Abstract

THE EFFECTS OF TEMPERATURE ON MICROCYSTIN-LR TOXICITY TO *BOSMINA LONGIROSTRIS*: FOOD WEB IMPLICATIONS IN THE CHOWAN RIVER, NORTH CAROLINA

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In the Chowan River, North Carolina, the frequency of *Microcystis aeruginosa* blooms has increased over recent years with an average 1.9°C rise in June water temperatures since 1975. Zooplankton are an important trophic link for toxins to move up the food web. *Bosmina longirostris*, a dominant zooplankton in the Chowan River, consumes toxic *M. aeruginosa* cells. This study aimed to understand how microcystin-LR, produced from *M. aeruginosa* blooms, affects *B. longirostris* mortality under increasing temperatures. *B. longirostris* were resistant to microcystin-LR, demonstrating an LC_{50} of 26.3 μg/L. Therefore *B. longirostris* can survive typical bloom microcystin-LR concentrations ranging less than 0.1 μg/L to 2.0 μg/L. As temperatures were increased from 25°C to 35°C, microcystin-LR mortality increased approximately 18% from 25-27°C; demonstrating that microcystin-LR was more toxic with increasing temperatures. Above 27°C, mortality also increased, but this was due to the effect of temperature rather than increased toxicity of microcystin-LR. This signifies that during spring, when temperatures are below 27°C and *B. longirostris* are most abundant, microcystin-LR may be most toxic and may have the greatest influence on the Chowan River food web. Under climate

change conditions microcystin-LR may eliminate resistant zooplankton from the food web, putting pressure on larval fish and fisheries.

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CHAPTER ONE:

BACKGROUND OF CHOWAN RIVER AND *MICROCYSTIS AERUGINOSA* BLOOMS

INTRODUCTION

Thirty years ago, blooms of the cyanobacterium *Microcystis aeruginosa* were common in eastern North Carolina, particularly in the Chowan River (Witherspoon et al. 1979). The ban of phosphorus detergents in the early 1980s, combined with the shutdown of a Tunis fertilizer plant discharging nitrates (C.F. Industries 1980), resulted in a reduction in cyanobacteria blooms in the Chowan River. However, cyanobacteria blooms have been reoccurring in the Chowan River over the last decade (NCDPH 2005-2012). Cyanobacteria blooms can occur under highly eutrophic conditions, particularly in lower salinity waters (Paerl et al. 2001)—conditions already present in the Chowan River and Albemarle Sound. In August of 2013, a *Microcystis aeruginosa* bloom had a particularly high microcystin-LR toxin level of 68 µg/L, nearly 34 times typical toxicity concentrations in the Chowan River of less than 0.1 µg/L to 2.0 µg/L (Moorman et al. 2017).

M. aeruginosa blooms are an issue because they can pose a significant threat to human populations (Ferrão-Filho and Kozlowsky-Suzuki 2011). When *M. aeruginosa* cyanobacteria die, the cells lyse and the microcystin-LR toxin is released into the surrounding waters (Butler et al. 2009). The entire aquatic food web is then exposed to this toxin, including humans using the water as a drinking or recreational source. The World Health Organization has set the safe drinking water limit of microcystin-LR to 1 µg/L (Hitzfeld et al. 2000). However, many of the *M. aeruginosa* blooms in North Carolina over the recent years are already at a microcystin-LR concentration of 2 µg/L, with one bloom in particular as high as 68 µg/L in 2013 (Moorman et al. 2017). Therefore, drinking water contaminated with microcystin-LR toxin can be harmful and may lead to acute liver disease in humans (Falconer, Beresford, and Runnegar 1983). Most

common symptoms of drinking water contaminated with microcystin-LR are fatigue, diarrhea, centrilobular hepatic necrosis, intrahepatic hemorrhage, and hepatocyte necrosis (Hitzfeld et al. 2000).

Microcystin-LR is a cyclical peptide hepatotoxin, which enters the body through organic anion transporters primarily in the liver and causes death of hepatocytes (Hitzfeld et al. 2000). The most serious case of exposure to this toxin occurred in 1996 in Caruaru, Brazil where sixty hemodialysis patients died when malfunctions in their filtration system exposed them to microcystin-LR from the Tabocas Reservoir nearby (Hitzfeld et al. 2000). Again in Brazil in 1993, an unusually large *M. aeruginosa* bloom in the Itaparica Dam resulted in 2,000 gastroenteritis cases, with 88 total deaths of mostly children (Hitzfeld et al. 2000). Similarly, exposure of livestock and dogs to microcystin-LR-contaminated waters has also resulted in death. Fish kills may also be a result from higher levels of microcystin-LR in freshwater (Hitzfeld et al. 2000, Butler et al. 2009). The state of North Carolina has been urging communities to keep small children and pets away from areas experiencing *M. aeruginosa* blooms (NCDEQ 2015).

M. aeruginosa blooms are both a health hazard and a nuisance—they result in elevated pH and unsightly blue-green water (NCDPH 2005-2012). Current management practices include ultra-filtration and application of ozone to contaminated waters, which appear to be the only efforts successful in breaking down the microcystin-LR toxin (Hitzfeld et al. 2000). Application of UV light has also been shown to breakdown microcystin-LR rapidly, but may not be applicable on large scales (Hitzfeld et al. 2000). Therefore, *M. aeruginosa* blooms continue to pose a significant threat to humans and aquatic life.

During *M. aeruginosa* blooms, zooplankton serve as the major link between microcystin-LR toxins in the aquatic environment and the rest of the food web. They can take up *M. aeruginosa* cells containing microcystin-LR before they lyse and transfer it to higher trophic levels through lipid storage (Ferrão-Filho and Kozlowsky-Suzuki 2011), or they can also be exposed to microcystin-LR dissolved directly in the water column. Microcystin-LR could eliminate susceptible zooplankton from the food web, and without this zooplankton link, the toxin would have no means of reaching fish and higher predators: beneficial for keeping microcystin-LR out of fish that humans might consume. However, resistant zooplankton, defined as zooplankton that survived microcystin-LR exposure would be able to transfer microcystin-LR up the food web—evidence shows that bioaccumulation of microcystin-LR has been seen at low trophic levels in planktivorous fish (Ferrão-Filho and Kozlowsky-Suzuki 2011), especially silver carp in China (Xie et al. 2005). Bioaccumulation can be defined as the continuous accumulation of a substance, in this case microcystin-LR toxin, in an organisms' lipids that can be passed unaltered to higher trophic levels. This is significant because bioaccumulation of microcystin-LR in planktivorous fish may make them unsafe for human consumption and use.

Additionally, risk of bioaccumulation of toxins increases with temperature (Ferrão-Filho and Kozlowsky-Suzuki 2011), which poses a threat to aquatic food webs and humans since microcystin-LR can be prevalent for up to two weeks after a bloom and may be found in higher concentrations at higher temperatures (Sharma et al. 2011). Optimum growth temperatures for *M. aeruginosa* cells are 27.5°C (Christian, Bryant Jr, and Stanley 1986); so warming climates suggest that *M. aeruginosa* blooms may be more frequent in the Chowan River, NC during summer months under eutrophic conditions.

An analysis of the zooplankton community of the Chowan River and Albemarle Sound showed that the dominant zooplankton in terms of abundance are rotifers, copepod nauplii, Cyclopoida, Chydoridae, Calanoida, and *Bosmina* spp. including *Bosmina longirostris* (Binion 2012, Leech and Piehler 2009, Lichti 2014). *Bosmina* spp. are cladocerans and are seen throughout the Chowan River and Albemarle Sound in April through June, seasonally (Lichti 2014). *Bosmina* spp. appear to reach peak abundance in April in the mid-Chowan River and in June in the upper Chowan River (Lichti 2014). Because of their ubiquitous distribution and abundance over other zooplankton, *Bosmina* spp., like *B. longirostris,* are good candidates for algal research of *M. aeruginosa* blooms and food web implications in the Chowan River system.

Although cyanobacteria strains of *M. aeruginosa* are both toxic and low in nutritional content, cladocerans like *Bosmina* spp. and *Daphnia* spp. are non-selective feeders and will readily consume this cyanobacteria as well as other algae in the surrounding environment during non-bloom conditions (De Bernardi and Giussani 1990, Benndorf and Henning 1989). However, studies have shown that *M. aeruginosa* is toxic to *Daphnia* spp. and often results in death (Ferrão-Filho, Azevedo, and DeMott 2000, Fulton and Paerl 1987a). Competition studies have shown that *B. longirostris* in the presence of *M. aeruginosa* toxins will outcompete *Daphnia pulex* and at higher temperatures (Jiang et al. 2014). *B. longirostris* are a species of cladoceran that has shown resistance to toxic *M. aeruginosa* blooms, demonstrated by their ability to survive and reproduce (Fulton and Paerl 1987a). A similar response has been seen in rotifers (Fulton and Paerl 1987a), but *B. longirostris* have actually fed on these blooms regardless of their poor nutritional value or algal morphology (unicellular, filamentous, or colonial) (Fulton and Paerl 1987b, Fulton 1988a).

The cladoceran *B. longirostris* is capable of consuming toxic strains of *M. aeruginosa* regardless of cell morphology and nutritional value. This may indicate additional survival and species dominance at higher temperatures under a warming climate, and makes it an ideal species for microcystin-LR research. In addition, *B. longirostris* are a significant source of food for larval and planktivorous fish that are part of several important fisheries within the Chowan River (Binion 2011, Mullen, Fay, and Moring 1986). Response of *B. longirostris* to microcystin-LR toxins under increasing temperatures due to climate change is important for making inferences on how these fisheries are affected during *M. aeruginosa* blooms in the Chowan River.

Several studies have examined the effect of temperature variations on the sensitivity of different consumer groups to toxic strains of *M. aeruginosa* (Ferrão-Filho, Azevedo, and Mott 2000, Hietala, Laurén-Määttä, and Walls 1997, Huang et al. 2012); however, no studies involving temperature and microcystin-LR have been conducted on *B. longirostris.* Higher temperatures increase the sensitivity of some zooplankton, like rotifers, to microcystin-LR toxins and negatively affect their life history (Zhang and Geng 2012). At warmer temperatures, *D. pulex* clones had reduced survivorship and reproduction when exposed to microcystin-LR over short time intervals (Hietala, Laurén-Määttä, and Walls 1997), but still no studies have been conducted analyzing the combined effects of microcystin-LR and temperature on the cladoceran *B. longirostris.*

Increasing sea surface temperatures as a result of climate change has led to concern of changes in harmful algal bloom taxa and frequency (Edwards et al. 2006), like the increasing frequency of *M. aeruginosa* blooms within the Chowan River. Evidence has already shown that the Chowan River in North Carolina has warmed by a total of 0.71°C (yearly average), and has

increased seasonally by an average of 1.9°C in June water temperatures since 1975 (EPA 2017). Similarly, occurrence and duration of cyanobacteria blooms are also higher in areas where temperature is greater (Ferrão-Filho and Kozlowsky-Suzuki 2011), demonstrating that water temperature increase may lead to longer, and more frequent *M. aeruginosa* blooms within the Chowan River. Research on the effects of microcystin-LR, in conjunction with rising temperatures due to climate change, on *B. longirostris* is therefore necessary and important for making implications for the planktonic food web in the Chowan River.

Rationale and Significance

Temperature changes during the summer months, as well as increasing sea surface temperatures due to global warming, could increase the sensitivity of zooplankton to toxins produced from harmful algal blooms. Within the Chowan River, average water temperature during the months of June have increased 1.9°C, along with an overall average year increase of 0.71°C since 1975 (EPA 2017). The reoccurring frequency of *M. aeruginosa* blooms in the Chowan River, along with temperature rise, suggests the need for research on how microcystin-LR toxins impact dominant zooplankton, like *B. longirostris,* with increasing temperatures.

This significantly impacts the food web in a negative way during *M. aeruginosa* blooms that produce microcystin-LR. Microcystin-LR eliminates non-resistant zooplankton from the food web, which could result in larval fish starvation—leading to less juvenile fish reaching maturity. A reduction in mature fish negatively affects important fisheries existing in the Chowan River. Survival of resistant species of zooplankton during toxic *M. aeruginosa* blooms reduces prey diversity for larval and planktivorous fish. Surviving *B. longirostris* zooplankton also have the potential to transfer microcystin-LR up the food web via lipid storage, leading to bioaccumulation in planktivorous fish.

Implications for the Chowan River food web have not been assessed using the combination of temperature and microcystin-LR on *B. longirostris.* The purpose of this study is to investigate the effect of temperature on microcystin-LR toxicity to *B. longirostris* in order to make implications for the Chowan River food web during toxic *M. aeruginosa* blooms under warming water conditions due to climate change.

Goals and Objectives

The goal of this study was to understand how cyanotoxins produced from algal blooms in the Chowan River affect the food web under warming conditions. *M. aeruginosa* blooms produce the toxin microcystin-LR to which zooplankton within the water column are exposed. The objectives of this study were to:

1). Calculate the effect of microcystin-LR on *B. longirostris* mortality.

2). Identify how temperature affects microcystin-LR toxicity to *B. longirostris*.

3). Elucidate the implications for the Chowan River food web under climate change temperatures and high microcystin-LR concentrations.

Hypotheses

The hypotheses corresponding to the objectives above are as follows:

1). Microcystin-LR increases *B. longirostris* mortality with increasing concentrations.

2). Increasing temperatures during exposure to microcystin-LR at the LC_{50} increases the mortality of *B. longirostris.*

3). Under climate change temperature and microcystin-LR toxin conditions, mortality of *B. longirostris* has negative implications for the Chowan River food web.

Statistical Analysis

All statistical analyses were conducted with SAS University. For toxicity dose-response experiments and temperature experiments, a regression analysis was completed to test the strength of the data fit to a trend line for all of the toxicity dose-response or temperature experiments conducted. A Probit Analysis was used to calculate the LC_{50}. A single factor ANOVA (Analysis of Variance) followed by a Fisher's LSD pairwise comparison *a posteriori* test was used for each experiment to determine which treatments were statistically different from each other. An ANCOVA (Analysis of Covariance) was conducted for temperature experiments to determine the significance of the interaction between microcystin-LR (26.3 µg/L) and temperature on *B. longirostris* percent mortality.

CHAPTER TWO: MICROCYSTIN-LR LC_{50} FOR *BOSMINA LONGIROSTRIS*

INTRODUCTION

Microcystin-LR is a cyanotoxin produced by *M. aeruginosa* cyanobacteria cells and is lysed into the water column when these cells die (Butler et al. 2009). Typical microcystin-LR concentrations within the Chowan River are less than 0.1 µg/L to 2.0 µg/L (Moorman et al. 2017). Concentrations of microcystin-LR have been shown to increase in warmer temperatures (Sharma et al. 2011). While other cyanotoxins are produced by *M. aeruginosa*, microcystin-LR is the most common and best studied. In addition to being a cyanotoxin, microcystin-LR is a hepatotoxin, which attacks liver cells once it has entered into the body (Hitzfeld et al. 2000). Microcystin-LR gains entry primarily through organic anion transporters in the cell membrane, most of which are located in liver cells (Hitzfeld et al. 2000). Ultimately, microcystin-LR inhibits protein phosphates, causing deformation of hepatocytes and leads to cell death (Hitzfeld et al. 2000, Ferrão-Filho and Kozlowsky-Suzuki 2011). Microcystin-LR refers to the specific structure of the toxin, in which "LR" refers to L-amino acids leucine and arginine (Hitzfeld et al. 2000). Other congeners of microcystin-LR produced by *M. aeruginosa* include: microcystin-LA, microcystin-YR, and microcystin-RR (Hitzfeld et al. 2000).

An LC_{50} refers to the lethal concentration of a solution fatal to 50% of a population (Hodgson and Roe 2014). The LC_{50} for a particular toxic solution is often calculated from a quantal dose-response curve in which a population is exposed to concentrations of this toxin at different increments, as the dose, and a response is measured for each increment (Hodgson and Roe 2014). Typically, the response measured is percent mortality of the population. Acute dose-response experiments can be carried out for 24 hours, 48 hours, 72 hours, or 96 hours. The dose-response relationship exhibits a sigmoidal curve and a linear regression can be used to best fit the

data after the data is transformed. A probit analysis is a common method to transform non-linear data to a linear relationship (Finney 1952). At 50% mortality, the corresponding dosage, or toxin concentration, is calculated from the regression line. The LC_{50} is then the concentration that corresponds to 50% of mortality. LC_{50} is the typical endpoint chosen to measure the lethal effect of a toxin on a population. Because 50% of the population is killed at the LC_{50}, concentrations above this value would be fatal to the majority of the population.

The LC_{50} for microcystin-LR toxicity on *B. longirostris* has not been previously determined. Organisms for which the LC_{50} for microcystin-LR toxicity has been determined include: ciliates, brine and fairy shrimp, copepods, and cladocerans related to *B longirostris* such as species of *Daphnia* (Ger, Teh, and Goldman 2009, DeMott, Zhang, and Carmichael 1991, Reinikainen et al. 2002, Ward and Codd 1999, Lahti et al. 1995, Keil et al. 2002). Fulton (1988b) claimed that *B. longirostris* were very resistant to toxic *M. aeruginosa* cells. *B. longirostris* are thought of as one of the most resistant zooplankton to microcystin-LR producing *M. aeruginosa*, compared to other zooplankton; however, the LC_{50} for microcystin-LR on *B. longirostris* is entirely unknown.

