GENETIC INVESTIGATIONS OF A NOVEL, TOXIC, EPIPHYTIC CYANOBACTERIUM, *AETOKTHONOS HYDRILLICOLA*

by

AMANDA HOWARD

(Under the direction of Susan Wilde)

ABSTRACT

Aetokthonos hydrillicola is a newly described epiphytic cyanobacterial species growing on dense aquatic plant infestations in southern US reservoirs. It produces a novel neurotoxin that causes Vacuolar Myelinopathy, neuropathy and mortality in waterbirds, fish, amphibians, reptiles, and birds of prey. On its primary host *Hydrilla verticillata*, *A. hydrillicola* lives in an acidic microenvironment with a consortium of heterotrophic bacteria, including some species attached to the mucilaginous sheath of its filaments. My research addressed three aspects of *A. hydrillicola* and its associated bacteria: (1) To sequence the *A. hydrillicola* genome, the associated heterotrophic bacteria must first be removed because their DNA confounds its genome assembly. Methods were developed to reduce contaminating bacterial levels and to quantify contaminating bacterial levels (2). The microbiome of hydrilla leaves was characterized. *A. hydrillicola*-colonized leaves from different locations and seasons were examined. (3) The genetic diversity of *A. hydrillicola* across sites in the southern US was examined by comparison of rDNA-ITS sequences. The degree of genetic diversity provides insight into whether it is native to the US or an exotic species imported on hydrilla.

GENETIC INVESTIGATIONS OF A NOVEL TOXIC EPIPHYTIC CYANOBACTERIUM, *AETOKTHONOS HYDRILLICOLA*

by

AMANDA HOWARD

BS, University of Georgia, 2014

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2019

GENETIC INVESTIGATIONS OF A NOVEL TOXIC EPIPHYTIC CYANOBACTERIUM, *AETOKTHONOS HYDRILLICOLA*

by

AMANDA KALYNN HOWARD

Major Professor:	Susan Wilde
Committee:	Dayton Wilde
	Travis Glenn
	Erin Lipp

Electronic Version Approved:
Ron Walcott
Interim Dean of Graduate School
University of Georgia
December 2019

ACKNOWLEDGEMENTS

I would first like to acknowledge my advising professor, Dr. Susan Wilde. She took a chance on me as a graduate student and supported me throughout the entire process. I would also like to acknowledge Dr. Dayton Wilde, who equally supported and guided my research. Without them, I would never have been able to achieve this goal. I would also like to acknowledge my committee members, Dr. Travis Glenn and Dr. Erin Lipp, and my lab mates, Wes Gerrrin, Karsen Weems, Heather Gladfelter, Tabby Phillips, Natalia Bayona-Vásquez and Lav Yadav for helping contribute to my research in various ways. My sister, Marissa Howard, deserves special recognition for the large contributions that she has made to my research not only in the lab, but also in the experimental designing and writing stages. My husband, Matthew Sullivan, also deserves special recognition for all of the advice and support that he provided throughout the entire process. I truly would not have been able to get through this without him. Finally, I would like to acknowledge my parents, Shawn and Monica Howard, for always being there when I needed a helping hand. I am truly grateful for all of the contributions that each person named here has made to my research. Words will never be enough to express my gratitude.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

CHAPTER 1

Introduction

Aetokthonos hydrillicola is a novel cyanobacterium about which little is known, other than its association with Vacuolar Myelinopathy. Vacuolar Myelinopathy, previously known as Avian Vacuolar Myelinopathy, was originally documented in 1994 when bald eagles were found dead for unknown reasons (Wilde et al. 2014; Wiley et al. 2007; Williams et al. 2007; Thomas, Meteyer, and Sileo 1998). Subsequently, extensive research was conducted to determine the cause of death and characterize the disease (Birrenkott et al. 2004; Wilde et al. 2014; Wilde et al. 2005; Wiley et al. 2007; Williams et al. 2007). This novel cyanobacterium causes Vacuolar Myelinopathy through the production of a neurotoxin that is passed through the food web (Bidigare et al. 2009; Dodd et al. 2016; Mercurio et al. 2014; Wilde et al. 2014). *A. hydrillicola* typically adheres to the underside of hydrilla leaves (*Hydrilla verticillata*), though it has been known to grow on other aquatic macrophytes. When hydrilla is consumed concurrent with *A. hydrillicola* producing the neurotoxin, it results in the formation of vacuolar lesions in the optic tectum and cerebellar tracts of the brains of waterfowl and bald eagles that prey upon them (Birrenkott et al. 2004; Wilde et al. 2014; Wilde et al. 2005; Williams et al. 2007; Williams et al. 2008; Fischer et al. 2002; Larsen et al. 2002). Laboratory and field trials documented neuropathy, vacuolar myelinopathy and mortality in additional herbivorous taxa and their predators (turtles, tadpoles, salamanders) after consuming hydrilla with *A. hydrillicola*. (Birrenkott et al. 2004; Haynie et al. 2013; Fischer, Lewis-Weis, and Tate 2003; Larsen et al. 2002; Mercurio et al. 2014; Maerz et al. 2019). Furthermore, a food-chain link between epiphytic *A. hydrillicola* on hydrilla, waterfowl and bald eagles has been outlined, revealing the severity of

the environmental risk (Birrenkott et al. 2004; Wilde et al. 2014; Dodd et al. 2016). However, the disease is still not fully understood, including whether it affects humans and other mammals.

In the past, many cyanobacteria have been studied due to their many pharmacological applications (Alvarenga, Fiore, and Varani 2017; Hughes et al. 2017; Niedermeyer 2015). There have been investigations surrounding the genes that are responsible for the biosynthesis of tolyporphin-A. This molecule is synthesized by cyanobacteria strain HT-58-2 and is known for its anti-cancer potential (Hughes et al. 2017). The anti-infection, bioactive compounds frequently made by cyanobacteria have also been a popular area of study (Niedermeyer 2015). However, there has been an increasing number of toxic cyanobacterial blooms and this has caused researchers to turn their attention towards this increasing incidence (Alvarenga, Fiore, and Varani 2017; Wells et al. 2015). *A. hydrillicola* has only emerged in the last few decades and has yet to have its genome sequenced. There is a high potential for the *A. hydrillicola* genome to contain yet undiscovered genes, which could have beneficial applications. Furthermore, if the genome were to be sequenced, then the genes responsible for the biosynthesis of the toxin could potentially be identified.

The microbiome of *A. hydrillicola* could also give insight into this emerging cyanobacterium. Cyanobacteria can have symbiotic relationships with their associated microbes. Often, these assemblages of microbes adhering to the mucilaginous sheath of cyanobacteria aid cyanobacteria with their metabolic processes by providing nutrients and contributing to their nutrient cycling (Penn et al. 2014; Litchman et al. 2010). These metabolic processes could potentially include the biosynthesis of *A. hydrillicola's* neurotoxin. Tracking the microbiome of *A. hydrillicola* throughout its toxin producing season could illuminate if there are any interactions occurring. Additionally, there is the potential for *A. hydrillicola's* microbiome to

contain microbes that can break down the toxin. There have been a large number of microbes implicated in the biodegradation of microcystins (Ho, Sawade, and Newcombe 2012). Identifying microbes that have the ability to break down *A. hydrillicola's* neurotoxin could potentially lead to bioremediation protocols to prevent Vacuolar Myelinopathy outbreaks.

The origin of *A. hydrillicola* in the southeastern United States is unknown. Information about its genetic diversity is needed to determine whether it is native to the region or an exotic species that was imported from Asia on hydrilla. Previous studies have used the 16S-23S Internal Transcribed Spacer (ITS) region of the rRNA operon within various cyanobacteria to infer their evolution and population dynamics (Boyer, Flechtner, and Johansen 2001; Ohbayashi et al. 2013; Sabart et al. 2009; Chonudomkul et al. 2004). These studies would serve as models for a study that compared the 16S-23S ITS region of the rRNA gene of *A. hydrillicola* at different locations throughout the Southeast.

As discussed above, there are many questions about *A. hydrillicola* that still need to be addressed such as: Can the whole genome sequence provide further information about *A. hydrillicola,* such as the genes that produce the toxin? Does the microbiome associated with *A. hydrillicola* remain consistent throughout *A. hydrillicola's* toxin season and if not, what does that indicate? Finally, have the negative impacts of *A. hydrillicola's* neurotoxin only recently come to light because it is newly introduced? Genetic investigations of the *A. hydrillicola* could help answer some of these more difficult questions. Here I will discuss the ways in which I have attempted to answer these questions.

CHAPTER 2

Methods for Extracting High Molecular Weight Genomic DNA from *Aetokthonos hydrillicola*

Background

A. hydrillicola is a novel, toxic cyanobacterium that was recently discovered due to its ability to induce a wildlife disease called Vacuolar Myelinopathy, formerly known as Avian Vacuolar Myelinopathy (Fischer et al. 2002; Larsen et al. 2002; Thomas, Meteyer, and Sileo 1998; Wilde et al. 2005; Williams et al. 2007). This disease is characterized by the vacuolar lesions that it forms in the brain (Fischer et al. 2002; Rocke et al. 2002; Thomas, Meteyer, and Sileo 1998; Wilde et al. 2014; Wilde et al. 2005). In 2002, it was determined that these lesions form after the neurotoxin produced by *A. hydrillicola* is consumed (Wilde et al. 2014; Wilde et al. 2005; Williams et al. 2007). Originally, it was observed to affect only bald eagles and waterfowl, however, further research determined that many herbivorous aquatic and their predators taxa are negatively affected (Birrenkott et al. 2004; Haynie et al. 2013; Fischer, Lewis-Weis, and Tate 2003; Larsen et al. 2002; Mercurio et al. 2014; Maerz et al. 2019). It also has the potential to biomagnify within aquatic foodchains (Birrenkott et al. 2004; Haynie et al. 2013; Fischer, Lewis-Weis, and Tate 2003; Larsen et al. 2002; Mercurio et al. 2014; Maerz et al. 2019). The deleterious effects of *A. hydrillicola's* neurotoxin on mammals are still unknown. With the growing number of cyanobacterial blooms, it is important that the genes that are responsible for biosynthesis of the toxin are identified.

The genes of cyanobacteria are often studied for their pharmacological applications. Many different species biosynthesize compounds that have anticancer and anti-infective activity (Hughes et al. 2017; Niedermeyer 2015). There is also discussion among researchers about using

whole genome sequencing to remedy the flaws in the current taxonomic classifications of cyanobacteria (Alvarenga, Fiore, and Varani 2017). In fact, *A. hydrillicola* was originally believed to be in the genus *Stigonema*, but upon further investigation of the 16S rRNA gene sequence it was found that it was a separate genus (Wilde et al. 2014). Nascent technologies make it much easier to sequence the genomes of cyanobacteria, which could, in turn, make it much easier to classify cyanobacteria properly (Alvarenga, Fiore, and Varani 2017; Driscoll et al. 2017).

Whole genome sequencing can give insight into the evolution, metabolism and endosymbiosis of cyanobacteria (Alvarenga, Fiore, and Varani 2017; Raven and Allen 2003; Rocap et al. 2003). Using whole genome sequencing, researchers have theorized that the chloroplasts used by plants today, originated through an endosymbiotic relationship with cyanobacterium (Raven and Allen 2003; Alvarenga, Fiore, and Varani 2017; Chaffron et al. 2010; Martin et al. 2002). Limited genetic investigation of *A. hydrillicola* has been done, with only the 16S-23S ITS region being studied (Wilde et al. 2014). A whole genome sequence of *A. hydrillicola* could give insight into specific genes that are responsible for the biosynthesis of the toxin. Once the genes responsible for biosynthesis are determined, their expression levels could be used as biomarkers to determine when the toxin is being produced and in what quantity. Furthermore, this could aid in understanding of the mechanism of action.

While there could be many benefits to performing whole genome sequences of different cyanobacteria, there are many obstacles that present themselves when trying to sequence their genomes. First, the genomes of cyanobacteria typically contain a high number of GC repeats, which makes genome assemble more difficult (Alvarenga, Fiore, and Varani 2017; Hughes et al. 2017). Additionally, cyanobacteria typically have an associated microbial community that

adheres to their mucilaginous sheath (Alvarenga, Fiore, and Varani 2017; Chaffron et al. 2010; Hughes et al. 2017). The added DNA from these associated microbes can confound the sequence assembly. Because growing cyanobacteria in axenic culture is quite difficult, researchers would benefit from a filtration protocol that removes enough of the contaminating bacteria for the genome of a cyanobacteria species to be assembled (Alvarenga, Fiore, and Varani 2017; Chaffron et al. 2010; Hughes et al. 2017). This protocol, combined with the longer reads from PacBio sequencing, should allow for researchers to more easily assemble the genomes of their desired cyanobacteria (Driscoll et al. 2017).

