An and Carmichael 1994, van Apeldoorn, van Egmond et al. 2007).Microcystins and nodularins are the only protein phosphatase-inhibiting cyanotoxins and, therefore, the only cyanotoxins that PPIA's can successfully detect. Some PPIA methods use radioactive material and require special equipment (van Apeldoorn, van Egmond et al. 2007). An and Charmichael describe detection ranges very similar to ELISA, but potential cross-reactivity and non-specific phosphatases as potential drawbacks from their PPIA methods (An and Carmichael 1994).

Polymerase chain reaction (PCR) techniques have also been used both qualitatively and quantitatively on cyanotoxins. Microcystins have been genetically described and can, therefore, be identified using these advanced analytical techniques (Al-Tebrineh, Gehringer et al. 2011). Originally derived from the genus *Microcystis*, a group of *mcy* genes coding for the microcystin synthetase complex have been identified as responsible for the production of microcystins in the genomes of producing species (Hisbergues, Christiansen et al. 2003). Assays have been developed that are capable of identifying microcystin-producing genotypes from *Anabaena, Microcystis,* and *Planktothrix*, such as a two-step PCR and RFLP (restriction fragment length polymorphism) method developed by Hisbergues et. al.(Hisbergues, Christiansen et al. 2003). Rudi et. al. developed a high-throughput PCR method utilizing paramagnetic beads for DNA separation that was sensitive to 250 cyanobacterial cells in a 0.5mL sample (Rudi, Larsen et al. 1998). The identification of cyanobacterial or toxin-producing genes is not, however, always indicative of toxicity for multiple reasons; environmental factors influence the production of microcystin in toxin-producing species, non-toxic variants of microcystin have been found,

amplification could be from old DNA present in the sample, or the organism may have lost other key genes necessary in the biosynthetic pathway of toxin production (Al-Tebrineh, Gehringer et al. 2011, Martins and Vasconcelos 2011). Consequently, additional non-genetic testing or clinical symptoms is necessary to prove toxicity.

Real-time, or quantitative, PCR (qPCR) has also been used as a means to quantify the potential toxin production within cyanobacterial samples taken from blooms. Al-Tebrineh et. al. developed a qPCR assay using SYBR-green dye for the detection of potentially hepatotoxic cyanobacteria spanning all known microcystin and nodularin producing taxa using primers for the *mcyE* and *ndaF* genes from the microcystin and nodularin synthetase complex (Al-Tebrineh, Gehringer et al. 2011). Real-time amplification provides not only a quantification of genes present, but is also capable of comparing *mcy* genotypes with use of the proper methods and dyes (Martins and Vasconcelos 2011). After determining the number of genes present, Al-Tebrineh et. al. assumed 1 copy of the respective genes per cell to provide a simple reflection of the number of organisms within a sample that are potentially capable of toxin synthesis (Al-Tebrineh, Gehringer et al. 2011). This is debatable, however, because even within potential microcystin-producing species not all are capable of toxin synthesis, and not all cyanobacteria within a bloom produce the same toxin, therefore, the assumption of 1 copy of *mcy* or *nda* genes per cell used in these methods overlooks true bloom quantity and the presence of additional toxins, and may result in poor bloom characterization. PCR methods are highly sensitive, but limited in their use on HABs by their inability to prove toxicity and the difficulty in converting gene quantities into quantities of cells that have those genes (Martins and Vasconcelos 2011). Additionally, the required concentration and isolation of DNA, and subsequent susceptibility to contamination may make qPCR methods a less than ideal HAB detection in some labs.

Technologies involving high performance liquid chromatography (HPLC) and mass spectrometry (MS) are also frequently used to classify cyanobacteria and their toxins. Although HPLC has lower detection limits than microscopy, it is not as specific in species identification (Wright, Thomas et al. 1996). Methods described using liquid chromatography (LC) and MS technologies are for the detection of cyanotoxins, or other cellular components such as chlorophyll and carotenoid pigments that can be used to characterize algal classes abundance within phytoplankton communities (Mackey, Mackey et al. 1996, Wright, Thomas et al. 1996). Both cyanotoxins and photosynthetic pigments used in HPLC can confirm the presence of potentially toxic cyanobacteria in a sample, but because both are usually found in multiple cyanobacterial genera, their use is unable to produce species-specific identification. For example, Wright et. al. describe that the interpretation of pigment HPLC is not straightforward; some pigments are unambiguous for particular classes of phytoplankton, but many are found in several classes (Wright, Thomas et al. 1996). Liquid chromatography techniques also depend on standards to perform accurate tests. This creates another potential limitation in its use, because a large number of microcystin variants exist, many of which lack the necessary commercial standards (van Apeldoorn, van Egmond et al. 2007).

Initially, LC was used as a stand-alone method for these characterizations, but because separation of chemicals can be difficult with some HPLC methods, coupling with more selective methods provided better results and reliability (Meriluoto 1997). The use of fluorescence with HPLC has been described as selective, rapid, and practical, but LC/MS methods provide more data and are more confirmative (van Apeldoorn, van Egmond et al. 2007). LC/MS methods are used today for cyanotoxin detection in both water and tissues, and are capable of identifying cyanotoxin concentrations as low as parts per billion or less (Bruno, Melchiorre et al. 2009).

Many variations of these methods exist, particularly due to the variety of mass spectrometry equipment and methods available today. Consistently reported in the literature is the use of liquid chromatography with tandem mass spectrometry (LC-MS/MS). Meriluoto describes LC/MS technologies as being more reliable than other methods because LC/MS separates different chemicals during the analysis, allowing for better classification of individual toxins, especially in mixed samples or those containing multiple toxins (Meriluoto 1997). While widely used and very reliable, the use of this method is relatively expensive (van Apeldoorn, van Egmond et al. 2007).

All of these detection methods are capable of providing some characterization to cyanobacterial communities, but they all have limitations. Microscopy is limited by its ability to only prove the presence of cyanobacterial cells while providing no proof of toxicity beyond the identification of species that are known to have toxin-producing capabilities. The limitations of ELISA are opposite of microscopy in that ELISA is only capable of chemical detection of toxins without regard to cyanobacterial presence. Furthermore, ELISA is currently limited to certain cyanotoxins because antibodies required for these assays have not yet been developed for any many cyanotoxins. Specific limitations for other advanced molecular methods such as ELISA, PCR, and LC/MS exist for specific equipment and protocols, but the major limitation to these techniques is that they all require expensive equipment and reagents, a highly trained staff, and often have extensive and timely sample preparations.

Fourier-Transform Infrared Spectroscopy

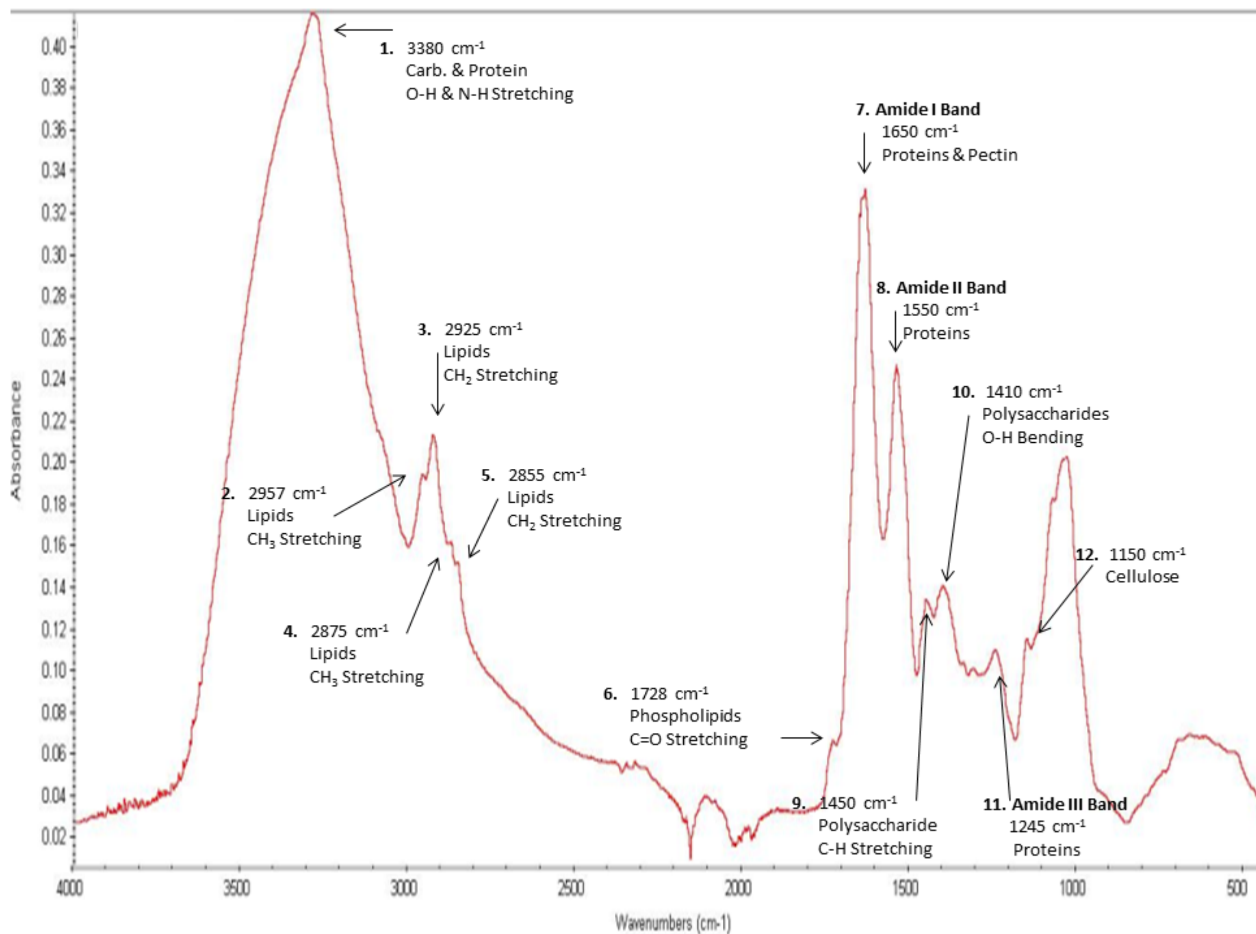
While many of the previously described methods are successful at identifying cyanobacteria and their toxins, they all share the same staff, time, and expense limitations. Fourier-Transform Infrared Spectroscopy (FTIR) is a simple, alternative method that is being

