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Abstract

In 2019, an unknown shell disease was reported in Northern red-bellied turtles (*Pseudemys rubriventris*), a freshwater turtle species with a range that mainly includes southern New Jersey through North Carolina. This study investigates the effects of harmful cyanobacterial blooms on the vulnerability to shell disease of freshwater Northern red-bellied turtles (*Pseudemys rubriventris*) in New Jersey by characterizing and enumerating cyanobacteria in lake water and within the epizoic community with a goal to provide data-driven recommendations for *P. rubriventris* conservation. Phytoplankton community composition and cyanotoxin concentration were measured in two affected lakes (Daretown Lake and Elmer Lake) and one unaffected lake (Lake Fred) in Salem and Ocean counties, New Jersey. These measures were examined to determine whether the presence of potentially toxin-producing cyanobacteria had any effect on the occurrence of shell disease in these turtles. In addition to water samples, epizoic community was examined and cyanotoxins were measured from samples collected from turtle shells. Results indicate that cyanobacteria may increase the vulnerability of *P. rubriventris* to the shell disease. In general, cyanobacteria density and cyanotoxin concentrations were greater in water and epizoic samples collected from the affected lakes than the unaffected lake. Additionally, epizoic samples collected from turtles afflicted with shell disease were found to contain significantly greater cyanobacteria density than samples collected from healthy turtles, suggesting epizoic cyanobacteria growth may be the cause of shell diseases of *P. rubriventris* in New Jersey.

MONTCLAIR STATE UNIVERSITY

Investigating the effects of harmful cyanobacterial blooms on the vulnerability to shell disease of
Northern red-bellied turtles (*Pseudemys rubriventris*) in New Jersey

by

Stephanie Getto

A Master's Thesis Submitted to the Faculty of

Montclair State University

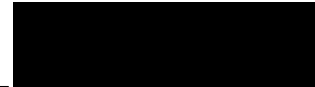
In Partial Fulfillment of the Requirements

For the Degree of Master of Science

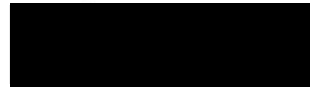
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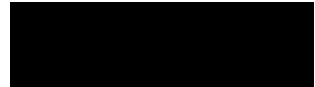
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INVESTIGATING THE EFFECTS OF HARMFUL CYANOBACTERIAL BLOOMS ON
THE VULNERABILITY TO SHELL DISEASE OF NORTHERN RED-BELLIED TURTLES
(PSEUDEMYS RUBRIVENTRIS) IN NEW JERSEY

Submitted in partial fulfillment of the requirements
For the degree of Master of Science

by
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2021

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1. Introduction

1.2 Harmful Cyanobacterial Blooms

Cyanobacteria are a complex and diverse group of prokaryotes capable of synthesizing and utilizing chlorophyll-a and other pigments to carry out photosynthesis, and can be found in nearly any habitat imaginable (Carr and Whitton, 1982; Whitton and Potts, 2007; Rastogi et al., 2014; Sciuto and Moro, 2015; Mishra et al., 2019). Cyanobacteria are often (somewhat incorrectly) referred to as blue-green algae due to their production of the phycobilin pigments, such as phycocyanin and phycoerythrocyanin, and their photosynthetic capability (Whitton and Potts, 2007).

Cyanobacteria fulfill diverse ecological roles in healthy aquatic ecosystems. As phytoplankton, they contribute substantially to primary production, helping to form the base of the food web and facilitate energy flows within their habitats (Stockner et al., 2000; Sciuto and Moro, 2015). Cyanobacteria are capable of forming extensive symbiotic relationships with other organisms (Sciuto and Moro, 2015); for example, evidence suggests that certain plant species are able to utilize the nitrogen-fixing capabilities of certain cyanobacteria by taking hormogonia into their own tissues; hormogonia are motile filaments of cells sometimes containing heterocytes, the nitrogen fixing structure of some cyanobacteria (Whitton and Potts, 2007).

Under certain environmental conditions, some species of bloom-forming cyanobacteria may produce a diverse array of harmful, and even deadly, toxins which are an increasing environmental and public health concern globally in both freshwater and marine ecosystems (Whitton and Potts, 2007; Neilan et al., 2012; California Office of Environmental Health Hazard Assessment, 2017; Nienaber and Steinitz-Kannan, 2018; Townhill et al., 2018; New York Department of Environmental Conservation, 2020; New Jersey Department of Environmental

Protection, 2021; United States Environmental Protection Agency, 2021). When blooms of toxin-producing cyanobacteria occur, they are referred to as Harmful Cyanobacterial Blooms (HCBs). A distinctive visual feature of many HCBs is the ‘spilled green paint’ or ‘pea soup’ look as phytoplankton accumulate to form green surface scums (New Jersey Department of Environmental Protection, 2021).

HCBs can be harmful to human and animal health, and severely impact the affected aquatic ecosystem in a negative manner (Sellner et al., 2003; Thangaraja et al., 2007; California Office of Environmental Health Hazard Assessment, 2017; United States Geological Survey, 2018; New York Department of Environmental Conservation, 2020; Centers for Disease Control and Prevention, 2021; Cui et al., 2021; New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021). High phytoplankton densities may cause breathing difficulties in gill-bearing organisms as their respiratory systems can become clogged, reducing oxygen exchange (Thangaraja et al., 2007; Centers for Disease Control and Prevention, 2021). Decomposition of algae and cyanobacteria during bloom conditions can deplete an aquatic ecosystem of the oxygen necessary for organisms to function, creating hypoxic or anoxic conditions sometimes referred to as ‘dead zones’ (Mackenthun et al., 1948; Norkko and Bonsdorff, 1996; Thangaraja et al., 2007; Díaz and Rosenberg, 2008; Piontkovski et al., 2016; Harrison et al., 2017; Centers for Disease Control, 2021; Cui et al., 2021). Hypoxic or anoxic conditions due to phytoplankton decomposition are thought to cause or contribute to fish kills, especially when accompanied by cyanotoxins (Mackenthun et al., 1948; Glibert et al., 2002; Thangaraja et al., 2007; Piontkovski et al., 2016; Harrison et al., 2017). For example, a large marine mortality event that occurred in St. Helena Bay, South Africa in 1996 was attributed to low dissolved oxygen content following an algal bloom (Matthews and Pitcher, 1996). These

blooms and subsequent mortality events affect nutrient cycling and other physiochemical processes essential to aquatic ecosystem health by altering water chemistry and ecosystem dynamics (Zhu et al., 2012; Han et al., 2015; Wang et al., 2016; Cui et al., 2021).

Oxygen depletion due to the decomposition of phytoplankton during bloom conditions may also lead to an abundance of anaerobic microbial populations which may trigger diseases in both farmed and wild populations of fish and other aquatic organisms (Glibert et al., 2002; Thangaraja et al., 2007; Díaz and Rosenberg, 2008). For example, a mortality event in an aquaculture pen located in the Kuwait Bay was initiated by anoxic conditions following the decomposition of algae after a *Ceratium furca* bloom, resulting in the deaths of 100-1000 gilthead sea bream (*Sparus auratus*) a day through the month of August in 2002 (Glibert et al., 2002). This mortality event had a cascading effect in the Kuwait Bay when the decomposition of dead fish helped spur environmental conditions that led to the proliferation of the bacterium *Streptococcus agalactiae*. This resulted in an even larger fish kill of more than 2,500 metric tons of wild mullet, *Liza klunzingeri* (Glibert et al., 2002). The presence of HCB species and other environmental factors predictive of a HCB, such as elevated temperatures and nutrient concentrations, during this event were also thought to increase the fishes' susceptibility to contracting the bacterial pathogen (Glibert et al., 2002). This is similar to a massive fish mortality event that occurred in the Caribbean in 1999, in which the pathogen *Streptococcus iniae*, in combination with other HCB related environmental stressors, resulted in the deaths of thousands of reef dwelling fishes (Ferguson et al., 2000). Additionally, toxic products of anaerobic bacteria that thrive under the conditions of depleted oxygen following a bloom, such as hydrogen sulfide, may cause the direct poisoning of aquatic animals (Matthews and Pitcher, 1996). Moreover, many people are reliant on the fishing industry for both food and/or

source of income, and thus HCBs present a significant concern for these groups. Anderson et al. (2000) released a report through the Woods Hole Oceanographic Institute which reported that between the years of 1987-1992, HCBs cost the United States an average of \$18,407,948 within the commercial fishing industry, \$22,202,597 in the area of public health, \$6,630,415 in terms of recreation and tourism, and \$2,088,885 in terms of the monitoring and management of blooms.

Public health concerns related to cyanotoxins can range from symptoms such as mild dermal rashes and vomiting to severe liver, respiratory, or neurological illness, and even death when the toxins are ingested, inhaled, or absorbed through the skin (Sellner et al., 2003; California Office of Environmental Health Hazard Assessment, 2017; Townhill et al., 2018; United States Geological Survey, 2018; Centers for Disease Control and Prevention, 2021; New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021). Cyanotoxins are produced as secondary metabolites by some cyanobacteria taxa, and these toxins may be classified into three main types: dermatotoxins (produce skin reactions and irritate various membranes), hepatotoxins (disrupt proteins that maintain liver function), and neurotoxins (cause rapid paralysis of skeletal and respiratory muscles) (Moe, 1996; Stewart et al., 2006; Clercin, 2012).

An example of dermatotoxins include lipopolysaccharides (LPS), a component of all gram-negative bacterial cell walls that may cause a variety of health issues in humans such as skin rashes, gastro-intestinal irritation, allergies, headache, fever, and respiratory disease (Stewart et al., 2006). Cyanobacteria are widely regarded as gram-negative prokaryotes (Stanier and Cohen-Bazire, 1977; Codd and Poon, 1988; Hunter, 1997; Codd, 1994; Holt et al., 1994; Duy et al., 2000; Stewart et al., 2006), and as such, LPS produced by cyanobacteria taxa may be responsible for various waterborne health incidents globally (Codd, 2000; Stewart et al., 2006). Other

dermatotoxins include aplysiatoxins (potent tumor promoters) and lyngbyatoxin-a (a powerful blister causing agent) (Clerc, 2012).

Microcystin congeners are an example of hepatotoxins produced by cyanobacteria, and represent some of the most widespread and well-studied cyanotoxins (Dawson, 1998; Rastogi et al., 2014; Nienaber and Steinitz-Kannan, 2018; New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021). Microcystins were named after the cyanobacteria genus in which they were first discovered, *Microcystis* (Nienaber and Steinitz-Kannan, 2018). Since then, these toxins have been found to be produced by such cyanobacteria genera as *Dolichospermum*, *Fischerella*, *Gloeotrichia*, *Nodularia*, *Nostoc*, *Oscillatoria*, and *Planktothrix* (Dawson, 1998; Rastogi et al., 2014; New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021). Microcystins can be detected globally in freshwater, estuarine, and marine waters, and even in the dried riverbeds of desert environments (Metcalf, 2012; Rastogi et al., 2014). Toxic effects of microcystins include disruption of the cellular cytoskeleton, inhibition of protein phosphatases, enlarged liver, and major hepatic hemorrhage (Dawson, 1998; Metcalf, 2012; Bouaïcha et al., 2019). Other hepatotoxins produced by some cyanobacteria spp. include cylindrospermopsin and nodularins (New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021).

Saxitoxins are an example of a potent neurotoxin found in both freshwater and marine environments, and these toxins are produced by cyanobacteria taxa such as *Aphanizomenon flosaquae*, *Dolichospermum circinalis*, *Lyngbya wollei*, *Planktothrix*, and *Raphidiopsis* (Christensen and Khan, 2020; World Health Organization, 2020; New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021).