This study attempts to quantify the effect that microcystin-LR has on *B. longirostris* by calculating the LC_{50}. Quantification of the LC_{50} for *B. longirostris* when exposed to this particular cyanotoxin will allow comparisons to be made between environmental toxicities in the Chowan River, North Carolina and zooplankton survivorship.

METHODS

Site description

The Chowan River is a freshwater estuary located in eastern North Carolina (Figure 1). The river system spans north to south over several counties: Hertford, Gates, Chowan, and

Bertie, and empties into the Albemarle Sound, which connects to the Atlantic Ocean at Oregon Inlet. Nottoway and Blackwater River convergences in southern Virginia form the origin of the Chowan River. Meherrin and Wiccacon Rivers are the two main tributaries that flow into the Chowan River. Several creeks flow into the Chowan River from the surrounding land, including Catherine's and Bennett's Creek. The area surrounding the Chowan River is dominated by agricultural farmland, punctuated by small towns: Gatesville, Edenton, Winton, Harrellsville, and Colerain (Figure 1).

Organism collection and storage

B. longirostris zooplankton were collected from Catherine's Creek within the Chowan River, North Carolina (N36.314706, W76.670449) in May/June of 2016 and April of 2017 using a 0.25 m diameter, 200 μm mesh plankton net towed for consecutive tows via kayak. *B. longirostris* were stored in 4 L plastic containers on ice in a cooler during transport from the field to East Carolina University. Zooplankton were fed unicellular Instant Algae® Isochrysis 1800™ (stock density 3.9 billion cells/mL) within unfiltered Chowan River water medium and kept at 4°C in the dark until experimental use. All collected *B. longirostris* were used in toxicity and temperature experiments within one week of collection from Catherine's Creek, NC. Chowan River water medium for toxicity experiments was collected simultaneously with organism collection from Catherine's Creek, Chowan River, NC using 10 L containers. Water for experiments was then vacuum-filtered immediately using 45 mm diameter, 0.7 μm Whatman glass-fiber filters. Filtered Chowan River water was then stored in the dark at 4°C until experimental usage.

Experimental design

To understand how toxicity from microcystin-LR affects *B. longirostris* mortality, toxicity dose-response experiments were conducted for 48 hours at 25°C. An exposure time of 48 hours was chosen because it is the most common exposure period, so comparisons between studies could be made. Additionally, preliminary studies showed that an exposure time of 96 hours allowed for an over abundance of reproduction and natural cell death of control treatments. During this exposure period, the effect of microcystin-LR on *B. longirostris* was difficult to determine, so a 48-hour exposure was used instead.

B. longirostris were separated from stock bottles and acclimated to 25°C for four hours prior to start of experiments. Microcystin-LR stock solution (1 mg/mL in 100% ethanol) was diluted with 100 mL of filtered Chowan River water to create seven microcystin concentrations: 0, 0.005, 0.05, 0.5, 5.0, 50, and 100 µg/L for LC_{50} determination for *B. longirostris.* Each microcystin-LR concentration experiment contained 10 *B. longirostris* and 10 µL of Instant Algae® Isochrysis 1800™ in 100 mL glass containers of filtered Chowan River water to control for natural cell death of *B. longirostris*. Triplicates were used for each microcystin-LR concentration and the entire experiment was repeated twice. After 48 hours, *B. longirostris* were filtered out using a 60 µm mesh sieve and assessed for mortality using microscopy by counting the number of dead. A single factor ANOVA followed by a Fisher's LSD pairwise comparison *a posteriori* test was used to determine which microcystin-LR treatments were statistically different from each other.

LC_{50} calculation

The LC_{50} was calculated using the probit analysis method described by Finney (1952): a frequent method used in the literature to calculate LC_{50} values from toxicology studies (Ger, Teh,

and Goldman 2009, DeMott, Zhang, and Carmichael 1991, Reinikainen et al. 2002). The probit analysis method transforms sigmoid dose-response data into a straight line in which a linear regression can be fit to calculate the LC_{50} (Finney 1952). Data from this experiment exhibited a normal distribution and therefore a probit analysis was used rather than a logit analysis, which assumes a non-normal distribution. Percent mortalities were transformed into empirical probits corrected for mortality from the controls and microcystin-LR concentrations logged. The LC_{50} was then calculated using a linear regression and solving for 5 probits (corresponding to 50% mortality) and taking the inverse log of the resulting microcystin-LR concentration.

RESULTS

The LC_{50} for *B. longirostris* exposure to microcystin-LR was calculated to be 26.3±6.4 µg/L for 48 hours at 25°C using Finney's (1952) probit analysis method (Figure 2). A relationship between percent mortalities and log of the microcystin-LR concentrations shows the sigmoidal shape of the dose-response curve and demonstrates how *B. longirostris* mortality changes with microcystin-LR concentration (Figure 3). Results from this experiment show that *B. longirostris* mortality increased with increasing microcystin-LR concentrations. The difference in percent mortality between treatments was significantly explained by the change in microcystin-LR concentration (Figure 3, ANOVA, $F_{5,30}$=139.96, p<0.0001). The ANOVA model explained 96% of the variability in *B. longirostris* mortality to microcystin-LR concentrations (R^2=0.96).

Microcystin-LR concentrations of 0.5, 5.0, and 50 µg/L were found to be statistically different from each other, with the LC_{50} concentration of 26.3 µg/L calculated in between 5.0 µg/L and 50 µg/L (Figure 2, Fisher's LSD, p<0.001). The *a posteri* test showed how different two average percent mortalities from treatments needed to be to indicate a statistical difference

based on a probability value less than 0.05. At 5.0 µg/L, *B. longirostris* experienced a 17.2% mortality resulting from the exposure to microcystin-LR, and at 50 µg/L there was an observed 66.0% mortality (Figure 3). At 100 µg/L, *B. longirostris* had a mortality of 71.5%, while concentrations below 0.5 µg/L had little effect on *B. longirostris* mortality. At 100 µg/L, mortality of *B. longirostris* (71.5%) was not significantly different than the mortality at 50 µg/L (66%) (Figure 3, $p>0.05$, Fisher's LSD). As the concentration of microcystin-LR increased *B. longirostris* mortality also increased.

A two-tailed, two sample t-test revealed that the LC_{50} of 26.3 µg/L was statistically different from 68 µg/L, the unusually high microcystin-LR concentration from a bloom in 2013, and typical concentrations of microcystin-LR in the Chowan River: less than 0.1 µg/L to 2 µg/L ($p<0.05$, Moorman et al. 2017).

DISCUSSION

Based on an LC_{50} of 26.3 µg/L, *B. longirostris* populations would be able to survive typical microcystin-LR concentrations in the Chowan River and surrounding Albemarle Sound, ranging from less than 0.1 µg/L to 2.0 µg/L (Moorman et al. 2017). Between the concentrations of 0.1-2.0 µg/L, *B. longirostris* experiences a mortality of 3.6% to 7.1%, compared to the 50% mortality at the LC_{50} (26.3 µg/L) (Figure 3). Therefore, approximately 93% of *B. longirostris* survive typical microcystin-LR concentrations in the Chowan River. Higher microcystin-LR concentration *M. aeruginosa* blooms in the Chowan River; however, may negatively affect *B. longirostris* populations and ultimately reduce survivorship. The 2013 bloom, for example, which had a microcystin-LR concentration of 68 µg/L (Moorman et al. 2017), could have killed 75% of the population of *B. longirostris* (Figure 3). Microcystin-LR concentrations that exceed

26.3 µg/L (LC_{50}) would therefore eliminate over 50% of the *B. longirostris* population and would result in negative implications for the Chowan River food web.

In this study, *B. longirostris* demonstrated resistance to microcystin-LR; the cyanotoxin produced from the cyanobacteria *M. aeruginosa,* as concentrations increase. Resistance here is defined as the ability of at least 50% of a population to survive exposure to microcystin-LR concentrations. Fulton (1988b) was one of the first to describe the resistance of *B. longirostris* to toxic *M. aeruginosa* cells. Previously, Fulton and Paerl (1987a) described how neither toxic nor nontoxic strains of *M. aeruginosa* provided any nutritional benefit to *B. longirostris.* This was later modified to show that *B. longirostris* does get some nutritional benefit from *M. aeruginosa* cells, but not enough to support an entire population because of negligible reproduction (Fulton 1988b). In this study, reproduction was seen in control treatments, but was strongly reduced in microcystin-LR treatments, based on observations. Additionally, *B. longirostris* do not strongly avoid different morphologies of *M. aeruginosa*, and will consume both unicellular and colonial strains (Fulton and Paerl 1987b). While *B. longirostris* were expected to be resistant to toxic strains of *M. aeruginosa,* the toxicity of the strain used by Fulton (1988b) was not confirmed. This is significant because the cyanotoxins produced by Fulton's (1988b) *M. aeruginosa* strain were not identified nor was the concentration of these cyanotoxins (either intracellular or in the medium) quantified. Without an analysis of the extent of the toxins produced by *M. aeruginosa*, it is difficult to assess the extent of *B. longirostris* survival to the cells. By quantifying the LC_{50} of microcystin-LR as 26.3 µg/L in this study, previously undone, the exact effect of microcystin-LR on *B. longirostris* is elucidated and inferences about how this population will respond to future toxic *M. aeruginosa* blooms can be made.

While the LC_{50} for *B. longirostris* has not been previously determined, additional literature has assessed the LC_{50} for other species of zooplankton. In comparison to other zooplankton, especially cladocerans, *B. longirostris* have the lowest resistance to microcystin-LR based on a low LC_{50} value of 26.3 µg/L. Larger-bodied cladocerans, like *D. pulex*, have an LC_{50} of 9600 µg/L when exposed to microcystin-LR (Table I, DeMott, Zhang, and Carmichael 1991). This is contested by Ferrão-Filho et al. (2000) who used an LT_{50} of 36 hours to determine that 50% of the population of *D. pulex* dies when exposed to a microcystin-LR concentration of 4.08 µg/L (Table II). Additionally, Hietala, Laurén-Määttä, and Walls (1997) determined an EC_{50} concentration of 2.983 µg/L, which is the effective concentration of microcystin-LR where *D. pulex* visibly had a negative response (e.g. lack of movement) to the cyanotoxin (Table I). Using LT_{50} and EC_{50} instead of LC_{50}; however, seems to result in high variability between estimates, making comparisons difficult for *D. pulex*. Other cladocerans, like *Daphnella hyalina* and *Daphnia pulicaria*, appear to have the highest LC_{50} values and demonstrate higher resistance to microcystin-LR compared to *B. longirostris* (Table I, DeMott, Zhang, and Carmichael 1991).

Copepods are another zooplankton group that demonstrate resistance to microcystin-LR. Copepod resistance varies by species, but ranges from an LC_{50} of 270 µg/L to 1550 µg/L (Table I, Reinikainen et al. 2002, DeMott, Zhang, and Carmichael 1991, Ger, Teh, and Goldman 2009). In this case, the resistance of copepods refers to the population's ability to survive by 50% microcystin-LR concentrations 270-1550 µg/L. Copepods, while showing a greater survival to microcystin-LR than *B. longirostris* (LC_{50} of 26.3 µg/L), are known for their chemosensory avoidance of *M. aeruginosa* cells, unlike *B. longirostris* (Fulton 1988a, Fulton and Paerl 1987a). Therefore, while copepods are very resistant to microcystin-LR, the likelihood that they would willingly ingest toxic *M.* aeruginosa cells is low, but they could still be exposed to lysed

microcystin-LR in the environment. In comparison, *B. longirostris* actually feeds on *M. aeruginosa* blooms regardless of poor nutritional value or algal morphology (unicellular, filamentous, or colonial) (Fulton and Paerl 1987b, Fulton 1988a). *B. longirostris,* therefore, would have a higher probability of being exposed to microcystin-LR both in the *M. aeruginosa* cells that they ingest and dissolved in the surrounding water column.

Determination of LC_{50} for microcystin-LR is important for making comparisons of zooplankton survivability to toxin concentrations within aquatic systems. These estimates of survivorship are necessary for making inferences about zooplankton prey availability for the rest of the food web. As water temperatures continue to rise due to climate change in the Chowan River, higher concentration microcystin-LR *M. aeruginosa* blooms may continue to be a problem for the zooplankton community (Sharma et al. 2011).

CHAPTER THREE: EFFECT OF TEMPERATURE ON TOXICITY

INTRODUCTION

Increasing global temperatures of 0.78°C since 1880 (NOAA 2016) and an increase in local Chowan River yearly water temperatures of 0.71°C since 1975 (EPA 2017, Witherspoon et al. 1979), leads to concern about the effects of temperature change on microcystin-LR toxicity. High microcystin-LR concentrations have been correlated with higher water temperatures in tropical regions (Sharma et al. 2011), which further increases concern about the effect of temperature increases on microcystin-LR blooms and zooplankton exposure. Higher Chowan River water temperatures are particularly important because zooplankton link microcystins produced from *M. aeruginosa* blooms to the rest of the food web and should be greatly affected by higher microcystin-LR concentrations due to temperature rise. The effect of increasing temperatures and toxic *M. aeruginosa* has already been established for some zooplankton species, such as rotifers and *D. pulex,* a large-bodied cladoceran related to *B. longirostris* (Jiang et al. 2014, Hietala, Laurén-Määttä, and Walls 1997, Zhang and Geng 2012). However, no studies have been conducted on the effects of temperature and microcystin-LR on *B. longirostris,* which is a dominant cladoceran in the Chowan River during the months of April and June.

B. longirostris have a wide thermal tolerance range of 11-36°C (Verbitsky, Verbitskaya, and Malysheva 2009), and should be able to survive future rises in river water temperature. Additionally, *B. longirostris* are able to survive typical *M. aeruginosa* bloom concentrations in the Chowan River ranging less than 0.1 µg/L to 2.0 µg/L (Moorman et al. 2017). However, populations of this species may not be able to survive conditions of higher temperatures and increased toxicity of microcystin-LR due to this temperature rise. At 25°C, when *B. longirostris* were exposed to microcystin-LR for 48 hours at the LC_{50} of 26.3 µg/L, 50% of the population

died. Therefore, if temperatures exceed 25°C, the increase in temperature may also increase the toxicity of microcystin-LR to *B. longirostris* at 26.3 µg/L, based on the fact that higher temperatures lead to higher concentrations of microcystin-LR (Sharma et al. 2011). It is then hypothesized that increasing temperatures during exposure to microcystin-LR at 26.3 µg/L would increase the mortality of *B. longirostris.*

METHODS

To test how temperature and microcystin-LR affect *B. longirostris*, mortality was assessed at 26.3 µg/L over a range of temperatures: 25°C, 27°C, 30°C, 32°C, and 35°C during a 48-hour exposure. *B. longirostris* for this experiment were collected in June of 2016 within Catherine's Creek of the Chowan River, North Carolina using a 200 µm plankton net via consecutive kayak tows. Each temperature treatment for the experiment was maintained using an incubator adjusted so that water temperature within each replicate reflected actual treatment temperature. Each temperature treatment contained 10 *B. longirostris*, 10 µL of Instant Algae® Isochrysis 1800™, and 26.3 µg/L of microcystin-LR (LC_{50}) in 100 mL glass containers of filtered Chowan River water. Triplicates were used for each temperature treatment and the entire experiment was repeated three times. After 48 hours, *B. longirostris* were filtered out using a 60 µm mesh sieve and assessed for mortality using microscopy by counting the number of dead.

This entire experiment was then repeated using a lower range of temperatures: 15°C, 17°C, 20°C, 22°C, and 25°C. This secondary study was conducted to determine if the population of *B. longirostris* exhibited different mortality results when exposed to microcystin-LR at 26.3 µg/L at lower temperatures. *B. longirostris* for this temperature range study were collected in April of 2017 again in Catherine's Creek of the Chowan River with a 200 µm plankton net over consecutive kayak tows. Each temperature treatment was maintained at that specific temperature

through the use of an incubator. Each temperature treatments had 10 *B. longirostris*, 10 µL of Instant Algae® Isochrysis 1800™, and 26.3 µg/L of microcystin-LR in 100 mL glass containers of filtered Chowan River water. Triplicates were used for each temperature and the entire experiment was repeated three times. After 48 hours of exposure to microcystin-LR, *B. longirostris* were filtered out using a 60 µm mesh sieve, and mortality was assessed via microscopy by counting the number of dead.

Microcystin-LR toxicity analysis

Evaluation of microcystin concentration (LC_{50}) used for the temperature experiments was conducted via an ELISA (Enzyme-linked immunosorbent assay) analysis at Greenwater Laboratories in Palatka, Florida. One sample from the 27°C temperature treatment and two samples from the 25°C treatments were analyzed for microcystin-LR content to determine the treatment effects on the LC_{50} concentration. Samples were sent to determine if the 48-hour exposure period degraded the microcystin-LR concentration used of 26.3 µg/L. Two 25°C samples, one replicate from the higher temperature range experiment (25°C-35°C) and one replicate from the lower range experiment (15°C-25°C) were analyzed to show that the microcystin-LR concentration used for the temperature range experiments was actually 23.3 µg/L. This concentration is within the range of the LC_{50} of 26.3±6.4 µg/L.

Statistical analysis

All statistical analyses were conducted with SAS University. A single factor ANOVA (Analysis of Variance) followed by a Fisher's LSD pairwise comparison *a posteriori* test was used for each experiment to determine which temperature treatments were statistically different from each other. An ANCOVA (Analysis of Covariance) was used to test the significance of the interaction between temperature and microcystin-LR for both temperature range experiments.