explored as a potential tool for cyanobacterial classification. Kansiz et. al. state that where microscopy fails to differentiate cyanobacterial strains or species, FTIR has the potential to do so (Kansiz, Heraud et al. 1999). Typically used for studying molecular structure, this technique has proven useful for efficient, sensitive, and inexpensive whole cell bacterial classification as well (Naumann, Helm et al. 1991, Sacksteder and Barry 2001). Sacksteder et. al. describe FTIR as a form of vibrational spectroscopy whose spectrum reflects both molecular structure and molecular environment (Sacksteder and Barry 2001). The resulting absorbance spectra acts as a unique infrared "fingerprint" for the chemical/sample and is comprised of information in the form of spectral peaks that each represent specific vibrational modes for functional groups found in a chemical or bacterial sample. The frequencies, reported in wave number (cm^{-1}), of specific peaks of cellular macromolecules are determined by the masses of vibrating atoms, the strength of chemical bonds, and by environmental factors (i.e. hydrogen bonding with solvent) (Coates 2000, Giordano, Kansiz et al. 2001, Sacksteder and Barry 2001, Stehfest, Toepel et al. 2005). Biological peaks of interest generally occur between 4000 cm^{-1} and 1000 cm^{-1}, with these peaks representing characteristic, non-overlapping protein, lipid, polysaccharide, and DNA components of a sample (Naumann, Helm et al. 1991, Kansiz, Heraud et al. 1999, Sacksteder and Barry 2001). Specific peak values and their respective molecular assignments used for cyanobacterial analysis are displayed in Figure 2.2**.**

These spectroscopy methods require a relatively small sample size, and because it can be performed on powdered, dehydrated, or aqueous samples it involves very simple sample preparation (Kansiz, Heraud et al. 1999, Sacksteder and Barry 2001). Multiple scans of a sample are used to provide the final spectrogram, with more scans providing better quality spectra. The more scans used on a sample will increase the time needed, but even a high number of scans can

be performed within minutes, providing a rapid, high quality result. The spectra can then be evaluated using basic library searches comparing spectra from identical experimental setups, or coupled with cluster analyses and dendrograms to provide further information and characterization (Naumann, Helm et al. 1991). Additionally, FTIR does not require standardization, unlike many other reliable techniques used for cyanobacterial characterization, providing an advantage over other commonly used methods (Naumann, Helm et al. 1991).

Figure 2.2 Important FTIR absorbance bands for cyanobacterial analysis.



Common FTIR wavenumbers used for examining plants, bacteria, and cyanobacteria. Each peak represents a specific molecular movement and includes the parts of functional groups from which they are derived. (Kansiz, Heraud et al. 1999, Coates 2000, Giordano, Kansiz et al. 2001, Stehfest, Toepel et al. 2005, Parikh and Madamwar 2006)

FTIR has previously been used on the bacterial level to investigate physiological effects of nitrogen deprivation on diatoms, to study nutrient related changes in various cyanobacteria and diatoms, as well as in the discrimination of cyanobacterial strains (Kansiz, Heraud et al. 1999, Giordano, Kansiz et al. 2001, Stehfest, Toepel et al. 2005, Dean, Martin et al. 2007). It has also been used to identify surface components responsible for metal absorption, but has been found to penetrate beyond the cell surface, therefore, the spectra cannot be attributed to cell surfaces alone (Jiang, Saxena et al. 2004, Pradhan, Singh et al. 2007). FTIR microspectroscopy studies of phytoplankton resulted in complex absorption spectra that provided both qualitative (via band assignments) and quantitative (via species differentiation) data on freshwater systems (Dean and Sigee 2006). These distinct patterns are highly reproducible and unique for different cells, allowing for differentiation between taxa to be performed (Kansiz, Heraud et al. 1999).

FTIR's development for the characterization of bacteria can easily be extended to other microorganisms such as virus, fungi, yeast, amoeba, and even mammalian cells (Naumann, Helm et al. 1991). Dean, Kansiz, and others have demonstrated the ability of FTIR to distinguish between cyanobacterial genera in the lab (Kansiz, Heraud et al. 1999, Dean, Martin et al. 2007). Regarding infrared spectra, however, John Coates describes that it is always helpful to study the sample as it occurs naturally, if possible, without any form of physical modification (Coates 2000). Future application of this method to environmental samples in the field has the potential to provide distinct information necessary for the characterization of HABs. If this spectroscopic technology proves reliable at distinguishing cyanobacteria from other phytoplankton and plant species, it may act as a rapid and reliable diagnostic tool that is not limited by the staff, time, and expense restraints seen with other detection methods.

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Chapter 3 - Classification of Potentially Toxic Cyanobacterial Blooms by Fourier-Transform Infrared Technology

Introduction

At least 2,654 species of cyanobacteria have been classified worldwide (Koksharova 2009). These cyanobacteria, or blue green algae, are regularly found in water systems across the world, and many are responsible for the release of cyanotoxins that are a health risk to animals and humans alike. Cyanobacteria are Gram negative photosynthetic prokaryotes that, unlike other typical pathogens, are unable to colonize or grow inside of animal and human hosts to cause disease, but rather, their presence in water bodies and release of toxins into water systems is how they are capable of posing hazards to health (Codd, Morrison et al. 2005, Gamboro, Barbaro et al. 2012). In fresh water bodies, favorable conditions can allow cyanobacteria to form large colonies known as blooms, and due to the buoyancy of some species they are often seen as bright blue-green scums on the water surface. These blooms may contain one dominant species or several genera of cyanobacteria, with both situations capable of producing more than one type of cyanotoxin (Oehrle, Southwell et al. 2010). Not all blooms are toxic, however, and while the release of cyanotoxins from blooms can be dangerous to animals and humans, the environmental parameters that influence the internal dynamics of these cyanobacterial communities and their toxin production remain poorly understood (Al-Tebrineh, Gehringer et al. 2011, Martins and Vasconcelos 2011).

Several types of cyanotoxins are produced by blue green algae and fall into three significant categories, including the cyclic peptides such as hepatotoxic microcystins, the alkaloid neurotoxins, and the lipopolysaccharides (Codd, Morrison et al. 2005, Gamboro,

Barbaro et al. 2012). Microcystins are very stable cyclic peptides; they are the most significant cyanotoxin worldwide and are the most commonly detected cyanotoxin in freshwater environments (Mackey, Mackey et al. 1996, Lowe, Souza-Menezes et al. 2012, Marie, Huet et al. 2012). Over 70 variants of microcystins have been characterized; they are known hepatotoxins and have also been shown to be tumor promoters and possibly carcinogenic to humans (Bruno, Melchiorre et al. 2009, Koksharova 2009). Aside from liver damage from microcystin exposure, other significant types of blue-green algae toxicosis include peracute neurotoxicosis from anatoxins and saxitoxins, and gastrointestinal disturbances from the less significant lipopolysaccharide toxins (Beasley, Dahlem et al. 1989). Cyanotoxins are of concern to human health if they are present in consumed water. This ingestion hazard has led to the World Health Organization setting a guideline of 1µg/L of microcystin in drinking water and cell count guidelines for recreational use waters warning of moderate health risks when greater than 100,000 cells/mL (Codd, Morrison et al. 2005, Oehrle, Southwell et al. 2010). This value is only a guideline, however, and many countries including the United States do not regulate drinking or recreational water to this level.