Saxitoxins are lipophilic and are thus a concern for potential bioaccumulation in animal adipose tissues (Negri and Jones, 1995; Galvão et al., 2009; Wiese et al., 2010; Christensen and Khan, 2020). These cyanotoxins block voltage-gated sodium channels along nerve cells, resulting in the depression of nerve impulses which leads to neurological symptoms such as numbness, paralysis, and in some instances, death by respiratory failure (Christensen and Khan, 2020; New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021). When shellfish feed on saxitoxin-producing phytoplankton, toxins bioaccumulate within shellfish body tissues and are then consumed by other marine life as well as humans (Negri and Jones, 1995; Galvão et al., 2009; Wiese et al., 2010; Christensen and Khan, 2020; World Health Organization, 2020; New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021). Galvão et al. (2009) detected saxitoxins in liver and muscle samples from tilapia, a fish regularly consumed by humans, demonstrating the public health concern these toxins present. Other neurotoxins produced by cyanobacteria include anatoxins and β -N-methylamino-L-alanine, referred to more commonly as BMAA (New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021). BMAA is thought to be causative of amyotrophic lateral sclerosis (Lou Gehrig's Disease) and parkinsonism-dementia complex (ALS/PDC) (Clercin, 2012).

There is no one definitive cause or environmental condition which initiates HCBs, and research suggests it may vary case by case (Roelke and Buyukates, 2012). One such process that is believed to facilitate excessive growth of bloom-forming, toxin-producing cyanobacteria is eutrophication, which can be defined as the process by which water is polluted by excess nutrients, such as phosphorus and nitrogen (Smith et al., 1999; Smith, 2003; Glibert et al., 2005;

Anderson et al., 2006; Díaz and Rosenberg, 2008; O’Neil et al., 2013; le Moal et al., 2019). Anthropogenic activities are believed to facilitate lake eutrophication as sewage, fertilizer, and livestock manure runoffs drive excessive nutrient loads into aquatic systems (Smith, 2003; Dawson, 1998; Schindler, 2012; Jankowiak et al., 2019; United States Environmental Protection Agency, 2021; World Health Organization, 2021). Other environmental factors that contribute to eutrophication include light availability and temperature (le Moal et al., 2019). Evidence already suggests that HCBs are increasing in number and species complexity (Anderson et al., 2000; Paerl and Huisman, 2008, 2009; O’Neil et al. 2013; Gobler, 2020; Griffith and Gobler, 2020). Into the future, the issue of eutrophication due to nutrient loading will likely only increase as urbanization and industrialization push onward to support the growing human population (Rabalais et al., 2010). Climate change may facilitate more frequent and severe HCBs as conditions such as increased precipitation and rising temperatures are thought to stimulate cyanobacterial growth (Paerl and Huisman, 2008; Nazari-Sharabian, 2018; Bouaïcha et al., 2019; Gobler et al., 2020). Increased precipitation provides the chance for runoff to enter into and pollute aquatic systems at an escalated rate (Paerl and Huisman, 2008; Nazari-Sharabian, 2018). Cyanobacteria are able to tolerate high temperatures and tend to have a wider range of optimal growth conditions than other groups of phytoplankton such as diatoms (Paerl and Huisman, 2008, 2009), allowing for cyanobacteria to outcompete other groups of phytoplankton.

Since HCB monitoring and response by the New Jersey Department of Environmental Protection (NJDEP) began in 2017, HCBs have been documented as an ongoing problem within New Jersey’s freshwater waterbodies. In 2017, 30 suspected HCB events within 24 different waterbodies were responded to, and of those 30 events, 22 were lab confirmed HCBs (New Jersey Department of Environmental Protection, 2019). In 2018, the number of suspected HCBs

in New Jersey waterbodies was reported as 32 within 25 different waterbodies, with 20 lab confirmed HCB events documented (New Jersey Department of Environmental Protection, 2019). In 2019, the number of suspected HCB events rose to 74 occurrences within 54 different waterbodies, with 39 of the 74 occurrences confirmed as HCBs (New Jersey Department of Environmental Protection, 2020). In 2020, officials responded to suspected HCBs in 84 different waterbodies, with 47 confirmed by laboratory analysis (New Jersey Department of Environmental Protection, 2020).

If a HCB event is suspected within a New Jersey waterbody, water samples will be collected and screened to determine whether further quantitative analysis is necessary to confirm the presence of a HCB (New Jersey Department of Environmental Protection Bureau of Freshwater & Biological Monitoring, 2020). The fluoresce of chlorophyll and fluorescence of phycocyanin are measured using a fluorometer in order to quickly assess the status of HCBs, and microscope analysis is conducted to determine the presence of toxin producing cyanobacteria species (New Jersey Department of Environmental Protection, 2020). If these preliminary screenings indicate a potential HCB event, cyanobacterial cell counts and cyanotoxins are both measured in order to confirm the presence of a toxic bloom. When cyanobacterial density in a recreational water body exceeds 20,000 cells/mL, a HCB event may be present and additional monitoring will occur (New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021). The NJDEP established a threshold of 2 µg/L for microcystins in recreational waters, and a threshold of 0.6 µg/L for saxitoxins; toxins measured above these concentrations indicate bloom conditions. (New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021).

In addition to occurring within the water column, toxin producing cyanobacteria can be found on benthic substrates and on the benthos, growing on virtually any submerged surface, such as rocks (epilithon), sand (epipsammon), or animals (epizoan) (Lamberti et al., 2007). Thus, epizoic algae and cyanobacteria collected from turtle shells can be seen as partially representative of the benthic algae community. The phenomenon of epizoic algal and cyanobacteria growth can occur in aquatic turtles (Ernst and Norris, 1978; Akgül et al., 2014; Fayolle, 2016; McKnight et al., 2020; Beau and Brischoux, 2021). Residing on top of an animal offers benefits to algae and cyanobacteria; for example, algae living on turtle shells may benefit by residing on an elevated position and thus avoiding the resuspension of sediments (Totti et al., 2011). Examining epizoic community structure may aid in the understanding of community structure and ecosystem health within an aquatic system.

1.2 Northern Red-bellied Turtles (Pseudemys rubriventris)

Northern red-bellied turtles (*Pseudemys rubriventris*) are a basking species of aquatic turtle. Besides a geographically and ecologically distinct population in southeastern Massachusetts, the native geographic range of *P. rubriventris* extends down the Atlantic coastal plain from central New Jersey to northeastern North Carolina, and westward up the Potomac River to eastern West Virginia (Browne et al., 1996; Swarth, 2004; Pearson, 2013; Gregoire, 2021). Within New Jersey, these turtles can be found in the central and southern parts of the state, and are especially abundant in the Pinelands (DiLio, 2016). The preferred habitat of *P. rubriventris* may range from slow moving, deep water with ample vegetation and basking locations (Swarth, 2004) to fast moving rivers and creeks (Clark, 2021; Chesapeake Bay Program, 2021). They may occasionally be found in estuarian habitats (Swarth, 2004; Chesapeake Bay Program, 2021), and as such, red-bellied turtles are sometimes regarded as

terrapins (Conant, 1951; Virginia Herpetological Society, 2021). The term terrapin refers to a non-taxonomic classification describing turtles that spend part of their lives on land and part in aquatic habitats that may contain brackish water (National Marine Life Center, 2016). *P. rubriventris* will overwinter in the mud beneath the rivers, lakes, and ponds they reside in (Clark, 2004; Chesapeake Bay Program, 2021). Adults typically measure 10-12 inches in length, can weigh up to 10 pounds, and reach sexual maturity around 10-20 years of age (Swarth, 2004; US Fish and Wildlife, 2006; Chesapeake Bay Program, 2021). Sexual dimorphism can be observed in *P. rubriventris*, with females growing larger than males and males displaying elongated nails and a cloaca opening that is located further down from the base of the tail than that of the female (Virginia Herpetological Society, 2021). Adults have a carapace that ranges from a dark olive brown to nearly black, and a plastron that ranges from pale pink to a distinctive brilliant red (Virginia Herpetological Society, 2021; Chesapeake Bay Program, 2021). Juveniles are omnivorous while adults are herbivorous (Pearson, 2013; Virginia Herpetological Society, 2021). Adults are diurnal, not territorial, and spend long hours basking (Clark, 2004; Virginia Herpetological Society, 2021).

Northern red-bellied turtles are currently classified as a Northeast Regional Species of Greatest Conservation Need and a New Jersey Priority Species of Greatest Conservation Need (New Jersey Department of Environmental Protection Division of Fish and Wildlife, 2018). The decline of this species has been ongoing throughout recent history (Waters, 1962; Pearson, 2013). Mating activities occur from April through June, and females can lay up to 2 clutches of around 9-13 eggs per year in the sand or soil a few hundred feet from their watery habitat (Virginia Herpetological Society, 2021). Hatchlings have a nearly 100% mortality rate mainly due to predation from skunks, raccoons, bullfrogs, snapping turtles, heron, and some species of

fish (US Fish and Wildlife, 2006). If they are able to survive the hatchling stage, these turtles may live up to 55 years (Clark, 2004; Chesapeake Bay Program, 2021). Adult red-bellied turtles are threatened by habitat degradation and loss, road vehicle mortality events, overharvesting, and introduction of non-native species as a result of the pet trade (Ernst et al., 1994; Hulse, 2001; US Fish and Wildlife, 2006; Pearson, 2013).

1.3 Shell Diseases

Though much information exists regarding maladies of the shell in captive turtles due to the widespread interest in keeping these charismatic animals as household pets, data regarding shell diseases in wild populations of freshwater turtles is scarce (Garner et al., 1997). This may be in part because turtles, like other reptiles, decompose quickly following death and thus it can be difficult to ascertain a cause of death once a deceased animal is found (Hunt, 1957). Shell disease, sometimes referred to as shell rot, is a blanket term for highly infectious conditions that affect the chitinous plates of the dorsal shell, referred to as the carapace, and/or the ventral shell, referred to as the plastron (Wallach, 1975). Affected individuals tend to die from secondary infection following development of shell disease (Wallach, 1975). Although symptoms can vary both intra- and interspecifically, this disease is generally characterized by lesions, pitted edemas, sloughing off of scutes, and necrosis of underlying bone (Hunt, 1957; Wallach, 1975; Lovich et al., 1996; Garner et al., 1997).

In the spring of 2019, the first reports of a debilitating shell disease found in Northern red-bellied turtles began to surface in Salem County, New Jersey. Turtles collected from Salem County showed the above listed characteristics of shell diseases. Though causative agents of shell diseases seem to rarely be determined throughout the literature, several potential factors are generally cited, the first of which is mycosis (Hunt, 1957). The second factor often cited is

bacterial invasion either of the shell's keratin layer (Lovich et al., 1996) which may occur as a result of mechanical injury to the epidermal laminae of the shell (Wallach, 1975), or of the circulatory system as a result of internal visceral diseases (Garner et al., 1997). Following the initial reports from Salem, 3 turtles were collected from Daretown Lake (DT), and 2 were collected from Elmer Lake (EL) by the NJDEP (Brian Zarate of NJDEP, personal communication). Affected individuals showed dark lesions specific to the plastron area of their shells and became lethargic before either succumbing to the disease entirely, or being humanely euthanized. Necropsies of diseased turtles showed a parasitic overburden in all turtle samples, and histology findings included compromised hepatocytes in addition to the presence of heterophils and other cells associated with inflammatory response in the liver (Brian Zarate of NJDEP, personal communication). The polymerase chain reaction (PCR) method was used to test turtle lesion samples for bacterial and fungal pathogens known to cause disease in turtles; all turtle samples were negative for *Endomyces*, and no known pathogenic bacteria were identified (Nicole Lewis of NJDEP, personal communication). Bacteria isolated from the shells of affected individuals was not necessarily atypical from what would be expected of wild freshwater turtles (Brian Zarate of NJDEP, personal communication). These early testing results were inconclusive and no clear cause of shell disease was identified.