RESULTS

Higher temperature range

During the higher temperature range experiment, increasing temperatures resulted in an increase in total percent mortality when *B. longirostris* were exposed to microcystin-LR at 26.3 µg/L. Specifically, as temperatures increased from 25°C to 35°C, the total average percent mortality of *B. longirostris* increased from 64.6% to 100% (Figure 4). The difference in percent mortality between treatments was significantly explained by the difference in temperatures (ANOVA, $F_{4,30}$= 15.92, p<0.0001), supporting the hypothesis that increasing temperature during exposure to microcystin-LR at 26.3 µg/L increases the mortality of *B. longirostris.* The ANOVA model explained 68% of the variability in *B. longirostris* mortality to temperature treatments (R^2= 0.68). The greatest increase in total percent mortality was seen between 25°C and 27°C, where there was a 30% increase in *B. longirostris* mortality. Total mortality of 100% was achieved at 32°C. Total percent mortality shows that when *B. longirostris* are exposed to both temperature increase and microcystin-LR (26.3 µg/L), the resulting mortality is a combination of both effects. In the Chowan River field environment, the effects of temperature and microcystin-LR would always coexist. An analysis of covariance showed that the interaction between the effects of temperature and microcystin-LR was statistically significant. The presence or absence of microcystin-LR significantly affected the mortality of *B. longirostris* at each temperature ($F_{1,68}$=23.18, p <0.0001). The ANCOVA model explained 79.5% of the variability in percent mortality (R^2= 0.795) and the variation in mortality was significantly explained by the variability in temperature (ANCOVA, $F_{3,68}$= 88.3, p<0.0001).

Control treatments (without microcystin-LR) for the higher temperature range experiment show that in the absence of microcystin-LR as temperature increases average percent mortality increases as well, demonstrating a 70.5% increase over the range of 25°C to 35°C (Figure 4,

ANOVA, $F_{4,30} = 40.23$, $p<0.0001$, $R^2= 0.84$). This signifies that as temperatures approach 35°C, the upper limits of *B. longirostris* thermal tolerance is reached as mortality approaches 100%. At 35°C, 87% mortality is observed, with the upper thermal limits for *B. longirostris* being 36°C (Verbitsky, Verbitskaya, and Malysheva 2009). Similarly, control treatments for the lower temperature range experiment showed that in the absence of microcystin-LR mortality significantly decreased with lower temperatures (Figure 5, ANOVA: $F_{4,40}= 2.72$, $p<0.05$, $R^2=0.214$). The lowest percent mortality over the entire 15°C-35°C is at 15°C, demonstrating that population of *B. longirostris* survives best at this temperature with a 97% survival rate.

While the effects of temperature and microcystin-LR would always coexist in the environment, a separation of these effects can show what their respective contributions are to *B. longirostris* total mortality. Additionally, this separation shows that temperature exacerbates the effect of microcystin-LR on *B. longirostris* mortality in this study. A separation of the effects of temperature and microcystin-LR (26.3 µg/L) for the higher range temperature experiment revealed an 18% increase in *B. longirostris* mortality between 25°C and 27°C due to microcystin-LR, alone (Figure 6). This 18% increase in mortality over a 2°C increase in temperature shows that, while the concentration of microcystin-LR remained the same at 26.3 µg/L, its toxicity to *B. longirostris* actually increased, resulting in the increase in percent mortality. At 27°C, microcystin-LR is the greatest contributor to total percent mortality attributing to 66.4% (Figure 6). At 25°C, microcystin-LR contributes to 48.4% (out of 65% total) mortality, demonstrating the difference of 18% between the 2°C range. An 18% increase in percent mortality due to just microcystin-LR is significant because it shows that while the actual concentration of 26.3 µg/L did not change as temperature increased from 25°C to 27°C, the resulting toxicity of microcystin-LR to *B. longirostris* increased. Between 25°C and 27°C,

microcystin-LR may be more toxic to *B. longirostris* than at any other temperature in this range. The percent mortalities attributed to microcystin-LR are significantly different from each other between 25°C and 27°C (Fisher's LSD, $p<0.05$).

As temperatures rose between 27°C and 35°C, the percent mortality due to microcystin-LR alone actually decreased, demonstrating that increasing temperature had a negative result on microcystin-LR mortality to *B. longirostris* (Figure 6). This suggests at temperatures above 27°C, the effect of microcystin-LR will be less of a contributing factor to *B. longirostris* total percent mortality compared to the effect of temperature, and ultimately may be less toxic.

Lower temperature range

When the experiment was repeated over a lower temperature range (15°C-25°C), with *B. longirostris* collected in April of 2017, total mortality increased with temperature as expected (Figure 5) The difference in percent mortality between treatments was again significantly explained by the difference in temperatures (ANOVA, $F_{4,37}=85.37$, $p<0.0001$). The initial hypothesis that mortality would increase with temperature when *B. longirostris* was exposed to microcystin-LR at 26.3 µg/L was supported by the results of the lower range temperature experiment. The ANOVA model explained 90% of the variability in *B. longirostris* mortality to temperature ($R^2= 0.90$). Total mortality increased by 83% over the 10°C range from 15°C to 25°C (Figure 5). Between 15°C and 17°C, total mortality was low, but then increased approximately 60% over 3°C from 17°C to 20°C (Figure 5). An analysis of covariance (ANCOVA) confirmed that the interaction between the effects of temperature and microcystin-LR was statistically significant. The presence or absence of microcystin-LR significantly affected the mortality of *B. longirostris* at each lower temperature treatment ($F_{1,83} = 92.48$, $p <0.0001$).

While the effects of temperature and microcystin-LR would be coexisting factors in the environment, a separation of the effects of temperature and microcystin-LR (at 26.3 µg/L) on total mortality revealed that temperature exacerbated the effects of microcystin-LR on *B. longirostris* mortality. Microcystin-LR became more toxic to *B. longirostris* as temperatures increased from 15°C to 25°C (Figure 7). A one-way ANOVA revealed that the difference in percent mortality due to microcystin-LR (26.3 µg/L) was significantly explained by the difference in temperature ($F_{4,37}$=50.16, p<0.0001). The model explained 84% of the variability in microcystin-LR derived mortality to temperature (R^2=0.84). At lower temperatures, 15°C and 17°C, the effect of microcystin-LR decreased, contributing to 11% and 19%, respectively (Figure 7). However, a significant increase in microcystin-LR percent mortality is seen at 20°C (Fisher's LSD: p<0.05). Between 17°C and 20°C, the percent mortality due to microcystin-LR alone increases by 56%, indicating an increase in toxicity while the actual microcystin-LR concentration remained constant at 26.3 µg/L. Mortality due to microcystin-LR continues to increase with temperature, reaching 83% at 25°C (Figure 7). This increase in percent mortality due to microcystin-LR of nearly 72% over the temperature range of 15°C to 25°C showed that while the concentration of 26.3 µg/L remained constant, the toxicity of microcystin-LR to *B. longirostris* increaseed with temperature over this range.

The 25°C treatment from the lower range study; however, was not comparable to the 25°C from the higher temperature range study. This suggests a seasonal effect of *B. longirostris* when collected during April compared to June of 2016. While total percent mortality was lower at 15°C and 17°C, total mortality increased nearly 60% over 3°C between 17°C and 20°C (Figure 5). At 25°C, total mortality was 97.7%. This is approximately 33% higher than the total mortality for 25°C in the higher temperature range experiment: 25°C to 35°C (Figure 4). Because

of this significant discrepancy between the 25°C treatments (Fisher's LSD, $p<0.05$), it is suggested that the *B. longirostris* collected in June of 2016 for the higher range study may be seasonally adapted to microcystin-LR. June zooplankton were collected from the Chowan River when *M. aeruginosa* blooms are present, compared to those collected in April for the lower range study, which could explain the lower total percent mortality at 25°C because they were more adapted.

DISCUSSION

The hypothesis that increasing temperatures increases the mortality *B. longirostris* when exposed to microcystin-LR was supported by results of the temperature range experiments. This study demonstrated that a 2°C change in temperature between 25°C and 27°C increased the toxicity of microcystin-LR at 26.3 µg/L, resulting in an 18% increase in mortality of *B. longirostris.* In this case, the microcystin-LR concentration of 26.3 µg/L remained constant, but its toxicity to *B. longirostris* increased. This is also shown between 15°C and 25°C, when mortality due to microcystin-LR increased 72%, demonstrating how microcystin-LR toxicity increased with temperature. In both temperature range experiments, the concentration of microcystin-LR of 26.3 µg/L to which *B. longirostris* were exposed remained unchanged throughout the duration of the experiment, while the toxicity of microcystin-LR to *B. longirostris* increased. This is significant because it showed at higher temperatures, that a 2°C change will lead to increased *B. longirostris* mortality even when the microcystin concentration stayed the same.

Seasonal adaptation to microcystin-LR by *B. longirostris* may also affect the ability of the zooplankton to survive microcystin-LR at higher temperatures based on the results of this study where zooplankton collected in April of 2017 had higher mortalities than those collected in

June of 2016. *M. aeruginosa* blooms are only seasonally in the Chowan River environment during the summer months of June through September (Moorman et al. 2017). Zooplankton abundant in the Chowan River during these months may have significant tolerance to toxins produced from *M. aeruginosa* blooms, because they have been previously exposed to microcystin-LR during a toxic bloom and are adapted to higher summer temperatures over zooplankton abundant before the blooms become prevalent. Majority of zooplankton abundant seasonally in the spring may not be as resistant to microcystin-LR as zooplankton abundant during the summer *M. aeruginosa* bloom months, because they are not adapted to the presence of microcystin-LR and warmer temperatures. This could then explain the laboratory results where the June 2016 *B. longirostris* population was less susceptible to the microcystin-LR toxin at 25°C compared to the April 2017 population, in which a 33% increase in mortality was observed.

B. longirostris may also show seasonal adaption to microcystin-LR as it becomes prevalent in the environment with warming temperatures. *B. longirostris* from the low range temperature experiment (15°C to 25°C) were collected during April 2017, where temperatures average 17.9°C (Figure 8, Figure 9C,D) and *M. aeruginosa* blooms are not yet prevalent in the environment (Witherspoon et al. 1979). *B. longirostris* used for the high temperature range experiment, in contrast were collected in June 2016, where temperatures average 27.3°C (Figure 8, Figure 9A,B) and *M. aeruginosa* blooms are prevalent (Witherspoon et al. 1979). The inability of *B. longirostris* collected in April to resist microcystin-LR (at 26.3 µg/L) at temperatures above 17°C, but the ability of those collected in June to survive by 50% at 25°C perhaps demonstrates seasonal adaptation against microcystin-LR in the environment (Figure 9). April 2017 *B. longirostris* are better adapted to lower temperatures near 17°C and an environment free of

microcystin-LR (Figure 9C). When they were exposed to microcystin-LR (at 26.3 μg/L) at temperatures above 17°C, they were unable to survive due to the presence of microcystin-LR (Figure 9D). Those zooplankton collected in June 2016; however, have been previously exposed to microcystin-LR toxins from preceding *M. aeruginosa* blooms within the Chowan River and are already conditioned to higher temperatures (Figure 9A,B). This could help explain the discrepancy between the 25°C treatments from the low and high temperature experiments (Figure 9B,D). June 2016 zooplankton exposed to microcystin-LR at 25°C only experienced 48.4% mortality due to microcystin-LR, whereas April zooplankton experienced 83% mortality due to microcystin-LR because they lacked the seasonal tolerance stemming from a warmer environment and adaptation to microcystins (Figure 9B,D). Because higher temperatures increase the mortality of *B. longirostris* to microcystin-LR, rising temperatures as a result of climate change, in conjunction with high microcystin-LR concentration *M. aeruginosa* blooms above typical concentrations, could lead to the removal of *B. longirostris* in the Chowan River.

Several other studies have looked at the effect of temperature and toxic *M. aeruginosa* cells on zooplankton mortality, but none have effectively described the interaction between temperature, microcystin-LR, and *B. longirostris.* Hietala, Laurén-Määttä, and Walls exposed *D. pulex*, a larger-bodied cladoceran related to *B. longirostris,* to microcystin-LR at two different temperatures: 19°C and 24°C (1997). *D. pulex* showed increased mortality to microcystin-LR at higher temperatures (Hietala, Laurén-Määttä, and Walls 1997), similar to *B. longirostris* in this study. Additionally, estimates of EC_{50} (effective concentration in which 50% of zooplankton had an inability to move) decreased with increasing temperature at 24°C, demonstrating that microcystin-LR became more toxic to *D. pulex* (Hietala, Laurén-Määttä, and Walls 1997). Similarly, exposure of rotifers to *M. aeruginosa* cells at different temperatures shows that as

temperature and concentration of cells increase, survivability and reproduction of rotifers decrease (Zhang and Geng 2012). Increasing temperatures therefore increase the sensitivity of rotifers to *M. aeruginosa* cells (Zhang and Geng 2012). It is important then that studies involving temperature change and *M. aeruginosa* toxins, like microcystin-LR, be conducted for various zooplankton species in order to understand how each species' mortality differs when exposed to a constant toxin concentration over a range of temperatures.

In a competition study between cladocerans *B. longirostris* and *D. pulex* over different temperatures, Jiang et al. (2014) estimates the effects of temperature on toxicity and found that *B. longirostris* outcompeted *D. pulex* at 20°C and 28°C in the presence of toxic *M. aeruginosa* cells. At 20°C, *B. longirostris* exhibited a higher growth rate than at 28°C, indicating that higher temperatures may negatively affect population growth (Jiang et al. 2014). In this current study, *B. longirostris* experienced a mortality of 28% at 27°C, compared to 7.7% mortality at 20°C (Figure 4, Figure 5). With the addition of microcystin-LR (26.3 µg/L) at 27°C, *B. longirostris* mortality increased by 66.5% (Figure 4). Comparatively, during exposure to toxic *M. aeruginosa* cells, *B. longirostris* experienced a greater mortality at 28°C than at 20°C (Jiang et al. 2014). This finding is supported by the data from this study: at 27°C, *B. longirostris* had a significantly higher mortality to microcystin-LR (26.3 µg/L) than at 25°C. From 25°C to 27°C, total mortality increased by nearly 30% (Figure 4). This study demonstrates that regardless of how *B. longirostris* are exposed, whether by direct exposure to microcystin-LR or to *M. aeruginosa* cells (Jiang et al. 2014), they experience a greater mortality at higher temperatures.

A competition-based study is useful in understanding the interaction between competitor cladocerans in an environment prone to toxic *M. aeruginosa* blooms. Quantifying the toxicity of *M. aeruginosa* cells and identifying their toxins that zooplankton may be exposed to allow for

the specific effect of the interaction between temperature and cyanotoxins to be understood and allows for exact estimates of population mortality to be made.

This study builds upon previous studies and fills critical gaps in information by demonstrating the mortality of *B. longirostris* at specific temperatures during exposure to the microcystin-LR concentration of 26.3 µg/L in order to make accurate implications for the population and food web. Based on the results of this study, *B. longirostris* had an increased mortality due to higher temperatures during exposure to microcystin-LR. Toxicity of microcystin-LR to *B. longirostris* increases between 25°C and 27°C, and over the 15°C-25°C ranges. Lastly, discrepancies between total mortalities and mortality attributed to microcystin-LR at 25°C between temperature studies suggest that the season in which *B. longirostris* are collected may impact their ability to resist the microcystin-LR toxin in the environment, based on seasonal adaptation and previous exposure to the toxin and seasonal temperatures.

CHAPTER FOUR: ENVIRONMENTAL AND FOOD WEB IMPLICATIONS

B. longirostris reaches peak abundance in the upper Chowan River in June (Lichti 2014) where water temperatures average 27.3°C (Table II, Figure 8), and *M. aeruginosa* blooms are present in the environment (Table III). Under these conditions, *B. longirostris* have the greatest potential to be affected by toxic *M. aeruginosa* blooms due to the increased toxicity of microcystin-LR at 27.3°C (Figure 6). Between 25°C and 27°C, microcystin-LR at 26.3 µg/L was most toxic to *B. longirostris*, resulting in an 18% increase in mortality. At 27°C, *B. longirostris* experienced a total mortality of 94.4% due to the combined effects of microcystin-LR at 26.3 µg/L and temperature (Figure 4, Figure 9B). Results from this study suggest that under climate change microcystin-LR concentrations above 26.3 µg/L and current average temperatures of 27.3°C in June *B. longirostris* would be effectively removed from the ecosystem and the aquatic food web, as 94.4% died.

Current typical microcystin-LR concentrations in the Chowan River and surrounding Albemarle Sound only range between less than 0.1 µg/L to 2.0 µg/L (Moorman et al. 2017), which is significantly lower than the LC_{50} for *B. longirostris* of 26.3 µg/L. Based on the results of this study, between microcystin-LR concentrations of 0.1-2.0 µg/L, *B. longirostris* mortality ranges 3.6-7.1% (Figure 2). Therefore, *B. longirostris* should be able to survive typical microcystin-LR concentrations. However, water temperatures in June are already at 27.3°C. Because microcystin-LR is most toxic to *B. longirostris* between 25°C and 27°C, mortalities resulting from exposure to 0.1-2.0 µg/L blooms could still be greater because of the higher temperatures. This study has shown that temperature can exacerbate *B. longirostris* mortality due to microcystin-LR even when concentrations remain unchanged and temperature is increased (Figure 6, Figure 7). Therefore, if climate change leads to increased Chowan River water

temperatures, even a 2°C increase in temperature can increase microcystin-LR toxicity to *B. longirostris*, resulting in higher mortality.