Methods

Filtration

A hydrillicola was scraped from hydrilla leaves obtained from Lake J. Strom Thurmond, near the Keg Creek inlet. We resuspended about 100mg – 200mg of *A. hydrillicola* in a 50ml conical tube with approximately 50 ml of sterile BG-11 solution (17.7mM $NaNO_3$, 0.23mM K_2HPO_4, 0.3mM $MgSO_4 \cdot H_2O$, 0.24mM $CaCl_2 \cdot H_2O$, 0.031mM Citric Acid$\cdot H_2O$, 0.021mM Ferric Ammonium Citrate, 0.0027mM $Na_2EDTA \cdot 2H_2O$, 0.19mM Na_2CO_3, 1mM Sodium Thiosulfate Pentahydrate, 1% [vol/vol] BG-11 Trace Metals Solution). Three drops of Triton X-100 were added to the solution, which was then vigorously mixed. Cells were then allowed to settle to the bottom of the conical tube and the supernatant was removed. The supernatant was fixed by adding formalin to the sample for a final concentration of 1% and stored for later quantification of microbes per wash via staining and enumeration under an epifluorescence microscope. After supernatant was removed cells were washed by adding approximately 50ml of sterile BG-11 solution. Solution containing wash and *A. hydrillicola* was then separated via a

filtration apparatus as described by Heaney and Jaworski (Heaney and Jaworski 1977). The filter within the filtration apparatus contains 8 µm pores. This allowed contaminating microbes to pass through the filter, while *A. hydrillicola* remained on top of the filter. Remaining *A. hydrillicola* was washed twice more with 50ml TE Buffer (10 mM Tris pH 8, 0.5 mM EDTA pH 8). Each wash was formalin fixed and stored in a 50ml conical tube for later quantification of removed contaminating microbes. After washing *A. hydrillicola,* filaments were washed off the filter with 1ml TE Buffer for DNA extraction. A 10mg subsample of *A. hydrillicola* was taken before the filtration, after the supernatant was removed and after the washes. These subsamples were then observed under an epifluorescence microscope to confirm whether cyanobacteria were present within the sample.

Quantification of Heterotrophic Bacteria Removed with Each Wash via Direct Counting Using Epifluorescence Microscope

Each filtration process had the supernatant, 1st wash, 2nd wash and 3rd wash removed and stored in 1% formalin for subsequent microbial quantification. Supernatant and washes for each sample were stained using an aquatic prokaryotic enumeration protocol that utilizes a SYBR Green stain and an epifluorescence microscope by Patel et al. (Patel et al. 2007). Once samples were stained on an Acrodisc 0.02 µm filter, they were quantified using an epifluorescence microscope with a 0.4mm field of view under 100X magnification with oil immersion. Each filter was quantified, via direct counting, 10 times in randomly selected areas. This entire quantification process was repeated six times with different samples each time. Finally, these counts were averaged and the total number of microbes removed for each sample were quantified.

DNA Extraction

Each extraction began with approximately 50mg of *A. hydrillicola* suspended in 1ml of TE Buffer. Cells were spun down in a centrifuge for 5 min at 14,800 rpm and then the supernatant was poured off. Using liquid nitrogen, *A. hydrillicola* cell were frozen and then ground with an Eppendorf tube pestle. The cells were resuspended in 4 volumes of CTAB Extraction Buffer (100mM Tris – HCl, 1.4M NaCl, 0.5M EDTA, 1% [wt/vol] CTAB, 2% [wt/vol] PVP, 0.1% [vol/vol] ß-mercaptoethanol). RNAse was added for a final concentration of 10mg/ml and then samples were incubated for 30 min at 65°C. Following incubation, the samples were homogenized and then put through one freeze/thaw cycle using liquid nitrogen. Samples were left to warm to room temperature. Then one volume of chloroform:isoamyl alcohol was added and mixed gently. Samples were allowed to precipitate on ice for about 10 minutes, then centrifuged at 14,800 rpm for 10 min at 4°C. The supernatant was transferred to new tube and 2.5 volumes of 100% ethanol were added. Samples were incubated at -20°C for about an hour and then centrifuged at 14,800 rpm for 15 min at 4°C. Ethanol was poured off and pellet was washed with 70% ethanol. After washing the pellet, it was left to dry in laminar flow cabinet, followed by a resuspension in 30µl of 10mM Tris.

Real-time qPCR and comparison

Methods to quantify how much of the proportion of *A. hydrillicola* DNA increased within the filtered sample were done using the qPCR protocol from Heck et al. (2016). The DNA extracted from both *A. hydrillicola* and its associated bacteria were quantified using Stratagene Mx3000P and two sets of specific primers. To quantify *A. hydrillicola* primers CYA359F (5'-GGGGAATYTTCCGCAATGGG-3') and CYA781-a (5'-

GACTACTGGGGTATCTAATCCCATT-3') were used to amplify a region of the 16S rRNA gene unique to cyanobacteria, with 35 cycles at 94°C for 1 min, 47°C 1 min and 72° 1 min. For all other associated bacteria, the universal primers P1-F (5'-CCTACGGGAGGCAGCAG-3') and P2-R (5'-ATTACCGCGGCTGCTGG-3') which also target a portion of the 16S rRNA gene were utilized, with 25 cycles at 95°C for 30 sec, 55°C 30 sec and 72° 30 sec. Both sets of primers were used with the PowerUp SYBR Green Master Mix by ThermoFisher Scientific. Dissociation curves were run for each set of primers using 1 cycle at 95°C 1 min, 55°C 30 sec and 95°C 30 sec. Each set of primers were run with a sample of *A. hydrillicola* that had not been filtered and a sample of *A. hydrillicola* that had been filtered. This was done in duplicate. The threshold values from the filtered *A. hydrillicola* samples were then compared to the threshold value from the non-filtered *A. hydrillicola* samples using the relative quantification method with normalization of the starting amount of nucleic acid for each sample.

Results

Quantification of Heterotrophic Bacteria Removed with Each Wash via Direct Counting Using Epifluorescence Microscope

The average of the total number of heterotrophic bacteria removed by the filtration process was 15,275 (Table 1). The average number of bacteria removed during supernatant removal and the first wash of the filter is very similar; with an average 42% of the overall bacteria being removed with the supernatant and 36% being removed in the first wash. After that the average bacteria removed in the second wash was about 14% and the average overall bacteria removed in the third was around 8% (Figure 1).

Table 1. Quantification of Heterotrophic Bacteria Removed. The number of bacteria removed per 50 mL of solution (i.e. Supernatant, First Wash, etc.).

	Rep 1	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Rep 6**	**Average**
Supernatant	6,457	7,400	6,509	4,479	8,562	5,727	**6,522**
1st Wash	2,484	4,376	2,605	3,996	9,591	9,861	**5,486**
2nd Wash	1,052	1,110	1,179	1,507	4,008	3,996	**2,142**
3rd Wash	650	633	707	1024	1,921	1,823	**1,126**
Total Bacteria Removed	**10,643**	**13,509**	**11,000**	**11,006**	**24,082**	**21,407**	**15,275**

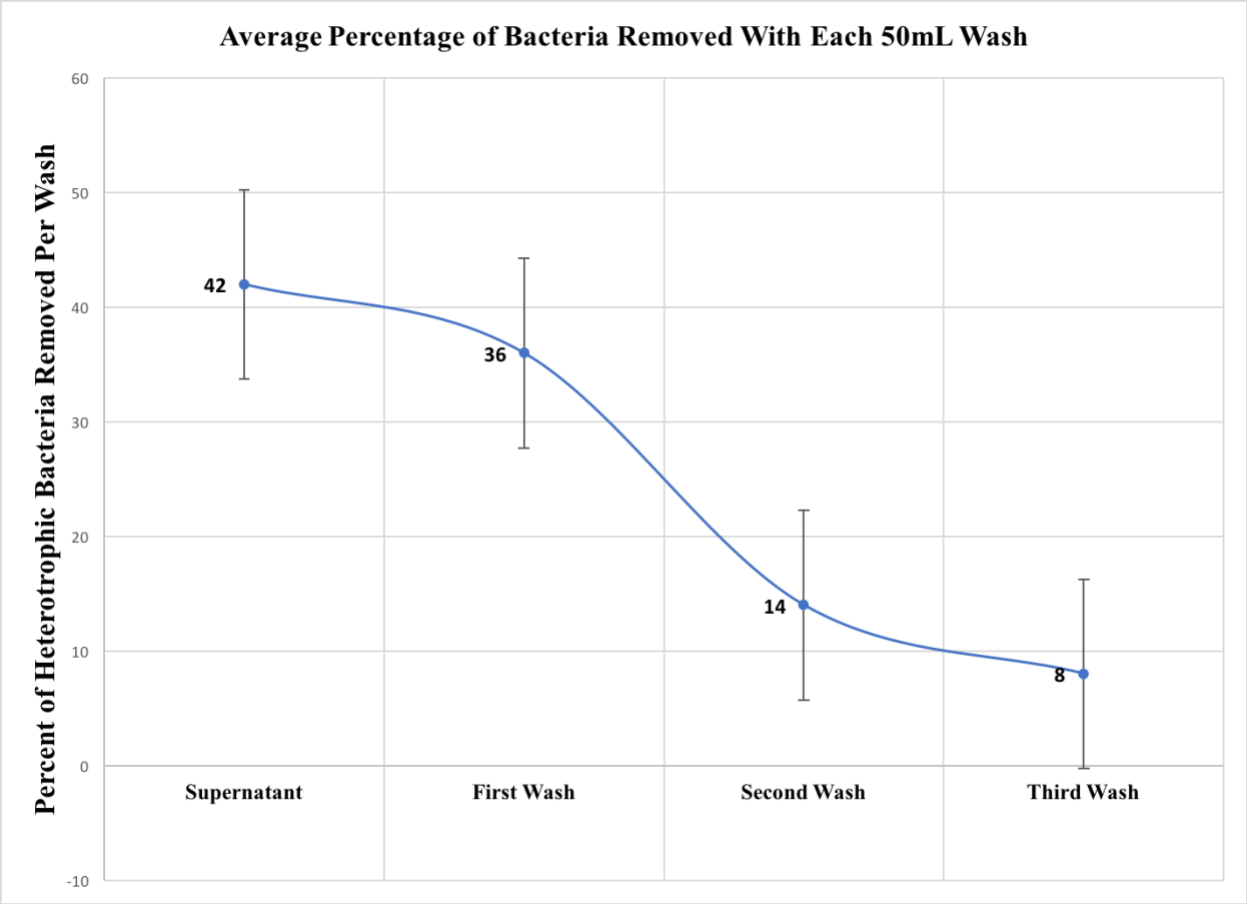

Figure 1. Average Percentage of Bacteria Removed Per 50mL Wash. This line graph shows the average percentage of heterotrophic bacteria removed with each 50mL wash with standard error bars for each wash.

DNA Yields for Filtered and Non-Filtered Samples

On average, 3131.5 ng of DNA were extracted from the non-filtered samples of *A. hydrillicola*, whereas an average of 1512.5 ng of DNA were extracted from the filtered samples of *A. hydrillicola*. This means that a little more than twice as much DNA was obtained from the non-filtered sample of *A. hydrillicola*.

Comparison of Real-time qPCR

The relative quantification method was used to compare the non-filtered samples of *A. hydrillicola* to the filtered samples of *A. hydrillicola*. The relative quantification method using the cyanobacteria specific primers to compare the non-filtered and filtered samples yielded ratios of 2.61 for the first run and 2.23 for the duplicate run (Table 2). This indicates that the amount of *A. hydrillicola* DNA in the filtered samples was more than double the amount of *A. hydrillicola* DNA in the non-filtered sample. The relative quantification between non-filtered *A. hydrillicola* and filtered *A. hydrillicola* using the results from the universal bacteria primers gave ratios of 0.71 for the initial run and 0.72 for the duplicate run (Table 2). This reveals that the total starting DNA in the filtered sample was lower than the total starting DNA in the non-filtered sample for both runs. Finally, when comparing the total amount of *A. hydrillicola* DNA in the filtered sample versus the total amount of *A. hydrillicola* DNA in the non-filtered sample, there were higher ratios of *A. hydrillicola* DNA observed in the filtered sample, 0.02 and 0.07, than the non-filtered sample, <0.01 and 0.01 (Table 2).