Cyanotoxins can also have a severe effect on domestic animals and livestock that drink from private lakes and ponds that may not be regulated or monitored for the presence of cyanobacteria and their toxins. Livestock and other animals do not avoid drinking from cyanobacterial scums or their surrounding water, which is likely cause for the numerous reports of livestock mortality in the literature (Briand, Jacquet et al. 2003). Some animals, such as dogs, may even be attracted to eating cyanobacterial surface scums due to their taste or odor (Codd, Edwards et al. 1992). Toxic cyanobacterial blooms also create a public health risk to recreational users of public waters. While accidental ingestion does occur, primarily in children, the primary

exposure seen in humans is physical/dermal contact. Gamboro et. al. also describes that cyanobacterial cells and toxins can be aerosolized by boating or whitecaps and may pose a more indirect exposure threat to recreational lake users (Paddle 2003, Gamboro, Barbaro et al. 2012). Since these cyanobacterial toxins have the potential to cause severe illness and even death to animals and humans, efficient characterization of potentially toxic cyanobacterial blooms is needed to assist in the monitoring of environmental waters used by livestock or for recreation.

Many methods are currently used for the detection of cyanobacteria and their toxins, ranging from microscopy to chemical marker detection to genetic amplification and sequencing. Microscopy can be a relatively inexpensive method to identify cyanobacterial cells from water samples, and remains the standard diagnostic method compared to others. Some microscopy methods, such as electron microscopy, are impractical for large samples that require more efficient detection methods (Wright, Thomas et al. 1996). Two other detection methods generally employed are ELISA and Liquid Chromatography/Mass Spectrometry (LC/MS) to detect cyanobacterial toxins (Gamboro, Barbaro et al. 2012). ELISA has allowed for the detection of microcystins for which antibodies have been synthesized, but its use for other cyanotoxins is limited. This limitation is due to the need for specific antibodies for each bacteria or toxin of interest to be produced and tested (Rudi, Larsen et al. 1998). Regarding the use of liquid chromatography, Mackey et. al. have previously developed a program for calculating algal class abundances from measurements of chlorophyll and carotenoid pigments by HPLC (Mackey, Mackey et al. 1996). LC/MS methods are limited, however, since some pigments are unambiguous for certain classes, but many are present in several classes (Wright, Thomas et al. 1996). More advanced mass spectroscopy methods have been described for cyanotoxin detection that overcome some of the drawbacks of conventional LC/MS methods, i.e. response to

individual congeners, sensitivity between analyses, and linearity of the standard curves by the use of smaller column media and tandem mass spectrometry (Oehrle, Southwell et al. 2010).

More recent methods utilizing polymerase chain reaction techniques (PCR, qPCR) have been developed to detect the presence of microcystin synthesizing genes, such as mcy, that are present in cyanobacterial species capable of producing the microcystin toxin (Al-Tebrineh, Gehringer et al. 2011, Martins and Vasconcelos 2011). Identification of these genes is not always indicative of toxicity though, because qPCR is capable of determining the number of genes present, giving only a reflection of the number of organisms potentially capable of synthesizing microcystin (Al-Tebrineh, Gehringer et al. 2011, Martins and Vasconcelos 2011). This potential capability is important to acknowledge because, as previously stated, the environmental parameters influencing toxin production remain highly unknown, so the presence of toxin-producing genes such as mcy is not always indicative of current toxin production.

Protein phosphatase inhibition assays have also been developed for the detection of microcystins (An and Carmichael 1994). These methods are strictly limited to testing the mechanism of action for microcystins, however, and cannot be used for other cyanotoxins. While many of these methods are successful at identifying cyanobacteria and their toxins, they require highly trained staff, expensive equipment and reagents, and time consuming sample preparations.

Another method that has proven useful for efficient and inexpensive bacterial characterization is Fourier-Transform Infrared Spectroscopy (FTIR). Sacksteder et. al. describe FTIR as a form of vibrational spectroscopy whose spectrum reflects both molecular structure and molecular environment (Sacksteder and Barry 2001). FTIR has previously been used to investigate physiological effects of nitrogen deprivation on diatoms, to study nutrient related changes in various cyanobacteria and diatoms, as well as in the discrimination of cyanobacterial

strains (Kansiz, Heraud et al. 1999, Giordano, Kansiz et al. 2001, Stehfest, Toepel et al. 2005, Dean, Martin et al. 2007). FTIR microspectroscopy of phytoplankton results in complex absorption spectra that provide both qualitative and quantitative data on freshwater systems (Dean and Sigee 2006). The infrared spectra of cells produced by FTIR techniques give distinct patterns that are highly reproducible and unique for different cells, allowing for differentiation between taxa to be performed (Kansiz, Heraud et al. 1999). These infrared spectra are very complex, resulting from contributions of all cellular macromolecules, and because the exact frequency of each vibration depends on the strength of the bonds involved, the mass of the component atoms, as well as the chemical environment of the molecular groups, they create very characteristic and distinct vibration spectra for a given sample (Giordano, Kansiz et al. 2001, Stehfest, Toepel et al. 2005). Here, the practical use of FTIR technology as a rapid, reliable, and portable method to characterize and differentiate potentially harmful cyanobacteria from other forms of green algae in environmental samples is described.

Methods

Sample Collection and Preparation

Samples used were diagnostic samples from the Kansas State University Veterinary Diagnostic Lab (KSUVDL) from August of 2011 through September of 2012. These water samples were sent to the diagnostic lab from various locations throughout the Midwest that were suspected of containing toxic cyanobacteria (Figure 3.1). Additional non-diagnostic samples were collected from water bodies near Manhattan, KS. All water samples were examined under light microscope to determine the presence of cyanobacteria. All microscopy samples diagnosed as positive for the presence of cyanobacteria, as well as green algae and select negative samples were then used for the FTIR study.

Figure 3.1 Suspected cyanobacterial bloom material received at KSUVDL.

A small amount of the sample was filtered through Watman #4 filter paper to separate solid material from the water in the sample. Each filter containing sample was appropriately labeled with the sample number and microscopy diagnosis of the sample, then left to air dry overnight (Figure 3.2). Two data sets (OMNIC and OPUS) were analyzed based on the two different spectrometers used, with both consisting of 46 initial samples generated from the microscopically examined KSUVDL samples. Another 20 samples were prepared and labeled with only the letters A through T, as seen in Figure 3.2, and were used for a secondary analysis. This sample set contained various cyanobacterial and green algae samples, as well as other types

of green material similar in appearance to cyanobacteria when placed on filter paper, such as latex paint and pureed grass or leaves. From this set of 20 samples, 8 cyanobacterial and green algae samples were later added to the OMNIC data set, resulting in a total of 54 OMNIC samples and 46 OPUS samples.

FTIR Spectroscopy

The attenuated total reflectance IR spectrum of each sample was measured using both a Bruker Alpha-P (Billerica, MA), and a Nicolet 6700 (Thermo Fischer, USA) FTIR spectrometer (Figure 3.3, Figure 3.4). Absorbance spectra were measured over the wavenumber range of 4000-400 cm-1 with a spectral resolution of 4cm-1 on both spectrometers. The absorbance was measured over 24 and 32 scans on the Bruker and Nicolet, respectively.

The crystal aperture surface was cleaned with isopropanol and dried, followed by an uncovered crystal background run prior to the scanning of sample sets. Each sample was run dry on the Watman #4 filter paper, without any medium or coverslip, and with the samples oriented to completely cover the aperture crystal. All of the 46 initial samples for the primary analysis from both data sets were run and recorded in triplicate on both the Nicolet and Bruker instruments, creating the two separate data sets. Twelve samples were initially scanned once per sample in August of 2011 using only the Nicolet 6700, and were then included again in the group of 46 that was scanned in triplicate during 2012 to examine effects of degradation. The 20 samples used for the secondary analysis were each scanned once per sample using only the Nicolet 6700.