In addition to the ongoing decline of *P. rubriventris*, it is widely accepted that chelonians, and herpetofauna species in general, are increasingly diminishing (Lovich et al., 1996; Wilson and McCranie, 2004; Measy et al., 2009; Christiansen et al., 2012; Quesnelle et al., 2013; Tapilatu et al., 2013; Whitfield et al., 2014; Stanford et al., 2020). Therefore, it is of utmost importance to understand the etiology of a disease that appears to be threatening this already vulnerable species. Literature suggests that toxic or immunosuppressive chemicals within aquatic

habitats may either cause or predisposition turtles to developing shell disease (Lovich et al., 1996; Garner et al., 1997); HCBs and cyanotoxins may increase turtle's vulnerability to shell disease. The objective of this study aims to investigate the effects of harmful cyanobacterial blooms on the vulnerability to shell disease of freshwater Northern red-bellied turtles (*Pseudemys rubriventris*) in New Jersey by characterizing and enumerating cyanobacteria in lake water and within the epizoic community with a goal to provide data-driven recommendations for *P. rubriventris* conservation.

2. Methods

2.1 Study Sites

A total of three lakes in two New Jersey counties were selected for this study (**figure 1**). Shell disease was reported in Daretown Lake (DT) and Elmer Lake (EL), and absent in Lake Fred (LF), therefore the latter functioned as the control site. DT is located in Upper Pittsgrove, Salem County, and is a part of the Salem River drainage system. DT is 16 acres, and is bordered on two sides by farms (one of which houses cattle). DT is primarily used for recreational purposes such as fishing and boating. DT was first confirmed with a HCB event in 2019 (New Jersey Department of Environmental Protection, 2019), and again in 2020 (New Jersey Department of Environmental Protection, 2020). As of the time of this report, no HCB alert has been put into place at DT for 2021.

EL is 51 acres in size, and is located in Salem County within the 385.80-acre Elmer Lake Wildlife Management Area. This body of water is located within the Muddy Run drainage area, and is primarily surrounded by woody wetlands. Country highway 611 borders the lake on the south side and US-40 runs across its northern point. Like DT, EL was also first confirmed with a HCB event in 2019 (New Jersey Department of Environmental Protection, 2019). No HCB alerts were put in place for EL in 2020, and at the time of this report, none have been enacted for 2021.

Lake Fred (LF) has a large and seemingly healthy population of *P. rubriventris*. LF stretches about half-mile long and a quarter mile wide, nestled within the New Jersey Pinelands National Reserve on the Stockton University campus in Galloway, New Jersey. A former cranberry bog, LF is surrounded by walking trails, oak and pine dominated forestland, and various campus and dormitory buildings. No HCB alerts or events have been documented at LF.

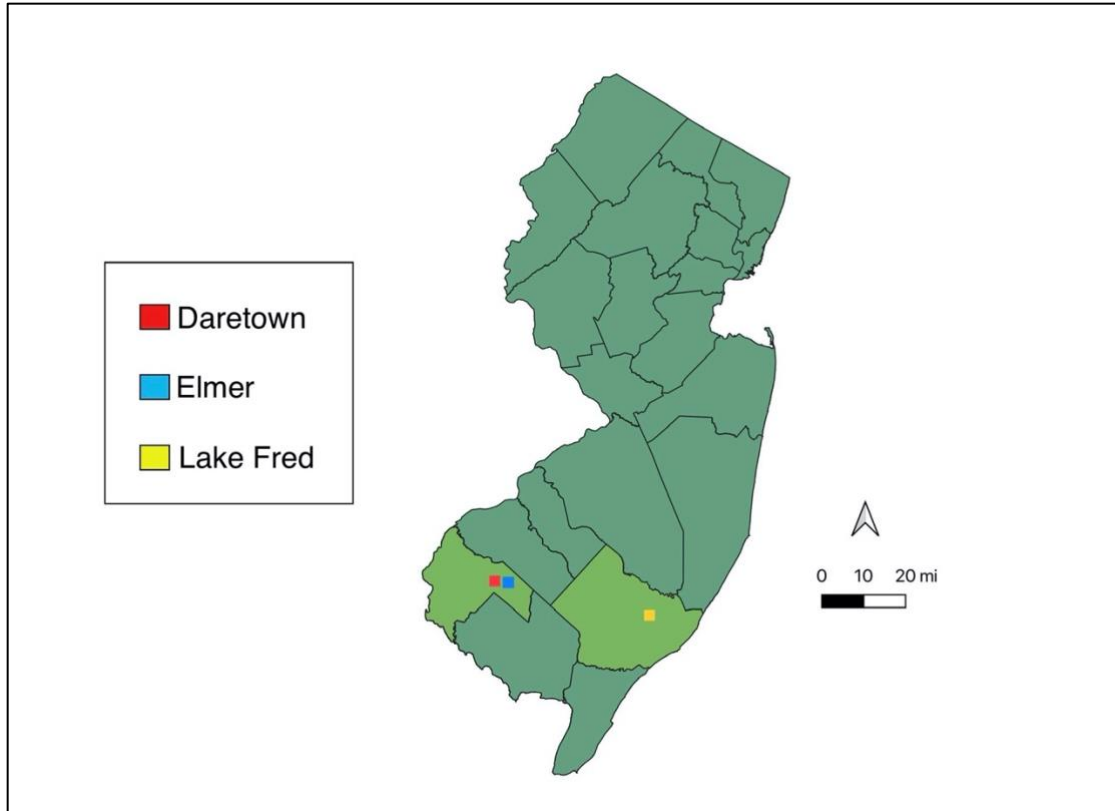


Figure 1: Location of the three lakes selected in the study. Elmer Lake and Daretown Lake are located in Salem County, New Jersey, and were observed with shell disease on Northern red-bellied turtles, (*P. rubriventris*). Lake Fred, located in Ocean County, had no turtle shell disease observed.

2.2 Sample Collections

All samples were collected during summer 2020 and spring 2021. These seasons were chosen as they represent the summer HCB season and the spring breeding season for *P. rubriventris*.

Due to COVID-19, sampling efforts were restricted to twice in the summer (August and September, 2020) and twice in the spring (March and April, 2021).

Three accessible water sampling locations were selected at each lake, and grab samples at a depth of 1 meter were collected. Following collection, samples were kept in ice and immediately transported back to a Montclair State University laboratory. Water samples were preserved with Glutaraldehyde and stored at 4°C for later identification and enumeration. Additional water

samples were also collected using amber glass bottles and stored at -20°C for later cyanotoxin analysis.

All turtle trapping was conducted by NJDEP and Stockton University using a combination of basking traps, hoop net traps, and hand-capture nets. Trapping was conducted during three separate events occurring in late July 2020, September 2020, and March 2021. Blood and tissue samples were collected by NJDEP, and turtles were fitted with radio telemetry chips. NJDEP also recorded size, weight, and sex of each turtle. All turtles included in this study were adults, with carapace sizes of 10 inches or greater. Due to the distinction between eukaryotic algae and prokaryotic cyanobacteria, the term ‘epizoic community’ will be used to refer to both algal and cyanobacterial genera identified and enumerated from turtle shell samples. Epizoic community samples were collected from turtle shells following a modified periphyton collection standard operating procedure from the West Virginia Department of Environmental Protection (2018). A modified PVC ring collection device with a surface area of 17.82cm^2 was placed on top of the turtles’ carapace, and 100mL of water was dispensed into the PVC tube. A toothbrush was used to gently loosen algal cells adhered to the carapace, and a turkey baster was then used to collect the sample. Epizoic community samples were preserved with Glutaraldehyde and stored at 4°C for later phytoplankton identification and enumeration. After enumeration samples were then multiplied by the 100mL of water used to collect samples, and divided by the collection device’s surface area of 17.82cm^2 to arrive at final counts in cells/cm^2 . Epizoic community samples were also collected using amber glass bottles and stored at -20°C for later cyanotoxin analysis. Cyanotoxins results in $\mu\text{g}/\text{L}$ were then multiplied by the 100mL of water used to collect samples, and divided by the collection device’s surface area of 17.82cm^2 to arrive at final toxin concentrations in $\mu\text{g}/\text{cm}^2$.

Field sampling was conducted following the Montclair State University's Institutional Animal Care and Use Committee (IACUC), protocol # 2020-062.

2.3 Phytoplankton Enumeration and Identification

Photosynthetic microorganisms from both water and epizoic samples were identified and enumerated using a nanoplankton chamber (PhycoTech) under a brightfield microscopy (Fisher Scientific AX800 series, model #11350112). The NJDEP criteria for determining HCB watches and advisories were used; cell counts above 20,000 cells/mL were used to indicate a HCB event.

2.4 Cyanotoxin Analysis

Microcystins and saxitoxins from both lake water and epizoic community samples were detected and quantified by New Jersey Center for Water Science and Technology (NJCWST) using the Enzyme-Linked Immunosorbent Assay (ELISA) kits for microcystins and for saxitoxins (Eurofins Abraxis). The minimum detection limit for microcystins and saxitoxins by ELISA is 0.3 µg/L and 0.022 µg/L, respectively. The NJDEP's threshold for microcystins in recreational waters is 2 µg/L, and the threshold for saxitoxins is 0.6 µg/L (New Jersey Department of Environmental Protection, 2021). The term 'ND' was used to describe samples in which no cyanotoxins were detected.

2.5 Statistical Methods

Statistical analyses were performed using IBM SPSS (version 28) and R (version 3.6.1). The Shapiro–Wilk test for normality was used to determine the distribution of the data. The epizoic community data collected did not satisfy the assumption of normal distribution, and thus the non-parametric Mann-Whitney U Test and Kruskal-Wallis One Way Analysis of Variance were used to compare samples. Mann-Whitney tests were used to examine differences between

epizoic community samples collected from turtles with shell disease compared to samples collected from turtles without shell disease. Kruskal-Wallis One Way Analysis of Variance was used in conjunction with Dunn's Multiple Comparison Test with Bonferroni adjustments to compare water samples across the 3 lakes studied. A mixed effects model which accounted for variation within data due to month of sampling was used to compare water samples collected from each lake. Lake site was used as the fixed factor with phytoplankton density, cyanobacteria density, saxitoxins, and microcystins functioning as response variables. The slopes and intercepts of the model were allowed to vary by including site and month as random factors. For the purposes of statistical testing only, half of the minimum detection limit were used.

3. Results

3.1 Phytoplankton Community and Cyanotoxins in Lake Water

Cyanobacteria density varied significantly by lake ($P=0.002$) (**table 2**), and taxa recorded at each lake varied by month, in accordance with season (**figures 2 & 3**). Overall, DT contained the greatest density of cyanobacteria each month sampled, and cell counts well exceeded 20,000 cells/mL during the summer months (August 2020 and September 2020). Cell counts from DT reached a maximum value of 867,397 cells/mL (**table 1**). EL also contained cyanobacteria taxa, though cell counts did not exceed 20,000 cells/mL at any sampling event. The maximum cyanobacterial cell count from EL samples was much less than the maximum value reached in DT samples, at 16,977 cells/mL. LF showed very minimal cyanobacteria taxa presented with the maximum cyanobacteria density at 2,267 cells/mL.