Table 2. Ratios yielded from the relative quantification method. The ratios used to compare different amounts of DNA within the filtered and non-filtered *A. hydrillicola* samples.

Comparisons	**Primers**	**Rep 1 Ratio**	**Rep 2 Ratio**
A. hydrillicola DNA in filtered vs. non-filtered samples	CYA359 & CYA781-a	2.61	2.23
Total DNA in filtered vs. non-filtered samples	P1-F & P2-R	0.71	0.72
Amount of *A. hydrillicola* in filtered samples	CYA359, CYA781-a, P1-F & P2-R	0.02	0.07
Amount of *A. hydrillicola* in non-filtered samples	CYA359, CYA781-a, P1-F & P2-R	< 0.01	0.01

Discussion and Conclusion

There is a steady increase in the number of cyanobacteria having their genomes sequenced. It would be advantageous for researchers to have a filtration and extraction process that helped cyanobacteria from associated bacteria. This need is further supported by the fact that many cyanobacteria cannot be grown in an axenic culture within a lab due to heterotrophic bacteria adhering to their mucilaginous sheaths (Alvarenga, Fiore, and Varani 2017; Chaffron et al. 2010; Heck et al. 2016; Hughes et al. 2017). Using the filtration method detailed above, an average of 15,275 heterotrophic bacteria were removed from each 100-200mg sample of *A. hydrillicola*. Additionally, the relative quantification ratios using the CYA359 and CYA781-a primers indicate that the filtration process more than doubled the amount of *A. hydrillicola* DNA within the extracted DNA sample. Therefore, the amount of *A. hydrillicola* may have been more than double, since the relative quantification ratios using the P1-F and P2-R primers indicate that there was were higher amounts of starting DNA in the non-filtered *A. hydrillicola* samples than there were in the filtered *A. hydrillicola* samples. The validity of this protocol is further supported when taking this finding into consideration. Since there was less DNA yield from the filtered samples, but they were enriched of *A. hydrillicola* DNA. Furthermore, when comparing the ratios of *A. hydrillicola* DNA in the filtered sample to the ratios of *A. hydrillicola* DNA in the non-filtered sample, larger ratios were observed in the filtered sample. This confirms that the amount of *A. hydrillicola* DNA is higher in the filtered sample than in the non-filtered sample.

The filtration and extraction process developed here will facilitate faster and easier whole genome sequencing for cyanobacteria that have an associated microbial community adhering to its mucilaginous sheath. The filtration process takes significantly less time to implement than

attempting to grow and purify a cyanobacterial culture in the lab. With this method, one could filter and extract a cyanobacteria's DNA within a day, as opposed to multiple days.

CHAPTER 3

Observing the Microbiome of *Aetokthonos hydrillicola* Spatially, Temporally and On Varying Macrophytes

Background

Following its introduction to new environments, hydrilla can wreak havoc by outcompeting native macrophytes and altering the habitat of many aquatic species (Blackburn 1969; Gordon-Bradley, Li, and Williams 2015; Gordon-Bradley, Lymperopoulou, and Williams 2014; Langeland 1996). However, these are not the only effects that hydrilla's presence has on the ecosystem. Not long after the introduction of hydrilla, a new wildlife disease, Vacuolar Myelinopathy, emerged (Augspurger et al. 2003; Fischer et al. 2002; Rocke et al. 2002; Thomas, Meteyer, and Sileo 1998). This new wildlife disease is characterized by the vacuolar lesions that are formed in the myelin of the optic tectum and cerebellar tracts of vertebrate brains (Fischer et al. 2002; Larsen et al. 2002; Rocke et al. 2002; Thomas, Meteyer, and Sileo 1998). Although Vacuolar Myelinopathy it was originally discovered due to an increased mortality in bald eagles, further research has determined that bald eagles are not the only species it effects. Vacuolar Myelinopathy has been confirmed in amphibians, reptiles, fish and other birds (Augspurger et al. 2003; Larsen et al. 2002; Maerz et al. 2019; Mercurio et al. 2014; Rocke et al. 2002; Thomas, Meteyer, and Sileo 1998; Wilde et al. 2014).

The culprit of this new wildlife disease is a novel, epiphytic cyanobacterium, *Aetokthonos hydrillico*la (Wilde et al. 2014; Wilde et al. 2005; Williams et al. 2007; Bidigare et al. 2009; Williams et al. 2008). This newly discovered cyanobacterium, adhering to the leaves of hydrilla, has been documented as neurotoxic from late summer until winter when hydrilla dies back completely in temperate reservoirs (Bidigare et al. 2009; Wilde et al. 2014; Wilde et al. 2005;

Williams et al. 2007; Williams et al. 2008). Temporal sampling conducted to determine the seasonal production of *A. hydrillicola's* toxin confirmed Ah toxin from July to December with a peak in November in J. Strom Thurmond Reservoir (SC/GA) (Unpublished work by Susan Wilde's lab at the University of Georgia). While *A. hydrillicola* most frequently grows on hydrilla leaves, it has also been confirmed to grow on other native macrophytes. Once *A. hydrillicola* adhering to hydrilla (or other macrophytes) is consumed in high enough quantities, vacuolar lesions will form in the brain (Bidigare et al. 2009; Wilde et al. 2014; Wilde et al. 2005; Williams et al. 2007; Williams et al. 2008). There has also been a food chain link established which could indicate a potential human health risk (Dodd et al. 2016; Wilde et al. 2014; Maerz et al. 2019).

Hydrilla has been known to change the natural microbiome of the water column in which it inhabits (Gordon-Bradley, Li, and Williams 2015; Gordon-Bradley, Lymperopoulou, and Williams 2014). It has been speculated that hydrilla's presence could create a disorder in the ecosystem that it has invaded (Gordon-Bradley, Li, and Williams 2015). There is also evidence that the epiphytic microbiome of hydrilla differs from that of native aquatic plants (Gordon-Bradley, Lymperopoulou, and Williams 2014). While the microbiome of hydrilla alone has been well researched, the microbiome of hydrilla containing *A. hydrillicola* has not been assessed. Furthermore, cyanobacteria have been known to produce their own "cyanosphere", which can be described as a cyanobacteria dominated community and their associated heterotrophic bacteria (Alvarenga, Fiore, and Varani 2017). The potential of these two species in combination to alter the natural microbiota of their environment could have adverse ecological affects. It is imperative to track the changes that these species are making in the environment around them, so that researchers can be aware of detrimental or irreversible shifts in fragile ecosystems.

Though cyanobacteria are primary producers that generally create their own "cyanosphere", they also have help from associated microbes that provide nutrients that cyanobacteria need for certain metabolic processes (Penn et al. 2014; Litchman et al. 2010; He, Ren, and Wu 2012). These associated microbes aid in nutrient cycling and could, therefore, aid in *A. hydrillicola's* metabolic processes that create its neurotoxin. Identifying these specific microbes could potentially help researchers better understand *A. hydrillicola's* neurotoxin and its biosynthesis. This could open doors to the development of management strategies. Conversely, the associated microbes within *A. hydrillicola's* microbiome could be breaking down its neurotoxin. Studies have identified microbes that can break down microcystins and other cyanobacterial toxins (Ho, Sawade, and Newcombe 2012). There has also been research that confirms the ability of certain microbes to break down specific toxic cyanopetides (Briand et al. 2016). Identifying microbes that have the capability to break down *A. hydrillicola's* neurotoxin could be extremely helpful in the creation of bioremediation protocols. Such protocols have already been theorized to remove microcystins from freshwater environments (Ho, Sawade, and Newcombe 2012), highlighting the potential for developing bioremediation practices for *A. hydrillicola's* neurotoxin.

Here, a structural microbiome analysis was conducted for *A. hydrillicola's* microbiome at different time points throughout its toxin season, at different locations in the Southeast and on different macrophytes. The structural analysis from each of these comparisons could give insight into the relationships that *A. hydrillicola* is forming with the microbes with in its' "cyanosphere" and if *A. hydrillicola's* presence is shifting the microbe of its environment. These insights could lead a better understanding of the mechanisms within *A. hydrillicola* that allow for toxin biosynthesis and to the future development of bioremediation practices.

Methods

Sample Sites

Twelve samples that were assessed for their microbial community. There was a control site at Lake Seminole, GA (30.733, -84.879) which has historically never had *A. hydrillicola* present. There were four samples of hydrilla with *A. hydrillicola* taken at Lake J. Strom Thurmond (33.72606, -82.29483), near the Keg Creek inlet. These samples were taken throughout the toxin-producing season, approximately a month apart (August 2018 – November 2018). In addition to the samples of hydrilla with *A. hydrillicola*, samples of *Potamogeton pusillus* with *A. hydrillicola* and *Najas guadalupensis* with *A. hydrillicola* were also taken at Lake J. Strom Thurmond. These three macrophyte samples were taken at the same time. There are five additional sites where hydrilla with *A. hydrillicola* samples were taken, corresponding to different lake and reservoir locations throughout the Southeastern United States: Tussahaw Reservoir, GA (33.38165, -83.99580), Lake Tohopekaliga, FL (28.26649, -81.40620), Lake Hatchineha, FL (28.03045, -81.36732), Lake Hunt, NC (36.32678, -79.72756) and Sam Rayburn Reservoir, TX (31.13946, -94.13722). These samples were collected by colleagues with specific sampling instructions and shipped to the Wilde lab at the University of Georgia for processing. Samples arrived in late October and early November of 2018. These additional sites were compared with one another and with Lake Seminole, GA (30.733, -84.879) and Lake J. Strom Thurmond (33.72606, -82.29483). The sampling for all site were carried out in the same manner.

Sample Collection

Most of the samples taken consisted of hydrilla with *A. hydrillicola* adhering to its leaves. However, there was also a control sample of hydrilla with no *A. hydrillicola* adhering to its

leaves (Lake Seminole sample) and two samples that were native macrophytes with *A. hydrillicola* adhering to it, *Najas guadalupensis* and *Potamogeton pusillus* (both from J. Strom Thurmond). All hydrilla samples were *Hydrilla verticillata*, with every sample being dioecious apart from J. Strom Thurmond samples which were monoecious. Each macrophyte sample was taken between late October of 2018 and early November, apart from samples that were taken through *A. hydrillicola's* toxin producing season (August 2018 – November 2018). Macrophyte samples were taken from a boat and stored in plastic bags on ice until they could be further assessed. Samples from states outside of Georgia were priority shipped on ice. Each sample was replicated three times. Once samples were back in the lab, they were screened for the presence of *A. hydrillicola*. Once *A. hydrillicola* was found, approximately 25mg of macrophyte material with *A. hydrillicola* adhering to it was frozen in TE solution for later DNA extraction.

Observing the Abundance of A. hydrillicola For Comparison Through Toxin Season

In addition to screening for the presence of *A. hydrillicola,* its abundance upon the hydrilla leaves was also observed and recorded as percent leaf coverage. Ten hydrilla leaves were taken from each sample and observed under an epifluorescence microscope. The percentage of *A. hydrillicola* covering each leaf was recorded and then the percentages of all ten leaves were averaged. This confirmed that the abundance of *A. hydrillicola* increased through its toxin season.

Quantifying A. hydrillicola's Toxin Throughout its Toxin Season

For each of the samples that were taken throughout *A. hydrillicola's* toxin season, a subsample of hydrilla with *A. hydrillicola* was extracted to determine toxin concentration.

Samples were dried and weighted, then ground and submerged in HPLC-grade methanol for approximately one hour. Samples were agitated every 15 min while incubating. Once, samples were done incubating the HPLC- grade methanol was gravity-filtered through Whatman #41 ashless filter paper to remove the plant material. Samples were then concentrated into microtubes and sent to the EPA to be run on an Agilent Infinity 1260 HPLC with gradient elution over a reverse phase C18 column and an UV detector (Haram 2016, unpublished data). After samples were run on the HPLC, the concentration of toxin produced by *A. hydrillicola* was calculated in ppm.

DNA Extraction

A DNeasy PowerSoil® DNA Isolation Kit (MO BIO laboratories, Inc.) was used to extract the DNA from the samples. Each sample consisted of ~25mg of macrophyte material with *A. hydrillicola* present. The DNeasy PowerSoil® DNA Isolation Kit's instructions were followed with the following modifications. Before starting the extraction, 200µl of the bead solution was removed and replaced with 200µl phenol:chloroform:isoamyl alcohol. The aqueous layer created by the phenol:chloroform:isoamyl alcohol was transferred to a new tube and used for the rest of the extraction process. Additionally, before washing the spin column with the C5 solution provided, the spin column was washed with 650µl of 100% ethanol. DNA was eluted in 50µl of 10mM Tris pH 8. These modifications provided a higher DNA yield from each sample.