Figure 3.2 Select Samples Prepared for FTIR Spectroscopy

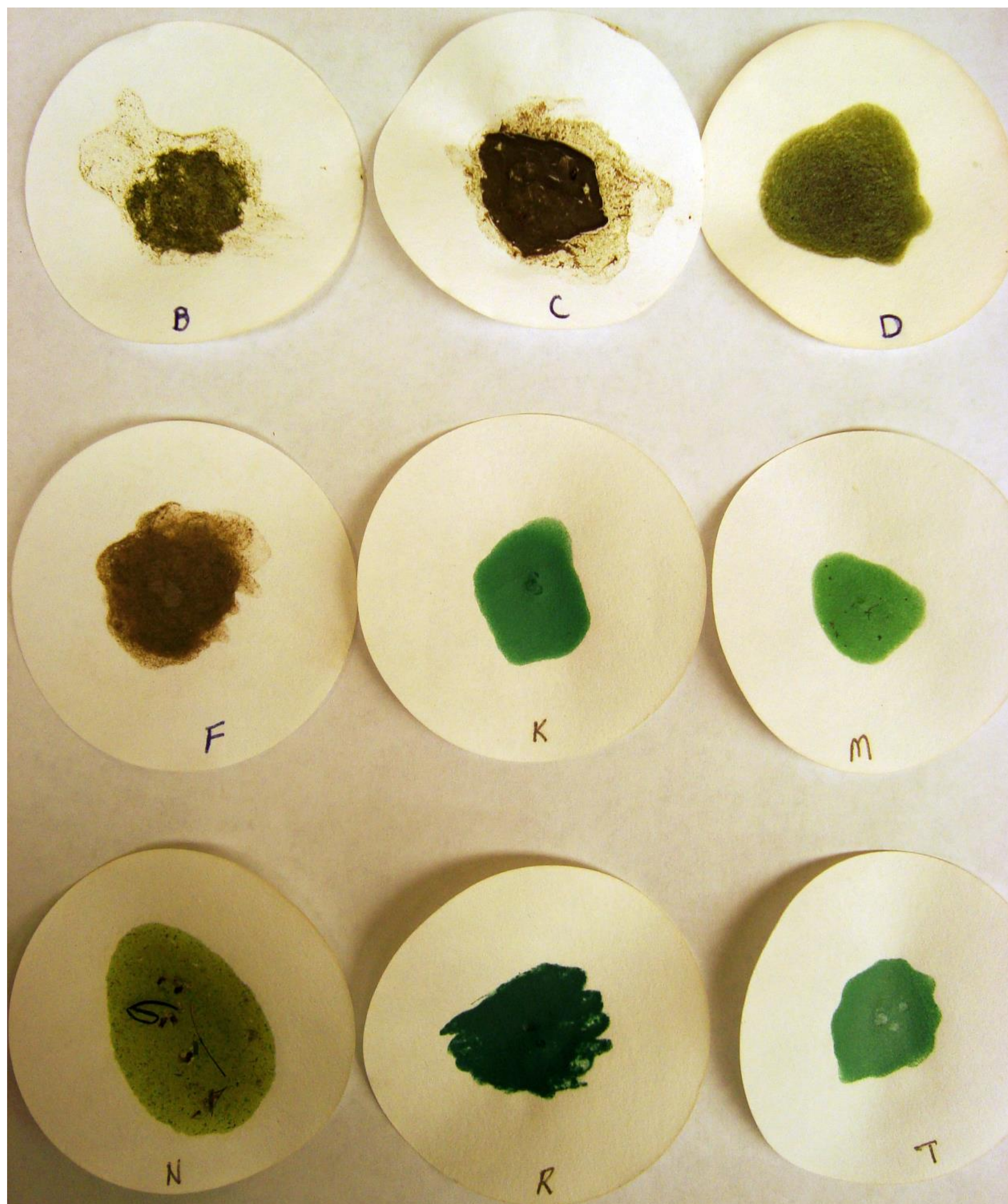


Figure 3.3 Bruker α-P with sample loaded.

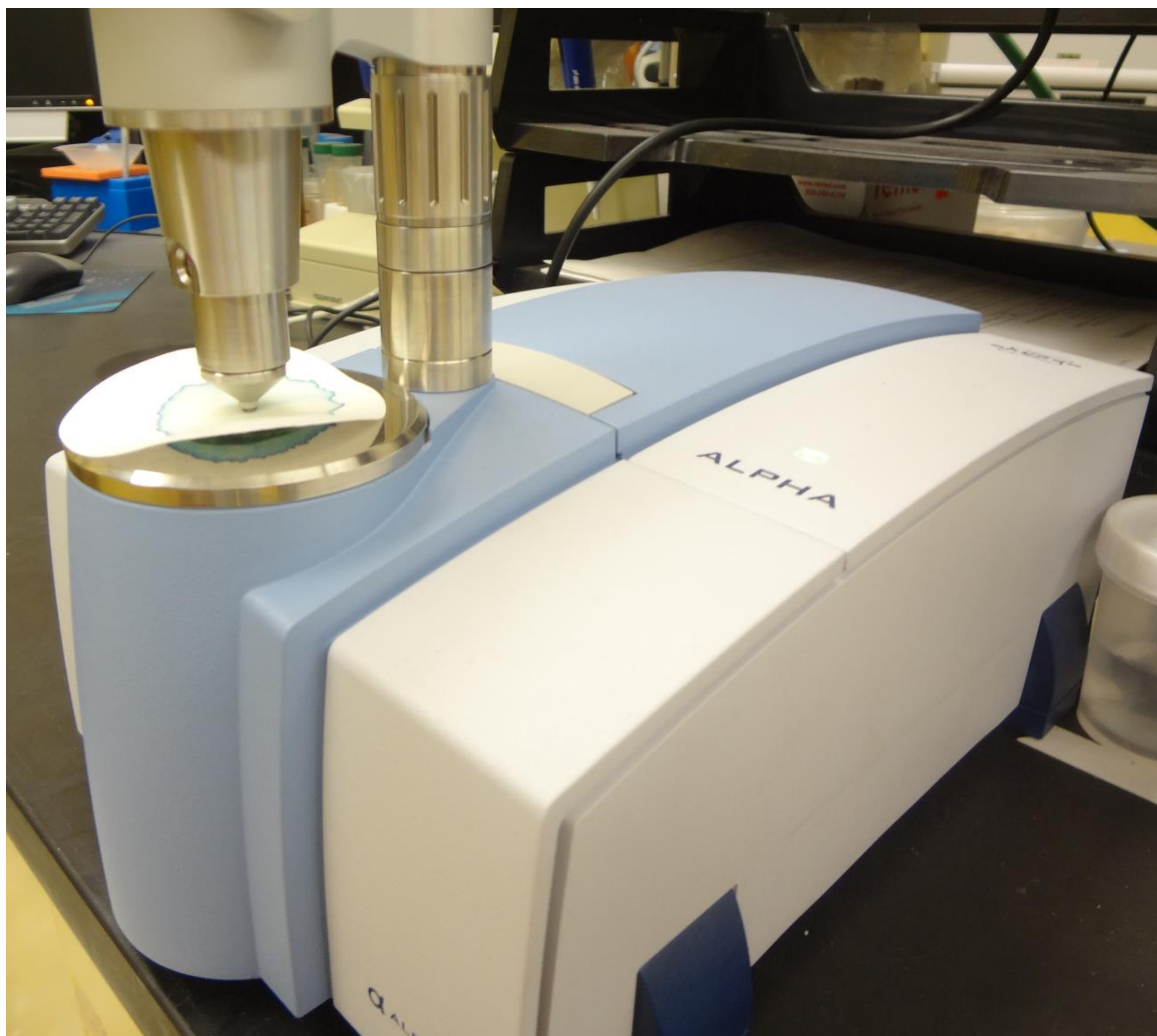
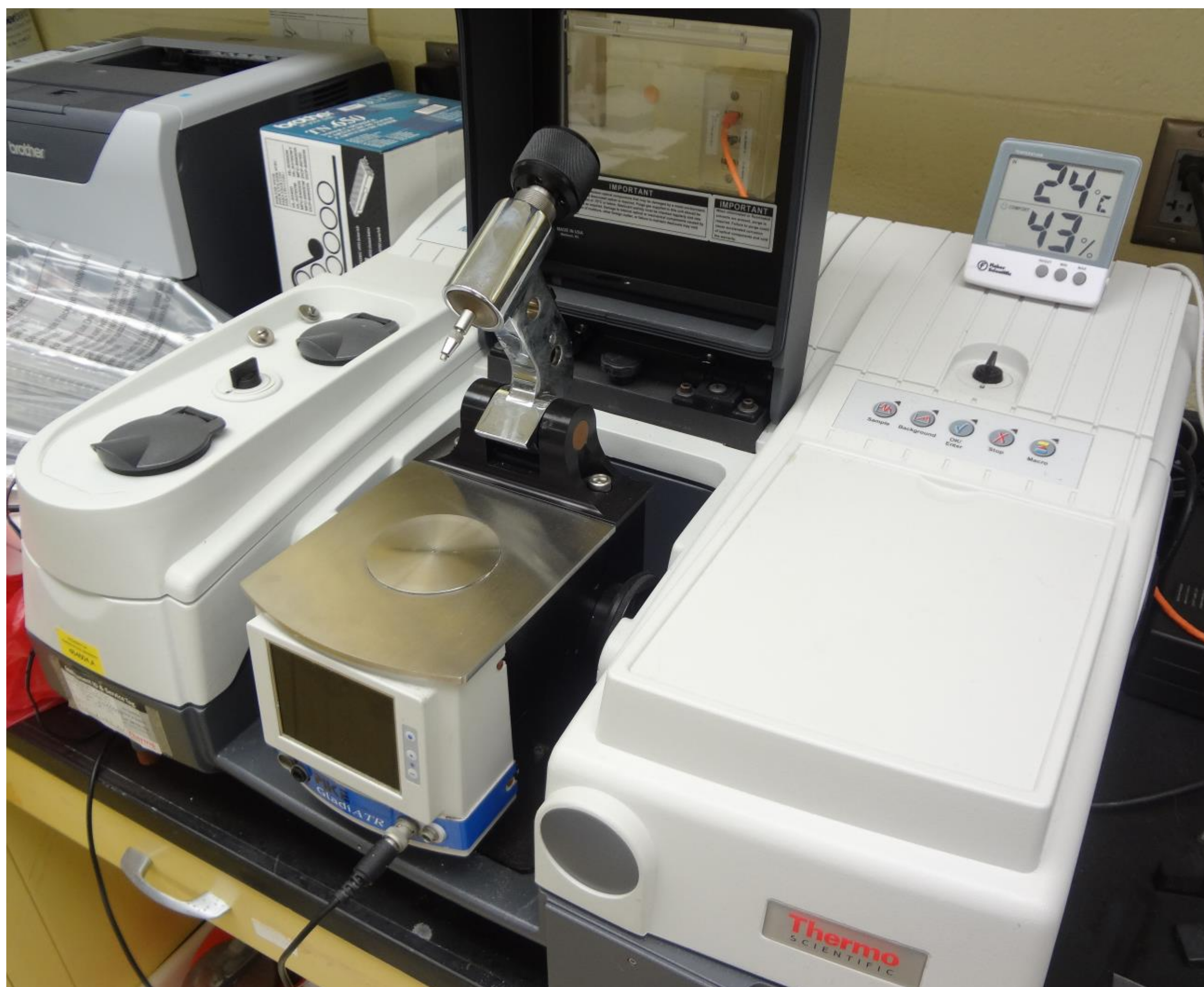


Figure 3.4 Nicolet 6700.