In addition to increasing temperatures, increases in microcystin-LR concentrations from *M. aeruginosa* blooms due to climate change have negative implications for *B. longirostris.* Another high microcystin-LR *M. aeruginosa* bloom, like the 2013 68 µg/L bloom would remove 75% of *B. longirostris* from the Chowan River.

If microcystin-LR concentrations (0.1-2.0 µg/L) remain consistently low in the Chowan River, zooplankton biological rates are still temperature dependent, so small increases in temperature could impact mortality (Heinle 1969). Current summer temperatures may continue to increase due to climate change. Globally, sea surface temperature has risen by 0.78°C since 1975 (NOAA 2016). Within the Chowan River, North Carolina, average yearly water temperature has risen by 0.71°C since 1975 (Figure 8). Over a time period of 41 years, the Chowan River has experienced an average water temperature increase of 2.7°C for the months of July and August compared to averages from 1975 (Table II) For the month of August, specifically, water temperatures have increased 1.8°C (Table II, Figure 8). At peak summer temperatures of 30.1°C in August, *B. longirostris* may experience approximately 50% mortality due to temperature alone (Figure 4). Therefore, as yearly and seasonal water temperatures continue to rise within the Chowan River, *B. longirostris* may not be able to survive higher temperatures due to climate change in the future.

Because *B. longirostris* mortality increased with increasing temperatures when exposed to microcystin-LR (26.3 µg/L), temperature rise and presence of toxic *M. aeruginosa* blooms increases the potential for *B. longirostris* mortality within the Chowan River. Since 2000, the Chowan River has experienced a return of *M. aeruginosa* blooms comparable in frequency of

occurrence to blooms from 1974 (Table III, Figure 10). *Microcystis* has been especially prevalent from 2000 to 2005 and more recently between 2011-2016 (Figure 10, Figure 11). Of all the cyanobacteria blooms seen in the Chowan River since 2000, 50% of the genus that could have produced cyanotoxins and were large, high-density blooms (Moorman et al. 2017). Similarly, in comparison to the *M. aeruginosa* blooms experienced in the Chowan River between 1975 and 1976, as the density of the total bloom biomass increased, so did the biomass attributed to *M. aeruginosa* cells (Figure 12). Density of *Microcystis* blooms in recent years has also been high (Figure 13). Return of *M. aeruginosa* blooms similar in frequency and cell density, therefore suggests that the eutrophic conditions favorable to blooms in 1975 may be present in today's river system. Changes in land usage from forestry to agricultural, combined with urbanization, might be contributing factors to *M. aeruginosa* bloom reoccurrence. If current water quality conditions are contributing to the prevalence of *M. aeruginosa* blooms, continual rises in river water temperature and higher microcystin-LR concentration blooms above 26.3 µg/L due to climate change may negatively affect aquatic life, like zooplankton *B. longirostris,* in the future.

The results of this study suggest that *B. longirostris* may be able to adapt seasonally to toxic *M. aeruginosa* blooms in the Chowan River if these zooplankton are abundant when blooms are prevalent—resulting in decreased mortality compared to zooplankton that may not have been previously exposed to these toxins (Figure 9). Toxic *M. aeruginosa* blooms are present in the Chowan River from June until September (NCDEQ 2015, Moorman et al. 2017). *B. longirostris* abundant during these months would be exposed to microcystin-LR toxins and could show the seasonal adaptation that was seen in the higher temperature range experiments: where *B. longirostris* collected during June of 2016 had significantly lower mortality at 25°C when exposed to 26.3 µg/L of microcystin-LR than those collected in April of 2017. Again, *B.*

longirostris collected in April of 2017 would not have been seasonally adapted to the presence of microcystin-LR and as a result, had higher mortalities when exposed to increasing temperature and microcystin-LR. The ability of *B. longirostris* collected in June to be more tolerant to microcystin-LR toxins over *B. longirostris* collected during non-bloom seasons (April) suggests the potential of these zooplankton to adapt to toxic *M. aeruginosa* blooms (of 26.3 µg/L). It is possible that *B. longirostris* could continue to adapt to higher microcystin-LR toxins from future *M. aeruginosa* blooms above 26.3 µg/L, but as future temperatures in the Chowan River approach their thermal tolerance they would not be able to survive.

Because *B. longirostris* will likely not survive high microcystin-LR *M. aeruginosa* blooms above 26.3 µg/L at 27.3°C, higher microcystin-LR blooms of 68 µg/L, or adapt to temperatures above 30°C, return of toxic *M. aeruginosa* blooms and continuation of those at higher toxin concentrations, along with future temperature increases in the Chowan River should effectively remove *B. longirostris* from the aquatic food web. These results have negative implications for the Chowan River food web during *M. aeruginosa* blooms of high microcystin-LR concentration above 26.3 µg/L for the existing zooplankton community and the fish reliant upon them as a food source.

Food web implications

Implications for the Chowan River food web under a *M. aeruginosa* bloom can be predicted under four different scenarios which encompass current Chowan River conditions and future climate change scenarios for what the Chowan River may experience, in which temperature and microcystin-LR concentrations will likely increase. The four scenarios are: current Chowan River conditions (microcystin-LR concentrations 0.1-2.0 µg/L and temperatures 27.3°C), future Chowan River conditions due to climate change where water temperatures do not

increase, but microcystin-LR concentrations increase above 26.3 µg/L, current microcystin-LR concentrations (0.1-2.0 µg/L) and climate change temperatures (30°C), and lastly future Chowan River conditions with increased microcystin-LR concentrations and temperatures (>26.3 µg/L and 30°C). Under these scenarios, the zooplankton community within the Chowan River is affected, which leads to changes at higher trophic levels for planktivorous fish and ultimately humans. Understanding the fate of *B. longirostris* during these scenarios is important for the Chowan River food web and *M. aeruginosa* bloom management aspects under four different conditions in which microcystin-LR and temperature interact now and in the future due to climate change.

During a cyanobacteria bloom zooplankton community tends to shift from large-bodied cladocerans to small-bodied cladocerans, copepods, and rotifers (Benndorf and Herring 1989, Fulton and Paerl 1988b). Within the Chowan River these three groups make up the dominant zooplankton from April until June: small cladocerans *Bosmina* spp., calanoid copepods, and rotifers (Lichti 2014). *B. longirostris* and rotifers are ubiquitously distributed throughout the Chowan River and have demonstrated survival to *M. aeruginosa* cells and microcystin-LR toxins (Fulton and Paerl 1988, 1987a, Fulton 1988b, Fulton and Paerl 1987b). Additionally, rotifers and *B. longirostris* are thought to get some nutritional benefits from low concentrations of *M. aeruginosa* cells (Huang et al. 2012, Fulton and Paerl 1987b). Calanoid copepods and nauplii are ubiquitously distributed in April and May, but adults are restricted to the lower river and Albemarle Sound in June (Lichti 2014). Calanoid copepods, in contrast to rotifers and *Bosmina* spp., are known for their chemosensory avoidance of toxic *M. aeruginosa* cells (Fulton and Paerl 1987b, Fulton and Paerl 1987a, Fulton 1988a, Fulton, Rolland, and Paerl 1988). *B. longirostris,*

copepods, and rotifers therefore are the available prey source for zooplanktivorous fish in the Chowan River environment.

Chowan River fish that are dependent upon a planktivorous food source include river herring, American shad, and Black crappie (Binion 2011, Mullen, Fay, and Moring 1986, Winslow, Mozley, and Rulifson 1985, Haskell, Tiffan, and Rondorf 2013). Juvenile Black crappie (*Pomoxis nigromaculatus)* and American shad (*Alosa sapidissima*) both rely on a zooplankton diet consisting of *Daphnia* spp. and copepods (Pope and Willis 1998, Haskell, Tiffan, and Rondorf 2013), but American shad have been also known to consume *Bosmina* spp., and rotifers (Binion 2011). River herring Alewife (*Alosa pseudoharengus*) and Blueback herring (*Alosa aestivalis)* rely either as juveniles or as adults, on small cladocerans like *B. longirostris*, copepods, and rotifers (Binion 2011, Mullen, Fay, and Moring 1986, Winslow, Mozley, and Rulifson 1985, Leech and Piehler 2009).

Blueback herring and Alewives are two historically important river herring for the Chowan River, North Carolina. Recent stock reports on both river herring species show that stocks are depleted in the Chowan River and Albemarle Sound areas; with stock reports along the east coast reaching historic lows (NCDMF 2014). The North Carolina Division of Marine Fisheries links this decline for these species to poor water quality, as well as other contributing factors including temperature rise (2014). Fisheries stock status reports in North Carolina for Black crappie and American shad list the stocks as viable and of concern, respectively (NCDMF 2016). This is especially important for humans who rely on these fisheries for recreation, food, or as a source of income.

Cyanobacteria blooms have been shown to significantly affect river herring size, resulting in significantly smaller fish in the Chowan River compared to nearby rivers (Winslow, Mozley,

and Rulifson 1985). It was concluded that this reduction in size may be a reflection of low food abundance, since zooplankton abundance shifts towards larger bodied cladocerans, rather than smaller bodied during a cyanobacteria bloom (Winslow, Mozley, and Rulifson 1985). However, this has been widely contested in the literature, and it is contrarily suggested that dominance shifts away from large bodied cladocerans to small bodied, copepods, and rotifers (Benndorf and Herring 1989, Fulton and Paerl 1988b). This shift is important because it shows that only certain zooplankton would be available as prey during a cyanobacteria bloom, like *M. aeruginosa* blooms in the Chowan River. Potential reduction in prey has negative implications for river herring, Black crappie, and American shad in the Chowan River, and in turn humans that rely upon them, when cyanobacteria blooms are prevalent in the environment.

Current concentrations and temperatures

Current *M. aeruginosa* bloom microcystin-LR concentrations in the Chowan River can be defined as typical concentrations ranging from 0.1 µg/L to 2.0 µg/L. Current temperatures in the Chowan River are the average water temperature for June at 27.3°C because this is the temperature when *B. longirostris* are dominant in the environment and *M. aeruginosa* blooms are present. This scenario represents the present conditions in the Chowan River without nutrient loading. Under this scenario where typical concentrations of microcystin-LR remain constant in the environment at 0.1-2.0 µg/L and temperatures remain at 27.3°C, the following implications for the Chowan River food web can be predicted.

An analysis of the LC_{50} for microcystin-LR toxin to these three zooplankton groups reveals greatest survival by rotifers, then copepods, and lastly *B. longirostris*. Rotifers have a demonstrated LC_{50} of 124,870 µg/L for 24 hours (Chen et al. 2001). In sharp contrast, copepod species *Diaptomus birgei* and *Eurytemora affinis* have an LC_{50} range of 270-520 µg/L for 48

hours (Reinikainen et al. 2002, DeMott, Zhang, and Carmichael 1991, Ger, Teh, and Goldman 2009). This study showed that *B. longirostris* had an LC_{50} of 26.3 µg/L for 48 hours. Based on these LC_{50} values, all three zooplankton groups would be able to survive current *M. aeruginosa* bloom microcystin-LR concentrations in the Chowan River (0.1-2.0 µg/L) because all of their LC_{50} values exceed this range.

Even though each zooplankton group is able to survive under current microcystin-LR concentrations, this survival could be affected by each zooplankton group's thermal tolerance range. *B. longirostris* is able to tolerate temperatures from 11-36°C (Verbitsky, Verbitskaya, and Malysheva 2009). Rotifer *Brachionus calyciflorus* tolerates similar temperatures ranging 8-35°C (Galkovskaja 1987) and copepod *E. affinis* tolerates temperatures 0-30°C, but has an optimal temperature range from 10-15°C (Bradley 1975). If temperatures in the Chowan River remain constant at 27.3°C, *B. longirostris* and rotifers would be able to survive this temperature, but copepod species would be greatly stressed and would likely not survive at this temperature because it exceeds their optimal thermal tolerance. Therefore, under the scenario in which temperatures and microcystin-LR concentrations remain at current conditions: 27.3°C and 0.1-2.0 µg/L, respectively, rotifers and *B. longirostris* are the survivng prey available for fish consumption.

Removal of copepods from the diet of planktivorous fish in the Chowan River may negatively affect Black crappie, which do not consume rotifers or cladocerans. American shad and river herring both rely upon copepods as well as part of their diets along with rotifers and *Bosmina* spp. However, because rotifers and *B. longirostris* would be available as prey during this scenario, American shad and river herring would have sufficient alternative sources of prey. Therefore, only Black crappie fisheries would be negatively affected within the Chowan River

and would suffer due to insuffient food source. Bioaccumulation of microcystin-LR in planktivorous fish is still a risk to humans under this scenario because both rotifers and *B. longirostris* are able to survive in this scenario and pass microcystin-LR up to higher trophic levels via planktivorous fish American shad and river herring.

Climate change concentrations and current temperatures

Climate change microcystin-LR concentrations refer to concentrations above *B. longirostris* LC_{50} of 26.3 µg/L because more than 50% of the zooplankton species would die. An example of climate change microcystin-LR concentrations would be the 2013 *M. aeruginosa* bloom in the Chowan River that produced a microcystin-LR concentration of 68 µg/L. Current temperatures are the average water temperature for June of 27.3°C. In this scenario, current Chowan River water temperatures remain constant at 27.3°C, but microcystin-LR concentrations increase. This scenario represents increased nutrient loading into the Chowan River generating high microcystin-LR concentration *M. aeruginosa* blooms, which have been seen in the Chowan River already.

As *M. aeruginosa* blooms become more frequent and produce higher concentrations of microcystin-LR due to climate change, the zooplankton available to fish species within the Chowan River could change. In a climate change situation where there is a *M. aeruginosa* bloom that produces high concentrations of microcystin-LR, like 68 µg/L from the 2013 bloom, only certain zooplankton groups will be able to tolerate these conditions. While *B. longirostris* and rotifers may be able to feed on the *M. aeruginosa* cells as a nutrient source, only rotifers would be able to survive such high microcystin-LR concentrations due to their high LC_{50} value of 124,870 µg/L for 24 hours (Chen et al. 2001). Majority of *B. longirostris* only survive microcystin-LR concentrations equivalent to their LC_{50} concentration of 26.3 µg/L or lower, and

therefore would die under climate change microcystin-LR concentrations. Because copepods actively avoid *M. aeruginosa* cells, they would most likely starve to death from a lack of food source even though they could in theory survive microcystin-LR concentrations up to their LC_{50} range of 270-520 µg/L (Reinikainen et al. 2002, DeMott, Zhang, and Carmichael 1991, Ger, Teh, and Goldman 2009). Additionally, copepods would likely not tolerate current water temperatures of 27.3°C. Therefore, under high microcystin-LR concentration *M. aeruginosa* blooms (>26.3 µg/L) and current temperature conditions (27.3°C), rotifers would be the only surviving prey source for planktivorous fish in the Chowan River.

Because rotifers are the only surviving source of prey, all dominant Chowan River fish (Black crappie, river herring, and American shad) would be negatively affected because of a loss of copepods and *B. longirostris* from their diet. Black crappie would be especially affected because it does not consume rotifers and would likely starve from no source of prey under this scenario. American shad and river herring would compete against one another for access to rotifers for food, leading to further decline of these species in the Chowan River from their current status of concern and depleted, respectively (NCDMF 2016).

Because the majority of copepods and *B. longirostris* would die under climate change conditions, the risk for bioaccumulation is significantly decreased because microcystin-LR would not be able to pass up to higher trophic levels in the food web through these zooplankton groups. Therefore, removal of this trophic link by copepods and *B. longirostris,* would prevent bioaccumulation of microcystin-LR in planktivorous fish, which in turn could be passed on to humans. On the other hand, some microcystin-LR may be passed on to planktivorous fish from rotifers. There are no experimental studies in the literature, though, that examine the potential for

rotifers to bioaccumulate microcystin-LR (Ferrão-Filho and Kozlowsky-Suzuki 2011). Therefore, the extend of this potential bioaccumulation in the food web can only be speculated.

Current concentrations and climate change temperatures

This scenario represents a rise in river water temperature due to climate change in the future without additional nutrient loading into the Chowan River creating high toxin concentration algal blooms. Current microcystin-LR concentrations refer to typical concentrations seen in the Chowan River of 0.1-2.0 µg/L. Climate change temperatures refer to water temperatures above the average for June of 27.3°C. A climate change temperature of 30°C would be an appropriate temperature for climate change within the Chowan River in future decades. This study showed that only a few degrees celcius rise in water temperature negatively impacted *B. longirostris* survival and resulted in higher mortalities (Figure 4, Figure 5). Zooplankton biological rates are temperature dependent (Heinle 1969), so other zooplankton groups could be negatively affected under climate change temepratures of 30°C in the Chowan River. Under this scenario, typical microcystin-LR concentrations remain constant at 0.1-2.0 µg/L, but water temperatures in the Chowan River increase to 30°C. Therefore, some *M. aeruginosa* blooms would have low microcystin-LR concentrations, even though water temperature has risen.

If microcystin-LR concentrations remain at 0.1-2.0 µg/L within the Chowan River, all zooplankton groups would be able to survive these concentrations because their LC_{50} values are greater than this range. *B. longirostris* and copepods have an LC_{50} value of 26.3 µg/L and 270-520 µg/L (Reinikainen et al. 2002, DeMott, Zhang, and Carmichael 1991, Ger, Teh, and Goldman 2009), respectively for 48 hours. Rotifers have an LC_{50} value of 124,870 µg/L for 24 hours (Chen et al. 2001).