Library Prep and Sequencing This Area Needs Work

Once DNA was extracted, universal 16S fusion primers and a KAPA HiFi HotStart PCR Kit (Roche) were used to amplify the V3 and V4 region within the 16S ribosomal RNA gene,

which yielded a 464bp amplicon using the methods of Glenn et al. (2019). These primers were adapted from the S-D-Bact-0341-b-S-17 forward (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 reverse (5'-GACTACHVGGGTATCTAATCC-3') primers used by Klindworth et al. (2013). These primers were fused with internal barcodes to ensure sample identification post sequencing and universal 5' TruSeq HT fusions (Appendix A) that make the primer compatible with the iTru7 and iTru5 adapters (Glenn et al. 2016). Once the desired region was amplified, the samples were cleaned with a 1.25X bead clean using speed beads (SpeedBead Magnetic Carboxylate Modified Particles 15ml, Azide 0.05%, GE Healthcare) Next, the amplicons were externally tagged with iTru7 and iTru5 primers using the ReadyMix, containing a polymerase and PCR reagents, from the NEBNext Ultra II DNA Library Prep Kit for Illumina. This was followed by a second 1.25X bead clean up. Once the samples had been cleaned, they were pooled and sent to be sequenced on a MiSeq PE300 at the Georgia Genomics and Bioinformatics Core (University of Georgia).

Analysis

Samples were analyzed for relative abundance in QIIME2 using the Greengenes database 13.8 and dada2 pipline (Bolyen et al. 2019; Desantis et al. 2006; Callahan et al. 2016). Chloroplast DNA was filtered out of the relative abundances that were generated. Once the relative abundance of microbes was generated for each sample, the ANCOM plugin within QIIME2 was employed to perform differential abundance tests between samples. Comparisons were done between samples taken throughout the toxin season, samples from different macrophytes, and samples throughout the southeastern United States. Finally, the alpha diversity for samples taken throughout the toxin season, samples from different macrophytes, and samples

throughout the Southeastern United States were assessed using the alpha diversity plugin in QIIME2.

Results

Comparison of A. hydrillicola Microbiome Throughout Toxin Season

There was an increase in the percent abundance of *A. hydrillicola* covering the hydrilla leaves at this J. Strom Thurmond site (Table 3). Additionally, there was an increase *A. hydrillicola's* toxin from August-November (Table 4). The average percent coverage of *A. hydrillicola* on the hydrilla leaves also increased during this time period. The alpha diversity analysis found that the month of August had the highest diversity out of all the months with a p-value of 0.05.

Table 3. The Average Percent Coverage of *A. hydrillicola* on Ten Hydrilla Leaves Collected from August-November

Month	**Average Percent Coverage of *A. hydrillicola***
August	28.5
September	35
October	43
November	52

Table 4. The Concentration of *A. hydrillicola's* Toxin in ppm From August-November in J Strom Thurmond Reservoir.

Month	Toxin Present (ppm)
August	0.015
September	0.035
October	0.558
November	1.419

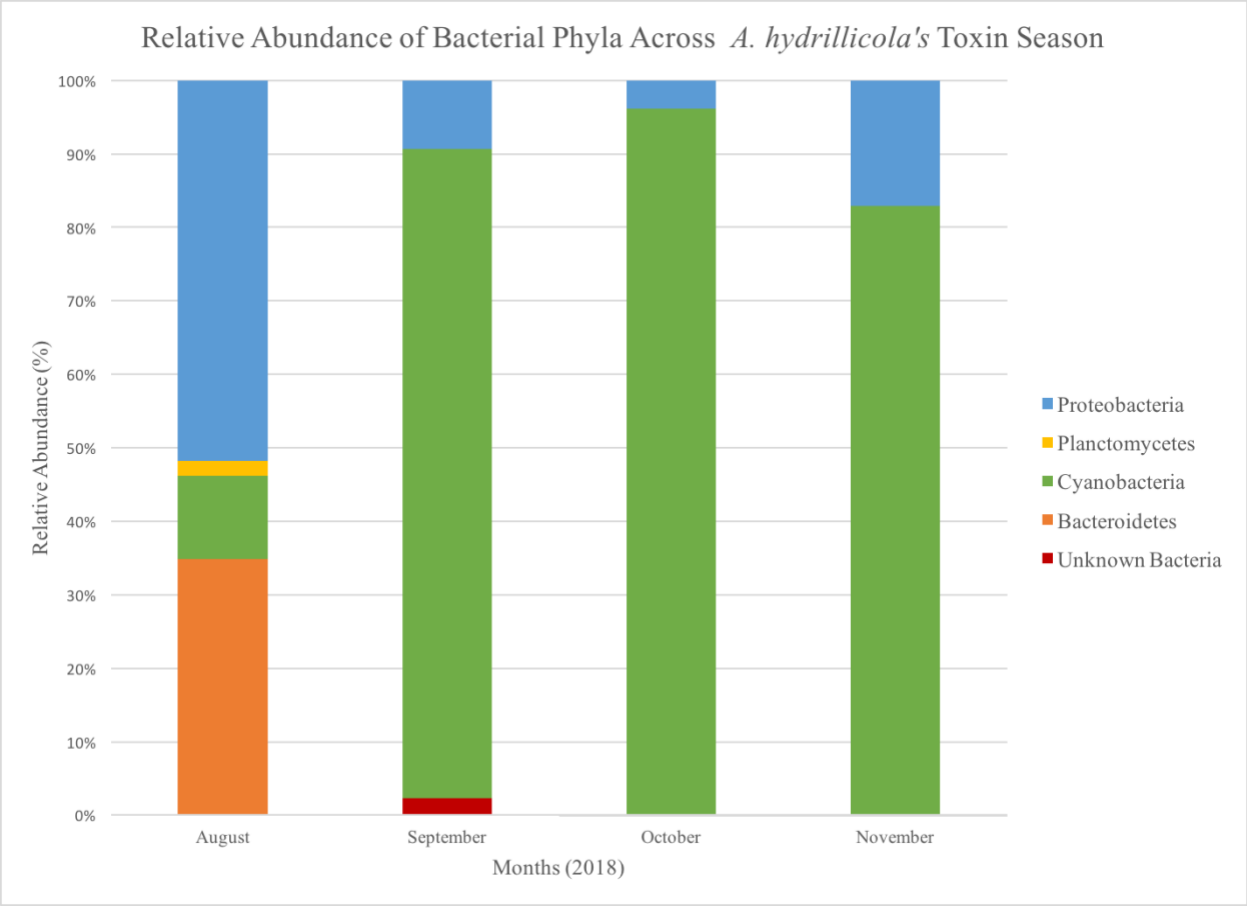

Figure 2. Relative Abundance of Bacterial Phyla Across *A. hydrillicola's* Toxin Season. The relative abundance of microbes (by phyla) on hydrilla samples taken at Lake J. Strom Thurmond throughout *A. hydrillicola's* toxin producing season (August – November) in 2018.

The relative abundance of microbes for each hydrilla sample taken in J. Strom Thurmond in different months through *A. hydrillicola's* toxin producing season are shown in the bar graph above (Figure 2). When compared using the ANCOM plugin in QIIME2, there was a significant difference found in August when compared to the other months. This significant difference was due to the strong presence of the phylum Bacteroidetes found in August, whereas there was no presence of Bacteroidetes in the subsequent months.

Comparison of A. Hydrillicola Microbiome on Different Macrophytes

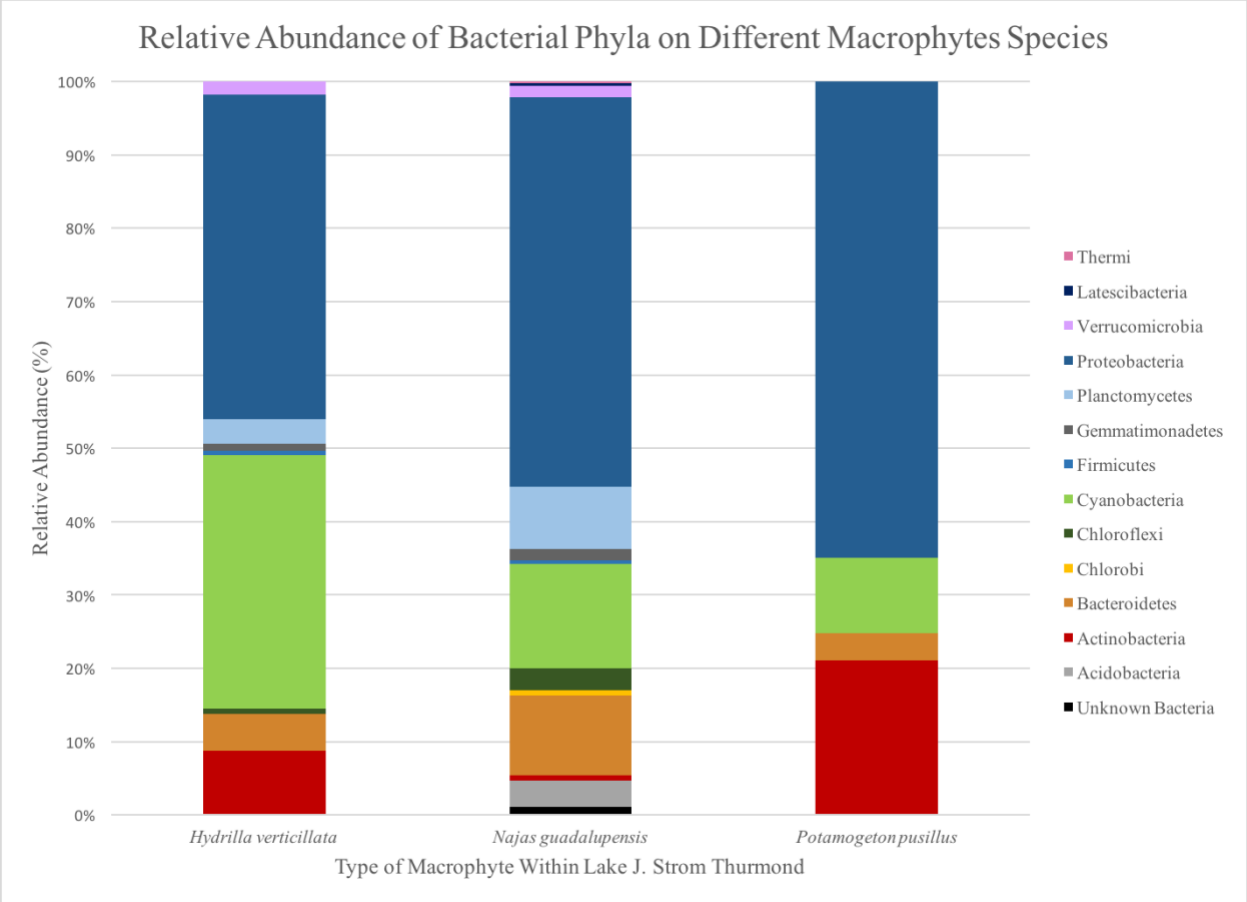

Figure 3. Relative Abundance of Bacterial Phyla on Different Macrophytes Species. The relative abundance of microbes (by phyla) on three different macrophytes samples taken at Lake J. Strom Thurmond in the month of October.

The relative abundance of microbes on the different types of macrophytes were determined (Figure ##). Relative abundance of microbiota among species were compared using ANCOM, demonstrating a significant difference among all three macrophytes for all the microbial phyla present. The alpha diversity analysis indicated that *Najas guadalupensis* had the highest diversity among the samples, though it was not found to be significant with a p-value of 0.08.