Software Algorithm Screening

Known high quality samples containing only a single genus of Microcystis, Aphanizomenon, Anabaena, Planktothrix, or green algae were added to default spectral libraries present in both Nicolet's OMNIC (Thermo Fischer, USA) and Bruker's OPUS (Billerica, MA) software programs. These environmental samples were used as controls in an attempt to maintain the same conditions as regular samples while being tested, such as natural lake/pond water nutrients and constituents, as well as an absence of laboratory reagents used to create optimal growth conditions in-house that may lead to cyanobacterial development that differs from natural conditions. All of the samples added to the libraries were clean and free of debris, making them sufficient environmental sample controls to be screened against in the software libraries.

Each triplicate scan for all samples was individually screened against these libraries and a list of the closest related library spectra was generated based on the default software algorithm settings from each software program. The OMNIC software default algorithm is a spectral correlation of wavenumbers 2600-400, while the OPUS software performs correlation on the entire spectrum (4000-400 cm^{-1}). Removal of three negative/debris samples that were neither green algae nor cyanobacteria resulted in a total sample size of 43 for both the OMNIC and OPUS data sets, 31 of which contained only one genus. The top two spectra generated and their calculated match values for each sample were then recorded and used in the analysis.

The twelve repeated samples in the OMNIC data set were used in a separate algorithm screening that was compared to the results of the same samples scanned in 2011. The final group of 20 samples labeled A-T was screened in the same manner, but to test the simplicity of the method they were scanned and screened by a lab member unassociated with the project.

Principle Component Analysis

To acquire the necessary data for multivariate spectral analysis, all spectra scanned and stored using the Bruker Alpha-P's OPUS software were transferred and analyzed using Nicolet's OMNIC software. Using the OMNIC software, 12 peaks between 3280 and 1150 cm^{-1} were chosen for statistical analysis (Figure 3.5). Raw peak absorbance values were recorded for all clearly defined peaks within the samples. These values from all sample triplicate scans, from both the Bruker Alpha-P and the Nicolet 6700, were normalized to the Amide I band and individually analyzed on their normalized peak height values using MATLAB® 7.7.0.471 (The Mathworks Inc., MA). Samples containing only a single genus were separated from mixed cyanobacterial samples for principle component cluster and dendrograms analyses. Poor quality sample spectra were also eliminated prior to these analyses based the raw absorbance intensity of the Amide I band. OMNIC spectra with an Amide I peak absorbance of >0.10 and OPUS spectra >0.06 were eliminated from the data sets, resulting in 40 single-genus OMNIC and 31 single-genus OPUS samples to be analyzed.

Principle component cluster analysis was performed on MATLAB software using all FTIR spectra (including all triplicates) for each data set. After application of the described exclusion criteria, the OMNIC data set used a total of 107 spectra including the twelve 2012 repeat spectra, and the OPUS data set used a total of 91. Because only clearly defined peaks were recorded from the FTIR spectra, there were some absent peak values within the data sets for wavenumbers in which peaks were present but masked by adjacent peaks and molecular influences that occur from neighboring molecules present in the complex whole organism samples. To prevent the software from using a value of zero for these peaks and skewing the data, absent peak values were replaced with the average value of that peak for each genus. The only time this method was not applied was for two peaks (peak 2 and 6) within the green algae

samples in which it was clearly visible that no peak was present (Figure 3.6). These two peaks, for green algae samples only, were allowed a value of zero due to no peak presence.

Figure 3.5 Absorbance peaks used for spectral analysis.

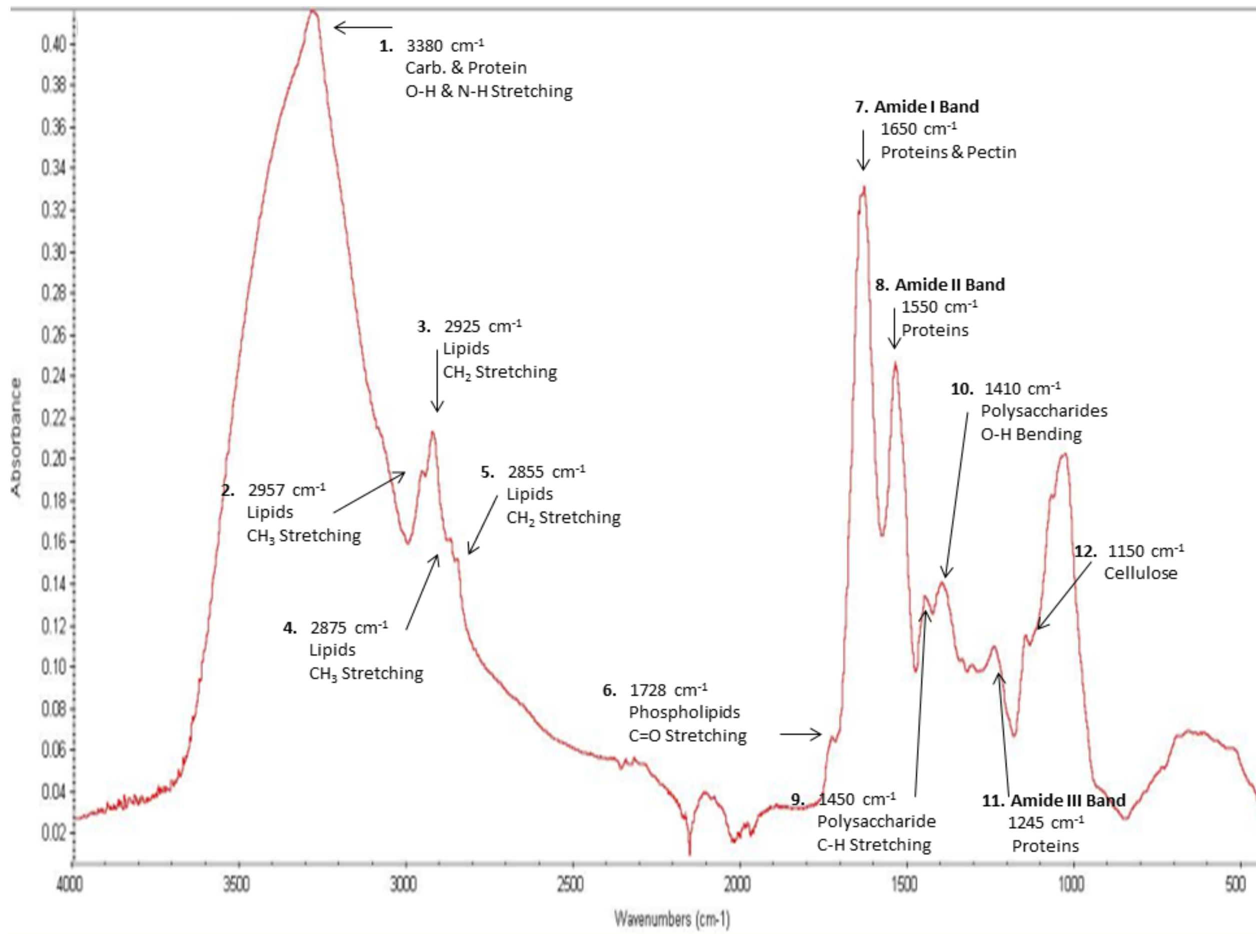
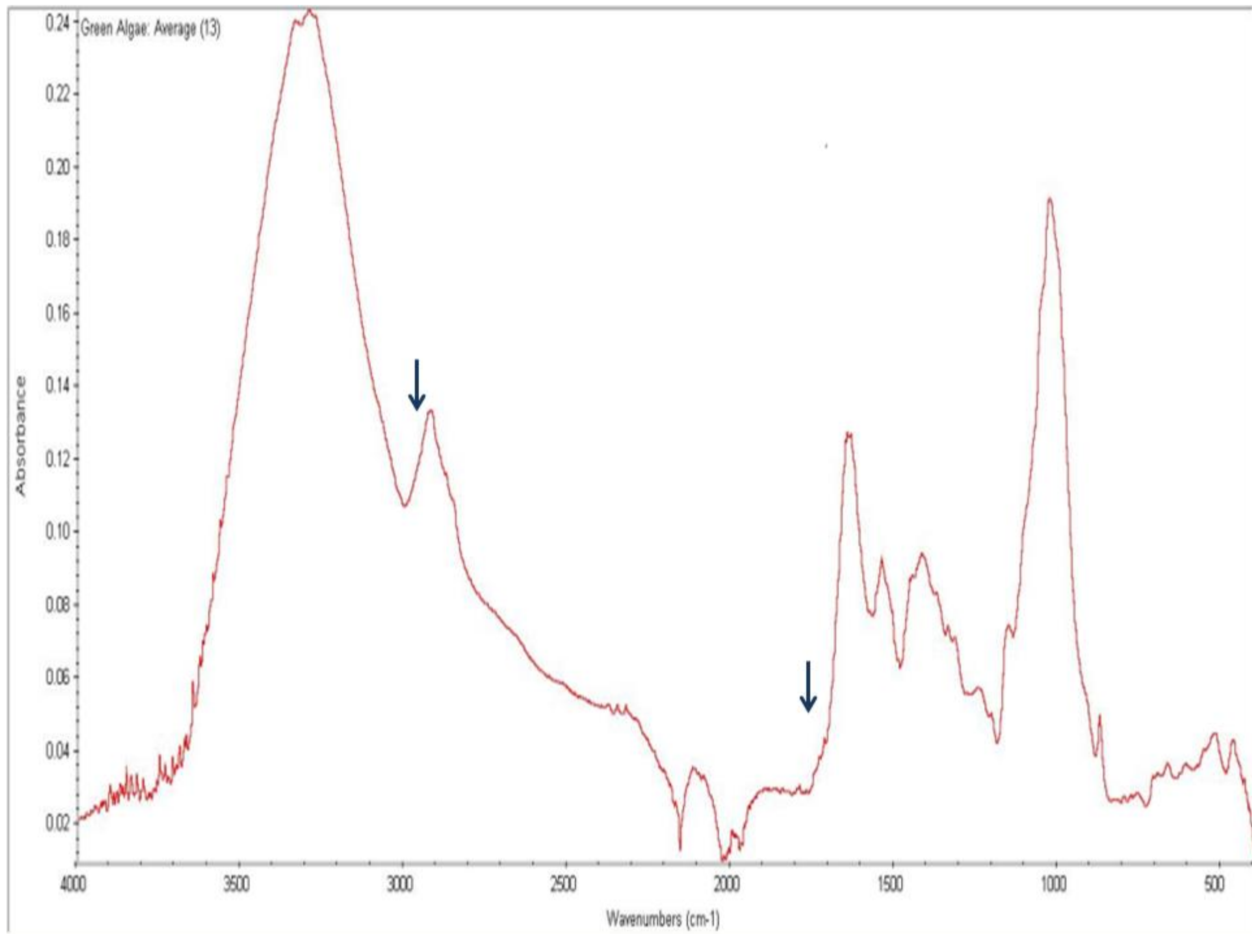