While all zooplankton groups survive current microcystin-LR concentrations, increasing Chowan River water temperatures to 30°C negatively affects some of these zooplankton species. At 30°C, *B. longirostris* has a mortality of nearly 50% (Figure 4). Rotifers are able to survive temperatures up to 35°C (Galkovskaja 1987), but the 30°C climate change temperture would be the limit of copepods thermal tolerance and well above their and optimal temperature range of 10-15°C (Bradley 1975). It can be expected, then under this scenario, that only rotifers and *B. longirostris* would be able to survive 30°C temperatures and microcystin-LR concentrations of 0.1-2.0 µg/L conditions within the Chowan River.

Again, Black crappie are most affected without copepods in their diet, but river herring and American shad should be able to sustain off of rotifers and *B. longirostris* zooplankton alone. Because 50% of *B. longirostris* would die due to higher climate change temepratures of 30°C, they would be a lesser part of river herring and American shad diet. Rotifers are still able to survive; however, and could also act as an alternative source of food for these fish species. Black crappie, which subsist on only copepods in the Chowan River, because *Daphinia* spp. are not present in dominant numbers, would starve and stocks would plumet.

Because bioaccumulation risk increases with increasing temperatures (Ferrão-Filho and Kozlowsky-Suzuki 2011), this risk could become more serious as water temperatures warm due to climate change. Rotifers and *B. longirostris* are able to tolerate waters up to 35°C (Galkovskaja 1987) and 36°C (Verbitsky, Verbitskaya, and Malysheva 2009), respectively, so they would be able to transfer microcystin-LR via bioaccumulation to planktivorous fish in warmer Chowan River waters of 30°C. Under this scenario, risk of bioaccumulation of microcystin-LR at higher water temperatures to humans is significant.

Climate change concentrations and temperatures

This scenario represents several decades into the future of the Chowan River if nutrient loading is increased and water temperatures are higher from climate change. Climate change microcystin-LR concentrations can be defined as concentrations above *B. longirostris* LC_{50} value of 26.3 µg/L, such as the 68 µg/L microcystin-LR concentration bloom in 2013. Climate change temperatures, as defined above, refer to water temperatures in the Chowan River of 30°C. Under this last scenario, both microcystin-LR concentrations from *M. aeruginosa* blooms and water temperatures increase above current conditions due to climate change. This scenario predicts food web implications in the Chowan River under the most extreme circumstances in the Chowan River during a toxic *M. aeruginosa* bloom and warmer temperatures.

Under climate change conditions, where microcystin-LR concentrations are above 26.3 µg/L and water temperatures are at 30°C, the following implications for the Chowan River food web can be predicted. Majority of *B. longirostris* die at microcystin-LR concentrations greater than 26.3 µg/L, their LC_{50}. Additonally, only 50% of *B. longirostris* surivive at 30°C. Therefore, the combination of higher temperatures and microcystin-LR concentrations effectively removes *B. longirostris* from the food web. Copepods are able to surive microcystin-LR concentrations above 26.3 µg/L up to their LC_{50} range of 270-520 µg/L (Reinikainen et al. 2002, DeMott, Zhang, and Carmichael 1991, Ger, Teh, and Goldman 2009), but would be stressed at temperatures of 30°C, which is far above their optimal temperature range. Therefore, under climate change temperatures and microcystin-LR concentrations, copepods would be eliminated because of their inability to survive at 30°C. Rotifers tolerate temepratures up to 35°C (Galkovskaja 1987) and have an LC_{50} value of of 124,870 µg/L for 24 hours (Chen et al. 2001). Because of this, rotifers are the only zooplankton group with the ability to survive both higher microcystin-LR concentrations from future *M. aeruginosa* blooms and higher temperatures

within the Chowan River, and therefore are the only zooplankton group available as a food source for planktivorous fish under climate change scenarios.

If toxic *M. aeruginosa* blooms continue and increase in microcystin-LR content above 26.3 µg/L and water temperatures increase to 30°C for June, river herring, American shad, and Black crappie would be without a significant food source with rotifers as the only available prey. Competition between American shad and river herring for rotifers would lead to starvation of larval and juvenile fish, resulting in less juveniles reaching maturity and hindered growth rates. This would ultimately lead to further reduction of river herring and American shad stocks, which are listed as depleted and of concern in the Chowan River (NCDMF 2016). Black crappie consume copepods within the Chowan River, which would be unavailable as a food source for this fish species. Black crappie would likely starve to death under this climate change scenario. Removal of two out of the three dominant zooplankton groups from the food web has negative implications for depleted Chowan River fisheries.

Under this climate change scenario of microcystin-LR concentrations above 26.3 µg/L and water temperatures at 30°C, risk of bioaccumulation is low to humans because river herring, American shad, and Black crappie would starve to death competing for rotifers as the only available food source and mature stocks would be exhausted. While risk of bioaccumulation of microcystin-LR is low to humans, the depletion of major fisheries in the Chowan River due to climate change microcystin-LR concentrations and temperatures poses a greater threat to the livelihood of people dependent upon these fisheries.

These four scenarios depict the interaction of zooplankton, fish, and humans when exposed to changing microcystin-LR concentrations produced from *M. aeruginosa* blooms and changing temperatures in the Chowan River as a result of climate change. Climate change

microcystin-LR concentrations and temperatures represents the most extreme and worst scenario for Chowan River fisheries, while bioaccumulation risk to humans is higher in scenarios where temperature is increased. The outcomes of each of these scenarios allows for implications to be predicted for the Chowan River food web now and in the future so that management strategies can be implemented to mitigate the negative consequences of climate change.

Bloom management and strategies

The reoccurrence of toxic *M. aeruginosa* blooms in the Chowan River over recent years and the increased production of microcystin-LR with increasing temperatures call for strategic management and prevention of future blooms. *M. aeruginosa* blooms occur under highly eutrophic conditions of high nitrogen and phosphorous content, and high temperatures (Paerl et al. 2001, Christian, Bryant Jr, and Stanley 1986). Current management practices include the application of ultra-filtration and ozone to filter out microcystin-LR (Hitzfeld et al. 2000). This is an effective removal strategy for drinking water, but may not be possible for filtration of an entire river system. Therefore other management strategies for cyanobacteria blooms need to be investigated.

Other management options prevent or reduce the eutrophic conditions conducive to cyanobacteria blooms in the Chowan River. The immediate watershed of the Chowan River consists of mostly land used for agricultural purposes. Nutrient or waste runoff from farmland could be supplying the nitrogen and phosphorous content needed for *M. aeruginosa* blooms to occur. Regulation of runoff from these areas could be an effective method for reducing nutrient loading into the river. Installations of holding ponds are one method typically used to prevent nutrient and waste runoff and allow them to be naturally broken down by the environment.

However, hurricanes and summer storms may be a problem for these holding ponds seasonally, leading to spill over into the Chowan River.

Finally, extension of riparian buffers, strips of forest surrounding agricultural land, could be done to decrease nutrient runoff into the Chowan River. The typical riparian buffer zone required by the North Carolina Environmental Management Commission around rivers is a minimum of 50ft of forest, with 30ft of natural vegetation and 20ft of managed vegetation (NC Conservation Network 2016). In order to reduce nutrient loading by 85% or greater; however, a riparian buffer width of 100ft to 165ft must be implemented (NC Conservation Network 2016). A nearly 50ft riparian buffer only reduces nitrogen runoff by 48%, in comparison and would help reduce nitrogen available for cyanobacteria blooms (Christian, Bryant Jr, and Stanley 1986). It is therefore, important to extend the riparian buffer zone around the Chowan River to best mitigate nutrient runoff and to prevent eutrophic conditions that could cause cyanobacteria blooms. Extension of the riparian buffer, along with other management practices of agricultural waste and runoff-holding ponds could help to prevent future cyanobacteria blooms due to eutrophic conditions. If the riparian buffer is instead reduced, nutrient runoff from surrounding agricultural land could be severe and increase cyanobacteria bloom duration, frequency, and toxicity. As Chowan River water temperatures continues to rise due to climate change, cyanobacteria blooms including *M. aeruginosa,* will only become more of a threat to the aquatic food web and action to prevent these blooms must be taken.

CONCLUSIONS

An analysis of the effect of microcystin-LR on *B. longirostris* demonstrated that mortality increased with toxin concentration. The concentration of microcystin-LR lethal to 50% of the *B. longirostris* population was determined to 26.3 µg/L. Because this LC_{50} of 26.3 µg/L is

greater than the typical Chowan River microcystin-LR concentrations of less than 0.1 µg/L to 2.0 µg/L, *B. longirostris* are expected to survive typical toxic *M. aeruginosa* bloom conditions. More than 50% of *B. longirostris* would have not been expected to survive the 2013 68 µg/L microcystin-LR *M. aeruginosa* bloom; however, because this particular bloom's concentration was above the $LC_{50.}$ Continued high microcystin-LR concentration *M. aeruginosa* blooms, resulting from temperature rise could negatively affect populations of *B. longirostris* in the Chowan River.

Temperature experiments with *B. longirostris* and microcystin-LR at 26.3 µg/L revealed that mortality increased with temperature when *B. longirostris* were exposed to a constant concentration of microcystin-LR. It was found that microcystin-LR was most toxic to April *B. longirostris* between the temperature range of 15°C-25°C and June *B. longirostris* between 25-27°C. Because the concentration of microcystin-LR at 26.3 µg/L did not change, this showed that the toxicity of microcystin-LR to *B. longirostris* did actually increase. The lower temperature range experiment demonstrated that microcystin-LR toxicity to *B. longirostris* also increased with temperature from 15°C-25°C, resulting in a 72% increase in mortality. This study additionally suggested that *B. longirostris* prevalent during *M. aeruginosa* bloom months (June-September) might be seasonally adapted to microcystin-LR at higher temperatures, compared to *B. longirostris* that were collected from non-bloom months (April). This suggestion of seasonal adaptation to microcystin-LR indicates the potential of *B. longirostris* to further adapt and survive higher concentrations in the future.

An evaluation of how temperature impacts *B. longirostris* at 26.3 µg/L allowed for food web implications to be made based on mortalities observed. Majority of *B. longirostris* populations may not be able to survive future temperature rise and high concentration

microcystin-LR in the Chowan River due to climate change. While *B. longirostris* could survive typical *M. aeruginosa* bloom concentrations (0.1-2.0 µg/L), current temperatures during peak *B. longirostris* abundance in June (27.3°C) have the potential to increase mortality from typical concentrations, because microcystin-LR is most toxic at this temperature. With the return of *M. aeruginosa* blooms to the Chowan River over recent years and temperature rises of 0.71°C since 1975, *B. longirostris* is effectively removed from the food web if temperatures and microcystin-LR concentrations continue to increase. Removal of *B. longirostris* and other zooplankton from the food web has negative implications for larval and planktivorous fish, such as river herring, that are dependent upon these zooplankton as a large part of their diet. Because *B. longirostris* are one of the dominant zooplankton in abundance within the Chowan River, removal of this significant source of prey for fish dependent on these small cladocerans puts substantial pressure on vital fisheries within the Chowan River. Return of cyanobacteria blooms, like *M. aeruginosa*, to the Chowan River, North Carolina calls for additional management strategies and prevention of eutrophic conditions, especially as environmental conditions worsen due to climate change.

FUTURE DIRECTIONS

In the future, it would be beneficial to explore the idea that *B. longirostris* could further adapt to microcystin-LR concentrations seasonally during *M. aeruginosa* blooms. To test seasonal adaption, *B. longirostris* should be collected during June, when *M. aeruginosa* blooms are prevalent in the environment and these zooplankton are at peak abundance within the Chowan River because they would be most likely have genes for microcystin-LR adaptation due to previous exposure. These *B. longirostris* could be exposed to a constant concentration of microcystin-LR for 48 hours at a constant temperature. After exposure, surviving *B. longirostris* and their offspring could then be recycled in repeat experiments at the same microcystin-LR

concentration and temperature, where they would be allowed to further reproduce. Any changes in population mortality could be observed to see if *B. longirostris* were able to adapt to microcystin-LR after several generations. Results from this study would be advantageous in determining the long-term survivorship of *B. longirostris* in the Chowan River during consecutive *M. aeruginosa* blooms of the same microcystin-LR concentration. A similar experiment could also be conducted exposing *B. longirostris* to sequentially higher microcystin-LR concentrations every 48 hours to determine their ability to adapt to consecutive *M. aeruginosa* blooms of increasing microcystin-LR concentration. This would be useful for determining *B. longirostris* response to climate change scenarios of higher microcystin-LR *M. aeruginosa* blooms within the Chowan River and its ability to adapt.

A two-factor experiment could also be conducted to demonstrate the effects of microcystin-LR over a variety of temperatures and concentrations concurrently. A range of microcystin-LR concentrations could be used pertaining to different lethal concentrations for *B. longirostris* (ie: LC_{10}, LC_{30}, LC_{50}, LC_{70}, etc.) over a range of temperatures suitable to *B. longirostris* survival (11-36°C) (Verbitsky, Verbitskaya, and Malysheva 2009). This would show how lethal concentrations of microcystin-LR change with temperature.

Lastly, future experiments could also be conducted on less common types of microcystin, such as microcystin-LA, microcystin-YR, and microcystin-RR, to see if the LC_{50} for *B. longirostris* for these congeners compares to that of microcystin-LR. Similarly, it could be worthwhile to conduct an experiment to see if increasing temperature at a constant concentration of these types of microcystin also resulted in similar increases in mortality of *B. longirostris* when exposed to microcystin-LR. This could help elucidate the effects of less common toxins

produced from *M. aeruginosa* blooms in the Chowan River on specific zooplankton as temperatures rise.

Table I: List of zooplankton species and their corresponding LC_{50} when exposed to microcystin-LR by reference. Results from this study in bold.

Zooplankton	Species	Cyanotoxin	LC50 (µg/L)	Duration	Reference
Cladoceran	***B. longirostris***	**Microcystin-LR**	**26.3**	**48 hours**	**This study**
Cladoceran	*Daphnia pulex*	Microcystin-LR	4.08	36 hrs (LT50)	Ferrão-Filho et al. 2000
Cladoceran	*D. pulex*	Microcystin-LR	2.983 (EC50)	48 hours	Hietala et al. 1997
Cladoceran	*D. pulex*	Microcystin-LR	9600	48 hours	DeMott et al. 1991
Cladoceran	*D. hyalina*	Microcystin-LR	11600	48 hours	DeMott et al. 1991
Cladoceran	*D. pulicaria*	Microcystin-LR	21400	48 hours	DeMott et al. 1991
Copepod	*E. affinis*	Microcystin-LR	1550	48 hours	Ger et al. 2009
Copepod	*P. forbesi*	Microcystin-LR	520	48 hours	Ger et al. 2009
Copepod	*E. affinis*	Microcystin-LR	270	48 hours	Reinikainen et al. 2002
Copepod	*Diaptomus birgei*	Microcystin-LR	450	48 hours	DeMott et al. 1991

Table II: Monthly average and standard deviation of water temperatures (°C) by reference for the Chowan River, NC during *M. aeruginosa* bloom months (June-September).

Reference	Witherspoon et al. 1979 1975-1976	Moorman et al. 2017 2012-2013	NCDEQ 2015-2016
June	22.4±3.1		27.3±1.3
July	25.7±0.8	30.2±0.7	28.6±0.7
August	28.3±0.5	28.2±0.1	30.1±0.7
September	23.5±0.9		
Grand Average	**24.2±3.1**	**29.6±1.1**	**28.2±1.3**

Table III: Frequency of *Microcystis* blooms by year. Data from 1974 taken from Appendix A of Witherspoon et al. 1979. Data from 2000-2016 from Moorman et al. 2017 and North Carolina Department of Environmental Quality (NCDEQ 2015), Water Resources.