Comparison of A. Hydrillicola Microbiome at Different Locations

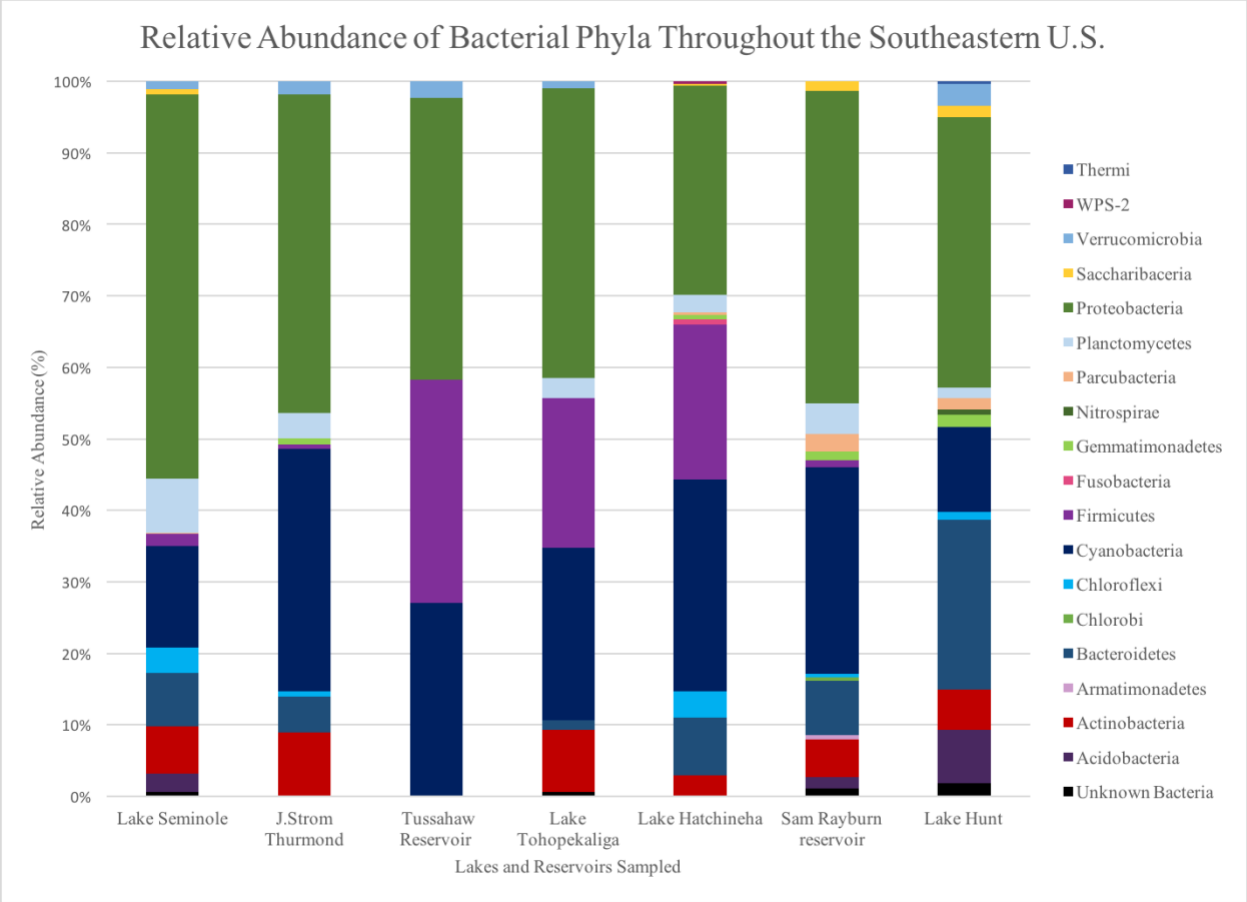

Figure 4. Relative Abundance of Bacteria Phyla Throughout the Southeastern U.S. The relative abundance of microbes (by phyla) on hydrilla samples taken at different locations throughout the southeastern United States in the months of October and November, with Lake Seminole being the control.

The relative abundance of microbes associated with hydrilla samples taken throughout the southeastern United States were determined (Figure 4). Microbial taxa abundance was significantly different between sample locations for the phyla Firmicutes, Cyanobacteria, Proteobacteria and Bacteroidetes. Lake Hatchineha and SamRayburn Reservoir had the highest diversity among the samples, though these two reservoirs were only significant when compared

to Lake J. Storm Thurmond, Tussahaw Reservoir and Lake Tohopekaliga ($p = 0.05$). Lake Hatchineha and Sam Rayburn Reservoir were also more diverse than Lake Seminole and Lake Hunt, though these differences were not significant ($p = 0.28$).

Discussion

Comparison of A. hydrillicola Microbiome Throughout Toxin Season

The microbiomes associated with toxic cyanobacteria often change as that cyanobacterial species begins to produce its toxin (Alvarenga, Fiore, and Varani 2017). The three main phyla that were seen among all the samples throughout *A. hydrillicola's* toxin season are Cyanobacteria, Proteobacteria and Bacteroidetes. These results are consistent with the results of Penn et al. (2014); where high numbers of Proteobacteria and Bacteroidetes were observed with cyanobacteria during its bloom. Penn et al. (2014) found that Proteobacteria tend to break down complex organic molecules, whereas Bacteroidetes generally broke down glycans . This could explain why there was Bacteroidetes at the beginning of *A. hydrillicola's* toxin season, but then it is no longer observed as *A. hydrillicola* moved further into its toxin season. Bacteroidetes would not benefit from the toxin, which is a complex compound, but the Proteobacteria may. This could also explain the increase of Proteobacteria in the last month of the toxin season, when there should be the most toxin being produced. Furthermore, the alpha diversity analysis found that the August samples were significantly more diverse than the other months. This may indicate that *A. hydrillicola* has an influence on its microbiome, though more sampling will need to be done through the toxin season to further support these results.

Comparison of A. hydrillicola Microbiome on Different Macrophytes

There were four phyla that were seen in high abundance in the three macrophyte samples: Cyanobacteria, Proteobacteria, Bacteroidetes and Actinobacteria. The only significant trend seen in the abundance of these microbes, was the much higher abundance of cyanobacteria on the hydrilla sample. *A. hydrillicola* has been known to prefer hydrilla, but the reasons are still unclear. Additionally, the alpha diversity analysis found *Najas guadalupensis* had the highest diversity among the samples, though it was not found to be significant. This could be due to the lower abundance of cyanobacteria present in the sample. Since there were no significant trends seen between the different macrophyte samples and the ANCOM test determined that the microbial structures for each of these macrophytes is significantly different for all phyla, there is not enough evidence to indicate that *A. hydrillicola* has a unique microbiome that influences the microbiome of the macrophyte that it is inhabiting.

Comparison of A. hydrillicola Microbiome at Different Locations

Actinobacteria, Firmicutes, Cyanobacteria, Proteobacteria and Bacteroidetes were observed in a much higher abundance than the other microbes for the samples that were taken at different locations throughout the Southeast. However, the ANCOM results showed that the Firmicutes, Cyanobacteria, Proteobacteria and Bacteroidetes abundances were significantly different between all the different sampling sites. Furthermore, Lake Hatchineha and Sam Rayburn Reservoir were considered the most diverse samples as estimated by the alpha diversity analysis. However, it was not significant for all the location comparisons. This would further confirm that there is no clear evidence to support *A. hydrillicola's* having its own "cyanosphere" that would influence the microbiome of the environment around it.

Conclusion

Though there was a slight trend in the microbial assemblages of the samples that were taken throughout *A. hydrillicola's* toxin producing season, there were no trends in microbial assemblages of the different macrophyte samples and the samples that were taken at different locations. This would indicate that *A. hydrillicola* does not have its own unique "cyanosphere". Furthermore, there is not sufficient evidence to demonstrate that *A. hydrillicola* has a significant influence over the microbiome of its environment, though it would be advantageous to repeat the methods for the samples taken through the toxin season to definitively come to this conclusion.

CHAPTER 4

Diversity in the 16S-23S rRNA Internal Transcribed Spacer Region of *Aetokthonos hydrillicola* Throughout the Southeastern United States

Background

At the turn of the century, a new wildlife disease, Vacuolar Myelinopathy, emerged (Fischer et al. 2002; Thomas, Meteyer, and Sileo 1998). This new wildlife disease was discovered due to a disease epizootic in DeGray Lake, Arkansas in 1994-1996 (Thomas, Meteyer, and Sileo 1998). Soon after its emergence, researchers began to investigate the physiological impacts of Vacuolar Myelinopathy and found that the disease is characterized by vacuolar lesions that form in the optic tectum and cerebellar tracts of the brain (Fischer et al. 2002; Larsen et al. 2002; Rocke et al. 2002). These lesions lead to mobility impairment and the symptoms can arise rapidly (Augspurger et al. 2003; Fischer et al. 2002; Larsen et al. 2002; Rocke et al. 2002). Finally, it was determined that the disease is induced by the consumption of a neurotoxin produced by a novel, epiphytic cyanobacteria growing on the dense hydrilla beds (Wilde et al. 2005; Williams et al. 2007; Williams et al. 2008).

It was also determined that bald eagles were not the only species of wildlife effected by the disease (Dodd et al. 2016; Fischer, Lewis-Weis, and Tate 2003; Maerz et al. 2019; Mercurio et al. 2014; Rocke et al. 2002; Wilde et al. 2014). Due to its deleterious effects on many species of wildlife, characterization of the cyanobacterium responsible for the toxin is imperative. Researchers began trying to classify this novel cyanobacterium in an attempt to better understand it. Based on morphology, it was believed that the cyanobacteria belonged to the genus *Stigonema*. However, upon sequencing the 16S-23S Internal Transcribed Spacer (ITS) region of the rRNA operon, researchers found that the cyanobacteria was far removed from any previously

sequenced cyanobacteria(Wilde et al. 2014). The novel cyanobacterium was termed *Aetokthonos hydrillicola*.

There are still more genetic investigations that need to be done with *A. hydrillicola.* While the 16S-23S ITS region the rRNA operon and other stable gene operons have been employed to classify new species, or organize species into clades, much more can be done with it (Lu et al. 2006; Neilan, Jacobs, and Goodman 1995; West and Adams 1997; Yoshida et al. 2005). Many techniques have been developed to infer evolution and population dynamics using the 16S-23S ITS region of the rRNA operon and other stable loci (Boyer, Flechtner, and Johansen 2001; Ohbayashi et al. 2013; Sabart et al. 2009; Chonudomkul et al. 2004). For example, Erwin and Thacker detected significant sequence divergences within clades of *Synechoccus spongiarium* using the 16S rRNA gene and its associated ITS region, giving insight into its environmental influences (Erwin and Thacker 2008).

Because the pernicious effects of *A. hydrillicola* have only arisen this century, the origins of this novel cyanobacteria are still unknown. There are two theories for *A. hydrillicola's* sudden emergence. One theory is that *A. hydrillicola* was introduced when the invasive aquatic macrophyte, hydrilla, was introduced to the Southeastern United States. The second theory is that *A. hydrillicola* was native to the southeastern part of the United States and when hydrilla was introduced it provided the perfect substrate for *A. hydrillicola* to grow on. These alternatives should be distinguishable by the degree of genetic diversity present in *A. hydrillicola* across the southeastern US. Genetic diversity would be expected to be high if *A. hydrillicola* was endemic to the region, but low if importation led to a genetic bottleneck (Ohbayashi et al. 2013). Gugger, et al. (2005) compared the 16S-23S ITS region of the ribosomal operon in *Cylindrospermopisis* strains isolated from four continents to determine the pattern in which it spread from tropical to

temporal environments (Gugger et al. 2005). Using this technique, three groups were formed: American, European and African-Australian. They could hypothesize the distribution patterns of their American and European groups, but are unable to do so with their African-Australian group (Gugger et al. 2005). These groupings were further supported by the findings of Haanade et al. (2008), however, they were not able to support any of the current hypothesis about the evolution or distribution of *Cylindrospermopisis* strains. In 2011 these groups were further validated by Piccini, et al. (2011), who hypothesized that the variation seen between all of the groups could be due to the existence of ecotypes within *C. raciborskii*. These ecotypes would give rise to the current strains of *C. raciborskii* based on their ability to survive within the environment that they were introduced (Piccini et al. 2011).

Here we use a similar technique, in which we will compare the 16S-23S ITS region of the rRNA operon of *A. hydrillicola* obtained from different locations throughout the southeastern United States. Polymorphisms within the sequence should give insight into whether *A. hydrillicola* has been newly introduced or if it has been a longtime resident of the southeastern United States.

Methods

Sample Collection

Samples of hydrilla with *A. hydrillicola* adhering to its leaves were collected between the months of September 2018 and November 2018, when *A. hydrillicola* is in highest abundance. Samples were collected from 12 sites which consisted of 10 different lakes and reservoirs. Hydrilla was then brought back to the lab to confirm presence of *A. hydrillicola.* Hydrilla was thoroughly washed to remove other cyanobacteria and debris. Slides of hydrilla leaves from each

site were made and observed under an epifluorescence microscope. Once *A. hydrillicola's* presence was confirmed, hydrilla leaves with the highest concentration of *A. hydrillicola* were selected for genetic analysis. Leaves with other cyanobacteria were not included in samples for genetic analysis. Approximately 5 leaves with *A. hydrillicola* were selected from each site. These leaves were put into 1mL of TE solution and then frozen until DNA could be extracted.