Figure 3.6 Green Algae average absorbance spectrum showing absence of Peak 2 and 6



Dendrograms

MATLAB was also used to create dendrograms using average Euclidean distance hierarchical clustering for each set of data to express the dissimilarities between the spectra. Average spectra were generated for each genus in both data sets to create dendrograms expressing the differences between the five cyanobacterial genera and green algae. These average spectra, however, do not express any variation seen within the samples of each genus. To observe this variation, dendrograms were also created for the each whole sample set using a single spectrum from each sample.

Results

Cyanobacterial Discrimination from Green Algae

Using the previously described environmental samples as controls, both software programs were highly successful at identifying the cyanobacterial and green algae samples through the standard library screening algorithms. To be considered a success, both of the top two matches for all three triplicate spectra for a sample had to be correctly identified as cyanobacteria by the software. The green algae samples required the same match requirements for all three triplicate spectra, but were matched to the green algae control. The only exceptions to this criteria, for both cyanobacteria and green algae, was if the second library match was notably low (>100 match value) compared to the first library match, signifying a much poorer spectral correlation.

The OPUS software correctly classified 36 of the 43 total samples (83.72%) and 29 of the 31 single genus samples (93.55%). The OMNIC software provided very similar results with 35 of 43 total samples (81.40%) and 28 of 31 single genus samples (90.32%) correctly classified. Percent agreement between the two spectrometers and their software packages was 89.13%. The 12 OMNIC samples that were re-scanned after one year had a 100% match with the results of the initially scanned samples. The same ten of the 12 samples were correctly classified in both 2011 and 2012.

Of the 20 samples (A-T) run on the Nicolet 6700, all 10 cyanobacterial samples and 3 of the 5 green algae samples were correctly classified. The remaining 2 green algae samples contained high debris and mud, and were not classified as either cyanobacteria or green algae by the library search algorithms. The remaining 5 samples consisted of green latex paint, a high density protist phytoplankton sample, a health food drink containing algae, pureed grass, and

pureed leaves. Of these 5 samples, only the health food drink and pureed grass had top matches with green algae from the spectral library, and none matched with any of the cyanobacterial genera.

Principle Component Cluster Analysis

Cluster analysis showed clear differentiation of cyanobacterial samples from green algae using the first three principle components determined by MATLAB software. For both the OMNIC (Figure 3.7) and OPUS (Figure 3.8) data sets, a tight clustering of cyanobacterial samples was seen with all green algae samples in a clearly different loose cluster. One sample initially labeled as green algae, however, was later found to be a species of protist phytoplankton rather than green algae. This sample was labeled as “other phytoplankton”, and due to its closer relation to cyanobacteria, was not separated from the cyanobacterial cluster seen in the OMNIC data set, but was differentiated to a small degree in the OPUS data set.

Dendrograms

Both the OMNIC and OPUS data sets resulted in dissimilarities between samples and genera that clearly distinguished cyanobacterial genera from green algae (Figure 3.9 & 3.10). Dendrograms created using average spectra for each genus expressed dissimilarities of small magnitudes between each cyanobacterial genera and a much higher magnitude of difference between the cyanobacteria and green algae.

Dendrograms created on the whole data sets were consistent with those using the average spectra in regards to cyanobacterial and green algae differentiation. For both data sets, all green algae samples were grouped together and showed a large order of magnitude of difference between the cyanobacterial samples. The dissimilarities between the cyanobacteria and the

“other phytoplankton” sample, however, were not enough to clearly differentiate it from the cyanobacterial samples in either data set.

Figure 3.7 OMNIC 3D principle component cluster analysis.

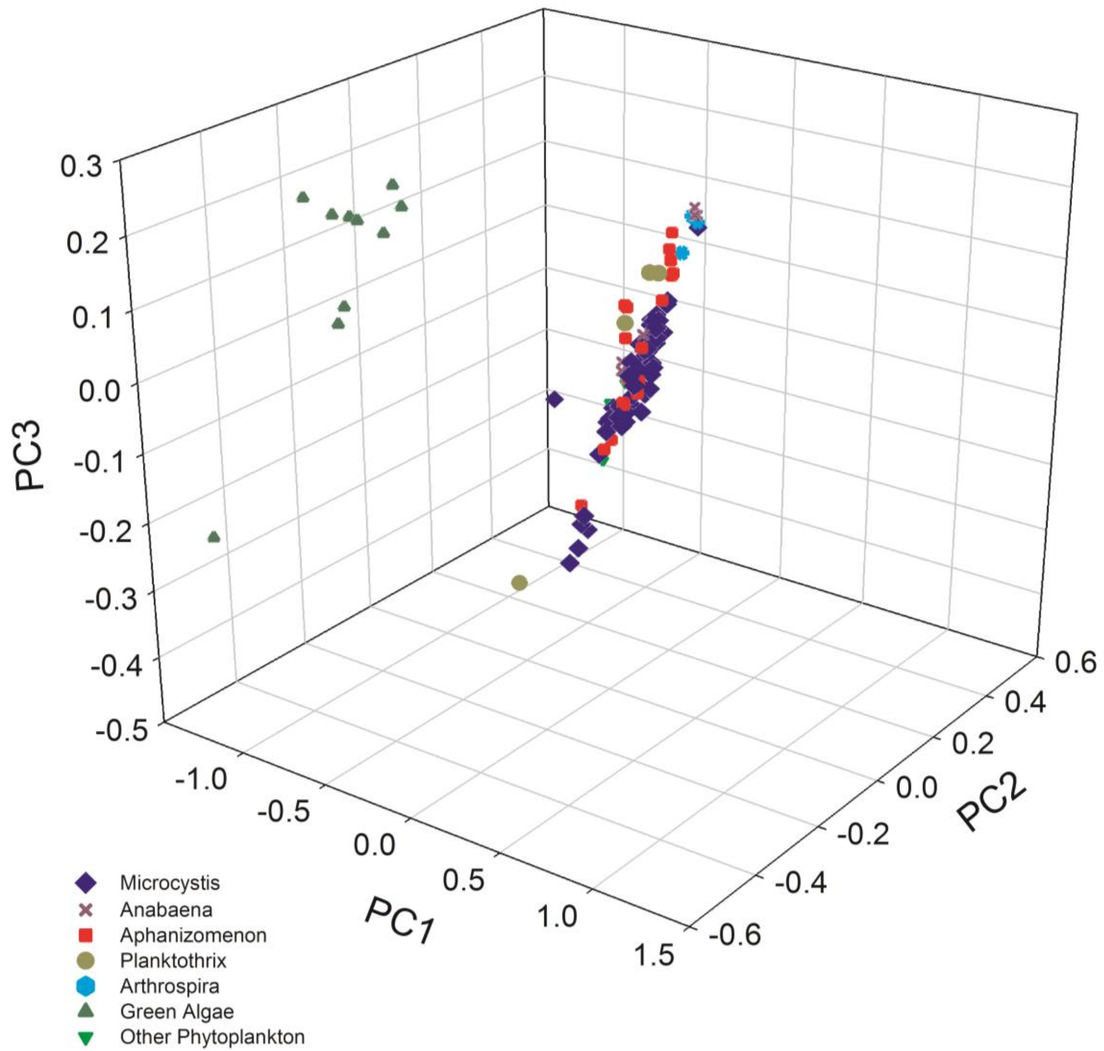


Figure 3.8 OPUS 3D principle component cluster analysis.