Microcystis Bloom Frequency by Year		
Month	**1974**	**2000-2016**
May	6	
June	5	3
July	6	5
August	1	9
September		7
October	1	

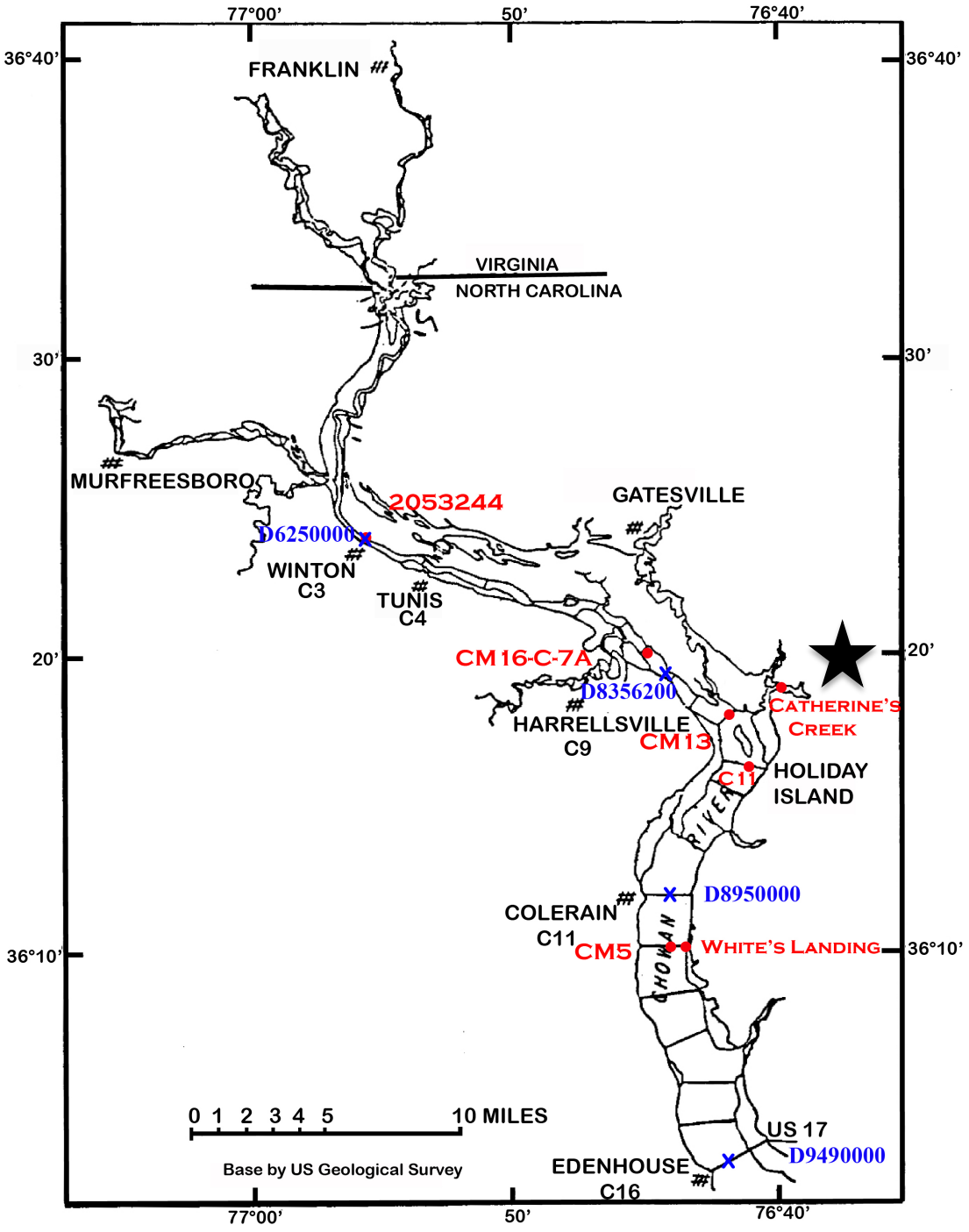

Figure 1: Sampling locations within the Chowan River, NC used by Witherspoon et al. 1979 (black), Moorman et al. 2017: 2012-2013 (red), and NCDEQ: 2015-2016 (blue). Image redrawn from Witherspoon et al. 1979. Zooplankton from this study were collected in Catherine's Creek (N36.314706, W76.670449).

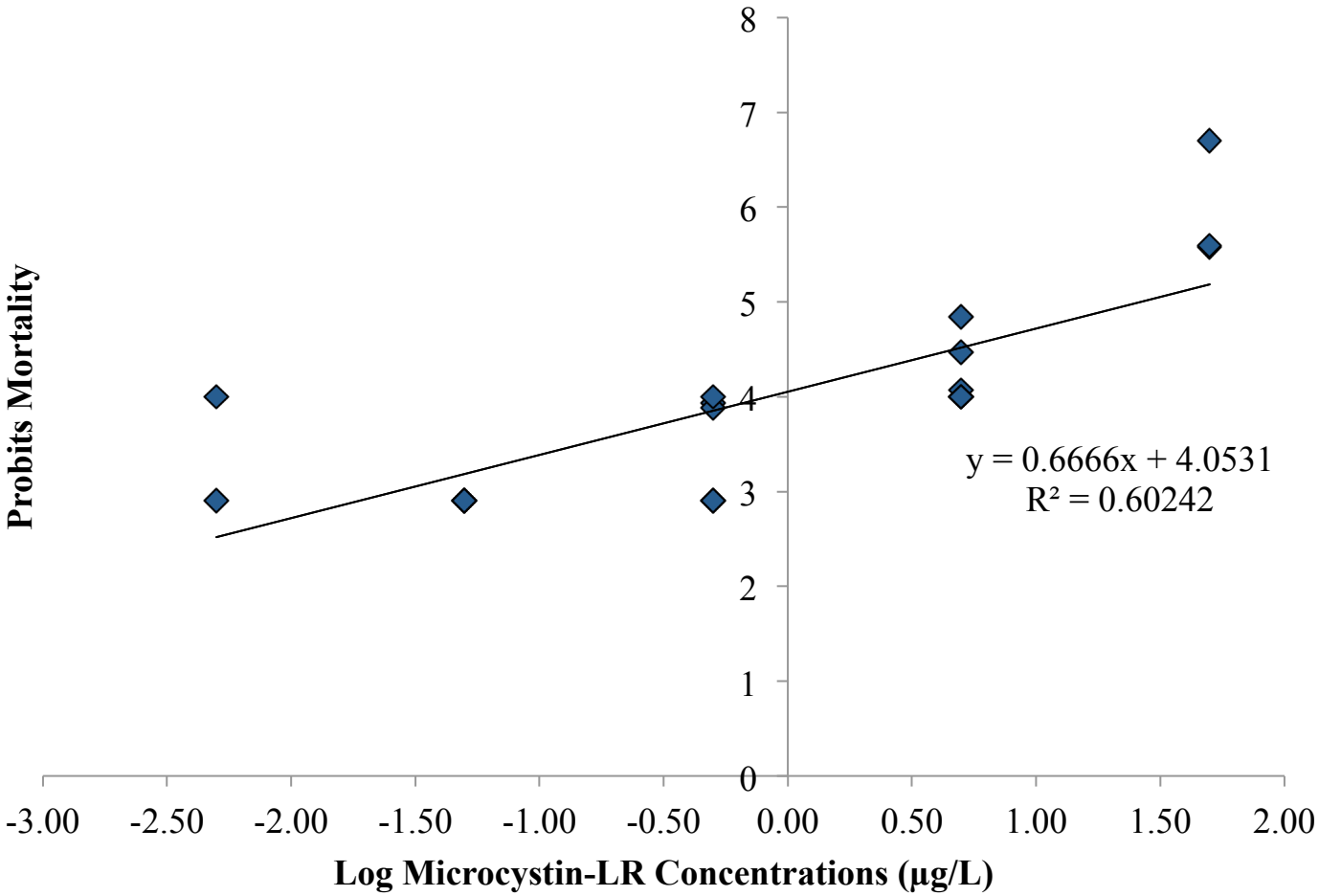

Figure 2: Probit analysis of the effect of microcystin-LR on *B. longirostris* mortality. Percent mortality converted to empirical probits versus log of the microcystin-LR concentrations based on (Finney 1952). Log-LC_{50}= 1.42, based on linear regression: y=0.6666x+4.0531. R^2=0.602. LC_{50}= 26.3±4.6 µg/L.

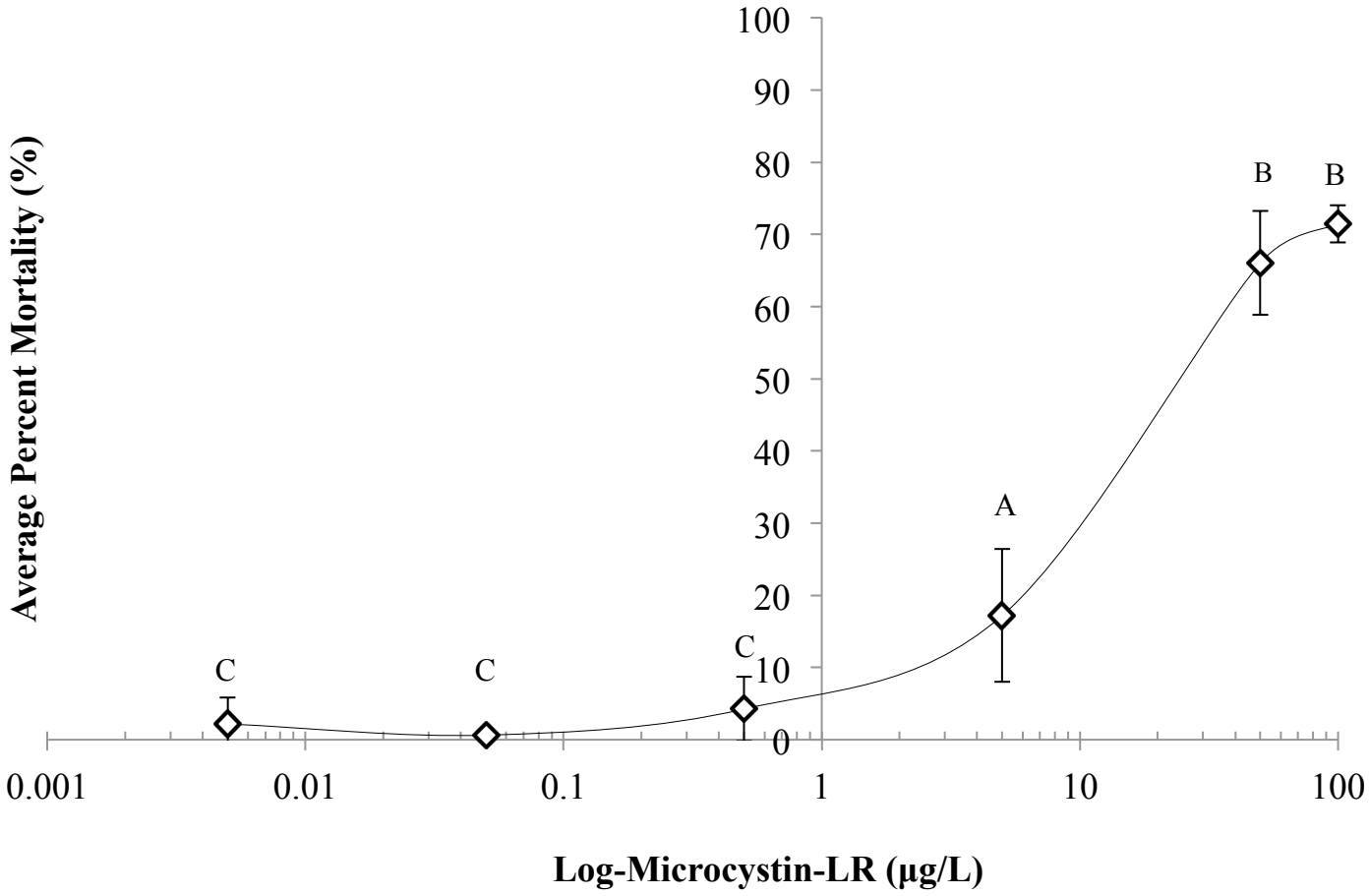

Figure 3: Average percent mortality of *B. longirostris* following a 48-hr exposure at 25°C to a large range of microcystin-LR concentrations. LC_{50} concentration calculated to be **26.3 µg/L**. Error bars represent standard deviation. Different letters indicate significant difference (p-value<0.05, Fisher's LSD). One-way ANOVA: $p<0.0001$. $F_{5,30}=139.96$. $R^2= 0.96$.

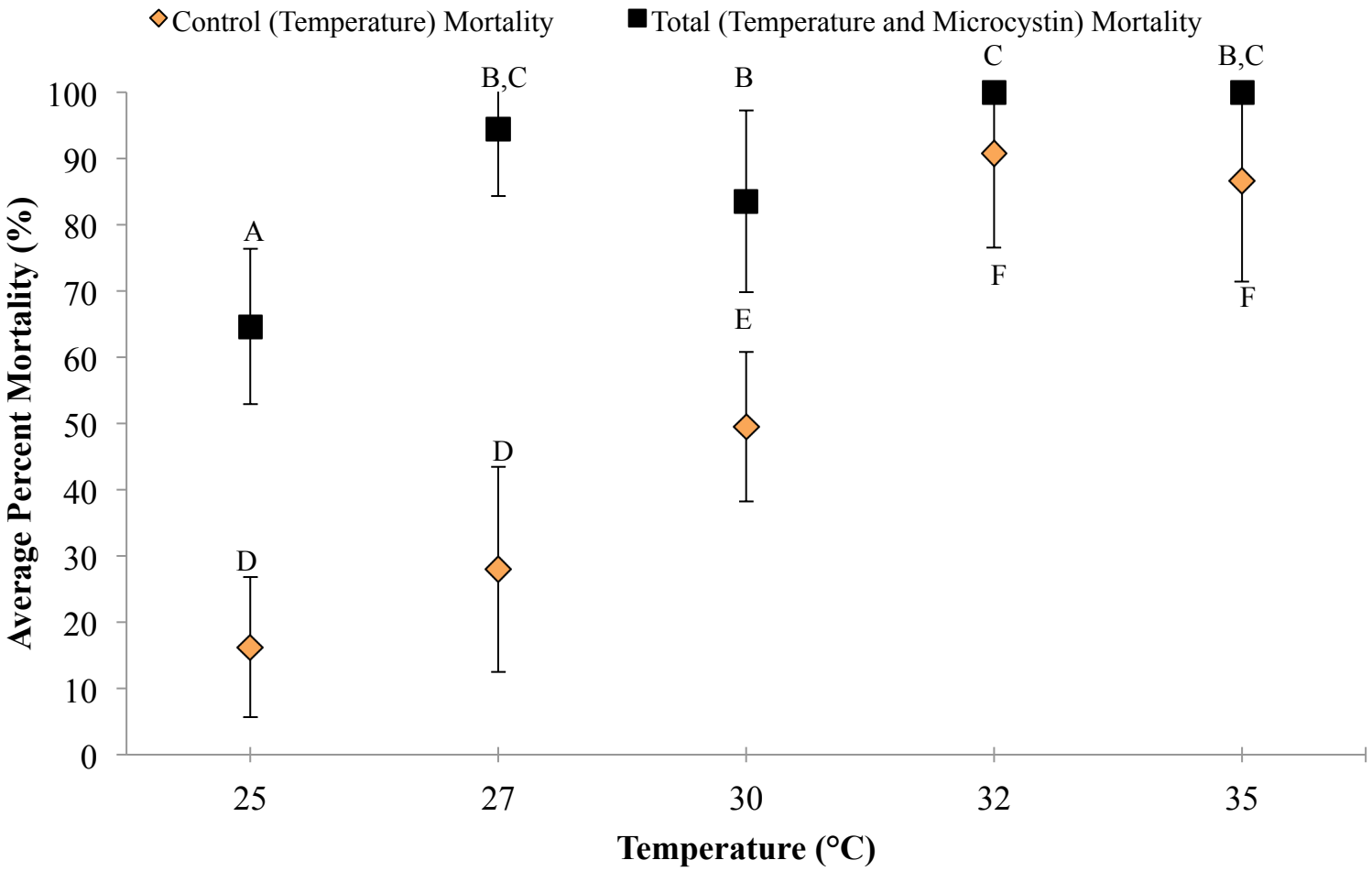

Figure 4: Average percent mortality of *B. longirostris* over a range of temperatures when exposed to a constant level of microcystin-LR: LC_{50} of 26.3 µg/L, for 48-hrs. Control treatments were without microcystin-LR. Total temperature and microcystin-LR was the combined effect of temperature and toxin on mortality. Error bars represent standard deviation. Different letters indicate significant difference ($p<0.05$, Fisher's LSD). ANCOVA: $p<0.0001$. R^2 =0.80. $F_{1,68}$= 23.18. Control one-way ANOVA: R^2= 0.84. $p<0.0001$. $F_{4,30}$ = 40.23. Total one-way ANOVA: R^2= 0.68. p <0.0001. $F_{4,30}$= 15.92.

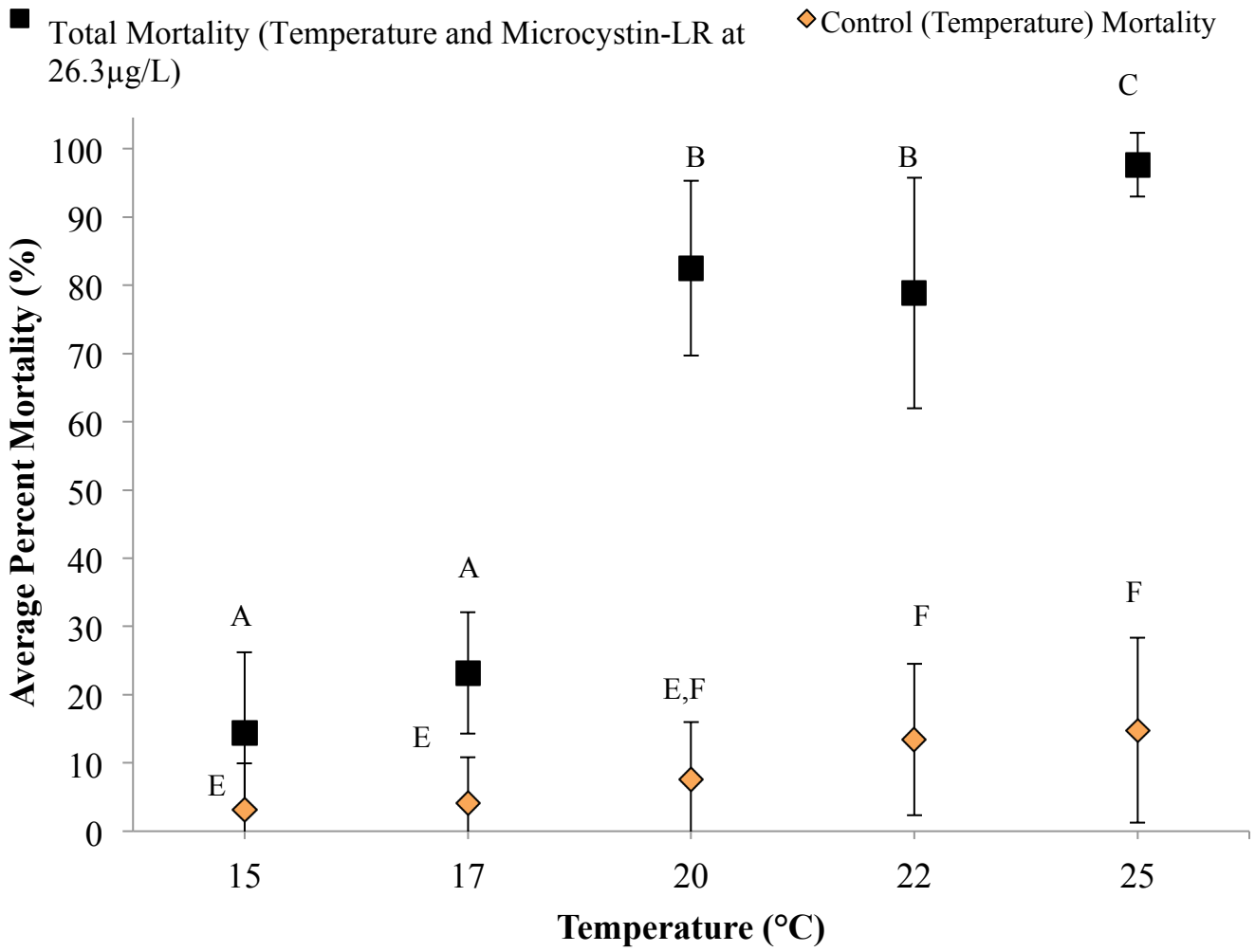

Figure 5: Average percent mortality of *B. longirostris* over a range of temperatures when exposed to a constant level of microcystin-LR: LC_{50} of 26.3 µg/L, for 48-hrs. Control treatments were without microcystin-LR. Total temperature and microcystin-LR was the combined effect of temperature and toxin on mortality. Error bars represent standard deviation. Different letters indicate significant difference (p <0.05, Fisher's LSD). ANCOVA: p <0.0001. R^2 =0.88. $F_{1,83}$= 92.48. Control one-way ANOVA: R^2= 0.21. p<0.05. $F_{4,40}$ = 2.27. Total one-way ANOVA: R^2= 0.90. p <0.0001. $F_{4,37}$= 85.37.

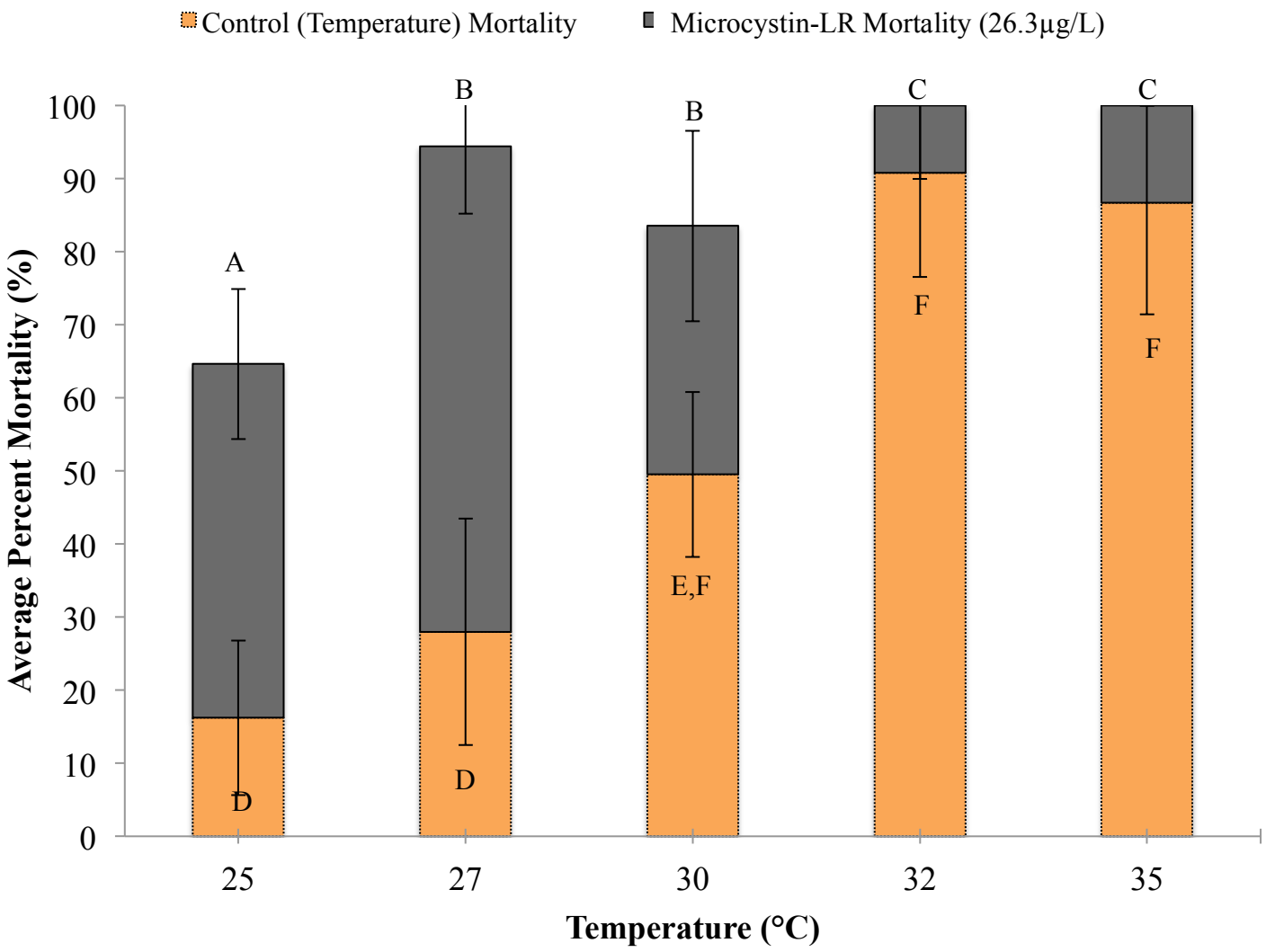

Figure 6: Average percent mortality of *B. longirostris* over a range of temperatures when exposed to a constant level of microcystin-LR: LC_{50} of 26.3 µg/L, for 48-hrs. Control treatments were without microcystin-LR. Error bars represent standard deviation. Different letters indicate significant difference (p <0.05, Fisher's LSD). Control one-way ANOVA: R^2= 0.84. p <0.0001. $F_{4,30}$ = 40.23. Microcystin-LR one-way ANOVA: R^2= 0.81. p <0.0001. $F_{4,31}$= 32.47.

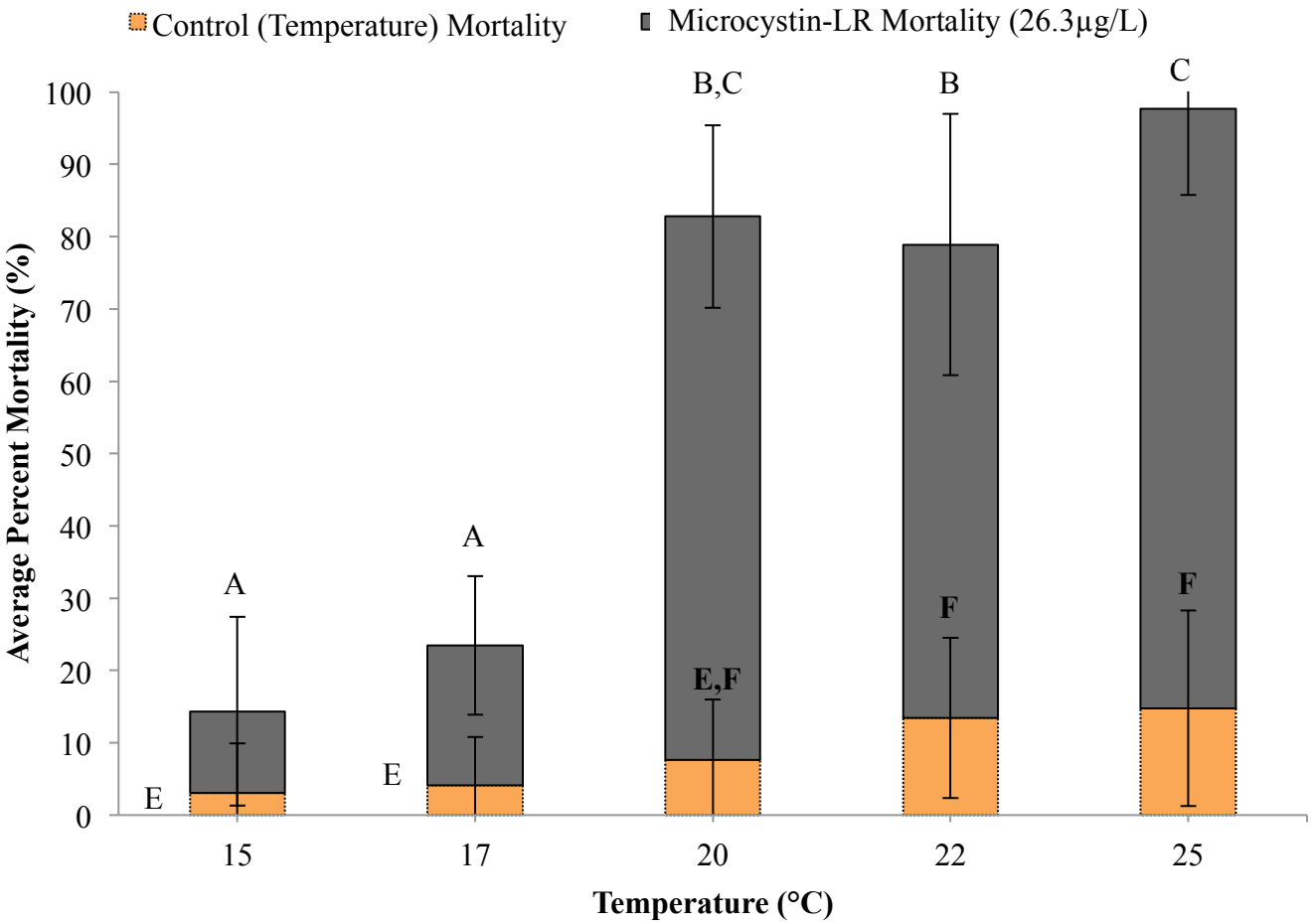

Figure 7: Average percent mortality of *B. longirostris* over a range of temperatures when exposed to a constant level of microcystin-LR: LC_{50} of 26.3 µg/L, for 48-hrs. Control treatments were without microcystin-LR. Error bars represent standard deviation. Different letters indicate significant difference ($p<0.05$, Fisher's LSD). Control one-way ANOVA: $R^2= 0.21$. $p<0.05$. $F_{4,40} = 2.27$. Microcystin-LR one-way ANOVA: $R^2= 0.84$. $p <0.0001$. $F_{4,37}= 50.16$.

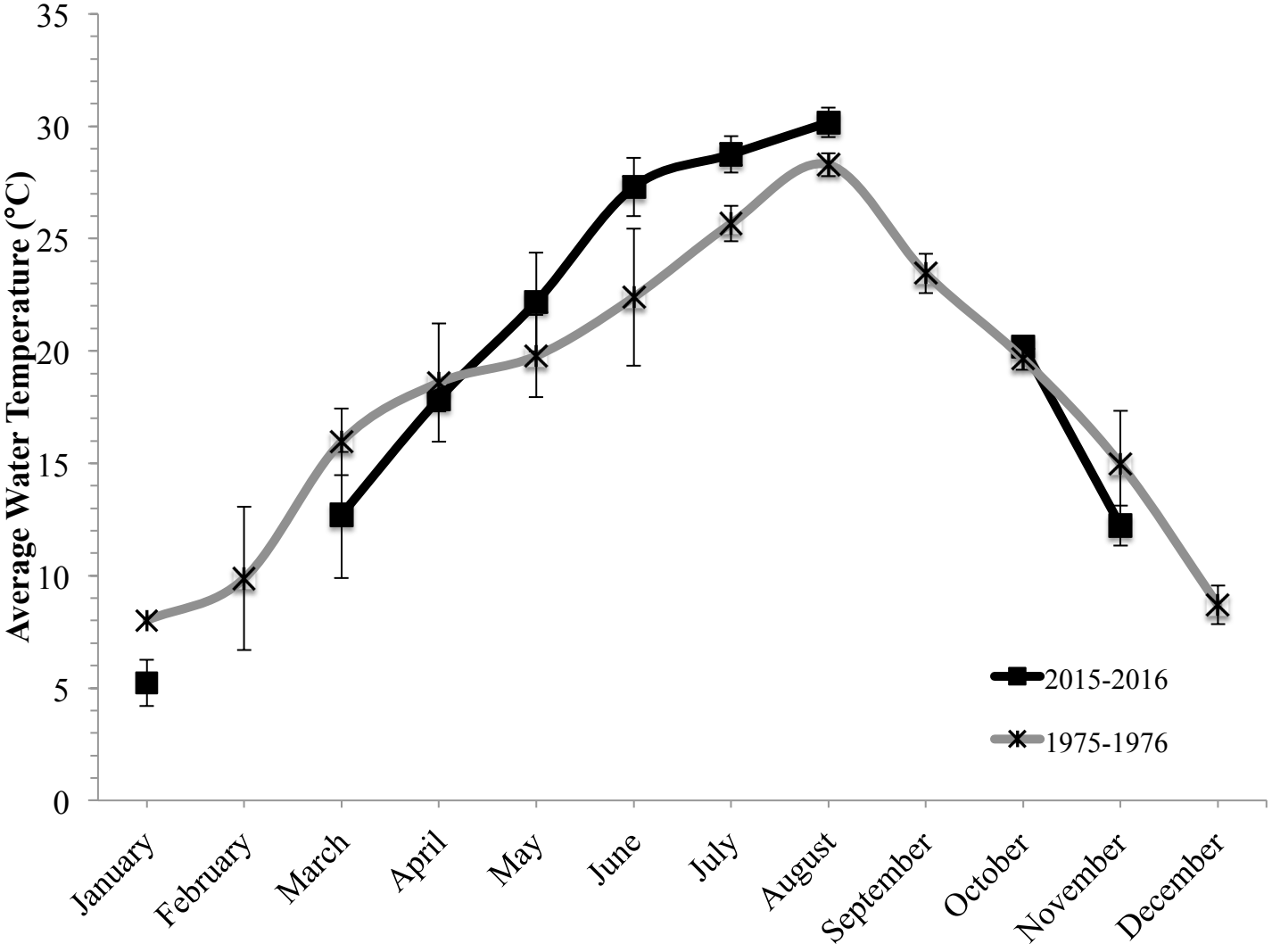

Figure 8: Average water temperature (°C) for 1975-1976 and 2015-2016 for the Chowan River, NC. Data taken from Witherspoon et al. 1979 and Department of Environmental Quality STORET database, respectively. For 1975-1976 data, R^2=0.904. For 2015-2016 data, R^2=0.889. Error bars represent standard deviation.

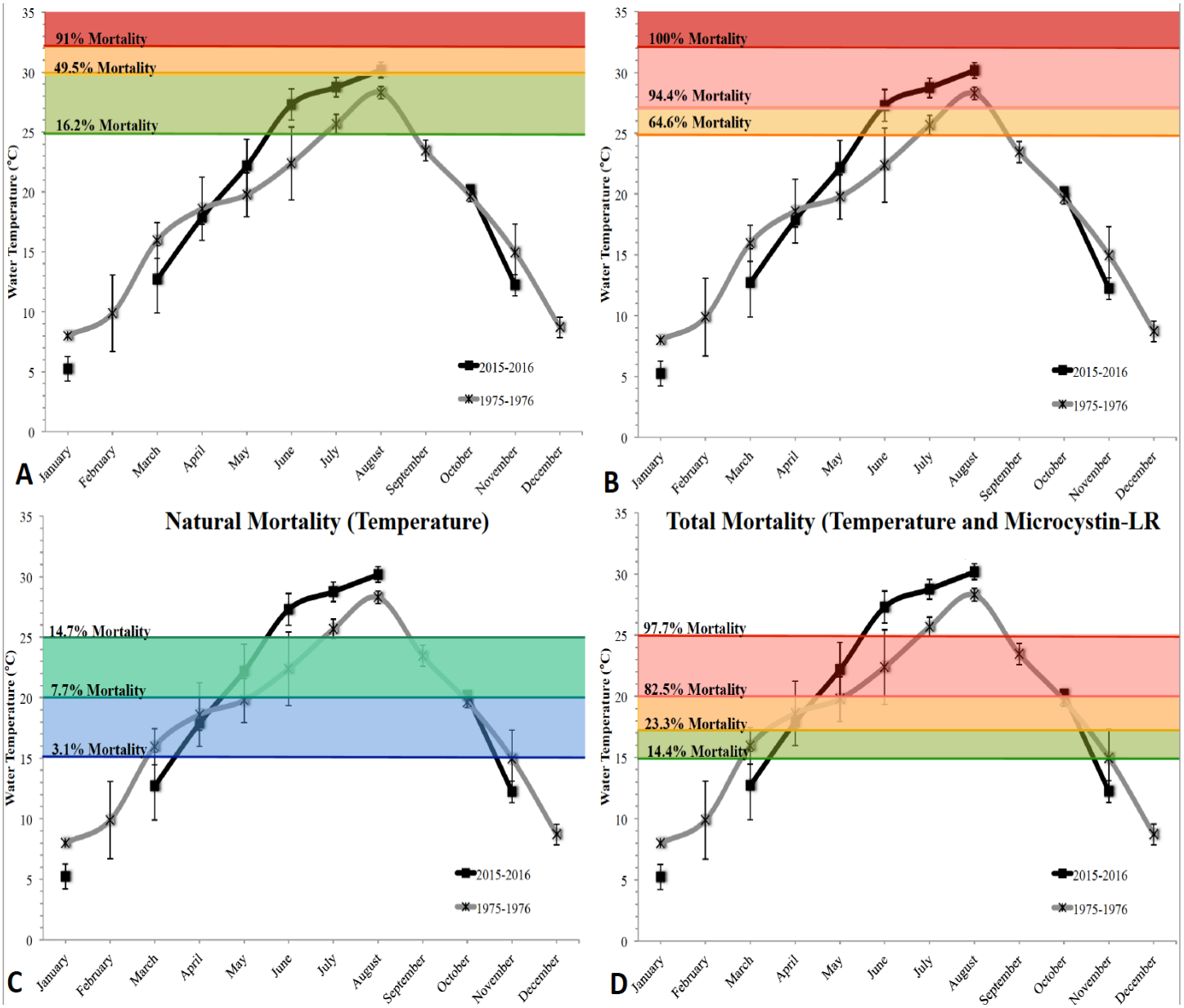

Figure 9: Seasonal adaptability of *B. longirostris* collected in April (C and D) versus June (A and B) to microcystin-LR (26.3µg/L) at 15°C-25°C and 25°C-35°C, respectively. Control (temperature) mortality represented in A and C, while total mortality (temperature and microcystin-LR) represented in B and D. Percent mortalities superimposed over yearly temperature trends for the Chowan River, North Carolina.

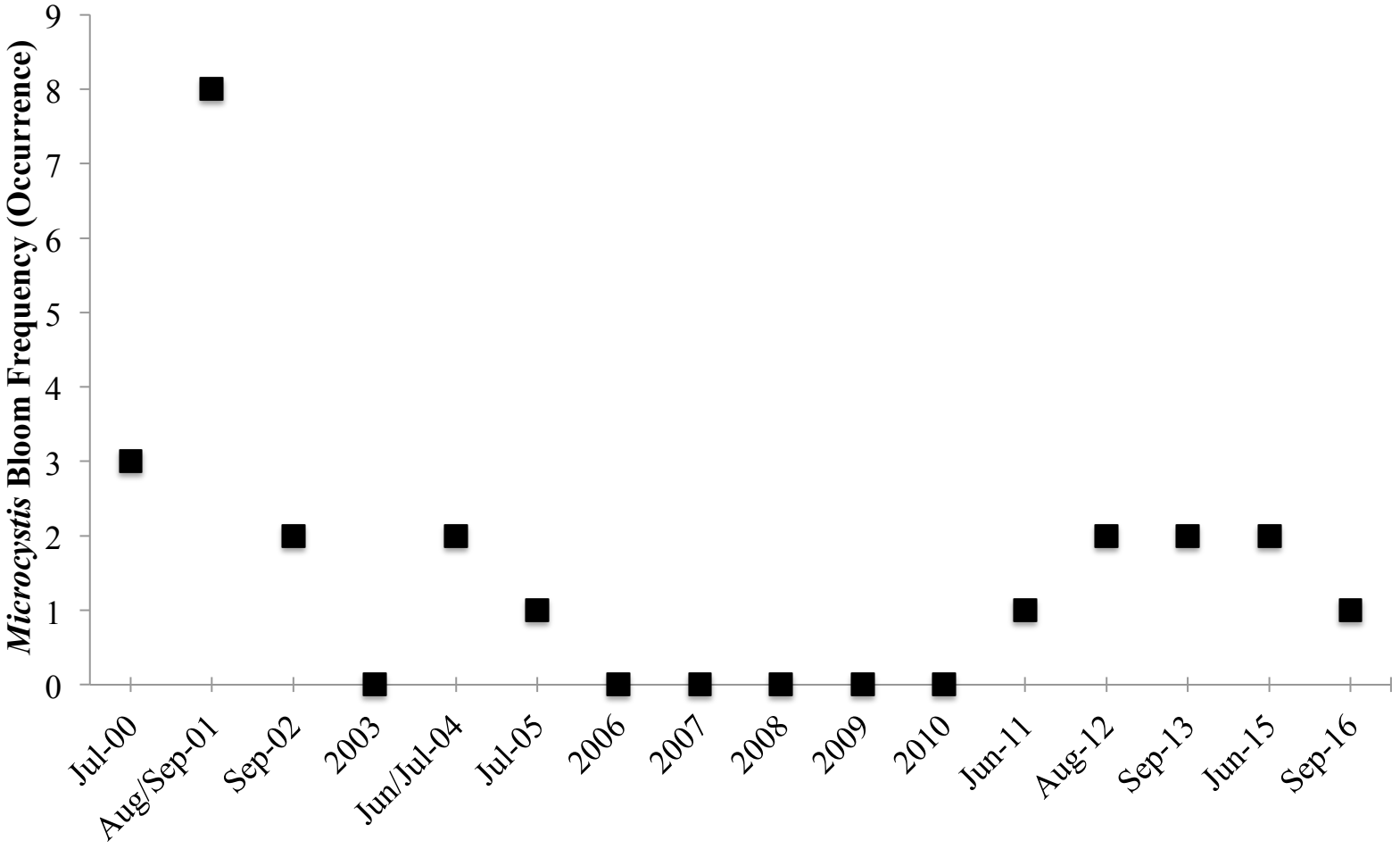

Figure 10: Frequency of *Microcystis* blooms in the Chowan River, NC from 2000-2016 by occurrence. Data collected by Moorman et al. 2017 and NCDEQ, Water Resources.

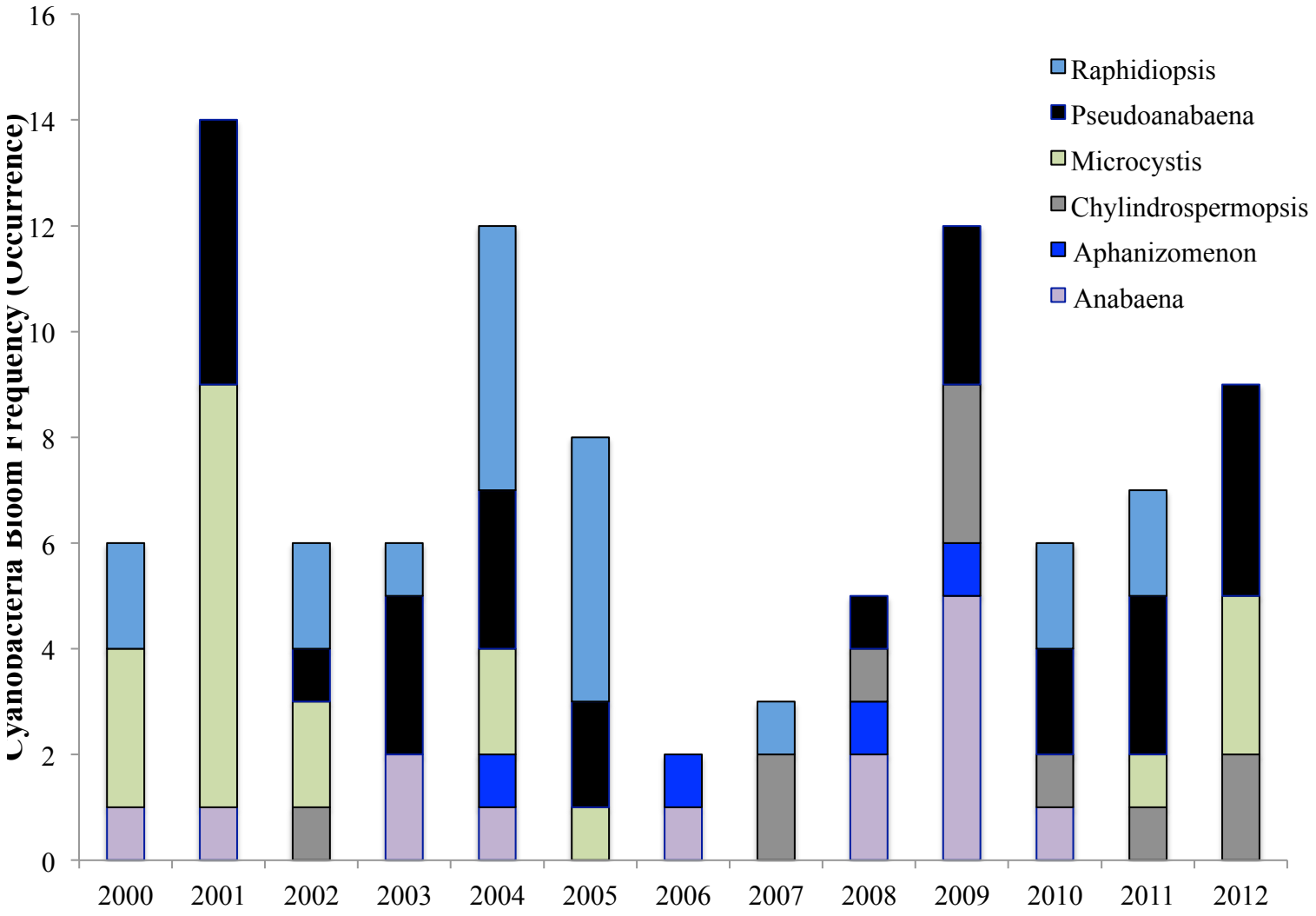

Figure 11: Frequency of cyanobacteria that produce cyanotoxins within the Chowan River, NC from 2000-2012. Original data taken by Moorman et al. 2017.

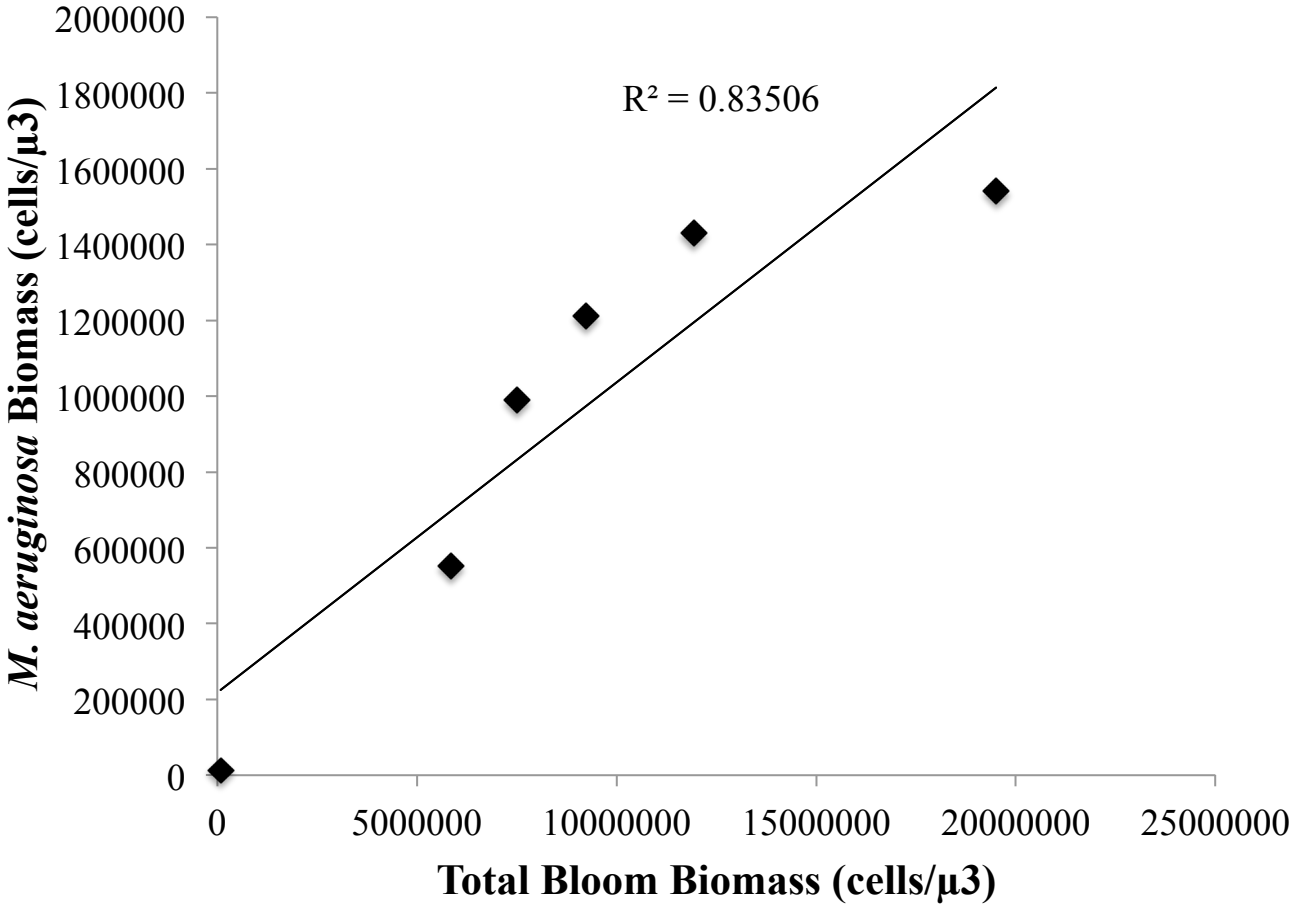

Figure 12: Linear regression between total bloom biomass and *M. aeruginosa* biomass from 1975 and 1976 algal blooms within the Chowan River, NC. Biomass of *M. aeruginosa* cells increases with total bloom biomass. Data taken from Appendix C of Witherspoon et al. 1979.

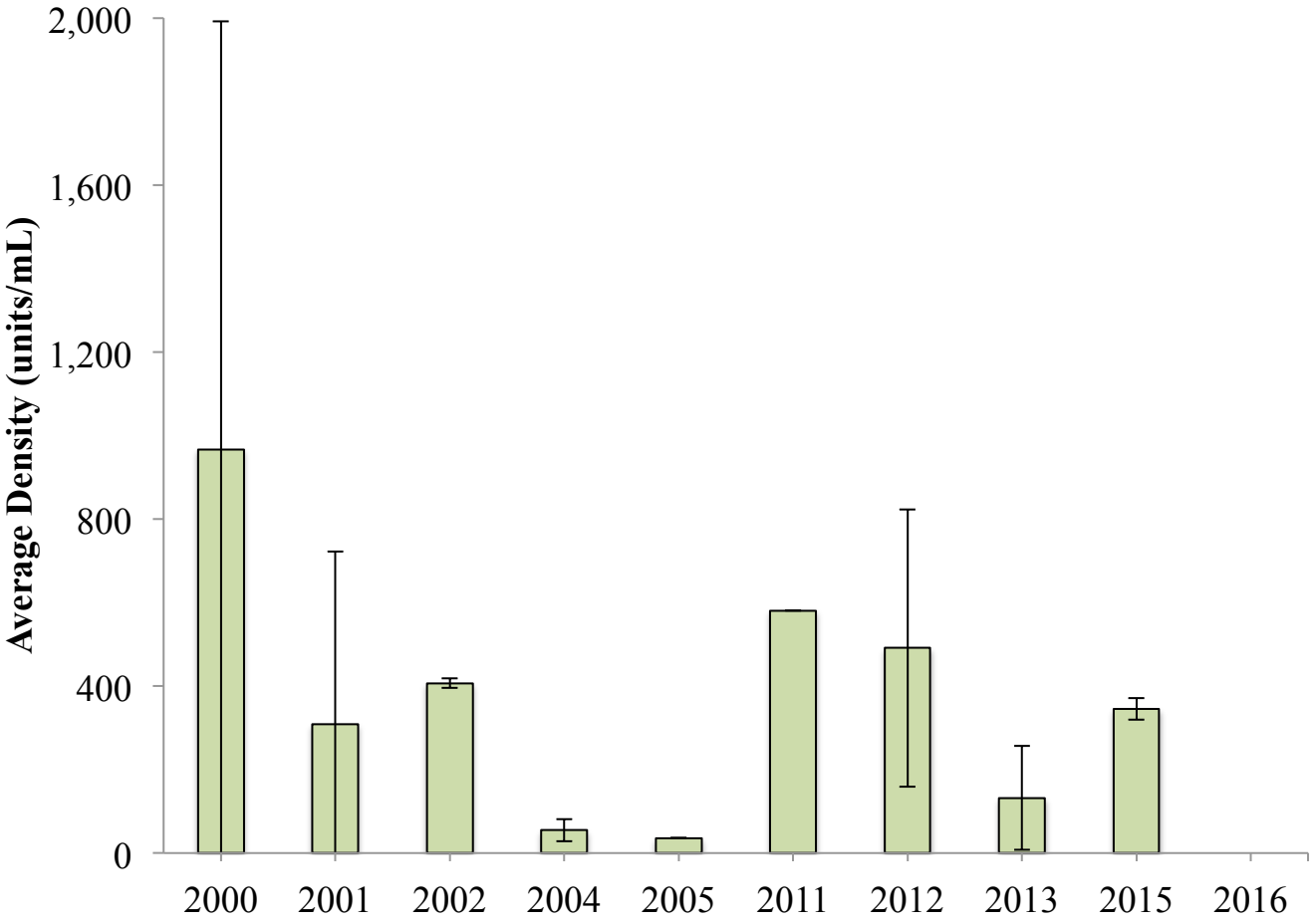

Figure 13: Average density of *Microcystis* blooms from 2000-2016, June-September in the Chowan River, NC. Data collected by Moorman et al. 2017 and North Carolina Department of Environmental Quality (NCDEQ), Water Resources.

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