Sample Sites

A total of ten samples and seven sites were included in this study (Figure 5). All samples were acquired within the months of October 2018 and November 2018 (Table 5).

Table 5. Sample Site Locations.

Site I.D.	Lake/Reservoir Name	State	Latitude	Longitude
D1	Lake J. Strom Thurmond	GA/SC	33.63866	-82.31079
D2	Lake J. Strom Thurmond	GA/SC	33.72606	-82.29483
D3	Tussahaw Reservoir	GA	33.35002	-83.99138
D4	Tussahaw Reservoir	GA	33.38165	-83.99580
D5	Long Branch Reservoir	GA	33.31907	-84.13657
D6	Lake Tohopekaliga	FL	28.26649	-81.40620
D7	Lake Ouachita	AK	34.61655	-93.29842
D8	Lake Hatchineha	FL	28.03045	-81.36732
D9	Sam Rayburn Reservoir	TX	31.13946	-94.13722
D10	Lake Nacogdoches	TX	31.61763	-94.83143

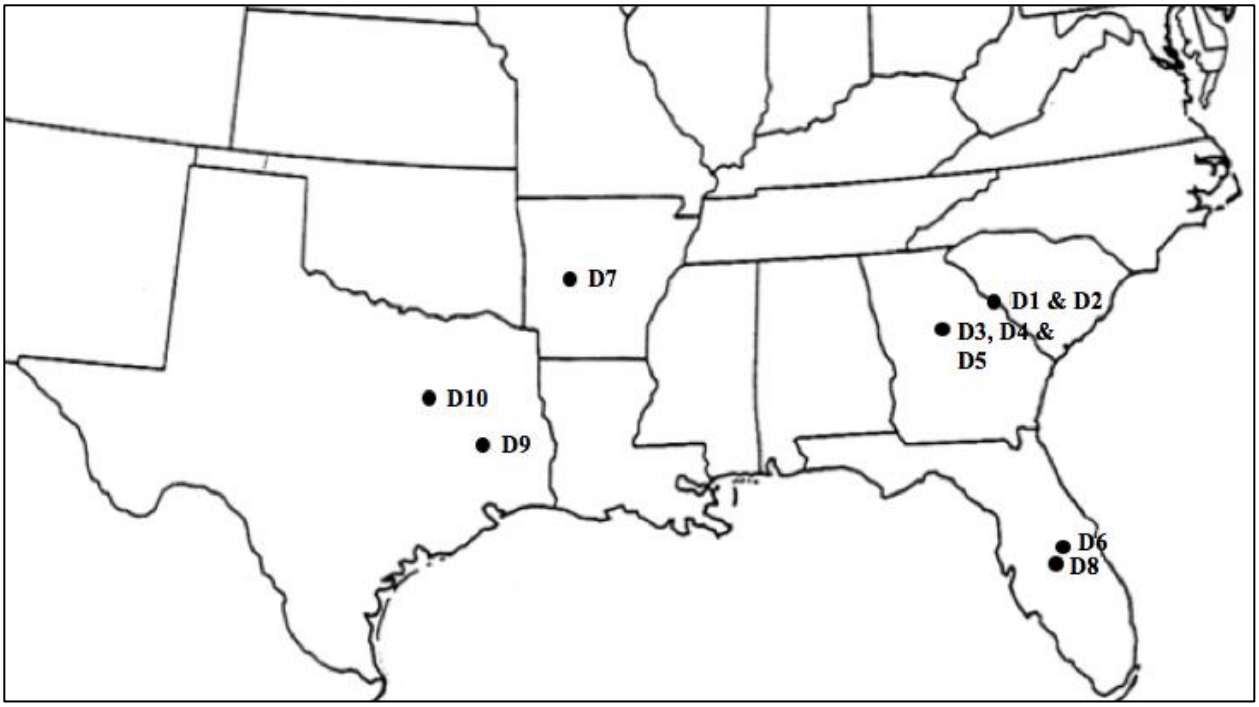

Figure 5. Map of the Sampling Sites Throughout the Southeastern United States.

DNA Extraction

For all samples, a DNeasy PowerSoil® DNA Isolation Kit (MO BIO laboratories, Inc.) was used to extract the DNA. There were some modifications the DNeasy PowerSoil® DNA Isolation Kit's protocol when using it. First, 200µl of the kit's bead solution was removed from the bead tube provided and was replaced with 200µl of phenol:chloroform:isoamyl alcohol. This was done before starting the extractions. The aqueous layer created by the phenol:chloroform:isoamyl alcohol was transferred to a new tube and used for the rest of the extraction process. Second, the spin column was washed with 650µl of 100% ethanol before washing with the provided C5 solution. Finally, the samples were eluted in only 50µl of 10mM Tris. Samples were stored in a -20°C freezer until the cloning process could take place.

Amplicon Cloning and Sequencing

The target amplicon was the 16S-23S ITS region of the rRNA operon. The CYA359 forward primer (5'-GGGGAATYTTCCGCAATGGG-3') utilized by Heck et al. and a uniquely designed reverse primer (5'-CTCTGTGTGCCTAGGTATCC-3') along with Taq 5X Master Mix (New England Biolabs) were used to amplify this desired region. The thermocycler conditions employed with these primers were as follows: 95°C for 2 minutes, then 35 cycles of 94°C for 45 seconds, 57°C for 45 seconds, 72°C for 2 minutes and 15 seconds, and finally a 5 minute incubation at 72°C. Once all the samples had the 1.6 Kb region amplified, a StrataClone PCR Cloning Kit (Agilent) was utilized per manufacturer instructions to clone *A. hydrillicola's* 16S-23S ITS region. Cells were grow for 48 hours before plasmids containing *A. hydrillicola's* 16S-23S ITS region were extracted using a QIAprep Spin Miniprep Kit (by Qiagen). The original primers used to amplify *A. hydrillicola's* 16S-23S ITS region of the rRNA gene were employed to confirm that each sample contained the correct amplicon. Once all samples were determined to contain the correct amplicon they were sequenced by the dideoxy method (Eurofins USA) using the universal M13 forward and M13 reverse primers. Each sample was done in replicates of four.

Analysis

Only one replicate per sample was recovered from sequencing data. Sequences were analyzed using Geneious Prime (version 2019.2.1). All barcodes, primers and low quality bases were removed from the raw sequences. Many N bases were edited to the most likely base using the provided peaks as a reference. After editing, the forward and reverse sequences were assembled *de novo*. Once all the sequences were assembled, the LASTZ program within

Geneious Prime determined the number of Single Nucleotide Polymorphisms (SNP's) for each sequence. Then, a phylogenetic tree was constructed using the alignment output from the LASTZ program. The neighbor-joining method was used to construct the phylogenetic tree with 2000 bootstrap replicates. Additionally, a Spearman's rank test was performed to correlate the number of SNPs in a sequence and the approximate distance (in kilometers) of the site in which sequence was acquired from the D1 site.

Results

Genotypic Composition

The sequences from each site ranged between 1524bp and 1640bp. Here, a unique genotype was defined as having 4 or more SNPs within a sequence when compared to our D1 site. Seven unique genotypes were found in this comparison. Below is a table containing the number of SNPs per sequence, as compared to our D1 site. Though seven unique genotypes were found, the genetic distances between samples were not high. When the percent identities were compared between each site, all percent identities were found to be 98.74% or higher (Table 7).

Table 6. Number of SNPs per Site When Compared to Site D1.

Site I.D.	Lake/Reservoir Name	State	Number of SNPs
D1	Lake J. Strom Thurmond	GA/SC	-
D2	Lake J. Strom Thurmond	GA/SC	2
D3	Tussahaw Reservoir	GA	13
D4	Tussahaw Reservoir	GA	4
D5	Long Branch Reservoir	GA	8
D6	Lake Tohopekaliga	FL	4
D7	Lake Ouachita	AK	8
D8	Lake Hatchineha	FL	0
D9	Sam Rayburn Reservoir	TX	15
D10	Lake Nacogdoches	TX	8

Table 7. The Percent Identity of Each Sample Site When Compared to One Another.

Sites	**D1 (GA/SC)**	**D2 (GA/SC)**	**D3 (GA)**	**D4 (GA)**	**D5 (GA)**	**D6 (FL)**	**D7 (AR)**	**D8 (FL)**	**D9 (TX)**	**D10 (TX)**
D1 (GA/SC)		99.90	99.16	99.77	99.53	99.74	99.61	100	99.08	99.61
D2 (GA/SC)	99.90		99.20	99.84	99.56	99.77	99.67	99.90	99.11	99.67
D3 (GA)	99.16	99.20		99.33	98.82	99.03	99.03	99.16	99.56	99.03
D4 (GA)	99.77	99.84	99.33		99.43	99.64	99.54	99.77	98.98	99.54
D5 (GA)	99.53	99.56	98.82	99.43		99.39	99.26	99.53	98.74	99.26
D6 (FL)	99.74	99.77	99.03	99.64	99.39		99.48	99.74	98.95	99.48
D7 (AR)	99.61	99.67	99.03	99.54	99.26	99.48		99.61	99.31	99.87
D8 (FL)	100	99.90	99.16	99.77	99.53	99.74	99.61		99.08	99.61
D9 (TX)	99.08	99.11	99.56	98.98	98.74	98.95	99.31	99.08		99.31
D10 (TX)	99.61	99.67	99.03	99.54	99.26	99.48	99.87	99.61	99.31	

Comparison of Genotypic Composition and Geographical Location

The Spearman's rank-correlation analysis yielded $\rho = 0.40$, $p=0.286$. Therefore, there was no signification relationship found between geographic location and number of SNPs in a sequence. When the sites were plotted on a graph it is apparent that there is no clear relationship between distance and number of SNPs. This graph can be seen below, in Figure 6.

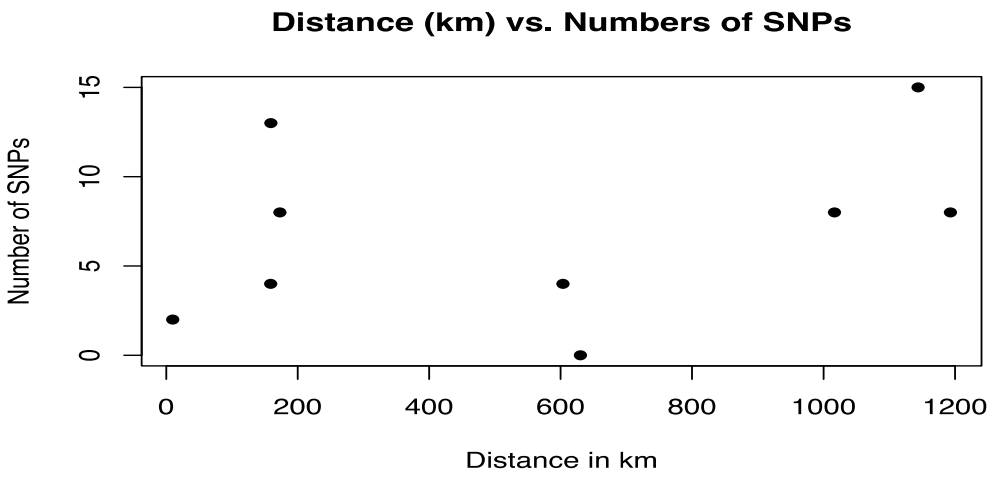

Figure 6. Distance (km) vs. Number of Single Nucleotide Polymorphisms. Relationship between geographical distance in kilometers and number of SNPs for each sequence.

Phylogenetic Comparison

While most of the sites were clustered together on the phylogenetic tree, the two sites with the highest number of SNPs (sites D3 and D11) were not included within that cluster. Apart from D3, there is an observed split between samples from sites that are further west and samples that are further east within the Southeast (Figure 7).