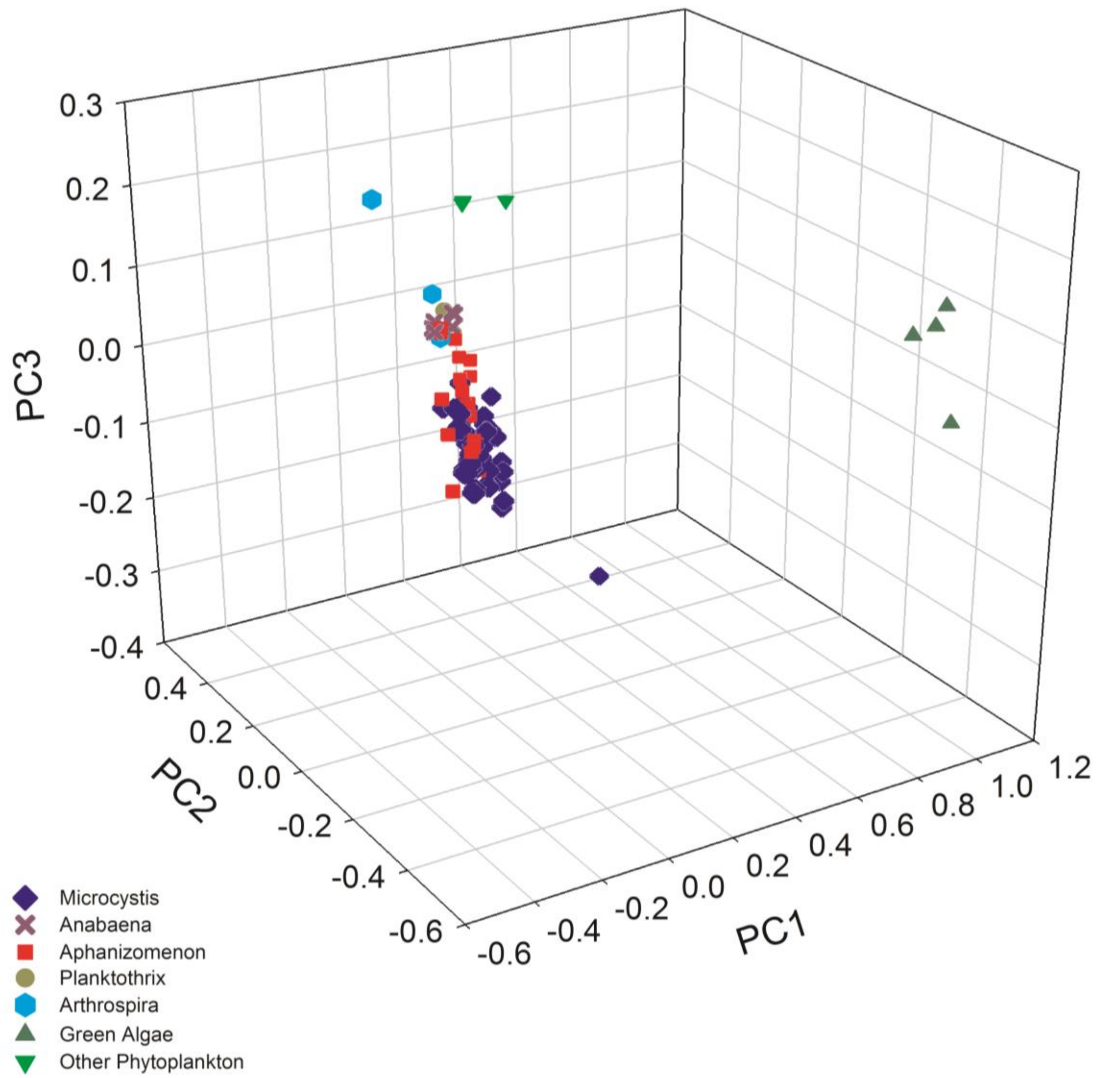
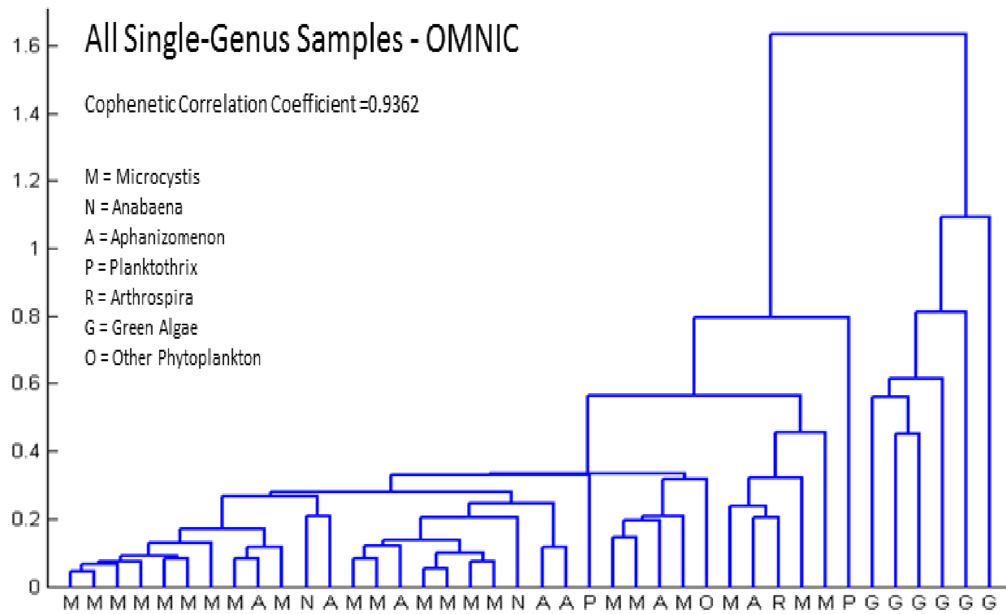


