# EXPANDING MOLECULAR DETECTION OF THE CYANOBACTERIUM PRODUCING 'EAGLE KILLER TOXIN' CAUSING VACUOLAR MYELINOPATHY

by

ERIKA MICHELLE KLAR

(Under the Direction of Caterina Villari & Susan Wilde)

## ABSTRACT

Vacuolar Myelinopathy (VM) is a neurological disease caused by exposure to aetokthonotoxin (AETX), a neurotoxin produced by the cyanobacteria *Aetokthonos hydrillicola* growing epiphytically on *Hydrilla verticillata*. Vacuolar Myelinopathy causes mortality in bald eagles and other wildlife in the Southeastern US by inducing vacuoles in white brain matter. Field molecular detection protocols allow for the detection of the cyanobacterium with minimal time and equipment. In this study, we developed a LAMP (Loop-Mediated Isothermal Amplification) protocol for the molecular detection of *A. hydrillicola* in a field setting. The primer set targets the toxin gene cluster associated with the production of AETX, which ensures high specificity to *A. hydrillicola*. Our results demonstrate that LAMP can effectively be utilized with crude DNA extracts to detect the cyanobacterial toxin gene clusters in the field at concentrations similar to conventional PCR. Once fully developed, this protocol could be used to create an AETX field test kit for scientists and managers to detect the potential for AETX, the eagle killer toxin.

INDEX WORDS: Harmful Algal Blooms, LAMP, PCR, *Aetokthonos hydrillicola*, Wildlife Disease, aetokthonotoxin

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# DEDICATION

I wish to dedicate this work to my fiancé Jamey, Mama and Dad, and my friends and family who supported me through the late nights, frustrating days, and encouraged me to push through and never give up. “Iron sharpens iron” and you all have definitely sharpened mine.

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# CHAPTER 1

# THESIS INTRODUCTION AND LITERATURE REVIEW

## Cyanobacteria Overview

Cyanobacteria are photosynthetic prokaryotes that are found in most environments around the world. They are considered one of the most ecologically important organisms on Earth due to their enormous diversity and the number of individuals observed throughout the natural world (Whitton and Potts 2012). Cyanobacterial harmful algal blooms (cyanoHABs), rapidly accumulating in nutrient-rich environments, can produce toxins that are a threat to aquatic ecosystems worldwide. This trend is expected to increase with eutrophication, the spread of non-native species, and climate change (Sukenik and Kaplan 2021). Cyanobacterial harmful algal blooms are common in inland freshwater ecosystems due to their preference for aquatic conditions including low water movement, neutral to alkaline pH, and waters that have high nutrient loads of nitrogen and phosphorous (Carmichael 1994). These conditions are often met in areas where human populations are high, and there are elevated levels of anthropogenic runoff. While most of the scientific attention has focused on the risks of toxic cyanobacteria to water quality and human health, cyanoHABs also present a threat to wildlife, including rare, threatened, and endangered species, or agricultural commodities (Carmichael and Boyer 2016; Sahoo *et al.* 2024)). Epiphytic cyanobacteria present an emerging threat to species and community dynamics through the introduction of novel toxins, with potentially far-reaching effects across taxa and trophic levels within aquatic communities (Haram *et al.* 2020; Paerl *et al.*

2018). An example of a disease linked to epiphytic cyanobacteria is Vacuolar Myelinopathy (VM) (Breinlinger *et al.* 2021), which is the focus of this thesis.

**Vacuolar Myelinopathy Overview**

Vacuolar Myelinopathy has been documented across the southeastern United States since its discovery in DeGray Lake, Arkansas in 1994 (Thomas *et al.* 1998). At its discovery, it was causing mass casualties among waterfowl, including American coots, *Fulica americana*, and bald eagles, *Haliaeetus leucocephalus* (Wilde *et al.* 2014). Since it was believed to only affect birds, the disease was originally named Avian Vacuolar Myelinopathy (AVM). However, since then it has been shown to affect other animal species and is now referred more generally to as Vacuolar Myelinopathy. Between 1994 and 2004, more than 70 bald eagle deaths had been documented from VM between Arkansas, Georgia, and South Carolina (Fischer *et al.* 2006). Since then, at least an additional 30 sites throughout the Southeast have observed cases of VM with the presence of *A. hydrillicola* (Woods *et al.* 2016), and there are likely many more observed cases of VM that have not yet been published. The disease is characterized by substantial intramyelinic edema, specifically in the optic tectum and cerebellar tracts, of birds, fish, reptiles, and amphibians, resulting in a loss of motor function and mortality (Breinlinger *et al.* 2021; Thomas *et al.* 1998; Wilde *et al.* 2014). Potential cases of VM are identified in the field when birds lack the ability to fly when startled, or when typical neurological signs appear such as unusual movement or behavioral patterns, sensitivity to light, or isolation from a group. For positive identification of the disease, histological analysis of the brain tissue would present with "holes" or vacuoles within the white brain matter of the affected individual (Breinlinger *et al.* 2021; Thomas *et al.* 1998; Williams *et al.* 2007). Although the disease has been classified since

the late 20th century, the cause of VM was not identified and named until 2021 (Adak *et al.* 2022; Breinlinger *et al.* 2021). Vacuolar myelinopathy is induced by the lipophilic cyanotoxin aetokthonotoxin (AETX), which is produced by a cyanobacterium, *Aetokthonos hydrillicola,* that grows epiphytically on a highly invasive aquatic plant, specifically *Hydrilla verticillata* (Hydrilla) (Breinlinger *et al.* 2021). Aetokthonotoxin induces disease through ingestion by waterfowl, fish, and other aquatic organisms of Hydrilla with toxin producing *A. hydrillicola* growing on the leaves and stems (Breinlinger *et al.* 2021). There has not been a confirmed case of mammals contracting VM, however, since AETX is lipophilic, it has the capability to bioaccumulate through the food web and hence potentially impact mammals, including humans (Breinlinger *et al.* 2021). Recent data have been collected on a disease presenting similar symptoms in Florida Panther, *Puma concolor coryi*, and named feline leukomyelopathy (FLM), however, a linkage between AETX and FLM has not yet been confirmed (Bayles 2023). Mice clinical trials to determine the possibility of mammalian susceptibility to AETX are currently being performed by the Susan Wilde Lab at the University of Georgia. Preliminary results suggest that VM can be induced in mice when exposed to AETX through ingestion, however, these results are unpublished and still being investigated further.

**Natural History of *Aetokthonos hydrillicola***

Much is still unknown about the origin of *A. hydrillicola* and its distribution throughout U.S. watersheds. At its discovery, the species was considered a novel cyanobacterium in a new genus with no known related species (Wilde *et al.* 2014). It was originally challenging to place the cyanobacterium in a specific family due to its variance in ITS and morphological features, however, it has since been reclassified as a true-branching epiphytic cyanobacteria in the Order

Nostacales, Family Scytonemataceae (Strunecký *et al.* 2023; Wilde *et al.* 2014). *Aetokthonos hydrillicola* prefers slightly different environmental conditions than other epiphytic cyanobacteria. This may be the reason why it can survive in large mats of Hydrilla, with low light and nutrients, while other cyanobacteria cannot. The species prefers lower light, low nutrients, and lower temperature than co-occurring cyanobacterial species (Wilde *et al.* 2014). Hydrilla is an invasive aquatic plant species originating from India and southern Asia (Jacono 2015; Patrick and Florentine 2021) which is found in many reservoirs throughout the southeastern U.S. as well as many other regions within the U.S. (Jacono 2015; Wilde *et al.* 2005). It grows in a tightly clumped pattern near the surface of the water in the reservoirs it inhabits (Jacono 2015). Hydrilla is difficult to eliminate once established, as it reproduces through tubers that can remain dormant for >7 years in the reservoir sediment. While the top leaves and stems of the Hydrilla may be killed, there may be tubers and turions that remain below the surface of the soil (Patrick and Florentine 2021). These remaining tubers can then regrow and reestablish Hydrilla within reservoirs. Hydrilla is also unique as the pH on the top part of the leaves is much less acidic than the underside of the leaves during active photosynthesis (Carmichael 1994; van Ginkel *et al.* 2001). This inducible pH polarity provides an ideal environment for the *A. hydrillicola* growth on the underside of the leaf, where pH is ~4, while on the upper leaf surface, pH is ~10 in still water.

As of Spring 2023, *A. hydrillicola* and the novel AETX had been documented in 44 locations including large (>10,000 hectares) hydropower/water supply reservoirs, county water source reservoirs, suburban recreational lakes, and farm ponds (Gerrin, unpublished: Figure 1.1). Since then, several new Hydrilla/*Aetokthonos* positive reservoirs in Kentucky, New York, Louisiana, Texas, and North Carolina have been identified by increased sampling efforts and the

use of new detection methods targeting both the toxin and *A. hydrillicola,* as performed by Susan Wilde's Lab at the University of Georgia, Athens, Ga. According to a recent model predicting the probability of suitable habitat for *A. hydrillicola*, the most high-risk watersheds are those in the Southeast and are associated with pine tree presence surrounding the reservoir (Gerrin, unpublished). Commercial pine plantations are a major commodity in the Southeast which could contribute to the increased suitable habitat and presence of the cyanobacterium in these watersheds.

The origin of *A. hydrillicola* has not been determined, thus it is still unknown whether it is native or introduced. Recent studies have detected a potentially unique genetic variant of *A. hydrillicola* growing on American water-willow, *Justicia americana* (Štenclová *et al.* 2023). This finding raises questions about the diversity and origin of the *Aetokthonos* species present in U.S. watersheds. While *A. hydrillicola* is commonly associated with *H. verticillata*, it has also been found growing on other aquatic vegetation (S. Wilde, personal observation). However, the presence of AETX is not always detected when *A. hydrillicola* is present, highlighting the need to better understand the factors that induce toxicity, including aquatic vegetation selectivity. The aetokthonotoxin producing gene cluster within *A. hydrillicola* is composed of 6 genes that combined produce the toxin: *aetA*, *aetB*, *aetC*, *aetD*, *aetE*, and *aetF* (Adak *et al.* 2022; Breinlinger *et al.* 2021). Of these genes, *aetA* and *aetF* share strong homology with known genes coding for enzymes of FAD-dependent halogenases, *aetB* is classified as a cytochrome P450, and *aetE* is homologous to PLP-dependent tryptophanases. Genes *aetC* and *aetD* did not show any strong homology with any known enzymes (Adak *et al.* 2022). Since AETX has been identified as a pentabrominated metabolite, *A. hydrillicola* can live in a given environment but not have a significant toxin production until the bromide content reaches a critical level (Breinlinger *et al.*

2021). In a previous study, *A. hydrillicola* was grown in a laboratory setting on media lacking any bromide source, and it did not produce AETX until potassium bromide was added to the growth media (Breinlinger *et al.* 2021). Because of this bromide dependency, increases of bromide in the environment from anthropogenic and geologic sources are crucial for predicting areas of VM outbreak. The reliance of *A. hydrillicola* on bromide for toxin production is also hypothesized to be the reason for the seasonality of AETX production, as when Hydrilla dies back seasonally or post chemical treatment, it provides a bromide-rich environment for AETX production (Breinlinger *et al.* 2021). This theory, however, remains untested. In many regions throughout the range of Hydrilla, the leaves and stems of the aquatic plant die back completely, which would limit the habitat available to *A. hydrillicola* during the wintering months. However, due to the tubers in the soil, Hydrilla becomes prolific again the following spring. It is unknown whether *A. hydrillicola* continues to grow on the tubers or in the soil during the winter months when Hydrilla leaves are not present.

Another challenge when working with *A. hydrillicola* is its thick cell wall with polysaccharide sheath, commonly found among cyanobacterial species (Hoiczyk 1998; Mehta *et al.* 2015). The purpose of the sheath is to aid in motility and its form varies based on the need of the cyanobacterium (Hoiczyk 1998). This, however, provides complications for molecular detection techniques that require DNA extraction due to the need for more aggressive cell lysis steps (Mehta *et al.* 2015). Because of the sticky nature of the polysaccharide sheath, it is often observed that cyanobacteria also carry their own microbiome on their outer cell wall (Chaffron *et al.* 2010). This complicates the capability to isolate and grow pure cultures of *A. hydrillicola*, as well as its slow growth on media and the risk of being overgrown by other competing cyanobacterial species (Wilde *et al.* 2014). Attempts have been made to remove external

cyanobacterial species and enrich the *A. hydrillicola* DNA present in the sample, to ultimately make whole genome sequencing less time-consuming (Howard 2019), but working with this organism remains challenging.

**Loop-Mediated Isothermal Amplification Overview**

Loop-Mediated Isothermal Amplification (LAMP) is a molecular amplification process that is optimized for speed, specificity, and can be more easily portable compared to other conventional DNA amplification approaches, such as polymerase chain reaction (PCR) and quantitative PCR (qPCR) (Notomi *et al.* 2015; Notomi *et al.* 2000). LAMP utilizes four primers that bind to six sites in the target DNA region: the forward inner primer (FIP) and backward inner primer (BIP) each comprise two associated primer sequences (i.e., F1/B1 and F2/B2) that recognize as many regions within the DNA, while the two external primers F3 and B3 bind to one region each (Notomi *et al.* 2015; Notomi *et al.* 2000). Since there are more recognized sites for the primers to attach to, LAMP has the potential to be more specific to its target sequence than traditional PCR techniques (Notomi *et al.* 2015). In the initial stages of the reaction, the FIP and BIP primers create the characteristic loop structures of LAMP products, with the F1/B1 sequence in their tail that hybridize to the F1c/B1compliment sequences as the new DNA strand is produced (Notomi *et al.* 2000). The polymerase utilized for LAMP is a *Bst* polymerase instead of the *Taq* polymerase used in conventional PCR. The *Bst* polymerase does not hydrolyze previously synthesized DNA but allows for displacement during replication through the unwinding of the nucleic acid strands. This feature allows for the exponential amplification that is observed in LAMP reaction as well as the production of long amplicons of various sizes with repeated self-priming sites (Park 2022). The loops within the product provide a useful region for the

attachment of fluorescent probes that can be used to measure changes in amplification in real time. The time needed to amplify enough product for successful visualization varies among reactions but is usually within the hour (can be less than 20 minutes; Meinecke *et al.* (2023)). Despite the rapidity of the reaction, its sensitivity is comparable to that of real- time PCR (Notomi *et al.* 2015). The *Bst* polymerase is also less susceptible to inhibition than *Taq*, which allows for the use of crude DNA extraction methods and lends LAMP to field portability (Kogovšek *et al.* 2017; Meinecke *et al.* 2023). LAMP reaction assembly still requires the addition of DNA, probes, and primers to a commercially available master mix, however, once assembled, the reaction mix is stable at room temperature, and the reaction *per se* runs at a constant temperature, contributing to LAMP field portability (Aglietti *et al.* 2021; Hamilton *et al.* 2020; Hodgetts *et al.* 2015; Jenkins *et al.* 2011; Kiddle *et al.* 2012; Meinecke *et al.* 2023; Notomi *et al.* 2015; Villari *et al.* 2013; Williams *et al.* 2017).

There are many different techniques for visualizing LAMP products, including loop-binding fluorescent probes (Aglietti *et al.* 2021; Jenkins *et al.* 2011; Kiddle *et al.* 2012; Kogovšek *et al.* 2017), double-stranded intercalating dyes, such as SyberGreen (Parida *et al.* 2008), other colorimetric changes (Hai-sheng *et al.* 2012; Lai *et al.* 2020; Lai *et al.* 2021; Sriworarat *et al.* 2015), and sometimes gel electrophoresis (Hai-sheng *et al.* 2012; Li *et al.* 2017; Savan *et al.* 2005). Gel electrophoresis is, however, discouraged because of the high risk of contaminating the working environment with the reaction product, which is highly stable and prone to self-replication (Kubota *et al.* 2011). The DNA extraction methods suitable for use in LAMP reactions also vary and will depend on the type of the sample being extracted and the need for portability. Field portable extraction methods include, among others, a dipstick approach with mechanical homogenization, boiling, use of Chelex, or a combination of several

methods (Aglietti *et al.* 2021; Hai-sheng *et al.* 2012; Hamilton *et al.* 2020; Hodgetts *et al.* 2015; Mason and Botella 2020; Meinecke *et al.* 2023; Nagai *et al.* 2012; Stehlíková *et al.* 2020; Zou *et al.* 2017). However, while all technically field portable, these extraction methods are not equal in terms of ease of deployment. The pipette-free dipstick and extraction buffer developed by Mason and Botella (2020), for instance, is an extraction method particularly suitable for field settings, as it requires minimal equipment. However, it does have the limitations of providing a low extraction yield and not allowing long-term conservation of the DNA extracts (Mason and Botella 2020; Meinecke *et al.* 2023). This method has been utilized in combination with mechanical homogenization of tissue via metal ball bearings or micro pestles designed for 1.5ml microcentrifuge tubes in previous LAMP protocols (Meinecke *et al.* 2023). Combining boiling and the use of Chelex for extraction has also been shown to successfully provide adequate DNA for LAMP reactions, however, the protocol requires additional equipment (such as pipettes, a boiler, and a centrifuge) that may not be easy to transport in the field (Hamilton *et al.* 2021; Hamilton *et al.* 2020). A recent study has shown that field portable extraction and visualization methods can be comparable in detection sensitivity and specificity to laboratory-based qPCR (Meinecke *et al.* 2023)

**Objectives of this Thesis**

The goal of this research was to develop a protocol that would allow for better detection and further understanding of a neurotoxin producing cyanobacterium growing on Hydrilla and causing VM outbreaks throughout the southeastern United States. Molecular detection of *A. hydrillicola* was previously limited to conventional PCR assays that target the AETX biosynthesis gene cluster and ITS region (Štenclová *et al.* 2023). The outcome of this research

will increase the capacity in which current molecular techniques can be applied to our target species and hence better assist management decisions. Since the sequence coding for the AETX biosynthesis gene cluster is not found in any other known organism, it shows promise for providing the capability to detect *A. hydrillicola* with great specificity. Moreover, by targeting the AETX cluster region, not only will the assay allow for early detection of *Aetokthonos*, but it will also inform management decisions for determining which reservoirs are at risk for AETX presence before the toxin presence can cause disease. If the gene coding region for the toxin is present, disease mitigation steps can take place before an outbreak occurs.

To achieve this goal, I established a reliable molecular detection protocol that could be widely applied to further detect where *A. hydrillicola* is present in the landscape. I accomplished this by creating a rapid, field-portable LAMP protocol to detect the *A. hydrillicola* AETX biosynthesis gene cluster, which comprises six genes that code for AETX. The LAMP primers I developed target the *aetC* and *aetD* genes and the intergenic region in between. The PCR primer sets currently published for *A. hydrillicola* are also on the toxin gene cluster as well as the ITS region (Štenclová *et al.* 2023). However, conventional PCR protocols require extensive laboratory space, equipment, and time. I also identified a suitable field extraction method for this system by testing the efficacy of different field-portable DNA isolation techniques to break through the mucilaginous sheath of *A. hydrillicola.* Finally, I validated the combined DNA extraction-LAMP protocol with field-collected samples from the ecologically significant Mississippi Flyway (Texas, Mississippi, and Louisiana). This study provides a replicable and reliable protocol for the detection of *A. hydrillicola* that can be utilized by managers to determine the risk for VM outbreaks within their reservoirs to aid in mitigation efforts.

With the establishment of a LAMP molecular detection protocol, state and federal agencies with concerns about VM outbreaks and the presence of *A. hydrillicola* on Hydrilla in their waterways will be able to rapidly test for the cyanobacterium. Many agencies already manage for Hydrilla, however, treatments for the aquatic plant include chemical sprays containing bromide. This is of concern for regions where the cyanobacterium is present since the input of bromide in the water column potentially allows for further production of AETX. This molecular test could serve as a safety procedure to test for the presence of *A. hydrillicola* before treatment of Hydrilla with brominated sprays to prevent the potential for VM outbreaks.

LITERATURE CITED

Adak S, Lukowski AL, Schäfer RJB, and Moore BS (2022) From tryptophan to toxin: Nature's convergent biosynthetic strategy to aetokthonotoxin. *Journal of the American Chemical Society* **144**, 2861-2866. doi: 10.1021/jacs.1c12778.

Aglietti C, Meinecke CD, Ghelardini L, Barnes I, Nest Avd, and Villari C (2021) Rapid detection of pine pathogens lecanosticta acicola, dothistroma pini and d. Septosporum on needles by probe-based lamp assays. *Forests* **12**. doi: 10.3390/f12040479 https://www.mdpi.com/1999-4907/12/4/479.

Bayles T (2023) A mysterious disease is leaving some florida panthers disoriented, wobbly, confused, and others dead. (WUSF)

Breinlinger S, Phillips TJ, Haram BN, Mareš J, Yerena JAM, Hrouzek P, Sobotka R, Henderson M, Schmieder P, Williams SM, Lauderdale JD, Wilde D, Gerrin W, Kust A, Washington JW, Wagner C, Geier B, Liebeke M, Enke H, Niedermeyer THJ, and Wilde SB (2021) Hunting the eagle killer: A cyanobacterial neurotoxin causes vacuolar myelinopathy. *Science (Washington)* **371**. doi: 10.1126/science.aax9050 https://science.sciencemag.org/content/371/6536/eaax9050.abstract.

Carmichael WW (1994) The toxins of cyanobacteria. *Scientific American* **270**, 78-86.

Carmichael WW and Boyer GL (2016) Health impacts from cyanobacteria harmful algae blooms: Implications for the north american great lakes. *Harmful Algae* **54**, 194-212. doi: 10.1016/j.hal.2016.02.002.

Chaffron S, Rehrauer H, Pernthaler J, and von Mering C (2010) A global network of coexisting microbes from environmental and whole-genome sequence data. *Genome Research* **20**, 947-959. doi: 10.1101/gr.104521.109.

Fischer JR, Lewis-Weis LA, Tate CM, Gaydos JK, Gerhold RW, and Poppenga RH (2006) Avian vacuolar myelinopathy outbreaks at a southeastern reservoir. *Journal of Wildlife Diseases* **42**, 501-510. doi: https://www.wildlifedisease.org.

Hai-sheng X, Lin H, Sun-jian L, Qi G, and Song L (2012) Establishment of universal loop-mediated isothermal amplification method (lamp) for rapid detection of pathogenic vibrio spp. In aquatic organisms. *African Journal of Microbiology Research* **6**, 3447-3454.

Hamilton JL, Fraedrich SW, Nairn CJ, Mayfield AE, and Villari C (2021) A field-portable diagnostic approach confirms laurel wilt disease diagnosis in minutes instead of days. *Arboriculture & Urban Forestry* **47**, 98-109. doi: 10.48044/jauf.2021.010.

Hamilton JL, Workman JN, Nairn CJ, Fraedrich SW, and Villari C (2020) Rapid detection of raffaelea lauricola directly from host plant and beetle vector tissues using loop-mediated isothermal amplification. *Plant Disease* **104**, 3151-3158. doi: 10.1094/PDIS-02-20-0422-RE https://apsjournals.apsnet.org/doi/10.1094/PDIS-02-20-0422-RE.

Haram BN, Wilde SB, Chamberlain MJ, and Boyd KH (2020) Vacuolar myelinopathy: Waterbird risk on a southeastern impoundment co-infested with hydrilla verticillata and aetokthonos hydrillicola. *Biological Invasions* **22**, 2651-2660. doi: 10.1007/s10530-020-02282-w.

Hodgetts J, Hall J, Karamura G, Grant M, Studholme DJ, Boonham N, Karamura E, and Smith JJ (2015) Rapid, specific, simple, in-field detection of xanthomonas campestris pathovar musacearum by loop-mediated isothermal amplification. *Journal of Applied Microbiology* **119**, 1651-1658. doi: https://doi.org/10.1111/jam.12959.

Hoiczyk E (1998) Structural and biochemical analysis of the sheath of phormidium uncinatum. *J Bacteriol* **180**, 3923-3932. doi: 10.1128/jb.180.15.3923-3932.1998.

Howard A (2019) Genetic investigations of a novel, toxic, epiphytic cyanobacteria: Aetokthonos hydrillicola. M.S. Thesis, (University of Georgia, United States -- Georgia.

Jacono CC, Richerson, M.M. , Howard Morgan, V. , and Pfingsten, I.A. (2015) Hydrilla verticillata (l. F.) royle. U.S. Geological Survey, Nonindigenous Aquatic Species Database)

Jenkins DM, Kubota R, Dong J, Li Y, and Higashiguchi D (2011) Handheld device for real-time, quantitative, lamp-based detection of salmonella enterica using assimilating probes. *Biosensors and Bioelectronics* **30**, 255-260. doi: https://doi.org/10.1016/j.bios.2011.09.020.

Kiddle G, Hardinge P, Buttigieg N, Gandelman O, Pereira C, McElgunn CJ, Rizzoli M, Jackson R, Appleton N, Moore C, Tisi LC, and Murray JAH (2012) Gmo detection using a bioluminescent real time reporter (bart) of loop mediated isothermal amplification (lamp) suitable for field use. *BMC Biotechnology* **12**, 15. doi: 10.1186/1472-6750-12-15.

Kogovšek P, Mehle N, Pugelj A, Jakomin T, Schroers H-J, Ravnikar M, and Dermastia M (2017) Rapid loop-mediated isothermal amplification assays for grapevine yellows phytoplasmas on crude leaf-vein homogenate has the same performance as qpcr. *European Journal of Plant Pathology* **148**, 75-84. doi: 10.1007/s10658-016-1070-z.

Kubota R, Alvarez AM, Su WW, and Jenkins DM (2011) Fret-based assimilating probe for sequence-specific real-time monitoring of loop-mediated isothermal amplification (lamp). pp. 81-100. (AMERICAN SOCIETY OF AGRICULTURAL AND BIOLOGICAL ENGINEERS: United States)

Lai MY, Ooi CH, Jaimin JJ, and Lau YL (2020) Evaluation of warmstart colorimetric loop-mediated isothermal amplification assay for diagnosis of malaria. *Am J Trop Med Hyg* **102**, 1370-1372. doi: 10.4269/ajtmh.20-0001.

Lai MY, Ooi CH, and Lau YL (2021) Validation of sybr green i based closed-tube loop-mediated isothermal amplification (lamp) assay for diagnosis of knowlesi malaria. *Malaria Journal* **20**, 166. doi: 10.1186/s12936-021-03707-0.

Li Y, Fan P, Zhou S, and Zhang L (2017) Loop-mediated isothermal amplification (lamp): A novel rapid detection platform for pathogens. *Microbial Pathogenesis* **107**, 54-61. doi: 10.1016/j.micpath.2017.03.016.

Mason MG and Botella JR (2020) Rapid (30-second), equipment-free purification of nucleic acids using easy-to-make dipsticks. *Nature Protocols* **15**, 3663. doi: 10.1038/s41596-020-0392-7.

Mehta KK, Evitt NH, and Swartz JR (2015) Chemical lysis of cyanobacteria. *J Biol Eng* **9**, 10. doi: 10.1186/s13036-015-0007-y.

Meinecke CD, Vos L, Yilmaz N, Steenkamp ET, Wingfield MJ, Wingfield BD, and Villari C (2023) A lamp assay for rapid detection of the pitch canker pathogen fusarium circinatum. *Plant Dis* **107**, 2916-2923. doi: 10.1094/pdis-04-22-0972-sr.

Nagai S, Yamamoto K, Hata N, and Itakura S (2012) Study of DNA extraction methods for use in loop-mediated isothermal amplification detection of single resting cysts in the toxic dinoflagellates alexandrium tamarense and a. Catenella. *Marine Genomics* **7**, 51-56. doi: https://doi.org/10.1016/j.margen.2012.03.002.

Notomi T, Mori Y, Tomita N, and Kanda H (2015) Loop-mediated isothermal amplification (lamp): Principle, features, and future prospects. *The Journal of Microbiology* **53**, 1-5. doi: 10.1007/s12275-015-4656-9 https://rd.springer.com/article/10.1007/s12275-015-4656-9.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, and Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic acids research* **28**, E63. doi: 10.1093/nar/28.12.e63.

Paerl HW, Otten TG, and Kudela R (2018) Mitigating the expansion of harmful algal blooms across the freshwater-to-marine continuum. *Environ Sci Technol* **52**, 5519-5529. doi: 10.1021/acs.est.7b05950.

Parida M, Sannarangaiah S, Dash PK, Rao PV, and Morita K (2008) Loop mediated isothermal amplification (lamp): A new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev Med Virol* **18**, 407-421. doi: 10.1002/rmv.593.

Park JW (2022) Principles and applications of loop-mediated isothermal amplification to point-of-care tests. *Biosensors (Basel)* **12**. doi: 10.3390/bios12100857.

Patrick AES and Florentine S (2021) Factors affecting the global distribution of hydrilla verticillata (l. Fil.) royle: A review. *Weed Research* **61**, 253-271. doi: https://doi.org/10.1111/wre.12478.

Sahoo D, Busari I, Nix H, and White SA (2024) Impacts of harmful algal blooms (habs) on agriculture: A short communication. *Journal of South Carolina Water Resources* **9**, 45-50. doi: 10.34068/jscwr.09.02.09.

Savan R, Kono T, Itami T, and Sakai M (2005) Loop-mediated isothermal amplification: An emerging technology for detection of fish and shellfish pathogens. *J Fish Dis* **28**, 573-581. doi: 10.1111/j.1365-2761.2005.00670.x.

Sriworarat C, Phumee A, Mungthin M, Leelayoova S, and Siriyasatien P (2015) Development of loop-mediated isothermal amplification (lamp) for simple detection of leishmania infection. *Parasites & Vectors* **8**, 591. doi: 10.1186/s13071-015-1202-x.

Stehlíková D, Luchi N, Aglietti C, Pepori AL, Diez JJ, and Santini A (2020) Real-time loop-mediated isothermal amplification assay for rapid detection of fusarium circinatum. *BioTechniques* **69**, 11-17. doi: 10.2144/btn-2019-0168.

Štenclová L, Wilde SB, Schwark M, Cullen JL, McWhorter SA, Niedermeyer THJ, Henderson WM, and Mareš J (2023) Occurrence of aetokthonotoxin producer in natural samples – a pcr protocol for easy detection. *Harmful Algae* **125**, 102425. doi: https://doi.org/10.1016/j.hal.2023.102425.

Strunecký O, Ivanova AP, and Mareš J (2023) An updated classification of cyanobacterial orders and families based on phylogenomic and polyphasic analysis. *Journal of Phycology* **59**, 12-51. doi: 10.1111/jpy.13304.

Sukenik A and Kaplan A (2021) Cyanobacterial harmful algal blooms in aquatic ecosystems: A comprehensive outlook on current and emerging mitigation and control approaches. *Microorganisms* **9**. doi: 10.3390/microorganisms9071472.

Thomas NJ, Meteyer CU, and Sileo L (1998) Epizootic vacuolar myelinopathy of the central nervous system of bald eagles (haliaeetus leucocephalus) and american coots (fulica americana). *Vet Pathol* **35**, 479-487. doi: 10.1177/030098589803500602.

van Ginkel LC, Bowes G, Reiskind JB, and Prins HB (2001) A co(2)-flux mechanism operating via ph-polarity in hydrilla verticillata leaves with c(3) and c(4) photosynthesis. *Photosynth Res* **68**, 81-88. doi: 10.1023/a:1011838215424.

Villari C, Tomlinson JA, Battisti A, Boonham N, Capretti P, and Faccoli M (2013) Use of loop-mediated isothermal amplification for detection of ophiostoma clavatum, the primary blue stain fungus associated with ips acuminatus. *Applied and Environmental Microbiology* **79**, 2527-2533. doi: 10.1128/AEM.03612-12
https://aem.asm.org/content/79/8/2527.abstract.

Whitton BA and Potts M (2012) Introduction to the cyanobacteria. In 'Ecology of cyanobacteria ii: Their diversity in space and time'. (Ed. BA Whitton) pp. 1-13. (Springer Netherlands: Dordrecht)

Wilde S, Johansen J, Wilde D, Jiang P, Bartelme B, and Haynie R (2014) Aetokthonos hydrillicola gen. Et sp. Nov.: Epiphytic cyanobacteria on invasive aquatic plants implicated in avian vacuolar myelinopathy. *Phytotaxa* **181**, 243-260. doi: 10.11646/phytotaxa.181.5.1.

Wilde SB, Murphy TM, Hope CP, Habrun SK, Kempton J, Birrenkott A, Wiley F, Bowerman WW, and Lewitus AJ (2005) Avian vacuolar myelinopathy linked to exotic aquatic plants and a novel cyanobacterial species. *Environmental Toxicology* **20**, 348-353. doi: 10.1002/tox.20111 https://www3.interscience.wiley.com/cgi-bin/abstract/110494261/ABSTRACT.

Williams MR, Stedtfeld RD, Engle C, Salach P, Fakher U, Stedtfeld T, Dreelin E, Stevenson RJ, Latimore J, and Hashsham SA (2017) Isothermal amplification of environmental DNA (edna) for direct field-based monitoring and laboratory confirmation of dreissena sp. *PLoS One* **12**, e0186462. doi: 10.1371/journal.pone.0186462.

Williams SK, Kempton J, Wilde SB, and Lewitus A (2007) A novel epiphytic cyanobacterium associated with reservoirs affected by avian vacuolar myelinopathy. *Harmful Algae* **6**, 343-353. doi: 10.1016/j.hal.2006.07.005.

Woods WH, Haram BN, Wilde SB, and Ozier J (2016) Avian vacuolar myelinopathy (avm). *The University of Georgia Warnell School of Forestry & Natural Resources Outreach*.

Zou Y, Mason MG, Wang Y, Wee E, Turni C, Blackall PJ, Trau M, and Botella JR (2017) Nucleic acid purification from plants, animals and microbes in under 30 seconds. *PLoS biology* **15**, e2003916.

Figures and Tables

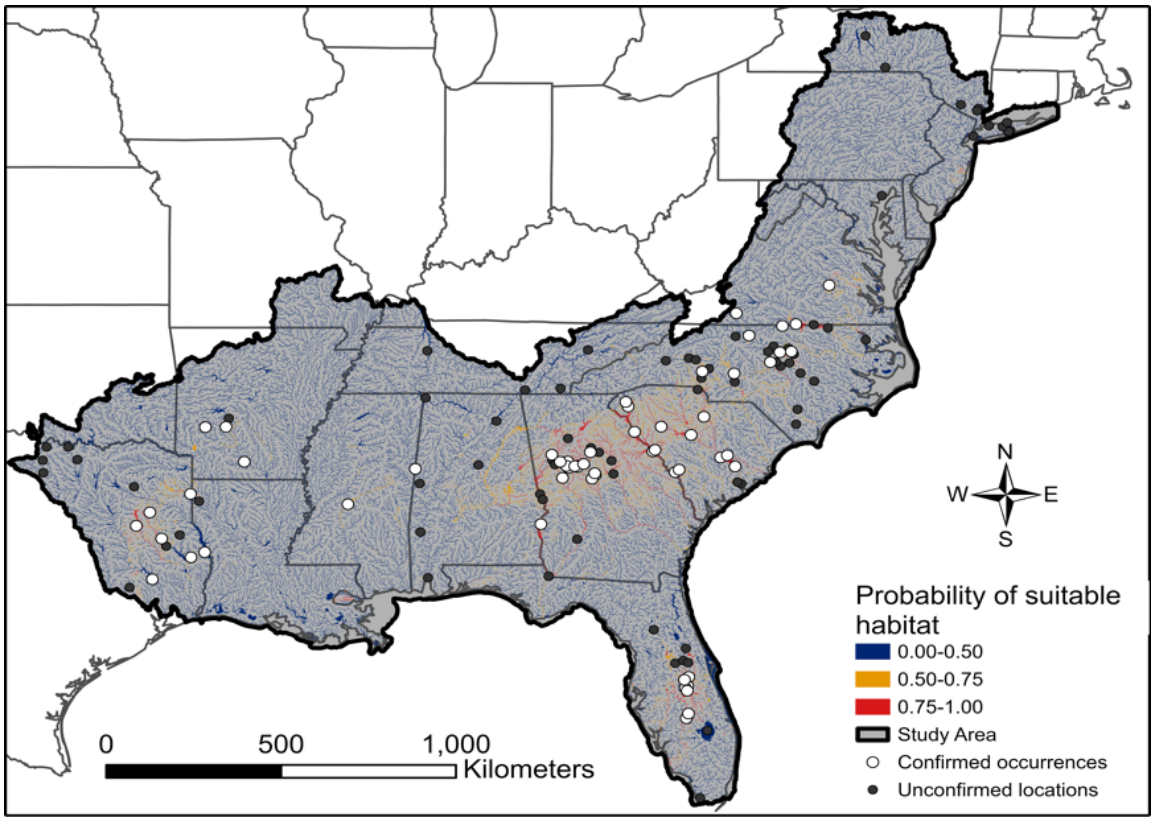


Figure 1.1 Species distribution model of occurrences and probability of suitable habitat for *Aetokthonos hydrillicola* within the model region (Gerrin, unpublished).

# CHAPTER 2

# RAPID AND FIELD PORTABLE MOLECULAR DETECTION OF TOXIGENIC *AETOKTHONOS HYDRILLICOLA*[1]

[1] Klar E., Meinecke C., Wilde D., Bothwell H., Wilde S., Villari C. To be submitted for publication to *Harmful Algae*.

**Abstract**

Vacuolar Myelinopathy (VM) is a neurological disease caused by exposure to aetokthonotoxin (AETX), a neurotoxin produced by the cyanobacteria *Aetokthonos hydrillicola,* and is a major cause of death for bald eagles and other wildlife in the Southeastern United States. Vacuolar Myelinopathy causes extensive brain vacuolation, and its symptoms include lethargy and inability to fly or evade predation. *Aetokthonos hydrillicola* is an epiphytic cyanobacterium that grows on *Hydrilla verticillata*, an invasive aquatic plant that has proliferated throughout the Southeast. Current detection methods for *A. hydrillicola* and AETX involve extensive lab time, space, and funds. Field molecular detection protocols have the potential to allow more rapid detection of the cyanobacterium with minimal equipment required. In this study, we developed a LAMP (Loop-Mediated Isothermal Amplification) protocol for the molecular detection of *A. hydrillicola* in a field setting. Our primer set targets the toxin gene cluster associated with the production of AETX, which ensures high specificity to *A. hydrillicola* and the capability of detecting the species even before it produces the toxin. Our results show that the LAMP assay can be utilized with crude DNA extracts to detect the cyanobacterial toxin gene cluster in the field at concentrations similar to conventional PCR, even though further work is still needed to optimize the crude DNA extraction protocol. Future applications of this approach could facilitate early detection of *A. hydrillicola* and guide management recommendations for rapid disease response for reservoirs at risk of developing VM outbreaks from AETX.

INDEX WORDS: Harmful Algal Blooms, LAMP, PCR, Wildlife Disease, aetokthonotoxin, Vacuolar Myelinopathy

## Introduction

Cyanobacterial harmful algal blooms (cyanoHABs) are a threat to aquatic ecosystems worldwide that are expected to increase with eutrophication, the spread of non-native species, and climate change (Sukenik and Kaplan 2021). Cyanobacterial harmful algal blooms are common in inland freshwater ecosystems, and while most of the attention has focused on the risks of toxic cyanobacteria to water quality and human health, cyanoHABs also present a threat to wildlife including rare, threatened, and endangered species (Carmichael and Boyer 2016). The negative impact of epiphytic cyanobacteria to wildlife species and community dynamics is linked to their introduction of novel cyanotoxins in the system, with potentially far-reaching effects across taxa and trophic levels within aquatic communities (Haram *et al.* 2020; Paerl *et al.* 2018).

Vacuolar Myelinopathy (VM) is a disease that has been documented across the southeastern United States since its discovery in DeGray Lake, Arkansas in 1994 (Thomas *et al.* 1998). At its discovery, it was causing mass casualties among waterfowl including American coots, *Fulica americana*, and bald eagles, *Haliaeetus leucocephalus* (Wilde *et al.* 2014). Since it was believed to only affect birds, the disease was originally named Avian Vacuolar Myelinopathy (AVM). However, since then it has been shown to affect other animal species and is now more broadly referred to as Vacuolar Myelinopathy. The disease is characterized by substantial vacuolization or "holes in the brain" of birds, fish, reptiles, and amphibians, resulting in a loss of motor function and mortality (Breinlinger *et al.* 2021; Thomas *et al.* 1998; Wilde *et al.* 2014). Although the disease has been classified since the late 20$^{th}$ century, the cause of VM was not identified and named until 2021 (Breinlinger *et al.* 2021). It is now recognized that VM is caused by the lipophilic cyanotoxin aetokthonotoxin (AETX) (Adak *et al.* 2022; Breinlinger *et al.* 2021), which is produced by the cyanobacterium *Aetokthonos hydrillicola* that grows epiphytically on

invasive aquatic plants, primarily *Hydrilla verticillata* (Breinlinger *et al.* 2021). Hydrilla is an invasive aquatic plant species originating from India and southern Asia (Jacono 2015; Patrick and Florentine 2021) which is found in many reservoirs throughout the United States, especially in the southeastern region (Jacono 2015; Wilde *et al.* 2005). The invasive aquatic plant grows in dense topped out mats in reservoirs and is difficult to eradicate because its turions buried in the sediment continue to sprout even after the above-ground biomass is removed (Jacono 2015). When waterfowl, fish, and other aquatic organisms feed on hydrilla with toxin producing *A. hydrillicola* growing on the leaves and stems, they ingest AETX and develop the disease (Breinlinger *et al.* 2021). While there has not been a documented case yet of mammals contracting VM, since AETX is lipophilic, it has the capability to bioaccumulate through the food web, and it thus has the potential to affect mammals, including humans (Breinlinger *et al.* 2021).

A recent species distribution model for *A. hydrillicola* characterized watersheds in the southeastern United States as being at high risk for colonization by the cyanobacteria and identified a strong association between suitable habitats for *A. hydrillicola* and both conifers and low cation soil capacity (Gerrin, unpublished). The fact that managed pine forests are a major commodity in the Southeast (Irby *et al.* 2020) could thus contribute to the identification of watersheds in this region as particularly suitable for the cyanobacterium. As of Spring 2023, *A. hydrillicola* and the novel AETX had been documented in 44 locations including large (>10,000 hectares) hydropower/water supply reservoirs, county water source reservoirs, suburban recreational lakes, and farm ponds (Gerrin, unpublished). Since then, sampling efforts led by the Wilde Aquatic Science Laboratory at the University of Georgia (Athens, GA) have continued to investigate and recover *A. hydrillicola* on hydrilla samples.

While *A. hydrillicola* is commonly associated with the highly invasive *H. verticillata*, it has also been found growing on other aquatic vegetation. However, the presence of AETX is not always detected when *A. hydrillicola* is present, suggesting a need to understand the factors that induce toxicity, including aquatic vegetation selectivity. *Aetokthonos hydrillicola* can be in the environment, but not have a significant toxin production until specific environmental conditions are met. In particular, the production of AETX, which comprises a pentabrominated structure (Adak *et al.* 2022; Breinlinger *et al.* 2021), has been shown to be linked with bromide presence. When hydrilla dies back either seasonally or because of a chemical treatment, it provides a bromide rich environment that is conducive to AETX production (Breinlinger *et al.* 2021). The implication of bromide might potentially explain the increasing cases of VM commonly observed in the late fall – early winter months (Breinlinger *et al.* 2021), when hydrilla releases stored bromide during senescing. This hypothesis, however, remains untested.

Molecular detection of *A. hydrillicola* is currently limited to a set of recently developed PCR assays that target the AETX biosynthetic gene cluster and the ITS region (Štenclová *et al.* 2023). The AETX producing gene cluster within *A. hydrillicola* is composed of 6 genes (i.e., *aetA*, *aetB*, *aetC*, *aetD*, *aetE*, and *aetF*) (Adak *et al.* 2022; Breinlinger *et al.* 2021). *aetA* and *aetF* share strong homology with known enzymes of FAD-dependent halogenases. *aetB* is classified as a cytochrome P450, and *aetE* is homologous to PLP-dependent tryptophanases. Genes *aetCD* and *aetD* did not show any strong homology with any known enzymes (Adak *et al.* 2022). Since the sequences for these genes in the cluster are not found in any other known organism, they show promise for providing the capability to detect *A. hydrillicola* with great specificity. Moreover, detection of the toxin gene cluster region would help identify which reservoirs are at risk for AETX presence even before toxin production and disease development, thus allowing for

preventative management interventions. The PCR-based molecular assay available to date, however, is laboratory-based, time-consuming, and requires space for specialized machinery to perform (Štenclová *et al.* 2023).

Loop-Mediated Isothermal Amplification (LAMP) is a molecular amplification process that is optimized for field portability (Notomi *et al.* 2015; Notomi *et al.* 2000). In fact, the LAMP polymerase is less susceptible to both high temperatures and amplification inhibition, which allow for isothermal reactions and the use of crude DNA extraction methods, thus removing the reliance on complex equipment and a laboratory setting (Kogovšek *et al.* 2017; Meinecke *et al.* 2023). The reaction is initiated by four primers that recognize six binding sites within the target DNA, allowing for higher specificity than traditional PCR techniques, despite being significantly more rapid (reaction times can range from 20 minutes to 1 hour) (Meinecke *et al.* 2023; Notomi *et al.* 2015). The use of LAMP for field diagnostics has already proven successful in different systems, including veterinary pathology (Filaire *et al.* 2024; Kumar *et al.* 2021), and plant pathology (Hamilton *et al.* 2021; Meinecke *et al.* 2023; Villari *et al.* 2017), and would be a valuable approach in the *A. hydrillicola* system as well. Here, we describe the development of a LAMP assay targeting the AETX biosynthetic gene cluster and its validation with crude DNA extracts from field-collected samples.

## Methods

### *Aetokthonos hydrillicola and other cyanobacteria cultures*

Freeze-dried pure cultured *A. hydrillicola* collected for a previous study (Breinlinger *et al.* (2021)) was used as a positive control. Twenty additional cyanobacterial species that are either phylogenetically close to our target species (Strunecký *et al.* 2023), coinhabiting species, or

previously tested species of interest for molecular studies involving *A. hydrillicola* (Štenclová *et al.* 2023) were compiled for the specificity testing of the developed LAMP primers . Cultures of each cyanobacterium were purchased from either UTEX Culture Collection of Algae at The University of Texas at Austin or Carolina Biology Supply, as indicated in Table 2.1. Four subcultures of each strain were created by transferring a portion of cells to fresh Blue Green-11 (BG-11) liquid media (Pandey *et al.* 2023), which was prepared according to the recipe provided by UTEX.

*Field Sample Collection and Location*

The Mississippi Flyway is a critical region for migratory birds traversing through the middle of the United States (Heim 2024). Hydrilla samples were collected by collaborators during 2022-2024 in this important flyway including Texas, Arkansas, Mississippi, and Louisiana. Samples from each site were sent via mail to the Wilde Lab at The University of Georgia. The sampling protocol included gathering a representative hydrilla sample in a resealable plastic bag. The plastic bag was then placed in a cooler box with ice packs and shipped overnight to the laboratory. To screen hydrilla samples for the presence of *A. hydrillicola*, a NIGHTSEA Xite Fluorescent Flashlight system emitting green light with wavelength between 510 - 540nm was used in coordination with red barrier filter glasses to visualize fluorescence from cyanobacteria. Once the region of interest was identified, leaves were screened again under an Amscope epifluorescent light microscope (FM800TC) with a green filter set (EX 510-560, DM 575, BA 590) to visualize phycocyanin pigments and enable *Aetokthonos* identification on hydrilla leaves. Following screening, samples were marked according to their *A. hydrillicola* status and stored frozen until further processing. Due to the ecological importance of the Mississippi Flyway for

migratory birds in this region and the abundance of *A. hydrillicola* present on hydrilla samples, these sites were selected for molecular confirmation. Two positive and two negative sites from Louisiana were chosen [Lake Claiborne and Caney Creek Reservoir (positive); Grand Bayou and Bayou D'Arbonne Lake (negative)], plus an additional positive site on the border between Texas and Louisiana (Caddo Lake). One positive site and three negative sites were chosen from Texas [Lake Naconiche (positive); Pinkston Reservoir, Lake Kurth, and Stillhouse Hollow Lake (negative)]. The final sample site was from a positive reservoir in Mississippi: Ross Barnett Reservoir (Figure 2.1).

*DNA Extraction*

Pure DNA from the freeze-dried *A. hydrillicola* and the cyanobacterial cultures was obtained utilizing a Qiagen PowerSoil Kit, following the manufacturer's instructions. The starting material for the freeze-dried *A. hydrillicola* was approximately 40 mg, while the starting material for the fresh cyanobacterial subcultures was approximately 80 mg. Pure DNA from field collected hydrilla samples (approximately 80 mg, which corresponds to 3-4 leaves) was obtained using a Qiagen DNeasy Plant Pro Kit, following the manufacturer's instructions. Three sub-sample replicates were taken from different areas within the total sample bag for extraction. All DNA extracted with pure extraction methods was quantified, standardized to 1.0 ng/µl, and stored in a -20°C freezer until further use.

Field-collected samples were additionally processed with a crude DNA extraction protocol. Crude DNA extractions were performed using a pipette-free dipstick method optimized for use in the field (Mason and Botella 2020; Meinecke *et al.* 2023; Zou *et al.* 2017). Hydrilla leaves were added to a 1.5 mL tube containing 600 µl of extraction buffer and homogenized

using a polypropylene pestle. A cellulose filter dipstick (Mason and Botella 2020) was then dipped fifteen times into the homogenate to saturate it, and the absorbed DNA was then washed by dipping the dipstick five times into 600 µl of wash buffer. Finally, the DNA was eluted directly into a 25 µl LAMP reaction mixture by further immersing the dipstick fifteen times (Mason and Botella 2020; Meinecke *et al.* 2023).

*LAMP Primer Design*

We designed the LAMP primer set using the PrimerExplorer V5 software with default parameters (Eiken Chemical Co., Tokyo, Japan). The set comprised two external primers (F3 and B3), two internal primers (FIP and BIP), and a forward-loop primer binding to the *aetC* and *aetD* genes, and the intergenic region in between, of the AETX producing gene cluster (Figure 2.2), which is considered unique to *A. hydrillicola* (Breinlinger *et al.* 2021). Of the two initial internal and external primer sets generated by the software, we selected the set with the most negative ΔG value for binding affinity and re-entered it in the software to generate and select a loop primer, following the same ΔG value criteria. We then used the selected forward loop primer to design a fluorescent labeled probe, as described in Kubota *et al.* (2011) and Meinecke *et al.* (2023). The resulting sequences for primers were inserted into BLAST searches to confirm no significant matches with other species of cyanobacteria.

*Molecular Amplification Parameters*

LAMP reactions were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems; Waltham, MA, U.S.). The 25µl LAMP reaction mastermix consisted of 15µl of Isothermal Master Mix (ISO-001nd; OptiGene Limited, Horsham, U.K.), 1.5µl of molecular

grade water, 2.8µl of primer mixture, 0.7µl of probe mixture, and either 5µl of pure DNA or an equivalent amount of molecular grade water for the reactions using the crude DNA extraction protocol, in which the DNA is added by submerging the dipstick directly in the reaction mixture (Meinecke *et al.* 2023). The final concentrations of primers and probes in the LAMP master mix were 2.8µM for each FIP and BIP and 0.28µM for each F3 and B3 in the primer mixture; 0.096µM for the loop primer and 0.184 for the quencher strand in the probe mixture (Meinecke *et al.* 2023). All LAMP reactions were run in eight-tube MicroAmp Fast Reaction Tube strips (Applied Biosystems; Waltham, MA, U.S.) at 65°C for 60 minutes with a final 85°C inactivation period for 5 minutes.

For end-point PCR reactions with pure DNA extracts, the 25µl master mix consisted of 5.5µl of molecular grade water, 12.5µl of GoTaq Green MM 2x (M7122; Promega; Madison, WI, U.S.), 2µl of primer mix, and 5µl of DNA. The conventional PCR primers used in this assay to detect *A. hydrillicola* target the same toxin gene cluster region as the developed LAMP primer set, however, they target the *aetA* gene of the toxin cluster (Štenclová *et al.* 2023). The final concentration of the primers within the 25µl PCR master mix was 0.4µM for *aetA* forward at 10µM and 0.4µM for *aetA* reverse at 10µM. PCR reactions were run in eight-tube 0.2mL Strip Tubes (ThermoFisher) with cycling parameters as published for the *aetA* primer set: initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, and elongation at 72°C for 40s, and finally a terminal elongation at 72°C for 10 min. Reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems; Waltham, MA, U.S.). A 1% agarose gel (100V, 30 minutes, 5µl PCR product per well) was used to visualize PCR results with a 1kb ladder and expected band size of 599bp (Štenclová *et al.* 2023)

qPCR reactions were performed on the same StepOnePlus Real-Time PCR System used for LAMP reactions. The primers 1055F and 1406R targeted the 16S rRNA ITS region conserved among members of the domain bacteria (Williams *et al.* 2007). The 20µl master mix consisted of 2.8µl of molecular grade water, 10µl GoTaq qPCR Master Mix 2x, 10µl primer mix (10µM each), 0.2µl of carboxy-X-rhodamine (CXR), and 5µl of DNA template per reaction. Cycling parameters were set based on Wilde *et al.* (2014): 35 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 2 min 15 s, followed by 5 min extension at 72°C and a 4°C hold for an indefinite amount of time.

*Sensitivity and Specificity Testing*

To test the primer set's ability to selectively and efficiently amplify our target organism, we performed a series of specificity and sensitivity testing. The sensitivity testing was completed using pure DNA of the freeze-dried *A. hydrillicola* culture with 1:10 DNA serial dilutions from 1.0ng/µl to 1.0fg/µl, each tested in triplicates. The sensitivity of the LAMP assay was compared to that of the end-point PCR assay developed by Štenclová et al. (Štenclová *et al.* 2023).

To assess the specificity of our primer set, we tested the assay using the clean DNA of the cyanobacterial species reported in Table 2.1. Specificity testing reactions were performed with both the LAMP assay, to test the new primer set, and qPCR, to verify the presence and quality of amplifiable DNA in the extracts from cyanobacteria cultures. Each reaction was run in triplicate, and each run included two no-template negative controls.

*LAMP comparison with PCR using field-collected samples*

Crude and pure DNA extracts of field-collected hydrilla samples were tested with both conventional PCR and LAMP assays for comparison of their ability to detect *A. hydrillicola*.

LAMP reactions and PCR reactions with pure DNA extracts were performed as described in the previous sections. For PCR reactions with crude DNA extracts, we used the same 25µl master mix formulation with the addition of 5µl of molecular water to compensate for the absence of the DNA volume. Three samples were tested from the total material collected at each site, for a total of 30 samples. Reactions for both PCR and LAMP were run in triplicates with two no-template negative controls per each run. For both the LAMP and PCR approaches, samples were determined to be positive if two out of the three reaction replicates showed amplification. The site was considered positive for the presence of *A. hydrillicola* if any of the site replicates yielded positive amplification results. Assuming the visual assessment of the presence of *A. hydrillicola* as a gold standard, we calculated the diagnostic sensitivity of both assays (i.e., true positive rate) as the number of correct positive predictions divided by the total number of positives (Shreffler and Huecker 2024).

**Results**

*LAMP Primer Design*

We designed a set of LAMP primers that bind to the *aetC* and *aetD* genes and their intergenic region of the AETX gene cluster in the *A. hydrillicola* genome. These consist of two outer primers, two inner primers, and a FAM-labeled forward loop primer as shown in Table 2.2.

*Sensitivity and Specificity Testing*

The results of the sensitivity test showed that the LAMP and the PCR assays have comparable sensitivity and can amplify the DNA of the target species *A. hydrillicola* at concentrations as low as 1.0 pg/µl (Figure 2.3 and 2.4). The LAMP amplification curve times ranged from ~13 – 24

minutes. Thus, we determined a cutoff time for detection of 35 minutes, which takes into consideration possible delays in amplification for crude DNA extracts.

All tested *A. hydrillicola* pure isolates yielded positive amplification with the LAMP assay, while none of the twenty other species of cyanobacteria did (Table 2.1), thus confirming the specificity of the developed LAMP assay. The presence of amplifiable DNA in all samples was confirmed via amplification using the universal 16S rRNA gene 1055F forward primer and the 1406R reverse primer (Wilde *et al.* 2014; Williams *et al.* 2007) with qPCR. All twenty cyanobacterial species tested contained amplifiable DNA at levels able to be visualized with qPCR, suggesting that the quality of DNA within the extracts was enough to be amplified in the LAMP reaction if a non-specific binding opportunity were to exist.

*Detection of A. hydrillicola with Pure DNA Extracts of Field Samples*

The reactions performed with pure DNA extracts showed that LAMP holds more promise in detecting *A. hydrillicola* in field-collected samples than PCR (Table 2.3). None of the samples visually assessed as negative yielded a positive reaction with either assay. All samples from Lake Claiborne and one sample from Lake Naconiche, which were visually assessed as positive, tested negative for both LAMP and PCR assays. All other samples visually assessed as positive tested positive with the LAMP and PCR assays, except for one sample from Ross Barnett Reservoir and two samples from Lake Naconiche, which tested positive with the LAMP assay but not with the PCR assay. The calculated diagnostic sensitivity for the LAMP assay was 73.33%, while that of the PCR assay was only 53.33%.

*Detection of A. hydrillicola with Crude DNA Extracts of Field Samples*

The reactions performed with crude DNA extracts from field samples showed an even more noticeable difference in results between PCR and LAMP, but the overall amplification success was very low (Table 2.3). The PCR did not detect *A. hydrillicola* DNA extracts in any of the samples, whether visually assessed as positive or negative. LAMP, however, detected *A. hydrillicola* in seven out of the 15 visually positive samples, including all samples from two of the sites (Caney Creek Reservoir and Lake Caddo). The average detection time for positive samples using crude extracts with LAMP was between 21-28 minutes. None of the samples from both Lake Naconiche and Lake Claiborne, which were visually assessed as positive, amplified with either reaction. All visually negative samples, except one from the Bayou D'Arbonne Lake, tested negative with the LAMP assay. The one false positive yielded 2/3 positive amplifications between 29-34 minutes, which is later than the other positive reactions observed for crude DNA extracts in this study (Table 2.3). The calculated diagnostic sensitivity of assays run with crude DNA extracts was 46.67% for the LAMP assay and 0% for the PCR assay.

**Discussion**

A new LAMP assay with the opportunity for field portability was established to detect the ecologically impactful AETX synthesis genes within the *A. hydrillicola* genome. The developed assay was similar in analytical sensitivity to the already available PCR assay (Štenclová *et al.* 2023), with a lower limit of detection of 1.0pg/µl of pure DNA in a 25µl reaction. The assay did not amplify with other closely related species or species that have been shown to grow in the same watersheds as *A. hydrillicola.* Excluding one site, Lake Claiborne, the results show that the developed LAMP assay can successfully detect *A. hydrillicola* in pure DNA extracts of field-

collected hydrilla leaves, while the assay efficiency decreased when combined with a crude DNA extraction protocol.

Detection of *A. hydrillicola* in pure DNA extracted from hydrilla collected at positive sites was achieved with both PCR and LAMP, but the LAMP assay had a higher diagnostic sensitivity. This may suggest that although the two assays show a similar sensitivity to DNA extracted from pure cultured *A. hydrillicola*, when introducing field samples, which will contain a greater number of inhibitors and other DNA in the mix, LAMP may be more suitable for detecting small amounts of the target species. All sites that screened negative for *A. hydrillicola* according to the visual assessment were successfully confirmed to be negative using the developed molecular screening with pure DNA from field samples. The average time frame for detection in pure extracts was between 17-27 minutes. As a comparison, amplification of pure cultured *A. hydrillicola* extracts occurred between 13-17 minutes. This delay in amplification time is expected for field samples, where the target DNA is combined with the DNA of hydrilla and other organisms, and where inhibitors are expected.

While successful for two of the sites, the coupling of the LAMP assay with the crude DNA extraction method overall significantly lowered the diagnostic sensitivity of the assay, which suggests that further testing is needed to optimize a field portable extraction method. Difficulty with establishing efficient and reliable extraction likely arose due to the mucilaginous sheath and thick sticky cell walls that some cyanobacteria, including *A. hydrillicola*, possess (Howard 2019). Various extraction methods, including metal ball bearings, glass beads, garnet beads, the addition of diatomaceous earth, and various sizes of pestles were tested (data not shown) before settling with the method used in this work. The polypropylene pestle for 1.5mL tubes was the most reliable form of homogenization for hydrilla leaves with or without *A.*

*hydrillicola* present. Despite the lower diagnostic sensitivity when using crude DNA extracts, however, the LAMP assay significantly outcompeted the PCR assay. PCR, in fact, failed to detect *A. hydrillicola* in any visually positive samples utilizing crude extracts. These results add to the existing body of literature reporting that LAMP is less susceptible to inhibitors than conventional PCR (Aglietti *et al.* 2021; Kaneko *et al.* 2007; Notomi *et al.* 2000) and exemplify why LAMP is such a powerful tool for utilization in the field compared to other established molecular techniques.

It is worth noting that in our analysis, we considered the visual assessment as the gold standard, and those cases in which we observed a discrepancy between the presence of colonies and both molecular testing negatively impacted the calculated diagnostic sensitivity of the molecular methods. However, further analyses are needed to confirm if the samples that tested negative with the molecular approach are true negatives. Lake Claiborne, for instance, is a Mississippi lake that is near to other positive sites tested in this study. When visually screened before DNA extractions, healthy cultures of what appeared to be *A. hydrillicola* were seen growing on the leaves. However, none of the samples from this site tested positive for *A. hydrillicola*, independently of the DNA extraction method or molecular approach, raising the question of whether this sample contained a strain variant or other physiologically similar cyanobacteria that do not contain the AETX producing gene cluster. Recent studies have detected a potentially unique genetic variant of *A. hydrillicola* growing on American water-willow, *Justicia americana*, when testing field samples with the ITS and AETX gene targeting PCR assay and hypothesized that there might be another subspecies of *Aetokthonos* with variations in its ITS region while containing the AETX gene clusters (Štenclová *et al.* 2023). More investigation into this site would need to be done before we can confidently claim speciation

among *Aetokthonos*. To determine if this site contained a new sub-species of *Aetokthonos*, whole genome sequencing with comparisons of both the ITS and AETX regions would be crucial to determine toxin production capability and phylogenetic placement. In the eventuality that the cyanobacteria colonies observed in samples from Lake Claiborne are confirmed to be a different, non-toxin producing variant of *A. hydrillicola*, the value of the calculated diagnostic sensitivity of the LAMP assay would dramatically improve in this study.

Due to our lack of understanding of the natural history of *A. hydrillicola*, as well as a recent observation of potential speciation within the genus, a study into the origin and genetic variation of this species within the U.S. is imperative. We do not know whether this species of neurotoxic cyanobacteria was introduced and is an invasive species, or if it is a naturally occurring species that is taking advantage of the perfect habitat on an aquatic invasive plant species, hydrilla. Understanding the genetic variation within samples from different reservoirs around the Southeast, which appears to be a vulnerable region, would be particularly beneficial in understanding the risk for disease and enhancing mitigation. If *A. hydrillicola* were to be identified as a naturally occurring cyanobacteria, it would not be feasible to eradicate the species. Instead, managing for hydrilla, and thus limiting its toxin-inducing habitat, would be the main priority. If, on the contrary, *A. hydrillicola* were to be classified as an introduced invasive species, directly managing and reducing the cyanobacteria species would also be crucial for preventing VM.

LITERATURE CITED

Adak S, Lukowski AL, Schäfer RJB, and Moore BS (2022) From tryptophan to toxin: Nature's convergent biosynthetic strategy to aetokthonotoxin. *Journal of the American Chemical Society* **144**, 2861-2866. doi: 10.1021/jacs.1c12778.

Aglietti C, Meinecke CD, Ghelardini L, Barnes I, Nest Avd, and Villari C (2021) Rapid detection of pine pathogens lecanosticta acicola, dothistroma pini and d. Septosporum on needles by probe-based lamp assays. *Forests* **12**. doi: 10.3390/f12040479 https://www.mdpi.com/1999-4907/12/4/479.

Breinlinger S, Phillips TJ, Haram BN, Mareš J, Yerena JAM, Hrouzek P, Sobotka R, Henderson M, Schmieder P, Williams SM, Lauderdale JD, Wilde D, Gerrin W, Kust A, Washington JW, Wagner C, Geier B, Liebeke M, Enke H, Niedermeyer THJ, and Wilde SB (2021) Hunting the eagle killer: A cyanobacterial neurotoxin causes vacuolar myelinopathy. *Science (Washington)* **371**. doi: 10.1126/science.aax9050 https://science.sciencemag.org/content/371/6536/eaax9050.abstract.

Filaire F, Sécula A, Lebre L, Croville G, and Guerin J-L (2024) A real-time colourimetric reverse transcription loop-mediated isothermal amplification (rt-lamp) assay for the rapid detection of highly pathogenic h5 clade 2.3.4.4b avian influenza viruses. In 'Avian pathology : journal of the W.V.P.A' pp. 93-100. (Informa Healthcare: England)

Hamilton JL, Fraedrich SW, Nairn CJ, Mayfield AE, and Villari C (2021) A field-portable diagnostic approach confirms laurel wilt disease diagnosis in minutes instead of days. *Arboriculture & Urban Forestry* **47**, 98-109. doi: 10.48044/jauf.2021.010.

Haram BN, Wilde SB, Chamberlain MJ, and Boyd KH (2020) Vacuolar myelinopathy: Waterbird risk on a southeastern impoundment co-infested with hydrilla verticillata and aetokthonos hydrillicola. *Biological Invasions* **22**, 2651-2660. doi: 10.1007/s10530-020-02282-w.

Heim M (2024) 5 things to know about the mississippi flyway as spring bird migration begins. (Milwaukee Journal Sentinel: WXPR)

Howard A (2019) Genetic investigations of a novel, toxic, epiphytic cyanobacteria: Aetokthonos hydrillicola. M.S. Thesis, (University of Georgia, United States -- Georgia.

Irby NE, França FJN, Michael Barnes H, Dan Seale R, and Shmulsky R (2020) Effect of growth rings per inch and density on compression parallel to grain in southern pine lumber. *BioResources* **15**, 2310-2325. doi: 10.15376/biores.15.2.2310-2325.

Jacono CC, Richerson, M.M. , Howard Morgan, V. , and Pfingsten, I.A. (2015) Hydrilla verticillata (l. F.) royle. U.S. Geological Survey, Nonindigenous Aquatic Species Database)

Kaneko H, Kawana T, Fukushima E, and Suzutani T (2007) Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *J Biochem Biophys Methods* **70**, 499-501. doi: 10.1016/j.jbbm.2006.08.008.

Kogovšek P, Mehle N, Pugelj A, Jakomin T, Schroers H-J, Ravnikar M, and Dermastia M (2017) Rapid loop-mediated isothermal amplification assays for grapevine yellows phytoplasmas on crude leaf-vein homogenate has the same performance as qpcr. *European Journal of Plant Pathology* **148**, 75-84. doi: 10.1007/s10658-016-1070-z.

Kubota R, Alvarez AM, Su WW, and Jenkins DM (2011) Fret-based assimilating probe for sequence-specific real-time monitoring of loop-mediated isothermal amplification (lamp). pp. 81-100. (AMERICAN SOCIETY OF AGRICULTURAL AND BIOLOGICAL ENGINEERS: United States)

Kumar S, Gali JM, Dutta TK, Roychoudhury P, and Subudhi PK (2021) Development of isothermal lamp assay for detection of intimin gene (eae) in e. Coli associated with piglet diarrhea. *Indian Journal of Animal Research* **55**, 956-959. doi: 10.18805/IJAR.B-4173.

Mason MG and Botella JR (2020) Rapid (30-second), equipment-free purification of nucleic acids using easy-to-make dipsticks. *Nature Protocols* **15**, 3663. doi: 10.1038/s41596-020-0392-7.

Meinecke CD, Vos L, Yilmaz N, Steenkamp ET, Wingfield MJ, Wingfield BD, and Villari C (2023) A lamp assay for rapid detection of the pitch canker pathogen fusarium circinatum. *Plant Dis* **107**, 2916-2923. doi: 10.1094/pdis-04-22-0972-sr.

Notomi T, Mori Y, Tomita N, and Kanda H (2015) Loop-mediated isothermal amplification (lamp): Principle, features, and future prospects. *The Journal of Microbiology* **53**, 1-5. doi: 10.1007/s12275-015-4656-9 https://rd.springer.com/article/10.1007/s12275-015-4656-9.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, and Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic acids research* **28**, E63. doi: 10.1093/nar/28.12.e63.

Paerl HW, Otten TG, and Kudela R (2018) Mitigating the expansion of harmful algal blooms across the freshwater-to-marine continuum. *Environ Sci Technol* **52**, 5519-5529. doi: 10.1021/acs.est.7b05950.

Pandey S, Narayanan I, Vinayagam R, Selvaraj R, Varadavenkatesan T, and Pugazhendhi A (2023) A review on the effect of blue green 11 medium and its constituents on microalgal growth and lipid production. *Journal of Environmental Chemical Engineering* **11**, 109984. doi: https://doi.org/10.1016/j.jece.2023.109984.

Patrick AES and Florentine S (2021) Factors affecting the global distribution of hydrilla verticillata (l. Fil.) royle: A review. *Weed Research* **61**, 253-271. doi: https://doi.org/10.1111/wre.12478.

Shreffler J and Huecker MR (2024) Diagnostic testing accuracy: Sensitivity, specificity, predictive values and likelihood ratios. In 'Statpearls'. (StatPearls Publishing Copyright © 2024, StatPearls Publishing LLC.: Treasure Island (FL) ineligible companies. Disclosure: Martin Huecker declares no relevant financial relationships with ineligible companies.)

Štenclová L, Wilde SB, Schwark M, Cullen JL, McWhorter SA, Niedermeyer THJ, Henderson WM, and Mareš J (2023) Occurrence of aetokthonotoxin producer in natural samples – a pcr protocol for easy detection. *Harmful Algae* **125**, 102425. doi: https://doi.org/10.1016/j.hal.2023.102425.

Strunecký O, Ivanova AP, and Mareš J (2023) An updated classification of cyanobacterial orders and families based on phylogenomic and polyphasic analysis. *Journal of Phycology* **59**, 12-51. doi: 10.1111/jpy.13304.

Sukenik A and Kaplan A (2021) Cyanobacterial harmful algal blooms in aquatic ecosystems: A comprehensive outlook on current and emerging mitigation and control approaches. *Microorganisms* **9**. doi: 10.3390/microorganisms9071472.

Thomas NJ, Meteyer CU, and Sileo L (1998) Epizootic vacuolar myelinopathy of the central nervous system of bald eagles (haliaeetus leucocephalus) and american coots (fulica americana). *Vet Pathol* **35**, 479-487. doi: 10.1177/030098589803500602.

Villari C, Mahaffee WF, Mitchell TK, Pedley KF, Pieck ML, and Hand FP (2017) Early detection of airborne inoculum of magnaporthe oryzae in turfgrass fields using a quantitative lamp assay. *Plant Disease* **101**, 170-177. doi: 10.1094/PDIS-06-16-0834-RE.

Wilde S, Johansen J, Wilde D, Jiang P, Bartelme B, and Haynie R (2014) Aetokthonos hydrillicola gen. Et sp. Nov.: Epiphytic cyanobacteria on invasive aquatic plants implicated in avian vacuolar myelinopathy. *Phytotaxa* **181**, 243-260. doi: 10.11646/phytotaxa.181.5.1.

Wilde SB, Murphy TM, Hope CP, Habrun SK, Kempton J, Birrenkott A, Wiley F, Bowerman WW, and Lewitus AJ (2005) Avian vacuolar myelinopathy linked to exotic aquatic plants and a novel cyanobacterial species. *Environmental Toxicology* **20**, 348-353. doi: 10.1002/tox.20111 https://www3.interscience.wiley.com/cgi-bin/abstract/110494261/ABSTRACT.

Williams SK, Kempton J, Wilde SB, and Lewitus A (2007) A novel epiphytic cyanobacterium associated with reservoirs affected by avian vacuolar myelinopathy. *Harmful Algae* **6**, 343-353. doi: 10.1016/j.hal.2006.07.005.

Zou Y, Mason MG, Wang Y, Wee E, Turni C, Blackall PJ, Trau M, and Botella JR (2017) Nucleic acid purification from plants, animals and microbes in under 30 seconds. *PLoS biology* **15**, e2003916.

Figures and Tables

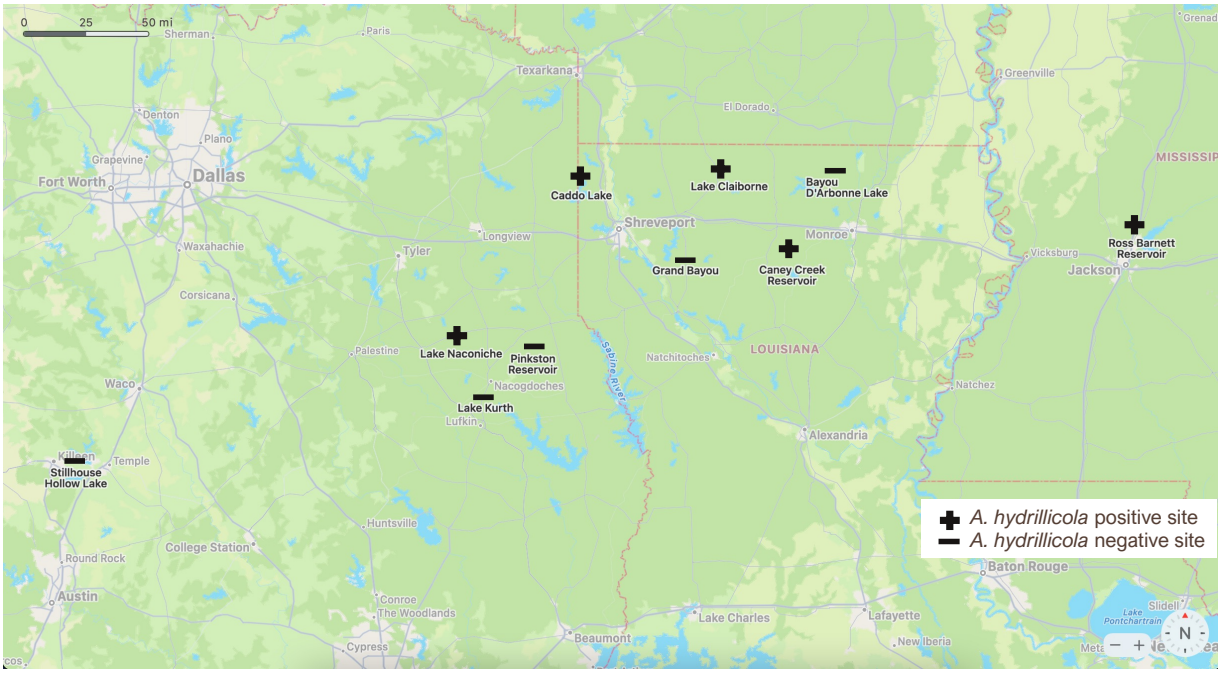


Figure 2.1 Relative site locations for visually assessed *Aetokhonos hydrillicola* positive and negative samples used in this study.

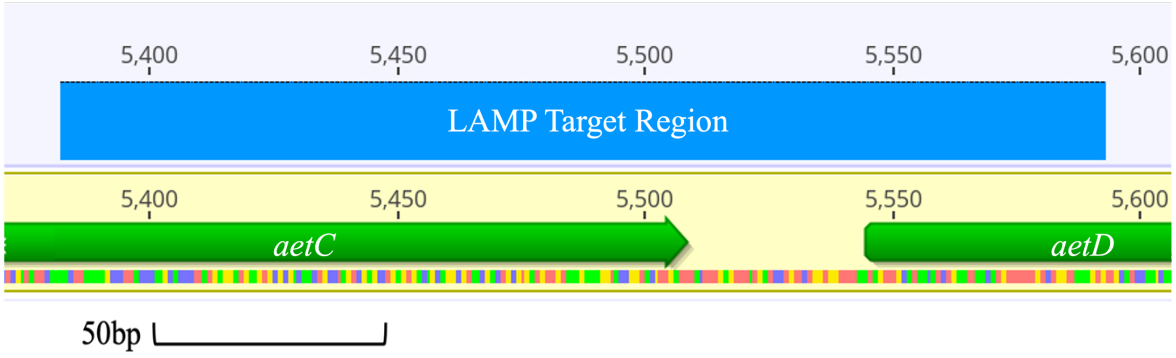


Figure 2.2 Diagram showing positioning of the Loop-Mediated Isothermal Amplification (LAMP) primer target binding region within the genome of *Aetokthonos hydrillicola* between the *aetC* and *aetD* coding genes.

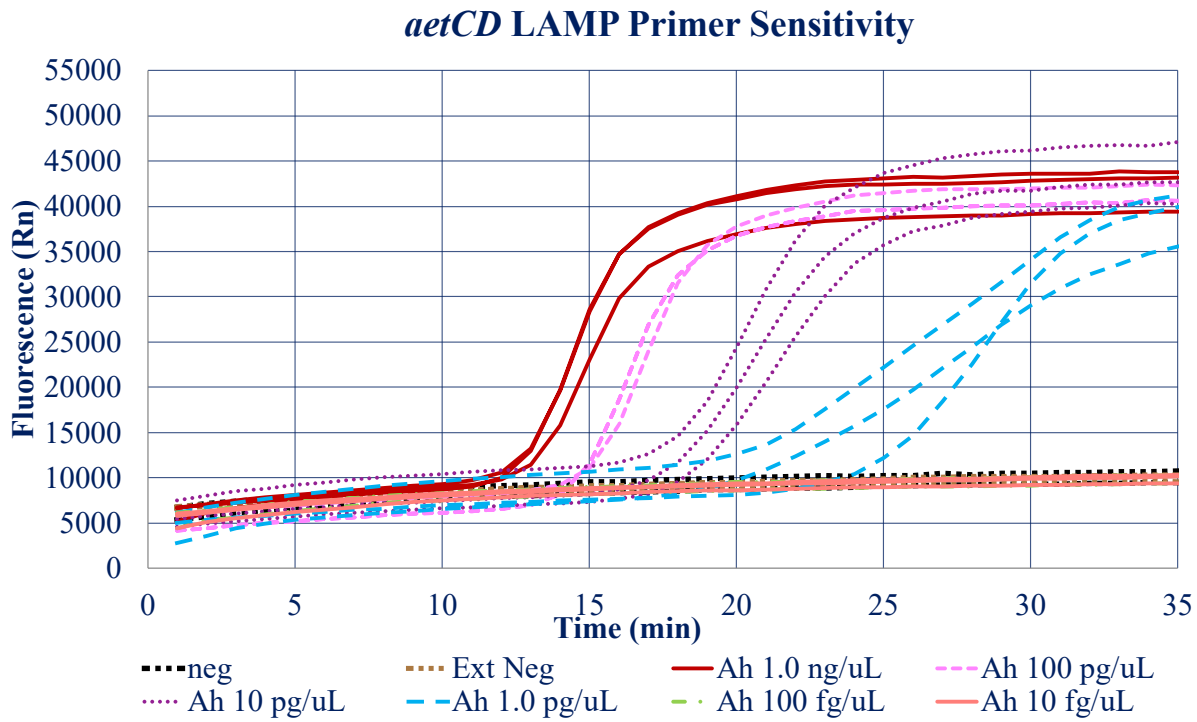


Figure 2.3 Amplification chart of the *aetCD* Loop-Mediated Isothermal Amplification (LAMP) primer sensitivity testing with serial dilutions from 1ng/µl to 10fg/µl of pure cultured *Aetokthonos hydrillicola* DNA. Two no-template and three mock extraction replicates were included for negative controls.

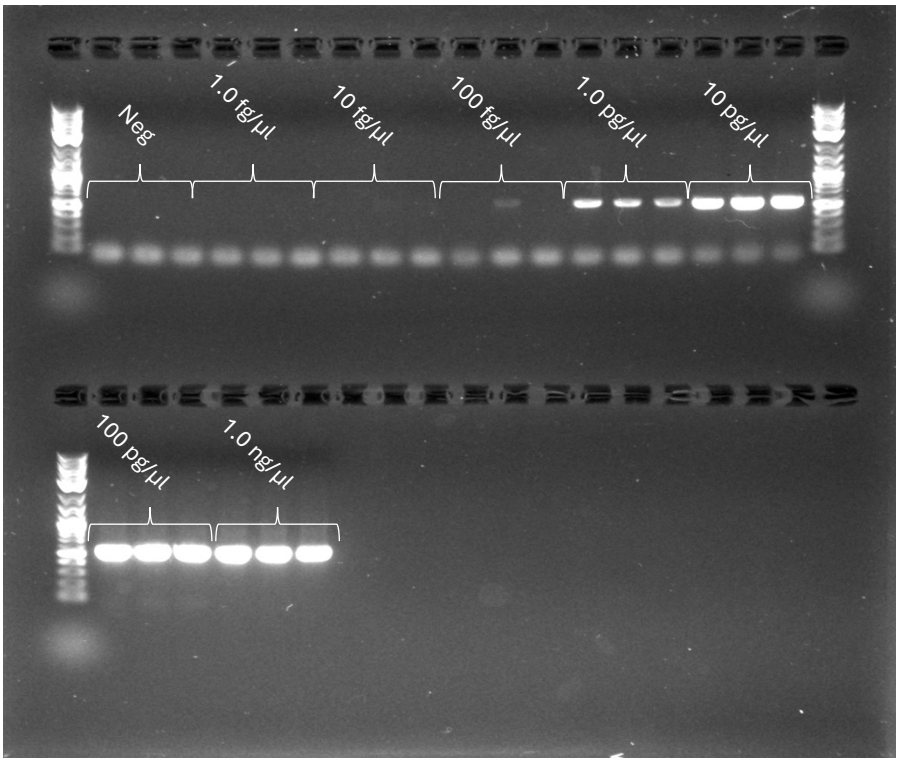


Figure 2.4 Agarose gel image (1% agarose; 100V, 30 minutes, 5µl per well) showing PCR sensitivity test results for the *aetA* primer set (Štenclová *et al.* 2023) detecting serial dilutions of pure DNA extracts of pure cultured *A. hydrillicola*. Reactions were performed in triplicates with a 1kb reference ladder. Expected band length = 599 bp.

Table 2.1 List of the cyanobacterial species used in the specificity test of the Loop-Mediated Isothermal Amplification (LAMP) primer set. Species were chosen for phylogenetic relatedness to *Aetokthonos hydrillicola*, co-inhabitance, and because they had been used in a previous molecular study of *A. hydrillicola* (Štenclová *et al.* 2023). The table reports the identification number from UTEX and Carolina Biology for each cyanobacterial strain, the results of the LAMP specificity test, and the results of the qPCR test to verify the presence of amplifiable DNA.

| Species | Culture Source | Identification Number | Positive LAMP Result | Positive qPCR Result |
|---|---|---|---|---|
| *Nostoc commune* | UTEX | B1621 | 0/3 | 3/3 |
| *Nostoc punctiforme* | UTEX | B384 | 0/3 | 3/3 |
| *Scytonema hofmannii* | UTEX | B2349, B1834 | 0/3 | 3/3 |
| *Cylindrospermum sp.* | Carolina Biology | 151755 | 0/3 | 3/3 |
| *Calothrix sp.* | UTEX | LB1319 | 0/3 | 3/3 |
| *Aphanothece sp.* | UTEX | SP25 | 0/3 | 3/3 |
| *Leptolyngbya sp.* | UTEX | B3193, B3192 | 0/3 | 3/3 |
| *Pseudanabaena sp.* | UTEX | B3194 | 0/3 | 3/3 |
| *Phormidium sp.* | UTEX | B1540 | 0/3 | 3/3 |
| *Synechococcus elongatus* | UTEX | 3055 | 0/3 | 3/3 |
| *Microcystis aeruginosa* | UTEX | LB3037 | 0/3 | 3/3 |
| *Fischerella musicola* | Carolina Biology | 151770 | 0/3 | 3/3 |
| *Anabeana sp.* | Carolina Biology | 151710 | 0/3 | 3/3 |
| *Tolypothrix distorta* | Carolina Biology | 151935 | 0/3 | 3/3 |
| *Lyngbya sp.* | Carolina Biology | 151830 | 0/3 | 3/3 |
| *Oscillatoria sp.* | Carolina Biology | 151865 | 0/3 | 3/3 |
| *Gloeocapsa sp.* | Carolina Biology | 151800 | 0/3 | 3/3 |
| *Spirulina sp.* | Carolina Biology | 151900 | 0/3 | 3/3 |
| *Merismopedia sp.* | Carolina Biology | 151835 | 0/3 | 3/3 |
| *Gleotrichia sp.* | Carolina Biology | 151810 | 0/3 | 3/3 |

Table 2.2 Loop-Mediated Isothermal Amplification (LAMP) developed primer and probe sequences for the amplification of the aetokthonotoxin producing gene cluster in *Aetokthonos hydrillicola*.

| **Primers** | **Sequence 5' – 3'** |
|---|---|
| ***aetCD* F3** | CAATCCCTTGCTCCCAAT |
| ***aetCD* B3** | CGAACAAATTCAAAGCAAGGTA |
| ***aetCD* FIP** | CTACTCGCTGACGAATCGCC-GTAGTTGAGCGCGTCTTC |
| ***aetCD* BIP** | AAGTGAAATAGTACCAGAACAGGAG-GCTTCTCAAATTCCTGTCTT |
| **Sequence Specific Probes** | |
| ***aetCD* Loop Primer (LF)[a]** | FAM-ACGCTGAGGACCCGGATGCGAATGCGGATGCGG ATGCCGAAGCAGACTCACTGGCACGTC |
| **Quencher Strand [b]** | TCGGCATCCGCATCCGCATTCGCATCCGGG TCCTCAGCGT-BHQ |

[a] The underlined fragment of the sequence acts as a loop primer
[b] Quencher strand designed as described in Kubota *et al.* (2011).
FAM = 6-carboxyfluorescein; BHQ = black hole quencher – 1 (Biosearch Technologies, Novato, CA, U.S.A.)

Table 2.3 Comparison of amplification results to detect *Aetokthonos hydrillicola* between PCR and LAMP assays using both crude and pure DNA extracts of field-collected samples of hydrilla. Positive results are highlighted in bold. Visual screening results are also displayed as the gold standard comparison.

| ***Sample Site*** | **Sample Number** | **Ah Visual Screening Result** | **Extraction Type** | **Positive PCR Result** | **Positive LAMP Result** |
|---|---|---|---|---|---|
| *Lake Kurth, TX* | 1 | - | Pure | 1/3 | 0/3 |
| | | | Crude | 0/3 | 1/3 |
| *Lake Kurth, TX* | 2 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Lake Kurth, TX* | 3 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Grand Bayou, LA* | 1 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Grand Bayou, LA* | 2 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 1/3 |
| *Grand Bayou, LA* | 3 | - | Pure | 1/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Bayou D'Arbonne Lake, LA* | 1 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 1/3 |
| *Bayou D'Arbonne Lake, LA* | 2 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 1/3 |
| *Bayou D'Arbonne Lake, LA* | 3 | - | Pure | 0/3 | 1/3 |
| | | | Crude | 0/3 | **2/3** |
| *Stillhouse Hollow Lake, TX* | 1 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Stillhouse Hollow Lake, TX* | 2 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Stillhouse Hollow Lake, TX* | 3 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Pinkston Reservoir, TX* | 1 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Pinkston Reservoir, TX* | 2 | - | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Pinkston Reservoir, TX* | 3 | - | Pure | 1/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Ross Barnett Reservoir, MS* | 1 | + | Pure | 1/3 | **2/3** |
| | | | Crude | 0/3 | **2/3** |
| *Ross Barnett Reservoir, MS* | 2 | + | Pure | **3/3** | **3/3** |
| | | | Crude | 0/3 | 1/3 |
| *Ross Barnett Reservoir, MS* | 3 | + | Pure | **3/3** | **3/3** |
| | | | Crude | 0/3 | 0/3 |
| *Lake Naconiche, TX* | 1 | + | Pure | 0/3 | **2/3** |
| | | | Crude | 0/3 | 0/3 |

| | | | | | |
|---|---|---|---|---|---|
| *Lake Naconiche, TX* | 2 | + | Pure | 0/3 | **3/3** |
| | | | Crude | 0/3 | 0/3 |
| *Lake Naconiche, TX* | 3 | + | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Caney Creek Reservoir, LA* | 1 | + | Pure | **3/3** | **3/3** |
| | | | Crude | 0/3 | **3/3** |
| *Caney Creek Reservoir, LA* | 2 | + | Pure | **3/3** | **3/3** |
| | | | Crude | 0/3 | **3/3** |
| *Caney Creek Reservoir, LA* | 3 | + | Pure | **3/3** | **3/3** |
| | | | Crude | 0/3 | **2/3** |
| *Lake Caddo, TX* | 1 | + | Pure | **3/3** | **3/3** |
| | | | Crude | 0/3 | **3/3** |
| *Lake Caddo, TX* | 2 | + | Pure | **3/3** | **3/3** |
| | | | Crude | 0/3 | **3/3** |
| *Lake Caddo, TX* | 3 | + | Pure | **3/3** | **3/3** |
| | | | Crude | 0/3 | **3/3** |
| *Lake Claiborne, LA* | 1 | + | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Lake Claiborne, LA* | 2 | + | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |
| *Lake Claiborne, LA* | 3 | + | Pure | 0/3 | 0/3 |
| | | | Crude | 0/3 | 0/3 |

# CHAPTER 3

# THESIS CONCLUSIONS

In this thesis project, I developed a rapid, field-portable, LAMP (Loop-Mediated Isothermal Amplification) molecular detection method for the cyanobacterium *Aetokthonos hydrillicola*, a freshwater epiphytic species that produces the neurotoxin aetokthonotoxin (AETX) and causes a wildlife disease known as Vacuolar Myelinopathy (VM) (Breinlinger *et al.* 2021). The toxin AETX causes vacuoles within the white brain matter of affected individuals, which leads to the inability to fly or move, sensitivity to light, and eventually death above a certain exposure threshold (Breinlinger *et al.* 2021; Thomas *et al.* 1998). Vacuolar Myelinopathy spreads through ingestion of *Hydrilla verticillata* with colonies of AETX producing *A. hydrillicola* present. Aetokthonotoxin is a lipophilic toxin that has the capability to affect primary consumers up the food chain through ingestion of fatty tissues containing toxin (Breinlinger *et al.* 2021). Aetokthonotoxin production has a high correlation with bromide availability within the environment, which lends to seasonal increases in VM cases in late fall – early winter, when *H. verticillata,* the preferred host for *A. hydrillicola,* is releasing stored bromide as it senesces (Breinlinger *et al.* 2021). *Hydrilla verticillata* is an aquatic invasive plant species prolific throughout the United States that grows in large mats on the surface of the water, providing an optimal habitat for cyanobacterial growth (Jacono 2015). Prior to this study, the molecular detection method for *A. hydrillicola* was limited to conventional PCR (Štenclová *et al.* 2023), however, the use of a more rapid and portable methodology for detection would allow for a more widespread surveillance of this dangerous species. The protocol developed in this study is a useful tool towards this goal since LAMP can be run with crude DNA extractions, occurs at a

single temperature, and can be performed using accessible materials, thus being particularly suitable for field implementation (Mason and Botella 2020; Meinecke *et al.* 2023; Notomi *et al.* 2000). A pipette-free field extraction method utilizing micro pestle homogenization and paper dipsticks (Mason and Botella 2020) was adapted for *A. hydrillicola* and tested for utilization with the newly developed LAMP primer set for the target species. While results are already promising, additional troubleshooting of the protocol might help further improve the amplification success. The primer set targets a region that spans across the *aetC* and *aetD* genes within the AETX producing gene clusters of *A. hydrillicola* to allow for the detection of the potential for toxin production before AETX is actually present on the landscape, and thus already causing disease. This region was also chosen for its unique specificity to *A. hydrillicola* (Adak *et al.* 2022; Breinlinger *et al.* 2021). The developed assay was able to detect *A. hydrillicola* with pure DNA extracts at a similar sensitivity to the already available PCR-based assay. Moreover, it was able to detect the target species in several positive sites even when coupled with the crude DNA extraction method, whereas the PCR-based assay could not. However, there was one site, Lake Claiborne, Louisiana, that was classified as positive for the presence of *A. hydrillicola* based on the visual observation of colonies of *A. hydrillicola*-like cyanobacteria, but that nevertheless failed to produce positive amplification with either the PCR or LAMP assay. This leads to the question of whether this site harbored an *A. hydrillicola* strain variant or other physiologically similar cyanobacteria that do not contain the AETX-producing gene cluster or that has variation within its ITS region, as previously hypothesized by other investigations (Štenclová *et al.* 2023). It also highlights the need to better understand the origin of the cyanobacterium and the diversity of the species in its distribution range.

It is currently unknown whether *A. hydrillicola* is a naturally occurring species within the United States or if it was introduced from another region with the movement of invasive plants, such as *H. verticillata*, other potential substrates, or even freshwater related human recreation. There is some evidence to suggest variation in the 16S-23S ITS region of the *A. hydrillicola* genome, but further studies would need to confirm this (Howard 2019; Štenclová *et al.* 2023). The development of our LAMP assay could potentially help answer some of these questions by aiding in sampling efforts throughout the distribution of *H. verticillata* to identify positive reservoirs quickly. A haplotype network analysis, developed from at least 10 different sites with multiple samples collected from each site, would then be a valuable next step for gathering more insight into the diversity of *A. hydrillicola* within the U.S. and southeastern reservoirs and potentially understanding the origin of the cyanobacterium (Doorenweerd *et al.* 2020; Leigh and Bryant 2015; North *et al.* 2021).

A cyanobacterium morphologically similar to *A. hydrillicola* has been reported growing on other submerged aquatic vegetation, specifically American water-willow, *Justicia americana,* however, the species did not amplify with a PCR assay targeting the *A. hydrillicola* ITS1 region. The cyanobacteria, however, did test positive for the AETX gene producing cluster, causing concern for the presence of a potential new toxigenic species (Štenclová *et al.* 2023). The discovery also raised concerns about whether *A. hydrillicola* could survive on other vegetation or substrate even if *H. verticillata* was eradicated in the waterway (Štenclová *et al.* 2023). This highlights the crucial need to understand where else *A. hydrillicola* can survive within a reservoir. Future studies investigating the presence of the cyanobacterium within soil substrate, leaves/pine needles that fall in the water, other aquatic vegetation, and *H. verticillata* tubers would greatly benefit from the use of the established field extraction and LAMP assay protocol.

These investigations would also help understand the current gaps in the life cycle of *A. hydrillicola,* especially regarding the overwintering phase, when *H. verticillata* is not present on the landscape in certain regions. It is unknown, for instance, if *A. hydrillicola* survives on the host plant tubers in the soil and then grows up with the host the following spring, or if it survives on other substrates within the reservoir, colonizing *H. verticillata* leaves only once they reemerge.

In conclusion, the LAMP assay developed in this study is a useful tool to aid in the understanding and future research of *A. hydrillicola*. This rapid and portable molecular detection assay can be used by state and federal agencies and researchers with simple, accessible materials and could assist in management decision making, thus helping prevent the occurrence of VM within southeastern waterways.

LITERATURE CITED

Adak S, Lukowski AL, Schäfer RJB, and Moore BS (2022) From tryptophan to toxin: Nature's convergent biosynthetic strategy to aetokthonotoxin. *Journal of the American Chemical Society* **144**, 2861-2866. doi: 10.1021/jacs.1c12778.

Breinlinger S, Phillips TJ, Haram BN, Mareš J, Yerena JAM, Hrouzek P, Sobotka R, Henderson M, Schmieder P, Williams SM, Lauderdale JD, Wilde D, Gerrin W, Kust A, Washington JW, Wagner C, Geier B, Liebeke M, Enke H, Niedermeyer THJ, and Wilde SB (2021) Hunting the eagle killer: A cyanobacterial neurotoxin causes vacuolar myelinopathy. *Science (Washington)* **371**. doi: 10.1126/science.aax9050 https://science.sciencemag.org/content/371/6536/eaax9050.abstract.

Doorenweerd C, San Jose M, Barr N, Leblanc L, and Rubinoff D (2020) Highly variable coi haplotype diversity between three species of invasive pest fruit fly reflects remarkably incongruent demographic histories. *Scientific Reports* **10**, 6887. doi: 10.1038/s41598-020-63973-x.

Howard A (2019) Genetic investigations of a novel, toxic, epiphytic cyanobacteria: Aetokthonos hydrillicola. M.S. Thesis, (University of Georgia, United States -- Georgia.

Leigh JW and Bryant D (2015) Popart: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6**, 1110-1116. doi: https://doi.org/10.1111/2041-210X.12410.

Mason MG and Botella JR (2020) Rapid (30-second), equipment-free purification of nucleic acids using easy-to-make dipsticks. *Nature Protocols* **15**, 3663. doi: 10.1038/s41596-020-0392-7.

Meinecke CD, Vos L, Yilmaz N, Steenkamp ET, Wingfield MJ, Wingfield BD, and Villari C (2023) A lamp assay for rapid detection of the pitch canker pathogen fusarium circinatum. *Plant Dis* **107**, 2916-2923. doi: 10.1094/pdis-04-22-0972-sr.

North HL, McGaughran A, and Jiggins CD (2021) Insights into invasive species from whole-genome resequencing. *Mol Ecol* **30**, 6289-6308. doi: 10.1111/mec.15999.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, and Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic acids research* **28**, E63. doi: 10.1093/nar/28.12.e63.

Štenclová L, Wilde SB, Schwark M, Cullen JL, McWhorter SA, Niedermeyer THJ, Henderson WM, and Mareš J (2023) Occurrence of aetokthonotoxin producer in natural samples – a pcr protocol for easy detection. *Harmful Algae* **125**, 102425. doi: https://doi.org/10.1016/j.hal.2023.102425.

Thomas NJ, Meteyer CU, and Sileo L (1998) Epizootic vacuolar myelinopathy of the central nervous system of bald eagles (haliaeetus leucocephalus) and american coots (fulica americana). *Vet Pathol* **35**, 479-487. doi: 10.1177/030098589803500602.