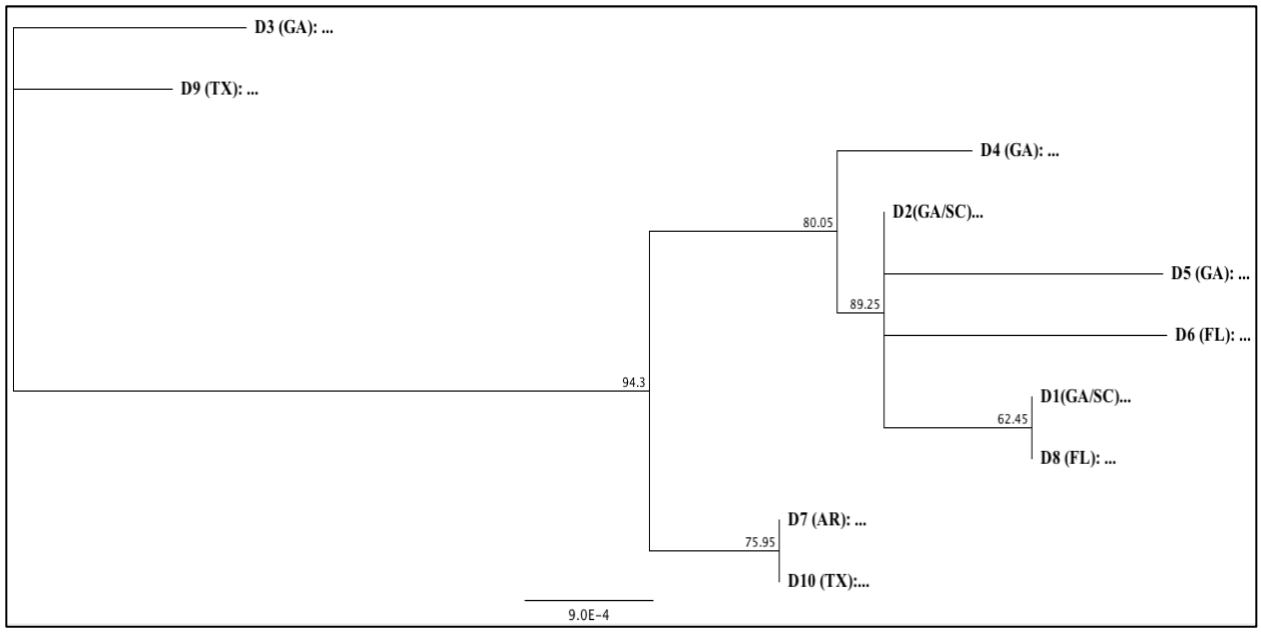

Figure 7. **Neighbor-joining Phylogenetic Tree.** The phylogenetic tree that was constructed using the 16S-23S ITS region of the rRNA gene of *A. hydrillicola* from different locations, with consensus support percentages at the nodes.

Discussion and Conclusion

There were only two sites, D2 and D8, that did not have a unique genotype when compared to the D1 site. Site D2 is located within the same lake as our D1 reference site. Therefore, Lake J. Storm Thurmond had the same genotype of *A. hydrillicola* within in it. This could be explained by site D2 being the closest in proximity to the D1 site. Aside from this, there were no other trends seen between number of unique genotypes and distance from our D1 site. Since the sequences were heavily edited, it is hard to determine whether the genotypes are true genotypes or just sequencing errors. There were also no trends to be seen in the genetic distances between sites were comparing their percent identities to one another. Since D3 and D9 were heavily edited, it is possible the *A. hydrillicola* from all the sites could still be closely related.

The two sites with the highest number of SNPs, D3 and D9, were not clustered with the other sites on the phylogenetic tree (Figure 7). This could indicate that sites D3 and D9 are more distantly related to the other sites. The phylogenetic tree also indicated that sites D7 and D10 were more distantly related to the remaining samples, which could be due to their distance from the other sample sites. However, more sequences will need to be isolated before we can confidently make these assumptions.

Finally, the Spearman's Rank Correlation test found no significant relationship between the number of SNPs in a sequence and the distance of that site when compared to our D1 site. This may have occurred due to some of the sites containing the same number of SNPs, which makes calculating the p-value of the Spearman's Rank correlation test more difficult. If there had been more sequences for each site, then a hierarchical clustering analysis could have been done between the sites. This would have given better insight into the relationship between number of SNPs and geographical location.

More research will need to be done to determine if *A. hydrillicola* is newly introduced or native to the southeastern United States. From this preliminary data, it appears there may be some variation in the 16S-23S ITS region of the rRNA gene of *A. hydrillicola* when looking at different locations. Though there is no clear trend in the number of SNPs and the distances of the sites. More sequences from each site will need to be isolated before it can be definitively stated whether *A. hydrillicola* is newly introduced or not.

REFERENCES:

Alvarenga, Danillo O., Marli F. Fiore, and Alessandro M. Varani. 2017. 'A Metagenomic Approach to Cyanobacterial Genomics', *Frontiers in Microbiology*, 8.

Augspurger, T., J. R. Fischer, N. J. Thomas, L. Sileo, R. E. Brannian, K. J. G. Miller, and T. E. Rocke. 2003. 'Vacuolar myelinopathy in waterfowl from a North Carolina impoundment', *Journal of Wildlife Diseases*, 39: 412-17.

Bidigare, R. R., S. J. Christensen, S. B. Wilde, and S. A. Banack. 2009. 'Cyanobacteria and BMAA: Possible linkage with avian vacuolar myelinopathy (AVM) in the south-eastern United States', *Amyotrophic Lateral Sclerosis*, 10: 71-73.

Birrenkott, A. H., S. B. Wilde, J. J. Hains, J. R. Fischer, T. M. Murphy, C. P. Hope, P. G. Parnell, and W. W. Bowerman. 2004. 'Establishing a food-chain link between aquatic plant material and avian vacuolar myelinopathy in mallards (Anas platyrhynchos)', *Journal of Wildlife Diseases*, 40: 485-92.

Blackburn, R.D., Weldon, L.W., Yeo, R.R., Taylor, T.M. . 1969. ' Identification and distribution of certain similar-appearing submersed aquatic weeds in Florida.'.

Bolyen, Evan, Jai Ram Rideout, Matthew R. Dillon, Nicholas A. Bokulich, Christian C. Abnet, Gabriel A. Al-Ghalith, Harriet Alexander, Eric J. Alm, Manimozhiyan Arumugam, Francesco Asnicar, Yang Bai, Jordan E. Bisanz, Kyle Bittinger, Asker Brejnrod, Colin J. Brislawn, C. Titus Brown, Benjamin J. Callahan, Andrés Mauricio Caraballo-Rodríguez, John Chase, Emily K. Cope, Ricardo Da Silva, Christian Diener, Pieter C. Dorrestein, Gavin M. Douglas, Daniel M. Durall, Claire Duvallet, Christian F. Edwardson, Madeleine Ernst, Mehrbod Estaki, Jennifer Fouquier, Julia M. Gauglitz, Sean M. Gibbons, Deanna L.

Gibson, Antonio Gonzalez, Kestrel Gorlick, Jiarong Guo, Benjamin Hillmann, Susan Holmes, Hannes Holste, Curtis Huttenhower, Gavin A. Huttley, Stefan Janssen, Alan K. Jarmusch, Lingjing Jiang, Benjamin D. Kaehler, Kyo Bin Kang, Christopher R. Keefe, Paul Keim, Scott T. Kelley, Dan Knights, Irina Koester, Tomasz Kosciolek, Jorden Kreps, Morgan G. I. Langille, Joslynn Lee, Ruth Ley, Yong-Xin Liu, Erikka Loftfield, Catherine Lozupone, Massoud Maher, Clarisse Marotz, Bryan D. Martin, Daniel McDonald, Lauren J. McIver, Alexey V. Melnik, Jessica L. Metcalf, Sydney C. Morgan, Jamie T. Morton, Ahmad Turan Naimey, Jose A. Navas-Molina, Louis Felix Nothias, Stephanie B. Orchanian, Talima Pearson, Samuel L. Peoples, Daniel Petras, Mary Lai Preuss, Elmar Pruesse, Lasse Buur Rasmussen, Adam Rivers, Michael S. Robeson, Patrick Rosenthal, Nicola Segata, Michael Shaffer, Arron Shiffer, Rashmi Sinha, Se Jin Song, John R. Spear, Austin D. Swafford, Luke R. Thompson, Pedro J. Torres, Pauline Trinh, Anupriya Tripathi, Peter J. Turnbaugh, Sabah Ul-Hasan, Justin J. J. Van Der Hooft, Fernando Vargas, Yoshiki Vázquez-Baeza, Emily Vogtmann, Max Von Hippel, William Walters, Yunhu Wan, Mingxun Wang, Jonathan Warren, Kyle C. Weber, Charles H. D. Williamson, Amy D. Willis, Zhenjiang Zech Xu, Jesse R. Zaneveld, Yilong Zhang, Qiyun Zhu, Rob Knight, and J. Gregory Caporaso. 2019. 'Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2', *Nature Biotechnology*, 37: 852-57.

Boyer, S. L., V. R. Flechtner, and J. R. Johansen. 2001. 'Is the 16S-23S rRNA Internal Transcribed Spacer Region a Good Tool for Use in Molecular Systematics and Population Genetics? A Case Study in Cyanobacteria', 18: 1057-69.

Briand, Enora, Jean-François Humbert, Kevin Tambosco, Myriam Bormans, and William H. Gerwick. 2016. 'Role of bacteria in the production and degradation ofMicrocystiscyanopeptides', *MicrobiologyOpen*, 5: 469-78.

Callahan, Benjamin J., Paul J. McMurdie, Michael J. Rosen, Andrew W. Han, Amy Jo A. Johnson, and Susan P. Holmes. 2016. 'DADA2: High-resolution sample inference from Illumina amplicon data', *Nature Methods*, 13: 581-83.

Chaffron, S., H. Rehrauer, J. Pernthaler, and C. Von Mering. 2010. 'A global network of coexisting microbes from environmental and whole-genome sequence data', *Genome Research*, 20: 947-59.

Chonudomkul, Duenrut, Wichien Yongmanitchai, Gunjana Theeragool, Masanobu Kawachi, Fumie Kasai, Kunimitsu Kaya, and Makoto M. Watanabe. 2004. 'Morphology, genetic diversity, temperature tolerance and toxicity of Cylindrospermopsis raciborskii (Nostocales, Cyanobacteria) strains from Thailand and Japan', *FEMS Microbiology Ecology*, 48: 345-55.

Desantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. 'Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB', *Applied and Environmental Microbiology*, 72: 5069-72.

Dodd, Shelley R., Rebecca S. Haynie, Susan M. Williams, and Susan B. Wilde. 2016. 'ALTERNATE FOOD-CHAIN TRANSFER OF THE TOXIN LINKED TO AVIAN VACUOLAR MYELINOPATHY AND IMPLICATIONS FOR THE ENDANGERED FLORIDA SNAIL KITE (ROSTRHAMUS SOCIABILIS)', 52: 335-44.

Driscoll, Connor B., Timothy G. Otten, Nathan M. Brown, and Theo W. Dreher. 2017. 'Towards long-read metagenomics: complete assembly of three novel genomes from bacteria dependent on a diazotrophic cyanobacterium in a freshwater lake co-culture', 12.

Erwin, Patrick M., and Robert W. Thacker. 2008. 'Cryptic diversity of the symbiotic cyanobacterium Synechococcus spongiarum among sponge hosts', *Molecular Ecology*, 17: 2937-47.

Fischer, J. R., L. A. Lewis, T. Augspurger, and T. E. Rocke. 2002. 'Avian vacuolar myelinopathy: A newly recognized fatal neurological disease of eagles, waterfowl and other birds.' in J. Rahm (ed.), *Transactions of the Sixty-Seventh North American Wildlife and Natural Resource Conference*.

Fischer, J. R., L. A. Lewis-Weis, and C. M. Tate. 2003. 'Experimental vacuolar myelinopathy in red-tailed hawks', *Journal of Wildlife Diseases*, 39: 400-06.

Glenn, Travis C., Roger A. Nilsen, Troy J. Kieran, Jon G. Sanders, Natalia J. Bayona-Vásquez, John W. Finger, Todd W. Pierson, Kerin E. Bentley, Sandra L. Hoffberg, Swarnali Louha, Francisco J. García-De León, Miguel Angel Del Río-Portilla, Kurt D. Reed, Jennifer L. Anderson, Jennifer K. Meece, Samuel E. Aggrey, Romdhane Rekaya, Magdy Alabady, Myriam Bélanger, Kevin Winker, and Brant C. Faircloth. 2016. "Adapterama I: Universal stubs and primers for 384 unique dual-indexed or 147,456 combinatorially-indexed Illumina libraries (iTru & iNext)." In.: Cold Spring Harbor Laboratory.

Glenn, Travis C., Todd W. Pierson, Natalia J. Bayona-Vásquez, Troy J. Kieran, Sandra L. Hoffberg, Jesse C. Thomas, Daniel E. Lefever, John W. Finger, Bei Gao, Xiaoming Bian, Swarnali Louha, Ramya T. Kolli, Kerin Bentley, Julie Rushmore, Kelvin Wong, Timothy I. Shaw,

Michael J. Rothrock, Anna M. McKee, Tai L. Guo, Rodney Mauricio, Marirosa Molina, Brian S. Cummings, Lawrence H. Lash, Kun Lu, Gregory S. Gilbert, Stephen P. Hubbell, and Brant C. Faircloth. 2019. "Adapterama II: Universal amplicon sequencing on Illumina platforms (TaggiMatrix)." In.: Cold Spring Harbor Laboratory.