Figure 3.9 OMNIC Dendrograms



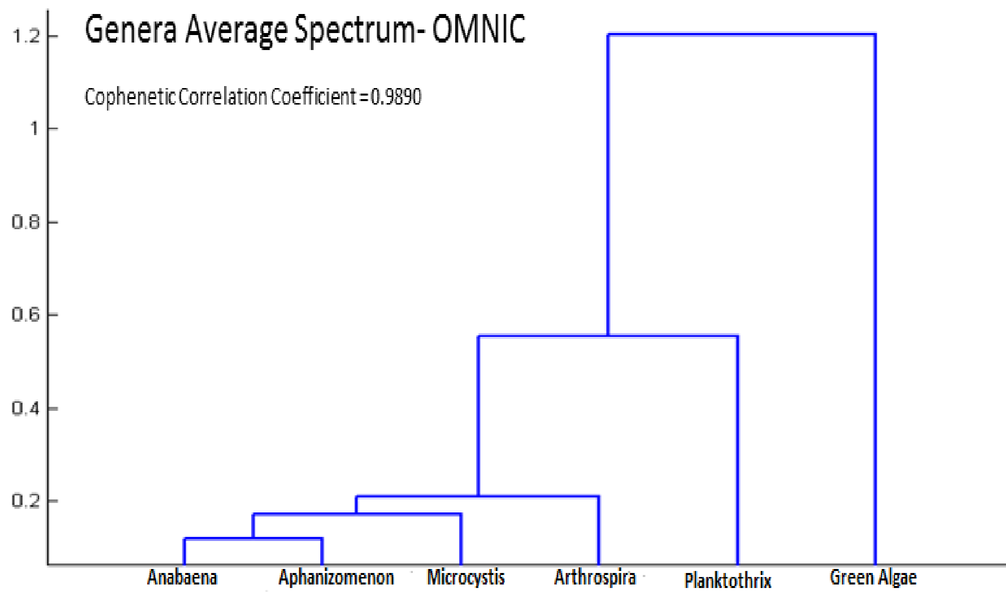
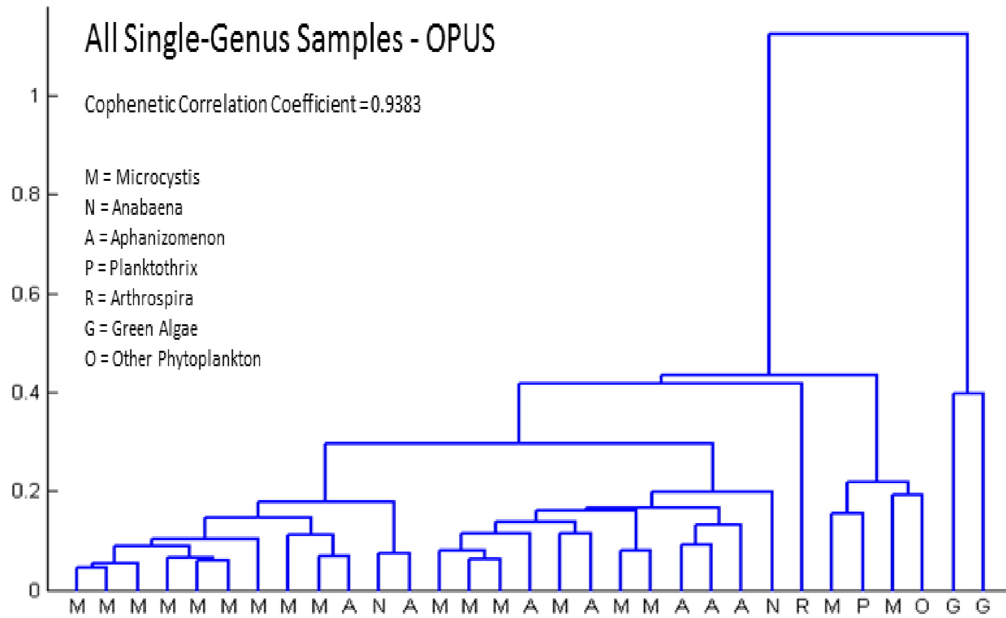
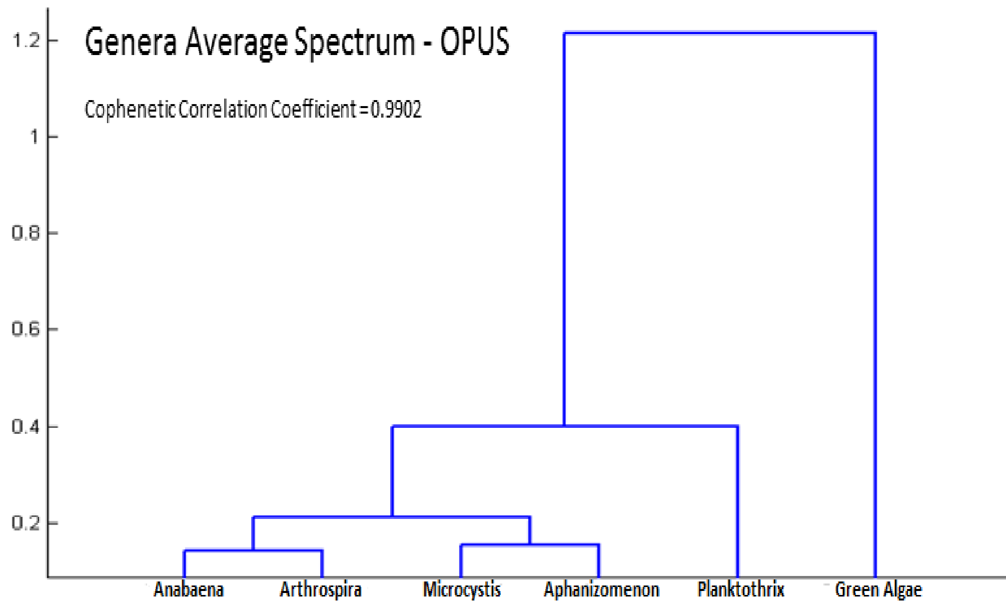


Figure 3.10 OPUS Dendrograms





Cophenetic correlation coefficients were recorded to verify that the dissimilarities expressed in the dendrograms are true representations of the original dissimilarities in the sample data. Dendrograms created from the average peak spectra resulted in high cophenetic correlation coefficients of 0.9890 and 0.9902 for the OMNIC and OPUS data sets, respectively. The values were slightly less for the whole sample set dendrograms, with the OMNIC data set cophenetic correlation coefficient being 0.9362 and the OPUS data set being 0.9383.

Discussion

Due to the nature of environmental samples used in this study, sample quality is inevitably variable. To show the true, practical use of the FTIR default software algorithms, sample exclusion criteria were not applied prior to the default algorithms screening, and samples of all quality were included. Therefore, many unsuccessful classifications were a result of poor sample quality, such as low cell density or samples containing environmental debris. The reproduced results seen between the 12 samples that were re-scanned one year after their initial scan, however, shows that any degradation occurring within the sample once it has dried on the filter paper does not negatively influence the quality enough to influence the method's capability.

The default software algorithms used were successful at differentiating between cyanobacterial samples and green algae, but were not found to be successful at characterizing specific cyanobacterial genera. While Dean and Kanzis both report similar methods being successful at species differentiation, our methods used on environmental samples have thus far not proved to be practical (Kansiz, Heraud et al. 1999, Dean and Sigee 2006).

More advanced analyses of spectral data using MATLAB demonstrate the true capability and reliability of FTIR analysis in cyanobacterial characterization. The principle component cluster analysis and dendrograms were highly successful at differentiating between potentially

toxic cyanobacterial samples and green algae on spectra from both spectrometers and software packages. Even with the reliable qualitative and quantitative data that these methods provide, FTIR as a cyanobacterial characterization tool must be performed in conjunction with other methods capable of determining toxicity if accurate HAB characterization is desired.

Conclusion

This research demonstrates that Fourier Transform Infrared technology can be used in a relatively simple fashion to determine the presence or absence of potentially toxic cyanobacteria in environmental algal samples. Given relatively clean samples, the use of the default software algorithms provided with the FTIR equipment described above is an inexpensive, rapid, and reliable method of algal classification. While these methods cannot be used independently to characterize HABs, they do show the potential and practicality of FTIR as a screening tool. The use of FTIR as an initial screening tool in large-scale cyanobacterial studies can reduce the time and cost of examining a large number of samples using other common methods. Further manipulation of spectral data is necessary to acquire more quantitative data and allow genus or species differentiation in environmental samples. Even without the capability of genus- or species-specific differentiation, however, FTIR is capable of being an important tool in animal and human health by efficiently classifying the potential toxicity of environmental waters.

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Chapter 4 - Conclusions and Future Work

The ability of FTIR to clearly distinguish cyanobacterial genera from green algae and other phytoplankton makes it a valuable tool in HAB classification. The methods described here overcome many limitations of other diagnostic methods; they are not limited to specific cyanobacterial genera or species, they are simple and require minimal staff training and labor, they are relatively cost-effective, they do not depend on antibody cross-reactivity or standards, and most importantly they are reliable across equipment, software, and most sample qualities. A major limitation of FTIR methods, however, is that they do not provide data on toxicity, but rather it is always assumed with the presence of toxin-producing genera. This makes FTIR inefficient as a stand-alone diagnosis for water samples, but does not make it invaluable as a diagnostic tool.

Coupled with toxin-detection methods such as ELISA, HPLC, or LC/MS, FTIR can act as valuable screening tool in a diagnostic or research laboratory. The specificity of the FTIR methods described here can screen for non-cyanobacterial samples, reducing the number of samples run on the more sensitive and toxin/species-specific methods. This will save time and money from being spent on running costly toxin-detection methods for non-cyanobacterial samples. Additionally, FTIR is advantageous compared to microscopy and cell count methods that are commonly coupled with the toxin-detection techniques. FTIR is less labor intensive than microscopy and cell counting, and requires less time, especially for large-scale studies. Due to the sample quality limitations observed in this study, low cell density samples and samples containing a large amount of environmental debris may still require microscopic examination if FTIR does not provide a conclusive result.

Previous work has been done on laboratory grown and laboratory collected specimens that was capable of genus and species differentiation. Further manipulation of the methods described here is necessary to increase the specificity and sensitivity to levels capable of genera differentiation. The ability to classify cyanobacterial genera in positive samples will also allow more efficient toxin-specific testing when FTIR is used as a screening tool. Coupling the results and methods described here with pure laboratory grown species may provide more insight into method adjustments that would allow genus- or species-specific identification of cyanobacteria in environmental samples.

Additional research is also being done to examine whether or not toxin-specific testing can be performed using the same air dried sample prepared for the FTIR analysis. This would further streamline the diagnostic process by eliminating an extra sample preparation step and decreasing the amount of sample necessary for a complete diagnosis.

This research demonstrates that Fourier Transform Infrared technology can be used in a relatively simple fashion to distinguish between green algae and potentially toxic cyanobacteria in environmental samples. Given relatively clean samples, FTIR methods offer a rapid, efficient method of cyanobacterial classification. Combined with the sensitivity of more advanced toxin-detection techniques, a complete classification of cyanobacteria and cyanotoxin is capable. As research on FTIR methods continues, its use with toxin-detection methods has the potential to streamline efficiency in research and diagnostic labs, providing a time- and cost-effective comprehensive diagnosis of environmental samples.