Overall, 96.84% of the phytoplankton density recorded within DT lake samples from August 2020 were composed of cyanobacteria taxa, with samples reaching a maximum of 186,144 cells/mL and a minimum of 130,555 cells/mL. Represented were identified as *Dolichospermum*, which represented 38.85% of the total phytoplankton community, *Microcystis* (24.62%), *Aphanizomenon* (17.97%), *Planktothrix* (14.83%), *Snowella* (0.33%), and *Oscillatoria* (0.23%). Other phytoplankton groups represented in DT sample this month were green algae (2.83%), diatoms (0.27%), and cryptophytes (0.06%). Cyanobacteria accounted for 20.02% of overall phytoplankton density within in EL samples from August 2020, with cell counts ranging from 255-3,478 cells/mL. *Microcystis* accounted for 10.56% of the total phytoplankton community, and *Aphanocapsa* accounted for 9.46%. Other phytoplankton groups represented were green algae (49.72%), diatoms (25.19%), golden algae (3.96%), and euglenoids (1.10%). Cyanobacteria were responsible for 18.20% of phytoplankton density within August LF samples,

all of which was represented by the genus *Anabaena*. Cyanobacteria cell counts ranged from 0-2,267 cells/mL. LF samples from this month were mostly composed of green algae (55.61%), followed by diatoms (23.69%). The remaining 2.49% of phytoplankton density was accounted for by golden algae.

During September 2020 sampling, cyanobacteria accounted for 99.40% of total phytoplankton density. Cell counts were highest during this month of sampling, with samples containing a maximum of 867,397 cells/mL and a minimum of 484,395.89 cells/mL. Represented taxa included *Dolichospermum*, which was responsible for 79.55% of the total phytoplankton community, *Planktothrix* (9.89%), *Aphanizomenon* (8.93%), and *Microcystis* (1.02%). Other phytoplankton groups represented in DT samples from this month were green algae (0.57%), diatoms (0.02%) and euglenoids (0.02%). Phytoplankton community composition in EL samples from September 2020 contained 6.50% cyanobacteria, represented entirely by *Aphanocapsa*. Cyanobacteria cell counts ranged from 0-1,646 cells/mL. September EL samples were dominated by green algae (68.42%), followed by diatoms (21.03%), euglenoids (2.45%), and golden algae (1.60%). No cyanobacteria taxa were observed in LF lake samples during September 2020 sampling. Phytoplankton community composition included green algae (73.43%) and diatoms (26.57%).

Cyanobacteria accounted for 53.68% of the phytoplankton density within DT lake samples from March 2021, represented by *Aphanocapsa* (51.67% of total phytoplankton community) and *Raphidiopsis* (2.01%). Total cyanobacteria cell density at DT sites in March 2021 were a maximum of 13,354 cells/mL and a minimum of 8,178 cells/mL for this month. Golden algae (28.09%), diatoms (10.20%), green algae (5.52%), and euglenoids (2.51%) accounted for the remainder of the phytoplankton density. March EL samples did not contain any

cyanobacteria taxa, and included mostly diatoms (53.66%). Green algae accounted for 39.02% of the phytoplankton density, and golden algae accounted for the remaining 7.32%. No cyanobacteria were present in March 2021 LF samples, which were composed mostly of synurophytes (85.48%). Green algae accounted for 8.47% of the samples, and diatoms were responsible for the remaining 6.05% of the samples.

DT lake samples from April 2021 contained 69.66% cyanobacteria, reaching a maximum of maximum of 16,149 cells/mL and a minimum of 12,629 cells/mL. Represented cyanobacteria taxa included *Aphanocapsa* (63.13% of total phytoplankton community), *Aphanothece* (6.04%), and *Raphidiopsis* (0.49%). Other phytoplankton groups found in samples were represented by green algae (12.23%), diatoms (8.32%), synurophytes (6.36%), golden algae (1.96%), and euglenoids (1.47%). April samples collected from EL were dominated by cyanobacteria (82.04%), reaching a maximum cell count of 16,977 cells/mL and a minimum of 8,695 cells/mL. *Aphanocapsa* (80.75% of total phytoplankton density) and *Chroococcus* (1.29%) were the cyanobacteria taxa identified. Other phytoplankton groups represented included diatoms (8.33%), green algae (4.47%), synurophytes (2.50%), golden algae (1.33%), and euglenoids (1.33%). No cyanobacteria were present in April 2021 LF samples, which contained mostly green algae (66.67%). Diatoms accounted for the remainder of the samples (33.33%).

Measurable saxitoxins varied across lake sites ($P=0.014$). Overall, this cyanotoxin was detected at both DT and EL lakes each month sampled, but was largely absent at LF, with toxins measured above the minimum detection limit occurring only in April 2021 at two out of three LF lake sites. At no point of sampling were saxitoxins measured above the NJDEP mandated threshold for recreational waters (0.6 µg/L). Saxitoxins were greatest at DT in August 2020, with values ranging from 0.115-0.151 µg/L. Within that sampling period, EL samples reported values

ranging from 0.025-0.091 µg/L. Measurable saxitoxins at DT and EL during September 2020 sampling efforts were similar, with a range of ND-0.064 µg/L within DT samples, and ND-0.048 µg/L within EL samples. Maximum saxitoxin values were greater at EL sites during both March 2021 and April 2021 sampling efforts. March samples displayed a range of 0.0264-0.0319 µg/L at EL sites, ND-0.023 µg/L at DT sites, and April samples contained a range of measurable saxitoxins from 0.0418-0.0891 µg/L at EL sites, and 0.0363-0.0473 µg/L at DT sites.

Measurable microcystins did not vary significantly across lake sites ($P=0.5439$). Microcystins were detected in all 3 DT lake sites during August 2020 sampling, and the maximum value measured (3.412 µg/L) exceeded the NJDEP established threshold for freshwater lake recreation (2 µg/L). Microcystins below the NJDEP threshold were measured at one DT site during September 2020 sampling (0.348 µg/L). No microcystins were detected at DT in March 2021 or April 2021, and microcystins were not detected at any EL or LF sites throughout all four sampling months.

Table 1: Phytoplankton density (cells/mL), cyanobacteria density (cells/mL), saxitoxins (µg/L), and microcystins (µg/L) measured in water samples collected from Daretown Lake, Elmer Lake, and Lake Fred during August 2020, September 2020, March 2021, and April 2021. ‘ND’ refers to values in which cyanotoxins were not detected, and ‘N/A’ is used for median values when only one site contained a measurable toxin

	Daretown Lake			Elmer Lake			Lake Fred		
	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>
<u>Aug. 2020</u>									
Phytoplankton	130555-186144	163722 ± 29311	174467	6708-11863	9410 ± 2586	9658	3416-4751	4151 ± 678	4286
Cyanobacteria	126860-182976	160089 ± 29453	170430	0-3478	1884 ± 1757	2174	0-2267	756 ± 1309	0
Saxitoxins	0.130-0.151	0.132 ± 0.018	0.130	0.025-0.091	0.056 ± 0.033	0.052	ND	ND	ND
Microcystins	0.331-3.412	1.449 ± 1.705	0.604	ND	ND	ND	ND	ND	ND
<u>Sept. 2020</u>									
Phytoplankton	484396-867397	21152 ± 5029	23188	1956-8447	15597 ± 4715	13354	1025-4503	1242 ± 621	1242
Cyanobacteria	479458-864726	14734 ± 1859	15424	0-1646	12802 ± 4141	12733	0	0 ± 0	0
Saxitoxins	ND-0.064	0.040 ± 0.027	0.044	ND-0.048	0.028 ± 0.019	0.025	ND	ND	ND
Microcystins	ND-0.348	0.216 ± 0.114	N/A	ND	ND	ND	ND	ND	ND
<u>Mar. 2021</u>									
Phytoplankton	16977-24016	24022 ± 9013	20910	2795-6211	4244 ± 1767	3727	1242-14906	8557 ± 6883	9524
Cyanobacteria	8178-13354	21738 ± 10480	11697	0-6211	4244 ± 1766	0	0	0 ± 0	0
Saxitoxins	ND-0.023	0.015 ± 0.006	N/A	0.026-0.031	0.029 ± 0.003	0.031	ND	ND	ND
Microcystins	ND	ND	ND	ND	ND	ND	ND	ND	ND
<u>April 2021</u>									
Phytoplankton	15424-24844	21152 ± 5029	23188	12422-21014	15597 ± 4715	13354	621-1863	1242 ± 621	1242
Cyanobacteria	12629-16149	14734 ± 1859	15424	8695-16977	12802 ± 4141	12733	0	0 ± 0	0
Saxitoxins	0.036-0.047	0.043 ± 0.006	0.062	0.042-0.089	0.064 ± 0.024	0.062	ND-0.039	0.025 ± 0.014	N/A
Microcystins	ND	ND	ND	ND	ND	ND	ND	ND	ND

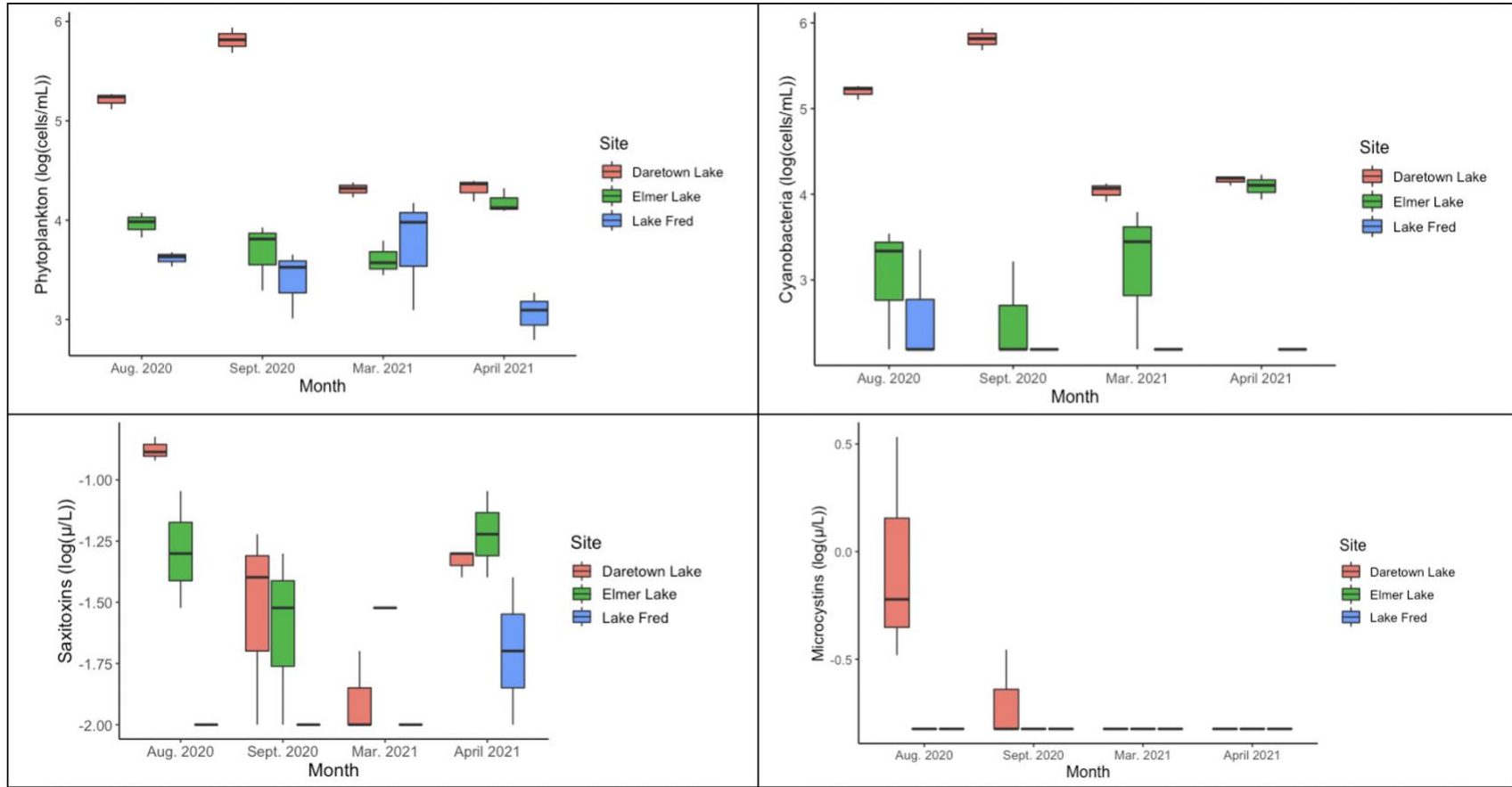


Figure 2: Boxplots showing phytoplankton density (cells/mL), cyanobacteria density (cells/mL), saxitoxins ($\mu\text{g/L}$), and microcystins ($\mu\text{g/L}$) measured in water samples collected from Daretown Lake, Elmer Lake, and Lake Fred during August 2020, September 2020, March 2021, and April 2021, plotted on a logarithmic scale

Table 2: Results of the linear mixed effects model via Satterthwaite's degrees of freedom method, comparing cyanobacteria density (cells/mL), phytoplankton density (cells/mL), saxitoxins (ug/L), and microcystins (ug/L) in water samples across lake sites, accounting for variation as a result of month sampled

	F value	P value
Cyanobacteria	53.112	0.00231*
Phytoplankton	7.5267	0.06779
Saxitoxins	15.54	0.0138*
Microcystins	0.7133	0.5439

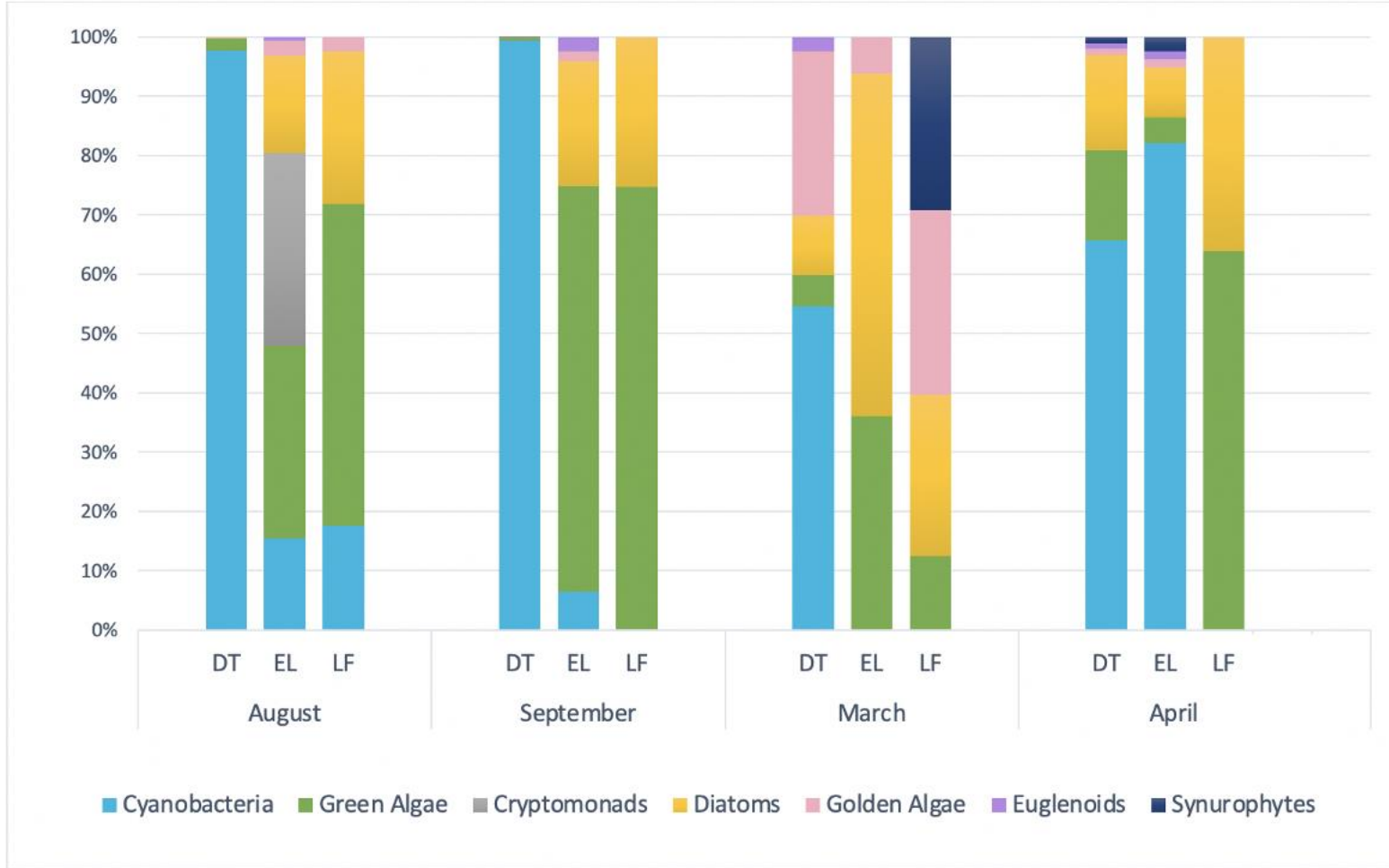


Figure 3: Phytoplankton community composition in water samples collected from Daretown Lake (DL), Elmer Lake (EL), and Lake Fred (LF). Relative abundance of cyanobacteria, green algae, cryptomonads, diatoms, golden algae, euglenoids, and Synurophyta are displayed for each lake during August 2020, September 2020, March 2021, and April 2021

3.2 Phytoplankton Community and Cyanotoxins in Turtle Epizoic Community

In total, 32 turtles were captured and examined (**table 3**). Healthy individuals showed relatively clean, evenly shaped carapaces and smooth, pink to dark red plastrons. In general, individuals affected with shell disease showed mishappen carapaces with visible epizoic algae and/or cyanobacterial growth. Lesions on affected turtles were mainly confined to the plastron area of the turtles and were characterized by dark, foul-smelling pits of necrotized tissue. In some affected individuals, the keratinized plates of the plastron were compromised enough that underlying bone was visible (**figure 4**). Of the turtles captured at DT, 1 out of 6 (17%) were affected by shell disease, and 7 of 16 (44%) turtles captured at EL were affected by shell disease as well. Of the 10 turtles caught at LF, no individuals showed any signs of being affected by shell disease.

Table 3: The numbers of Northern red-bellied turtles trapped at Daretown Lake, Elmer Lake and Lake Fred and the numbers of diseased turtles recorded

	Total	Diseased
Daretown Lake	6	1
Elmer Lake	16	7
Lake Fred	10	0

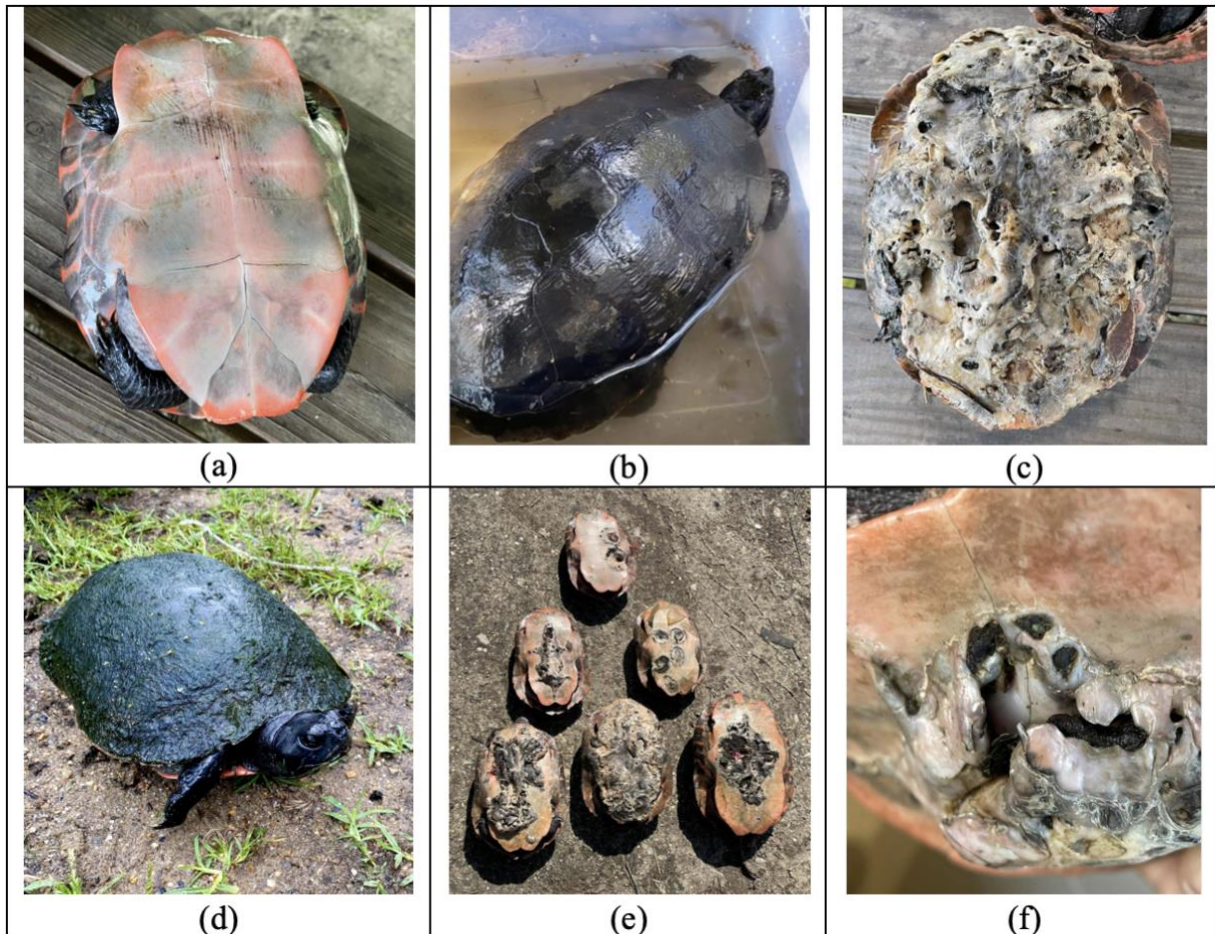


Figure 4: Healthy Northern red-bellied turtles and Northern red-bellied turtles affected by shell disease. (a): a healthy turtle's unblemished plastron, showing the distinctive red belly the species is known for; (b): carapace of a healthy turtle; (c): individual with shell disease, showing a plastron covered in lesions and necrotized tissue; (d) many individuals with lesions had visible algae and/or cyanobacteria growth on their carapace; (e): six individuals with shell disease, trapped during Spring 2021 sampling at Elmer Lake; (f) underlying bone is visible in some deeply necrotized lesions

3.2.1 Comparison between individuals based on lake of capture

Turtles sampled from EL showed the greatest density of total epizoic community growth on their shells, with a mean value of 668,124 cells/cm² (**table 4**). DT turtles had the next greatest density, with a mean value of 756,650 cells/cm², while turtles sampled at LF showed the lowest mean value of 60,654 cells/cm². Significant differences in total epizoic community densities were detected between turtles collected across all 3 sites ($P=0.002$) (**table 5**). Pairwise

comparisons revealed that DT and EL were not significantly different from one another in terms of total epizoic community density ($P=0.182$). However, densities measured from the study sites with shell disease (DT and EL) were both significantly greater than those measured from the control site without shell disease (LF) ($P < 0.011$ for DT & LF, $P=0.011$ for EL & LF).

A similar pattern was observed in epizoic cyanobacteria measurements across lake sites. Epizoic cyanobacteria densities varied between turtles collected across lakes ($P < 0.001$), and pairwise comparisons revealed that while values enumerated from DT and EL turtles were not different from one another ($P=0.495$), epizoic cyanobacteria measured from turtles sampled at study sites in which shell disease was present were significantly greater than those measured from the control site in which shell disease was not present ($P < 0.011$ for DT & LF, $P < 0.001$ for EL & LF). The highest density of epizoic cyanobacteria was measured in turtles collected from EL ($M=653,282$ cells/cm²), the second highest at DT ($M=390,707$ cells/cm²), and no cyanobacteria was identified in epizoic samples from LF turtles.

Saxitoxins measured from epizoic samples were highest in samples collected from DT turtles ($M=2.067$ µg/cm²), with a maximum value of 11.528 µg/cm². EL turtles showed the next highest saxitoxins measured within epizoic samples ($M=0.343$ µg/cm²), and LF turtles contained the least amount of saxitoxins within their samples, with only one out of ten epizoic samples detecting saxitoxins (0.167 µg/cm²). Statistical analysis showed the same pattern is once again present in saxitoxins measured from epizoic community samples; significant differences exist between the 3 lake sites ($P < 0.003$), with a greater amount of saxitoxins measured at lake sites positive for shell disease ($P=0.018$ for DT & LF; $P=0.001$ for EL & LF). Of the 16 turtles with measurable saxitoxins detected within their epizoic community samples, 15 of them were

captured from lakes positive for the presence of shell disease. DT and El were not significantly different from one another in terms of epizoic saxitoxins ($P=0.802$).

Table 4: Total epizoic community density (cells/cm²), cyanobacterial density (cells/cm²), saxitoxins (µg/cm²), and microcystins (µg/cm²) measured in epizoic community samples collected from turtle shells belonging to individuals trapped at Daretown Lake and Elmer Lake. ‘ND’ refers to values in which cyanotoxins were not detected, and ‘N/A’ is used for median values when only one site contained a measurable toxin

	Daretown Lake			Elmer Lake			Lake Fred		
	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>
Total community	179522-1533780	668124± 627407	319828	3486-3093705	756650 ± 994165	174293	1743-348586	60654 ± 104891	27016
Cyanobacteria	102833-1098047	390707 ± 388549	216124	ND-2954270	653282 ± 951031	156864	ND	ND	ND
Saxitoxins	ND-11.528	2.067 ± 4.636	0.222	ND-1.426	0.343 ± 0.365	0.238	ND-0.167	0.072 ± 0.365	N/A
Microcystins	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 5: *H* and *P* values of the one-way Analysis of Variance on ranks (Kruskal–Wallis test) carried out for total epizoic community density (cells/cm²), cyanobacteria density (cells/cm²), and saxitoxins (µg/cm²) measured in turtle epizoic community samples from Daretown Lake (DL), Elmer Lake (EL), and Lake Fred (LF). Pairwise comparisons of the three lakes for each variable are also given (Dunn’s method). Significant differences (*P* > 0.05) are indicated by bold asterisk (*)

	<i>H</i> value	<i>P</i> value	Pairwise comparison	<i>P</i> value	Relationship between groups
Total community	12.099	0.002*	DT – EL	0.182	DT > LF; EL > LF; DT = EL
			DT – LF	< 0.001*	
			EL – LF	0.011*	
Cyanobacteria	18.836	< 0.001 *	DT – EL	0.495	DT > LF; EL > LF; DT = EL
			DT – LF	< 0.001*	
			EL – LF	< 0.001*	
Saxitoxins	11.753	0.003 *	DT – EL	0.802	DT > LF; EL > LF; DT = EL
			DT – LF	0.018*	
			EL – LF	0.001*	

Epizoic community composition in turtle samples did not seem to vary much seasonally but rather vary by site of collection. Overall, epizoic community samples from turtles collected from EL were dominated by cyanobacteria (90.22%) (**figure 5**), the highest relative abundance of cyanobacteria in samples across all three sites. 5 cyanobacteria taxa were identified, including *Chroococcus*, which accounted for 43.25% of the total epizoic community, *Lyngbya* (35.46%), *Aphanocapsa* (8.52%), *Spirulina* (2.85%), and *Merismopedia* (0.14%) (**table 6**). 7.20% of the epizoic community in EL turtles were composed of diatoms, represented by *Aulacoseira* (2.66%), *Navicula* (1.64%), *Eunotia* (0.86%), *Cymbella* (0.32%), *Nitzschia* (0.27%), *Ulnaria* (0.23%), *Pinnularia* (0.13%), *Tabellaria* (0.07%), *Fragillaria* (0.06%), *Gomphonema* (0.01%), *Cyclotella* (0.01%), and *Cymatopleura* (0.01%). 0.91% of diatoms within epizoic community samples collected from EL turtles were unable to be identified. The remaining 2.58% of the epizoic community was accounted for by green algae: *Basycladia* (2.40%), and *Rhizoclonium* (0.17%).

Turtles collected from DT showed the next highest relative abundance of cyanobacteria within epizoic community samples collected (58.48%), with represented taxa were identified as *Chroococcus*, which accounted for 35.04% of the total epizoic community, and *Lyngbya* (23.43%). 33.48% of epizoic community samples from DT turtles were accounted for by diatoms, which included *Navicula* (13.65%), *Eunotia* (2.43%), *Ulnaria* (0.87%), *Nitzschia* (0.48%), *Cymbella* (0.39%), *Gomphonema* (0.17%), *Cyclotella* (0.09%), *Fragillaria* (0.09%), and *Asterionella* (0.04%). 15.26% of diatoms within epizoic community samples collected from DT turtles were unable to be identified. Green algae accounted for the remaining 8.04% of epizoic community samples: *Basycladia* (7.48%), and *Rhizoclonium* (0.57%).

No cyanobacteria taxa were identified in epizoic community samples from turtles collected from LF. Instead, the community was dominated by diatoms (87.36%) of the genus *Fragillaria* (39.08%), *Eunotia* (12.64%), *Asterionella* (7.76%), *Ulnaria* (6.90%), *Tabellaria* (4.89%), *Aulacoseira* (4.31%), *Cymbella* (2.87%), *Navicula* (2.01%), *Pinnularia* (0.57%), and *Placoneis* (0.57%). 5.75% of diatoms were unable to be identified to genus level. Green algae made up the remaining 12.64% of the phytoplankton community, with *Bacillaria* (5.46%) and *Spirogyra* (7.18%) represented.

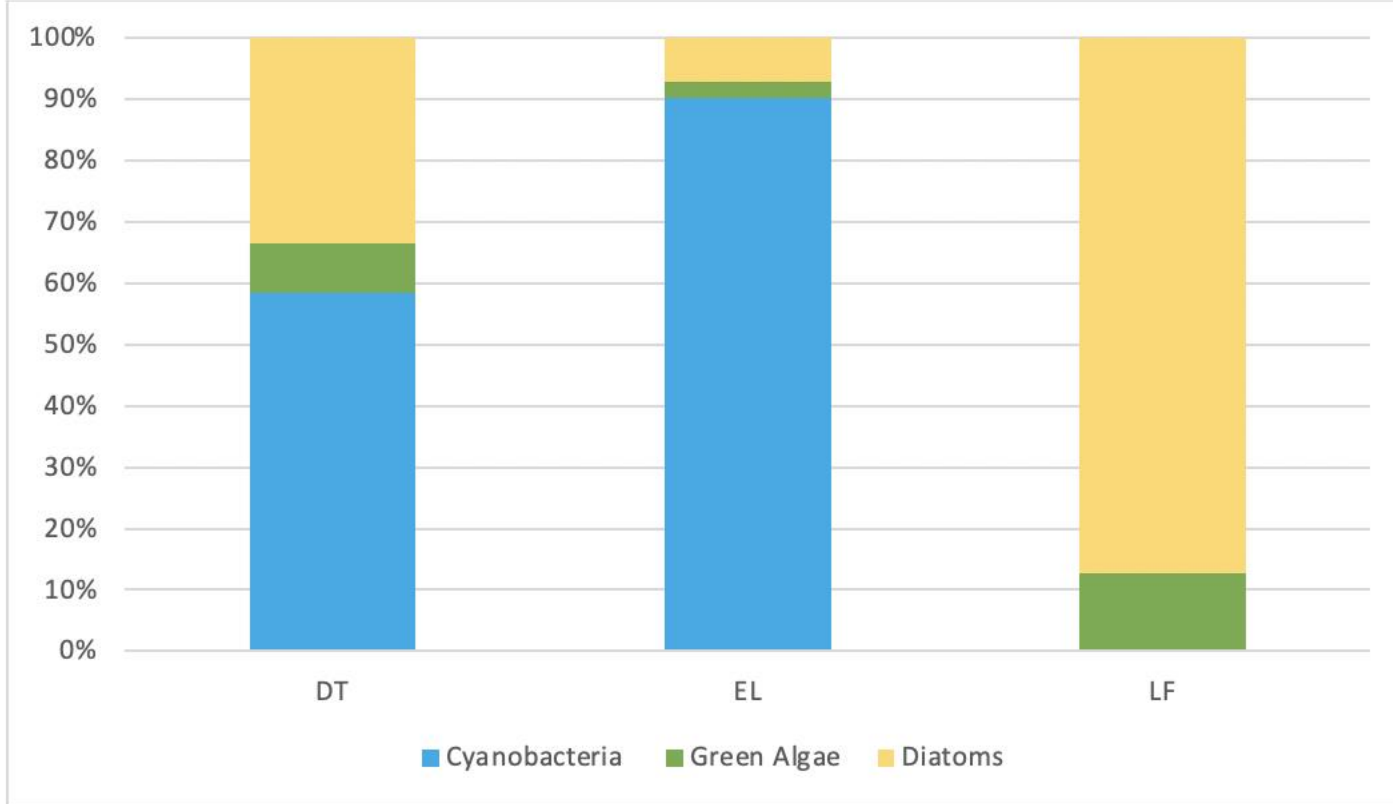


Figure 5: Relative abundance of cyanobacteria, green algae, and diatoms are displayed in epizoic community samples collected from turtles trapped at Daretown Lake (DL), Elmer Lake (EL), and Lake Fred (LF)

Table 6: Relative abundance of genera represented in epizoic community samples collected from turtles trapped at Elmer Lake Daretown Lake, and Lake Fred

<u>Elmer Lake</u>			<u>Daretown Lake</u>			<u>Lake Fred</u>		
Taxa	Group	%	Taxa	Group	%	Taxa	Group	%
<i>Chroococcus</i>	Cyanobacteria	43.25%	<i>Chroococcus</i>	Cyanobacteria	35.04%	<i>Basycladia</i>	Green Algae	336.78%
<i>Lyngbya</i>	Cyanobacteria	35.46%	<i>Lyngbya</i>	Cyanobacteria	23.43%	<i>Spirogyra</i>	Green Algae	0.00%
<i>Aphanocapsa</i>	Cyanobacteria	8.52%	<i>Basycladia</i>	Green Algae	7.48%	<i>Navicula</i>	Diatom	115.52%
<i>Merismopedia</i>	Cyanobacteria	0.14%	<i>Rhizoclonium</i>	Green Algae	0.57%	<i>Eunotia</i>	Diatom	0.00%
<i>Spirulina</i>	Cyanobacteria	2.85%	<i>Navicula</i>	Diatom	13.65%	<i>Cymbella</i>	Diatom	0.00%
<i>Basycladia</i>	Green Algae	2.40%	<i>Eunotia</i>	Diatom	2.43%	<i>Ulnaria</i>	Diatom	0.00%
<i>Rhizoclonium</i>	Green Algae	0.17%	<i>Cymbella</i>	Diatom	0.39%	<i>Fragillaria</i>	Diatom	0.00%
<i>Navicula</i>	Diatom	1.64%	<i>Ulnaria</i>	Diatom	0.87%	<i>Asterionella</i>	Diatom	0.00%
<i>Eunotia</i>	Diatom	0.86%	<i>Fragillaria</i>	Diatom	0.09%	<i>Aulacoseira</i>	Diatom	0.00%
<i>Cymbella</i>	Diatom	0.32%	<i>Asterionella</i>	Diatom	0.04%	<i>Pinnularia</i>	Diatom	0.00%
<i>Ulnaria</i>	Diatom	0.23%	<i>Nitzchia</i>	Diatom	0.48%	<i>Tabellaria</i>	Diatom	0.00%
<i>Fragillaria</i>	Diatom	0.06%	<i>Gomphonema</i>	Diatom	0.17%	<i>Placoneis</i>	Diatom	0.00%
<i>Aulacoseira</i>	Diatom	2.66%	<i>Cyclotella</i>	Diatom	0.09%	Unknown diatoms	Diatom	0.00%
<i>Pinnularia</i>	Diatom	0.13%	Unknown diatoms	Diatom	15.26%			
<i>Tabellaria</i>	Diatom	0.07%						
<i>Placoneis</i>	Diatom	0.00%						
<i>Nitzchia</i>	Diatom	0.27%						
<i>Gomphonema</i>	Diatom	0.01%						
<i>Cyclotella</i>	Diatom	0.01%						
<i>Cymatopleura</i>	Diatom	0.01%						
<i>Unknown diatoms</i>	Diatom	0.91%						

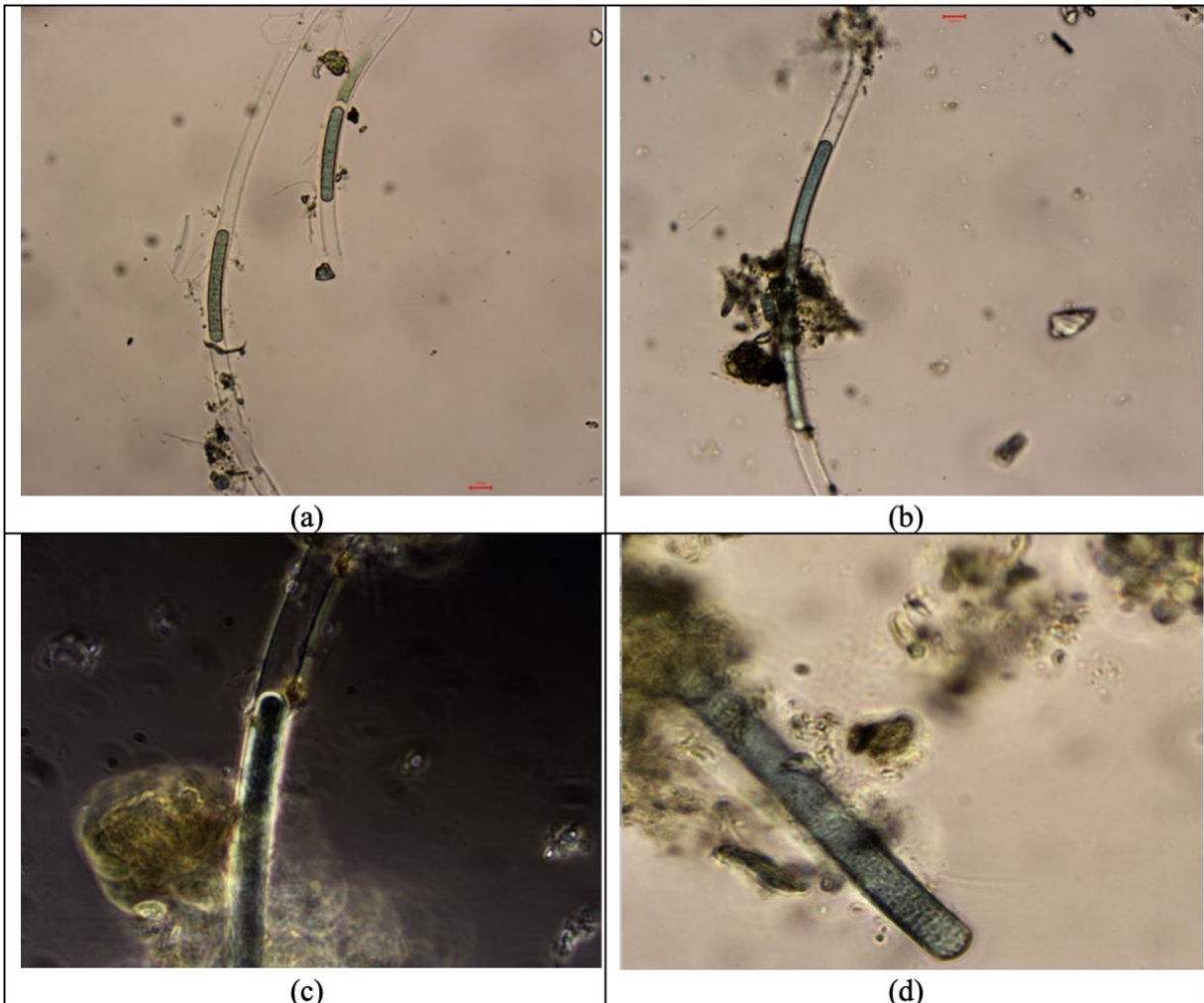


Figure 6: *Lyngbya* spp. found within the epizoic community in samples collected from turtle shells belonging to individuals trapped at Daretown Lake and Elmer Lake; (a & b): 400x, scale bar set to 10 μ m. (c) phase contrast image at 400x; (d) image under oil immersion at 1000x

3.2.2 Comparison between healthy individuals and individuals with shell disease

In general, total epizoic community density within diseased turtles ($M=535,734$ cells/cm²) (**table 7**) was marginally greater than that of healthy turtles ($M=518,159$ cells/cm²), though statistical testing did not determine significant differences between the two groups ($P=0.142$) (**table 8**). However, epizoic community samples collected from turtles with shell disease contained significantly greater cyanobacteria density ($M=412,567$ cells/cm²) when

compared to samples collected from healthy turtles ($M=361,876$ cells/cm²) ($P=0.031$). Despite this, relative abundancies of epizoic community did not differ greatly between healthy turtles (79.62% cyanobacteria, 16.43% diatoms, and 3.95% green algae) and turtles with shell disease (78.53% cyanobacteria, 16.35% diatoms, and 5.12 % green algae). No differences in saxitoxins were measured in epizoic community samples between healthy and diseased turtles ($P=0.158$), and no detectible microcystins were measured in any epizoic community samples.

Table 7: Total epizoic community density (cells/cm²), cyanobacterial density (cells/cm²), and saxitoxins (µg/cm²) measured in epizoic community samples collected from healthy turtles and turtles with shell disease, independent of collection site

	<u>Daretown Lake</u>			<u>Elmer Lake</u>			<u>Lake Fred</u>		
	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>	<i>RANGE</i>	<i>MEAN ± SD</i>	<i>MEDIAN</i>
Total community	179522-1533780	668124± 627407	319828	3486-3093705	756650 ± 994165	174293	1743-348586	60654 ± 104891	27016
Cyanobacteria	102833-1098047	390707 ± 388549	216124	ND-2954270	653282 ± 951031	156864	ND	ND	ND
Saxitoxins	ND-11.528	2.067 ± 4.636	0.222	ND-1.426	0.343 ± 0.365	0.238	ND-0.167	0.072 ± 0.365	N/A
Microcystins	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 8: Mann-Whitney U Test comparing epizoic community samples between healthy turtles and turtles with shell disease, independent of collection site, in terms of total community density (cells/cm²), cyanobacterial density (cells/cm²), and saxitoxins (µg/cm²). Significant differences ($P > 0.05$) are indicated by bold asterisk (*)

	U value	P value	Comparison between groups
Total community	59	0.113	Healthy turtles = diseased turtles
Cyanobacteria	46.5	0.029*	Diseased turtles > healthy turtles
Saxitoxins	110	0.158	Healthy turtles = diseased turtles

4. Discussion

4.1 Cyanobacteria as an Indication of Water Quality Degradation

Studying phytoplankton community composition in water samples may be a useful indicator of pollution within the ecosystem (Ziglar and Anderson, 2005; Akgül et al. 2014) as different phytoplankton have different pollution tolerances. Since phytoplankton taxa vary in their tolerance or resistance to pollution, selection pressure may result in less tolerant organisms being replaced by more resistant or tolerant ones (Bérard and Benninghoff, 2001).

Environmental conditions that may favor the growth of toxin producing cyanobacteria can severely impact aquatic organisms (Gorham, 1964; McCormick and Cairns, 1994) and so the presence of such taxa may be an early indication of water quality degradation. Many cyanobacteria taxa have the ability to survive polluted waters, specifically water with an excess of phosphorus or nitrogen (Smith, 2003; World Health Organization, 2020, 2021; United States Environmental Protection Agency, 2021) suggesting that the presence of cyanobacteria may indicate an impaired or stressed ecosystem for aquatic biota. Both DT and EL showed cyanobacteria as a dominant phytoplankton group during sampling events, with DT most affected. LF water samples rarely showed any presence of cyanobacteria within the phytoplankton community. This may indicate that DT is the most polluted system of the three lakes studied, with EL following behind, and LF may be the least polluted system.

4.2 HCB Conditions and Vulnerability of Organisms to Disease

Exposure to saxitoxins may render some organisms more vulnerable to degraded immune defenses, and both DT and EL water samples contained measurable saxitoxins each month sampled. Though there is currently no literature suggesting what threshold might cause an

immune response in freshwater turtles, in mollusks, saxitoxins have been demonstrated to affect cellular immunity in other aquatic organisms (Lassudrie et al., 2020; Abi-Khalil et al., 2017; Mello et al., 2013). One study showed that incubation of oyster hemocytes in combination with 2×10^4 cells/mL of the dinoflagellate *Alexandrium minutum* showed increases in transcription of the cytokine *Interleukin-17*, which is typically released upon introduction of a pathogen; when hemocytes were incubated with 0.05µg/L of *A. minutum*'s purified saxitoxin product, transcription levels of this cytokine dropped significantly (Mello et al., 2013). Hemocytes incubated with purified saxitoxin product also showed a significant decrease in cytochrome P450, an enzyme involved in the clearance of various xenobiotic substances, such as environmental pollutants, from the body (Mello et al., 2013). This suggests that cyanotoxins may increase the vulnerability of organisms to environmental stressors.

Within an aquatic community, environmental stressors may increase organisms' susceptibility to diseases. A 2021 study that investigated stress levels and immune function in reptiles residing in HCB impaired wetlands demonstrated that painted turtles exposed to HCB and eutrophic conditions had lowered immune function when compared to individuals in control populations (Refsnider et al., 2021). This study suggests that even if HCB conditions do not directly cause the mortality of the animal, these conditions can significantly impair the organism's defense against introduced pathogens (Refsnider et al., 2021). Johnson and Chase (2004) documented that eutrophication resulted in increased occurrences of amphibian malformations caused by the parasite *Ribeiroia ondatrae*. In their study, results indicated that eutrophic conditions allow snail hosts to outcompete other aquatic organisms with lower pollution tolerances, therefore increasing parasite load (Johnson and Chase, 2004).

Of the 3 lakes, DT showed the most cyanobacteria taxa each month sampled, with as many as 6 different taxa present. The taxa found at DT are capable of producing a diverse array of toxins. Various *Dolichospermum* spp. are capable of producing microcystins, anatoxins, saxitoxins, and cylindrospermopsin (Clercín, 2012; United States Environmental Protection Agency, 2020; New Jersey Department of Environmental Protection, 2021). Some *Microcystis* spp. are capable of producing microcystins and anatoxins; *Aphanizomenon* spp. may produce anatoxins, saxitoxins, and cylindrospermopsin; *Planktothrix* spp. may produce microcystins, anatoxins, and saxitoxins; *Snowella* spp. may produce microcystins, and some *Oscillatoria* spp. may produce microcystins and anatoxins (Clercín, 2012; Bukowska, 2017; United States Environmental Protection Agency, 2020; New Jersey Department of Environmental Protection, 2021). *Aphanocapsa* was also observed in DT water samples and, as mentioned previously, some members of this taxa are capable of producing microcystins (Clercín, 2012; Graham 2020). All of these taxa were found in DT water samples at points throughout the four months of sampling, and these taxa accounted for a large percentage of the total phytoplankton community in DT water samples.

EL water samples also had cyanobacteria taxa present, though in far less quantities and with less diversity; *Microcystis* and *Aphanocapsa* were found in samples collected from August 2020, and *Aphanocapsa* was found again in April 2021 water samples, with a high enough cell count to represent 80.75% of the entire phytoplankton composition in EL water samples that month. Despite the presence of these HCB taxa, cell counts did not exceed the 20,000 cell/mL threshold at any sampling event. No HCB taxa were identified in LF samples through four months of sampling, and cyanobacteria in general was largely absent, suggesting that LF did not experience HCB conditions at all through sampling efforts connected to this study. Though DT

showed the strongest indication of being impaired by a HCB, with cyanobacteria cell counts exceeding 20,000 cells/mL during two out of four sampling efforts, more diseased individuals were documented at Elmer Lake. This may have attributed to the study's small sample size; turtle trapping successes were greater at both EL and LF than at DT, and continued sampling of both *P. rubriventris*, phytoplankton composition, and cyanotoxins in water samples is necessary to understand the relationship between HCBs and shell disease.

As climate change progresses, eutrophication may continue to increase, thereby potentially increasing instances of HCBs. Climate models are showing that the Northwest Atlantic Ocean and continental shelf, the area encompassing the United States eastern coastline in which the Labrador Current and the Gulf Stream meet, is warming at a rate nearly three times as faster than the global average as a result of climate change (Saba et al., 2015; Neto et al., 2021). The New Jersey Scientific Report on Climate Change states that New Jersey in particular is warming faster than other states, and annual precipitation is expected to increase between 4% and 11% by 2050 (New Jersey Department of Environmental Protection, 2020). This may provide greater opportunity for increased nutrient runoff into New Jersey waterbodies, potentially increasing instances of eutrophication, animal disease, and public health concerns related to degraded water quality and toxic blooms. As the impacts of climate change continue to enhance environmental stressors that may lead to HCBs, continuous monitoring of areas showing signs of HCBs is essential to human, animal, and environmental health.

4.3 Turtle Epizoic Community

Studying epizoic communities in *P. rubriventris* may provide insight as to the health of the turtle, as the density of epizoic algal and cyanobacterial growth has been observed to be inversely related to the motility of the animal (Totti et al., 2011). Long term activity of sampled

turtles was not monitored in this study, though the general presence of greater epizoic algae and cyanobacteria density on turtles captured at DT and EL may suggest a higher degree of lethargy, which could be related to advanced infection with shell disease. Previous research regarding the impact of epizoic community has also shown that phytoplankton covering the carapace of European pond turtles (*Emys orbicularis*) may negatively affect the body condition and reproductive capabilities of otherwise healthy breeding females; Beau and Brischoux (2021) determined that females with algal cover had reduced body mass overall when compared to individuals with no algal cover, and a larger percentage of females without visible algal growth (19.72%) were reproductively successful compared to those with visible algal growth on their shells (13.69%). Continued monitoring of red-bellied populations at all 3 lake sites could provide insight on whether or not epizoic community densities may be related to reproductive success and overall body condition.

Research comparing epizoic community density and structure between healthy and diseased freshwater turtles is generally lacking; within this study, the confliction that exists between the significant difference detected in median epizoic cyanobacteria density between healthy and diseased turtles despite similar relative abundance values of cyanobacteria is likely explained by large variation within a relatively small data set, rendering the median as a poor representation of the population. More sampling of turtles is needed to reduce sampling error and provide greater clarity on the relationship between epizoic cyanobacteria and turtles.

Epizoic communities are cited as often overlooked in favor of other methods of water sampling to determine ecosystem health (Smith, 1996; Ziglar and Anderson, 2005; Akgül et al., 2014). Benthic communities may be less likely to fluctuate with changing environmental conditions such as nutrient availability; studies have found that benthic algae are nutritionally

replete, likely due to close access to sediment nutrients, whereas phytoplankton communities in the water column fluctuate based on nutrient availability (Bonilla et al., 2005; Loughheed et al., 2015; Steinman et al., 2016). Thus, benthic communities may remain more stable seasonally, and thus represent a more accurate community composition of phytoplankton within a lake when compared to planktonic algae. The results of this study suggest that epizoic community can potentially serve as a proxy for the phytoplankton community composition within an aquatic ecosystem, as phytoplankton density and community composition in water samples are likely to fluctuate rapidly but epizoic community seemed to stay relatively stable from month to month.

4.4 Cyanotoxins in Epizoic Community Samples

Of the cyanobacteria collected from DT and EL turtle epizoic community samples, *Lyngbya* accounted for 40.46% of the cyanobacteria on EL turtles, and 25.00% of the cyanobacteria on DT turtles. In addition to the lipopolysaccharide endotoxins common to all Gram-negative bacteria, *Lyngbya* is a freshwater cyanobacteria genus capable of producing such toxins as lyngbyatoxin-a, aplysiatoxins, and saxitoxins. (Graham, 2020; Wiese et al., 2010; New Jersey Department of Environmental Protection, 2021; United States Environmental Protection Agency, 2021); its abundance in epizoic community samples found at the two study sites may be the origins of the saxitoxins measured in epizoic community samples. *Aphanocapsa* is another cyanobacteria genus capable of toxin production that was seen in samples collected from turtles trapped at EL; some species of this genus are capable of producing microcystins (Clerc, 2012; Graham 2020). Though no microcystins were measured in epizoic community samples through the course of this study, this does not rule out the possibility that turtles at DT and EL may be exposed to microcystins nonetheless, given the presence of microcystin producing species both growing on their shells, and present within their habitat.

Epizoic distributions of cyanobacteria in freshwater turtles are relatively understudied. Only a limited number of studies documented the presence of epizoic cyanobacteria on turtles. The cyanobacteria species *Plectonema tenue* has been reported to grow on the common snapping turtle (*Chelydra serpentina*), and the cyanobacteria *Entophysalis rivularis* was reported to grow on both common snapping turtles and musk turtles (*Sternotherus odoratus*) (Edgreen et al., 1953). A 2011 study described the filamentous cyanobacteria *Komvophoron* as forming widespread colonies on the legs of Blanding's turtles sampled in Nova Scotia, and suggested this may represent an unknown symbiotic relationship between the turtle and the cyanobacteria (Garbury et al. 2007). Cyanobacteria of the genus *Lyngbya* was previously reported to occur epizoically in turtles, in addition to *Oscillatoria* spp. and *Trichodesmium* spp. (Ernst and Barbour, 1972). A 2020 study which aimed to record the complete external microbiome of shells and other outward body surfaces of freshwater turtles reported that cyanobacteria was common on external surfaces of Kreft's River Turtles (*Emydura macquarii krefftii*), a freshwater species found in Queensland, Australia (McKnight et al., 2020). However, in this study only seemingly healthy turtle individuals were examined, and cyanobacteria taxa were described simply as cyanobacteria without further identification to the lower taxonomic level, so the toxin producing potential of those cyanobacteria observed remains unknown.

At the time of this study, no current publications discuss cyanotoxin levels measured from epizoic community collected from turtle shells; research of this nature instead seems to focus on accumulation of cyanotoxins within tissues. Studying toxin levels in epizoic communities collected from turtle shells offers a non-invasive approach for assessing the vulnerability of freshwater turtles to cyanotoxins, as it does not require sacrificing the animal to obtain samples. Presently, there is no established lethal dose (LD₅₀) of cyanotoxins for

freshwater biota including turtles although the LD₅₀ of cyanotoxins is dependent on such variables as species, age, and body size (Suarez-Isla, 2015). Published literature on this subject seems to largely focus on sea turtle toxicity as a result of Paralytic Shellfish Poisoning (PSP) which occurs as a result of saxitoxin production via marine dinoflagellates (Ley-Quiñónez et al., 2020; Barrientos et al., 2019, Vilariño et al., 2018). Following a juvenile stage as omnivores, adult *P. rubriventris* individuals are herbivorous (Pearson, 2013; Virginia Herpetological Society, 2020); as such, they are primary consumers that feed directly on plankton and thus perhaps disproportionately susceptible to the negative effects of cyanotoxins (Ley-Quiñónez et al., 2020).

Research about how saxitoxins specifically affect the immune response in chelonians and other reptiles is currently lacking; it is still largely unclear how saxitoxins affect freshwater turtles in general, and more research is needed. *P. rubriventris* have demonstrated high nest-site fidelity (Swarth, 2004), and many turtle species in general are known to have high site fidelity (Gibbons et al., 2001; Bangma et al., 2019; Hauswaldt and Glenn, 2005). This is worth noting, as if toxic cyanobacteria are present within the habitat of *P. rubriventris* habitat, the turtles may be unlikely to remove themselves and establish elsewhere to a less degraded habitat. Understanding the etiology of this shell disease and understanding environmental stressors that may degrade the habitat of *P. rubriventris*'s habitat is valuable to turtle conservation.

5. Conclusions

The results of this study suggest that HCB may increase the vulnerability to shell disease in *P. rubriventris*. Both epizoic and lake phytoplankton samples showed significantly greater cyanobacteria density and saxitoxins at lakes positive for shell disease. Epizoic community samples showed that cyanobacteria density was greater in turtles with shell disease, suggesting epizoic cyanobacteria growth may be the cause of shell diseases of *P. rubriventris* in New Jersey. However, further research and long-term investigation is needed to provide clarity on the relationship between cyanobacteria, cyanotoxins, and shell disease. Assessing aquatic organism health and stress levels should be a part of HCB monitoring, and toxin thresholds for these organisms should be developed in order to more accurately determine at what level of toxin exposure their health may be threatened.

When investigating water quality of study lakes and ponds, benthic and epizoic communities should be examined in addition to phytoplankton within the water column to better understand the community dynamics at the ecosystem level. In order to confirm that cyanobacteria genera observed within epizoic community samples belong to toxin producing strains, future research should apply genetic analysis in tandem with morphologic identification under the microscope. Future studies should also develop a scale of shell disease severity, perhaps based on percent cover of shell with lesions in affected individuals, in order to make more detailed comparisons between individuals. Follow-up studies regarding this shell disease in *P. rubriventris* should also attempt viral isolation off of lesions of affected individuals, as up until this point, only bacterial and fungal etiologies have been explored. Cyanotoxin bioaccumulation should be analyzed in tissue samples collected from diseased individuals in

order to fill in the gap of current understanding between toxin producing cyanobacteria and turtle shell disease.

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