Gordon-Bradley, N., N. Li, and H. N. Williams. 2015. 'Bacterial community structure in freshwater springs infested with the invasive plant species Hydrilla verticillata', 742: 221-32.

Gordon-Bradley, Nadine, Despoina S. Lymperopoulou, and Henry Neal Williams. 2014. 'Differences in Bacterial Community Structure on Hydrilla verticillata and Vallisneria americana in a Freshwater Spring', 29: 67-73.

Gugger, M., R. Molica, B. Le Berre, P. Dufour, C. Bernard, and J. F. Humbert. 2005. 'Genetic Diversity of Cylindrospermopsis Strains (Cyanobacteria) Isolated from Four Continents', *Applied and Environmental Microbiology*, 71: 1097-100.

Haande, Sigrid, Thomas Rohrlack, Andreas Ballot, Kjetil Røberg, Randi Skulberg, Martin Beck, and Claudia Wiedner. 2008. 'Genetic characterisation of Cylindrospermopsis raciborskii (Nostocales, Cyanobacteria) isolates from Africa and Europe', 7: 692-701.

Haynie, R. S., W. W. Bowerman, S. K. Williams, J. R. Morrison, J. M. Grizzle, J. M. Fischer, and S. B. Wilde. 2013. 'Triploid Grass Carp Susceptibility and Potential for Disease Transfer when used to Control Aquatic Vegetation in Reservoirs with Avian Vacuolar Myelinopathy', *Journal of Aquatic Animal Health*, 25: 252-59.

He, Dan, Lijuan Ren, and Qinglong Wu. 2012. 'Epiphytic bacterial communities on two common submerged macrophytes in Taihu Lake: diversity and host-specificity', *Chinese Journal of Oceanology and Limnology*, 30: 237-47.

Heaney, S. I., and G. H. M. Jaworski. 1977. 'Simple Separation Technique For Purifying Micro-algae', *British Phycological Journal*, 12: 171-74.

Heck, Karina, Gabriela Silva Machineski, Danillo Oliveira Alvarenga, Marcelo Gomes Marçal Vieira Vaz, Alessandro De Mello Varani, and Marli Fátima Fiore. 2016. 'Evaluating methods for purifying cyanobacterial cultures by qPCR and high-throughput Illumina sequencing', *Journal of Microbiological Methods*, 129: 55-60.

Ho, Lionel, Emma Sawade, and Gayle Newcombe. 2012. 'Biological treatment options for cyanobacteria metabolite removal – A review', *Water Research*, 46: 1536-48.

Hughes, Rebecca-Ayme, Yunlong Zhang, Ran Zhang, Philip G. Williams, Jonathan S. Lindsey, and Eric S. Miller. 2017. 'Genome Sequence and Composition of a Tolyporphin-Producing Cyanobacterium-Microbial Community', *Applied and Environmental Microbiology*, 83: e01068-17.

Klindworth, Anna, Elmar Pruesse, Timmy Schweer, Jörg Peplies, Christian Quast, Matthias Horn, and Frank Oliver Glöckner. 2013. 'Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies', *Nucleic Acids Research*, 41: e1-e1.

Langeland, Kenneth A. . 1996. 'Hydrilla verticillata (L.F.) Royle (Hydrocharitaceae), "The Perfect Aquatic Weed"', *Castanea*, 61: 293-304.

Larsen, R. S., F. B. Nutter, T. Augspurger, T. E. Rocke, L. Tomlinson, N. J. Thomas, and M. K. Stoskopf. 2002. 'Clinical features of avian vacuolar myelinopathy in American coots', *Journal of the American Veterinary Medical Association*, 221: 80-85.

Litchman, Elena, Paula De Tezanos Pinto, Christopher A. Klausmeier, Mridul K. Thomas, and Kohei Yoshiyama. 2010. 'Linking traits to species diversity and community structure in phytoplankton.' in (Springer Netherlands).

Lu, Weiqun, E. Hilary Evans, Suzzanne M. McColl, and Venetia A. Saunders. 2006. 'Identification of cyanobacteria by polymorphisms of PCR-amplified ribosomal DNA spacer region', 153: 141-49.

Maerz, John C., Susan B. Wilde, Vanessa K. Terrell, Brigette Haram, R. Clay Trimmer, Chelsea Nunez, Erin Cork, Allan Pessier, Sue Lannoo, Michael J. Lannoo, and Sara L. Diamond. 2019. 'Seasonal and plant specific vulnerability of amphibian tadpoles to the invasion of a novel cyanobacteria', *Biological Invasions*, 21: 821-31.

Martin, W., T. Rujan, E. Richly, A. Hansen, S. Cornelsen, T. Lins, D. Leister, B. Stoebe, M. Hasegawa, and D. Penny. 2002. 'Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus', 99: 12246-51.

Mercurio, Albert D., Sonia M. Hernandez, John C. Maerz, Michael J. Yabsley, Angela E. Ellis, Amanda L. Coleman, Leslie M. Shelnutt, John R. Fischer, and Susan B. Wilde. 2014. 'Experimental Feeding of Hydrilla verticillata Colonized by Stigonematales Cyanobacteria Induces Vacuolar Myelinopathy in Painted Turtles (Chrysemys picta)', 9: e93295.

Neilan, B. A., D. Jacobs, and A. E. Goodman. 1995. 'Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the phycocyanin locus', *Applied and Environmental Microbiology*, 61: 3875-83.

Niedermeyer, THJ. 2015. "Anti-infective natural products from Cyanobacteria." In *Planta Medica* Thieme-connect.com.

Ohbayashi, Kako, Yoshikuni Hodoki, Yuki Kobayashi, Noboru Okuda, and Shin-Ichi Nakano. 2013. 'Genotypic composition and the relationship between genotypic composition and geographical proximity of the cyanobacterium Microcystis aeruginosa in western Japan', 59: 266-72.

Patel, Anand, Rachel T. Noble, Joshua A. Steele, Michael S. Schwalbach, Ian Hewson, and Jed A. Fuhrman. 2007. 'Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I', 2: 269-76.

Penn, Kevin, Jia Wang, Samodha C. Fernando, and Janelle R. Thompson. 2014. 'Secondary metabolite gene expression and interplay of bacterial functions in a tropical freshwater cyanobacterial bloom', 8: 1866-78.

Piccini, Claudia, Luis Aubriot, Amelia Fabre, Valentina Amaral, Mauricio González-Piana, Alessandra Giani, Cleber C. Figueredo, Leticia Vidal, Carla Kruk, and Sylvia Bonilla. 2011. 'Genetic and eco-physiological differences of South American Cylindrospermopsis raciborskii isolates support the hypothesis of multiple ecotypes', *Harmful Algae*, 10: 644-53.

Raven, John A., and John F. Allen. 2003. *Genome Biology*, 4: 209.

Rocap, Gabrielle, Frank W. Larimer, Jane Lamerdin, Stephanie Malfatti, Patrick Chain, Nathan A. Ahlgren, Andrae Arellano, Maureen Coleman, Loren Hauser, Wolfgang R. Hess, Zackary I. Johnson, Miriam Land, Debbie Lindell, Anton F. Post, Warren Regala, Manesh Shah, Stephanie L. Shaw, Claudia Steglich, Matthew B. Sullivan, Claire S. Ting, Andrew Tolonen, Eric A. Webb, Erik R. Zinser, and Sallie W. Chisholm. 2003. 'Genome divergence in two Prochlorococcus ecotypes reflects oceanic niche differentiation', 424: 1042-47.

Rocke, T. E., N. J. Thomas, T. Augspurger, and K. Miller. 2002. 'Epizootiologic studies of avian vacuolar meylinopathy in waterbirds', *Journal of Wildlife Diseases*, 38: 678-84.

Sabart, Marion, David Pobel, Delphine Latour, Joel Robin, Marie- J. Salençon, and Jean- F. Humbert. 2009. 'Spatiotemporal changes in the genetic diversity in French bloom-forming populations of the toxic cyanobacterium, Microcystis aeruginosa', 1: 263-72.

Thomas, N. J., C. U. Meteyer, and L. Sileo. 1998. 'Epizootic vacuolar myelinopathy of the central nervous system of bald eagles (Haliaeetus leucocephalus) and American coots (Fulica americana)', *Veterinary Pathology*, 35: 479-87.

Wells, M. L., V. L. Trainer, T. J. Smayda, B. S. O. Karlson, C. G. Trick, R. M. Kudela, A. Ishikawa, S. Bernard, A. Wulff, D. M. Anderson, and W. P. Cochlan. 2015. 'Harmful algal blooms and climate change: Learning from the past and present to forecast the future', *Harmful Algae*, 49: 68-93.

West, N J, and D G Adams. 1997. 'Phenotypic and genotypic comparison of symbiotic and free-living cyanobacteria from a single field site', *Applied and Environmental Microbiology*, 63: 4479-84.

Wilde, S. B., J. R. Johansen, H. D. Wilde, P. Jiang, B. A. Bartelme, and R. S. Haynie. 2014. 'Aetokthonos hydrillicola gen. et sp. nov.: Epiphytic cyanobacteria on invasive aquatic plants implicated in Avian Vacuolar Myelinopathy', *Phytotaxa*, 181: 243-60.

Wilde, S. B., T. M. Murphy, C. P. Hope, S. K. Habrun, J. Kempton, A. Birrenkott, F. Wiley, W. W. Bowerman, and A. J. Lewitus. 2005. 'Avian vacuolar myelinopathy linked to exotic aquatic plants and a novel cyanobacterial species', *Environmental Toxicology*, 20: 348-53.

Wiley, F. E., S. B. Wilde, A. H. Birrenkott, S. K. Williams, T. M. Murphy, C. P. Hope, W. W. Bowerman, and J. R. Fischer. 2007. 'Investigation of the link between avian vacuolar myelinopathy and a novel species of cyanobacteria through laboratory feeding trials', *Journal of Wildlife Diseases*, 43: 337-44.

Williams, S. K., J. Kempton, S. B. Wilde, and A. Lewitus. 2007. 'A novel epiphytic cyanobacterium associated with reservoirs affected by avian vacuolar myelinopathy', *Harmful Algae*, 6: 343-53.

Williams, S. K., S. B. Wilde, T. M. Murphy, C. P. Hope, A. Birrenkott, and A. J. Lewitus. 2008. 'Investigation of a novel epiphytic cyanobacterium associated with reservoirs affected by avian vacuolar myelinopathy.' in H. K. Hudnell (ed.), *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs*.

Yoshida, Mitsuhiro, Takashi Yoshida, Yukari Takashima, Ryuji Kondo, and Shingo Hiroishi. 2005. 'Genetic diversity of the toxic cyanobacteriumMicrocystis in Lake Mikata', *Environmental Toxicology*, 20: 229-34.

APPENDIX A.

iNext Fusion Primers with Internal Tags

Primer	Sequences 5' to 3'
iTru_1_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGAAGACTACHVGGGTATCTAATCC
iTru_2_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAGTGGGACTACHVGGGTATCTAATCC
iTru_3_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACGTCGACTACHVGGGTATCTAATCC
iTru_4_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTCTCAGCGACTACHVGGGTATCTAATCC
iTru_5_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTAGGGACTACHVGGGTATCTAATCC
iTru_6_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCTTAGACTACHVGGGTATCTAATCC
iTru_7_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCGAAGTGACTACHVGGGTATCTAATCC
iTru_8_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAATCCTATGACTACHVGGGTATCTAATCC
iTru_9_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCTGGACTACHVGGGTATCTAATCC
iTru_10_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAGACTGACTACHVGGGTATCTAATCC
iTru_11_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGATTCCGACTACHVGGGTATCTAATCC
iTru_12_16S_785_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTCAATCGACTACHVGGGTATCTAATCC
iTru_A_16S_341_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTACCCTACGGGNGGCWGCAG
iTru_B_16S_341_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACACCCTACGGGNGGCWGCAG
iTru_C_16S_341_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATCGGTTCCTACGGGNGGCWGCAG
iTru_D_16S_341_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGGTCAACCTACGGGNGGCWGCAG
iTru_E_16S_341_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGCGCCTACGGGNGGCWGCAG
iTru_F_16S_341_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCACACCTACGGGNGGCWGCAG
iTru_G_16S_341_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGGATGCCTACGGGNGGCWGCAG
iTru_H_16S_341_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGATTGACCCTACGGGNGGCWGCAG