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Antibiotics and Antibiotic-Resistant Bacteria in Coastal Plain Streams

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ANTIBIOTICS AND ANTIBIOTIC-RESISTANT BACTERIA IN COASTAL PLAIN STREAMS

by

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(Under the Direction of Risa A. Cohen)

ABSTRACT

Streams across the United States and globally are influenced by environmental contamination, including antibiotics, which enter streams due to widespread use and multiple pathways into the environment. Antibiotics are also likely to enter streams in mixture with other contaminants that alter the effects on aquatic organisms. Furthermore, antibiotic-resistant bacteria enter streams through similar pathways as antibiotics with implications for natural microbial communities. Therefore, understanding the presence and effects of antibiotic-contaminant mixtures and antibiotic-resistant bacteria in streams is important for resource management.

Chapter one describes an experiment that tested the hypothesis that the antibiotic tetracycline (TC) alone influences phytoplankton communities differently than in mixture with excess nutrients. Environmental conditions including season and flow regime were also manipulated to determine the effect of these variables. Artificial streams and ponds were inoculated with phytoplankton and exposed to one of four treatments: no-addition control, TC-only, nutrients-only, and TC + nutrients in fall 2015 and summer 2016. TC and nutrients individually had positive, negative, or neutral effects depending on the experiment, and TC-nutrient mixtures increased growth under certain conditions. Thus, the direction and scale of TC and nutrient effects are likely dependent upon seasonal changes in temperature and flow. Results from this study demonstrate the importance of considering antibiotic-nutrient mixtures and

environmental conditions in toxicity tests to more accurately predict how phytoplankton communities will respond to inputs of these contaminants.

Chapter two describes a field study that tested the hypotheses that: 1) land use influences TC-resistant bacteria abundance in southeastern coastal plain streams; 2) TC-resistant bacterial abundance is related to temperature, precipitation, and nutrient concentration; and 3) phytoplankton abundance and community composition are related to TC-resistant bacterial abundance. Water samples were collected from streams within the Ogeechee River Basin (ORB), southeastern GA, USA to examine relationships between land use, resistant bacterial abundance, phytoplankton communities, and environmental variables over time. Results suggested that agricultural locations are local sources of fecal bacteria and WWTPs are likely local sources of both fecal bacteria and resistant bacteria into surface waters. These findings also indicate that major precipitation events affect ARB delivery to streams. In addition, phytoplankton within ORB streams are influenced by light attenuation and nutrient concentration rather than altered competitive dynamics with resistant bacteria.

INDEX WORDS: Tetracycline, Phytoplankton, Land use, Contaminants, Nutrients, *E. coli*

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by

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CHAPTER 1

THE INFLUENCE OF TETRACYCLINE AND NUTRIENTS ON PHYTOPLANKTON ABUNDANCE AND COMMUNITY COMPOSITION UNDER DIFFERENT FLOW REGIMES AND SEASONS

INTRODUCTION

Contaminant mixtures enter aquatic systems in wastewater effluent and agricultural runoff with the potential to affect aquatic organisms differently than the individual contaminants (Christensen et al. 2006; Yang et al. 2008; Gonzalez-Pleiter et al. 2013). Contaminants in combination can have additive or synergistic effects, making mixtures more toxic than the individual contaminants (Pape-Lindstrom and Lydy 1997; Magnusson et al. 2010). Contaminant mixtures can also have antagonistic effects in which one contaminant counteracts toxic effects of another, resulting in a mixture that is less toxic than the individual contaminants (Franklin et al. 2002). Interactions between contaminants in mixture can be even more complex, with changes in toxicity that are unpredictable based on individual contaminant toxicity (Riedel et al. 2003; Christensen et al. 2006). For example, a mixture of typically toxic trace elements (As, Cu, and Cd) with excess nutrients stimulated growth of the diatom *Dactyliosolen fragilissimus* in stream mesocosms (Riedel et al. 2003). Therefore, evaluating contaminant mixture effects on phytoplankton that provide essential aquatic food resources to consumers is important.

Antibiotics enter surface waters globally due to widespread use preventing and treating bacterial infections in humans and livestock, with potential adverse effects to phytoplankton (reviewed by Halling-Sorensen et al. 1998; Sarmah et al. 2006; Kummerer 2009; Du and Liu 2012). In particular, tetracycline (TC) from wastewater treatment effluent and soil fertilized with liquid manure ultimately yields concentrations up to 1 µg L^{-1} in U.S. surface waters (Halling-Sorensen et al. 1998; Hamscher et al. 2002; Yang and Carlson 2003; Karthikeyan and Meyer

2006). Once TC enters aquatic systems, toxicity to phytoplankton occurs because both chloroplasts and bacterial ribosomes have a 30S subunit containing a binding site for TC (Brain et al. 2008). Laboratory-based toxicity tests confirmed phytoplankton sensitivity to TC, albeit at concentrations above the μg L^{-1} concentration range found in the environment. Growth inhibition occurred at 0.09-0.1 mg L^{-1} for cyanobacteria (*Microcystis aeruginsoa*) and 0.2-2.2 mg L^{-1} for green algae (*Selenastrum capricornutum*) after seven days of exposure to TC (Halling-Sorensen 2000; Yang et al. 2013). Although the magnitude of the effect concentrations (i.e. above the μg L^{-1} range in the environment) suggests that TC alone is not likely to affect aquatic organisms, TC in mixture with other contaminants could have negative effects at lower, environmentally relevant concentrations.

Mixtures of TC with other antibiotics can affect phytoplankton at lower concentrations than TC alone (Christensen et al. 2006; Yang et al. 2008; Gonzalez-Pleiter et al. 2013). For example, the green alga *Pseudokirchneriella subcapitata* experienced 23% growth inhibition when exposed to a mixture of antibiotics (including TC) in which each antibiotic was below its lowest No Observed Effect Concentration (NOEC) (Yang et al. 2008). Similarly, the combination of TC and erythromycin increased the growth inhibition EC_{50} for *P. subcapitata* by more than an order of magnitude compared to TC alone (Gonzalez-Pleiter et al. 2013). While TC toxicity to phytoplankton is likely to increase in combination with other antibiotics, toxicity changes in the presence of other contaminants is largely understudied.

In addition to TC, nutrients (i.e. nitrogen and phosphorus) enter aquatic systems from wastewater treatment plant discharges and agricultural runoff (Carpenter et al. 1998; Karthikeyan and Meyer 2006; Verhoeven et al. 2006). Although nitrogen and phosphorus are essential for phytoplankton growth, excess nutrients can increase phytoplankton biomass (Schindler 1977;

Henry et al. 1985; Mallin et al. 2004) and change competitive interactions among species, thereby altering species composition (Jacobsen et al. 1995; Munn et al. 2002). For example, green algae and diatoms generally have higher nutrient requirements than other phytoplankton groups, and therefore tend to increase with nutrient availability (Porter et al. 1977; Schindler 1977). Diatoms in particular are often more competitive than other groups when nutrients are abundant (Harding 1994; Piehler et al. 2004; Tozzie et al. 2004). Many cyanobacteria fix atmospheric nitrogen and therefore increase in abundance relative to other taxa in response to greater phosphorus availability (Schindler 1978; Porter et al. 1977; Tilman et al. 1982). Such changes in phytoplankton species composition affect trophic structure by influencing food availability due to differences in palatability and nutritional value to organisms feeding on them (Porter 1977; Wichard et al. 2007). For example, small, unicellular green algae are easily ingested by filter feeding zooplankton and are thus preferentially grazed on compared to large diatoms or colonial cyanobacteria (Porter 1977). Certain diatom species have high fatty acid content that allows for more efficient energy transfer to consumers compared to other taxa (Wichard et al. 2007). Several species of cyanobacteria are toxic to consumers, and under certain conditions the unrestricted growth of even non-toxic species of cyanobacteria and dinoflagellates can lead to harmful algal blooms that result in oxygen depletion, high turbidity, and ultimately fish kills (Hallegraeff 1993). Thus, consideration of the combination of species-specific nutrient requirements and differential sensitivity to TC is necessary to predict phytoplankton community response to TC-nutrient mixtures and potential effects on aquatic food webs.

Nutrient effects on contaminant toxicity to phytoplankton can be positive, neutral, or negative depending on the type of contaminant and species of phytoplankton. Nutrient addition in the presence of trace metals often has positive effects, such as increased cell density of an

estuarine centric diatom and reduced metal uptake by the freshwater green alga *Chlorella vulgaris* (Riedel et al. 2003; Qian et al. 2013). Nutrient effects on herbicide toxicity depend on the herbicide. For example, increased nutrient availability reduced growth inhibition of a green alga and cyanobacterium exposed to dinoseb (Tubea et al. 1981). No difference in toxicity of simazine and butachlor to the green alga *Selenastrum capricornutum* was observed, while aminotriazole became more toxic to *S. capricornutum* in mixtures with nutrients (Adams and Dobbs 1984; Kamaya et al. 2004). Phytoplankton responses to contaminant and nutrient additions can also be species specific; increased nutrient availability decreased crude oil toxicity to centric and pennate diatoms but not to dinoflagellates (Ozhan and Bargu 2014). It is possible that photosynthetic stress associated with TC-induced disruption of chloroplast protein synthesis can be alleviated by increased nutrient availability, which enhances uptake of beneficial ions such as Mg^{2+} (Chopra and Roberts 2001; Qian et al. 2013). Furthermore, increases in phytoplankton populations associated with excess nutrients (Schindler 1977; Henry et al. 1985; Mallin et al. 2004) could counteract losses caused by TC giving the appearance of reduced toxicity. These possibilities suggest that TC-nutrient mixture effects need to be studied under a variety of conditions on multiple species.

Environmental conditions including flowing water, the presence of multiple species, and seasonal differences might also influence TC and nutrient mixture effects on phytoplankton (Riedel et al. 2003; Wilson et al. 2004; Quinlan et al. 2011; Gonzalez-Pleiter et al. 2013). Flowing water can be stressful to non-motile phytoplankton taxa because they reproduce less effectively compared to reproduction in lakes and ponds (Reynolds 2000; Zhang et al. 2015). Flow may also increase TC toxicity to phytoplankton; Quinlan et al. (2011) observed a change in the dominant algal group in benthic algal communities exposed to just 0.5 µg L^{-1} TC

concentrations in artificial streams, well below the mg L^{-1} EC_{50s} calculated from static laboratory tests (Halling-Sorensen 2000; Yang et al. 2013). The presence of multiple species may also influence TC toxicity. Harass et al. (1985) grew cultures of the green alga *Scenedesmus obliquus* with the cyanobacteria *Anabaena cylindrica* and *S. capricornutum* and found that *S. obliquus* became competitively dominant only when the antibiotic streptomycin was added. Lastly, seasonal differences such as temperature have the potential to alter TC toxicity because toxin-induced growth inhibition may be decreased during periods of warmer temperatures that stimulate phytoplankton growth (Reidel et al. 2003).

The goal of this study was to evaluate the effects of TC and nutrients (N and P) alone and in combination on phytoplankton abundance and community composition under different flow conditions and seasons. I hypothesized that TC alone influences phytoplankton communities differently than in mixture with excess nutrients. Artificial streams and ponds were inoculated with communities of phytoplankton exposed to TC and/or nutrients to test the predictions that 1) TC-only reduces abundance of cyanobacteria and green algae more than diatoms, given their known sensitivity to TC, and 2) The presence of added nutrients reduces TC toxicity to phytoplankton. I also expected to see increased toxicity of TC to phytoplankton in the streams compared to the ponds, as well as decreased toxicity during the summer compared to the winter. Results from this study demonstrate the importance of considering antibiotic-nutrient mixtures and environmental conditions in toxicity tests in order to more accurately predict how phytoplankton communities will respond to inputs of these contaminants.

METHODS

Experimental Design

To evaluate the effects of TC and nutrients alone and in combination on phytoplankton abundance and community composition under flowing conditions, I conducted two artificial stream experiments in the summer and fall of 2016. In both experiments, phytoplankton communities were exposed to one of four treatments: no-addition control, TC-only, nutrients-only, and TC + nutrients with eight-fold replication. Artificial streams (N=32) consisted of black, polyethylene re-circulating tanks with 7.5 L of water and an average water velocity of 0.12 m s^{-1} controlled by powerhead pumps (King-160, PETCO, CA, U.S.A.)(Figure 1.1A). Streams were randomized by location in the Georgia Southern University Biological Sciences greenhouse (Statesboro, GA, U.S.A.; 32°25'31.6"N 81°47'23.6"W). Phytoplankton communities were created using taxa commonly found in rivers: cyanobacteria (*Microcystis aeruginosa*), diatoms (*Navicula* sp.), and green algae (*Scenedesmus* sp.) at a target density of 500-1,000 cells ml^{-1} to represent measured phytoplankton abundance in lotic systems (Soballe and Kimmel 1987; Marshall et al. 2009). Nitrogen (6 mg L^{-1} NO_3^-) and phosphorus (1 mg L^{-1} PO_4^{3-}) concentrations were added to the nutrient-only and TC + nutrient treatments to represent streams receiving a pulse of excess nutrients in the southeastern coastal plain (Mallin et al. 2004). TC was maintained at a concentration of 1 µg L^{-1} in the TC-only and TC + nutrients treatments by adding 1 µg L^{-1} every 48 hours to account for losses due to degradation (Brain et al. 2005). The experimental duration was 7 days in the summer (Sept 3-10) to allow sufficient time for changes in algal abundance and species dominance in response to TC (Quinlan et al. 2011) and 9 days in the fall (November 17-28) to compensate for slower phytoplankton growth due to cooler temperatures. Average ambient temperature in the greenhouse was 27.7±2.9˚C in summer and

19.5±5.7˚C in fall. An artificial pond component was included in the fall (to compare flow vs. no flow conditions) by replicating the stream treatments within the buckets in the center of each stream (Figure 1.1B). The same TC and nutrient additions as used in the stream experiments were included in the artificial pond component.

Sampling Regime

Phytoplankton samples were collected daily in summer and every three days in fall by compositing 0.5 ml subsamples from the surface, center, and bottom of the water column from each stream unit. Phytoplankton identification and enumeration were conducted using a BD Accuri C6 flow cytometer (Becton-Dickinson, CA, U.S.A.), which uses lasers to count cells and distinguish among populations based on size and pigment characteristics (Veldhuis and Kraay 2000). Identification of each taxon was based on comparisons to plots of the monocultures of *Navicula* sp., *Scenedesmus* sp., or *M. aeruginosa* used to create the initial phytoplankton community. Phytoplankton abundance was also estimated at the end of each experiment using chlorophyll *a* as a proxy (EPA method 445.0; Arar and Collins 1997). Water samples (100 ml) from each stream were filtered through Whatman GF/F glass fiber filters (nominal pore size 0.7 µm) to collect phytoplankton cells, followed by extraction of pigments from the cells in 90% acetone in the dark for 24 hours at -20°C. The concentration of the extracted pigments was determined using a Trilogy fluorometer (Turner Designs, CA, U.S.A.). Initial and final dissolved oxygen, conductivity, and pH were measured immediately after phytoplankton sampling (1200-1500 h) and using a hand-held YSI Pro Plus (YSI Inc., OH, U.S.A.) to ensure physicochemical variables remained within ranges acceptable for phytoplankton (Schindler et al. 1985; EPA 1986; Mouget et al. 1995; Padisak 2010). Initial environmental conditions were similar across all

stream and pond units in the summer and fall experiments (Table 1.1). Final measurements indicated greater conductivity in the nutrient treatments due to the presence of NO_3^- and PO_4^{3-} ions (Table 1.2). Only the ponds that received nutrient additions also had approximately 1 mg L^{-1} greater DO and 0.5 greater pH than no-nutrient treatments, likely due to increased photosynthesis (Table 1.2).

Data Analysis

Assumptions of normality and homogeneity of variances were tested using the Shapiro-Wilk *W* Test and Levene's Test, respectively. Data that did not meet assumptions were log or square-root transformed, or analyzed using non-parametric tests. Effects of treatment addition on total phytoplankton abundance and individual taxon abundances over time were analyzed using two-way repeated measures analysis of variance (rmANOVA). Data that did not meet the assumption of sphericity according to Mauchly's test were analyzed using degrees of freedom calculated with a Greenhouse-Geisser epsilon adjustment. All univariate tests were conducted using JMP Pro 10.0.0 (SAS Institute Inc., Cary, NC). To test for differences in species composition due to treatments over time, Bray-Curtis resemblance matrices were created for *M. aeruginosa*, *Navicula* sp. and *Scenedesmus* sp. cell density data followed by two-way repeated measures permutational multivariate analysis of variance (PERMANOVA+ add-on; PRIMER-E v.7, Plymouth Marine Laboratory, U.K.) (Clarke and Gorley 2006). Significance level was set at 0.05 ($\alpha = 0.05$) for all tests, and p-values of 0.06-0.1 were considered trends.

RESULTS

Total Cell Density and Chlorophyll a

Total cell density seemed to increase across all treatments throughout the summer stream and fall pond experiments, but generally did not exceed initial density in the fall stream experiment (Figure 1.2). The density increase in the fall ponds was at least an order of magnitude higher than in the summer stream experiment, with multiple treatments exceeding 100,000 cells ml^{-1} by the final day (Figure 1.2). Treatment effects on total phytoplankton abundance differed depending on season and flow conditions. Cell density was unaffected by TC or nutrient addition in the summer stream experiment (Table 1.3)(Figure 1.2A). In the fall stream experiment, an interaction between TC and time on total density occurred (Table 1.3), likely driven by a seemingly positive effect of TC alone on day 6 (Figure 1.2B). An interaction between TC and nutrients on total density also occurred in the fall streams; cell density in the nutrient-only treatment was 53% lower than TC-only and 70% lower than the TC+ nutrients treatment on the final day (Table 1.3)(Figure 1.2B). TC+ nutrient addition also appeared to increase cell density relative to the control by 20-60% throughout the experiment (Figure 1.2B). Total cell density in the ponds increased over time by 45-62-fold over initial density and trended toward higher density in the treatments receiving nutrients (Table 1.3)(Figure 1.2C).

Average final chlorophyll *a* concentration in the summer stream experiment was approximately twice the concentration in the fall stream experiment. The chlorophyll *a* concentration in fall pond experiment was highest, nearly doubling the concentration in the summer stream experiment (19 vs. 36 $\mu g\ L^{-1}$)(Figure 1.3). Although neither TC nor nutrients influenced chlorophyll *a* in the summer or fall streams, nutrient addition increased chlorophyll *a*

concentration by 33-50% (10-15 µg L^{-1}) relative to the other treatments in the fall ponds by day 9 (Table 1.4)(Figure 1.3).

Species Composition and Individual Taxon Densities

The dominant species differed by season and flow conditions. In the summer streams, *Scenedesmus* sp. appeared dominant (78-94%) by the end of the experiment, while *Navicula* sp. and *M. aeruginosa* comprised 5-20% and 1-4% of the community, respectively (Figure 1.4A). In the fall streams, *Scenedesmus* sp. and *Navicula* sp. each represented ~50% of the community with less than 1% *M. aeruginosa* across all treatments (Figure 1.4B). The fall pond community was dominated by *Navicula* sp. (90-93%), while *Scenedesmus* sp. represented 7-10% and *M. aeruginosa* comprised less than 1% of the community in all treatments (Figure 1.4C).

Treatments also affected phytoplankton species composition. Interactions between both TC and time and nutrients and time influenced stream communities in the summer due to both contaminants alone increasing and decreasing the proportion of *Navicula* sp. in unpredictable ways throughout the experiment (Table 1.5). Addition of both TC and nutrients appeared to increase the proportion of *Navicula* sp. by 10-15% relative to all other treatments by the end of the experiment (Figure 1.4A). In the fall, the presence of TC increased %*Navicula* sp. in the streams by 3-16%, while nutrients appeared to counteract TC effects, resulting in similar %*Navicula* sp. in the TC+ nutrient and nutrient-only treatments (Figure 1.4B). An interaction between nutrients and time affected fall pond species composition (Table 1.5)(Figure 1.4C), although differences across treatments from days 3-9 were always less than 10% and thus were not likely biologically relevant.

Changes in *Scenedesmus* sp. cell density in response to season, flow conditions and treatments contributed to the alteration of species composition. *Scenedesmus* sp. cell density increased from initial by 20,000-40,000 cells ml^{-1} during the summer stream experiment regardless of treatment (Table 1.6)(Figure 1.5A). In contrast, density of *Scenedesmus* sp. in the fall stream experiment did not exceed 2,000 cells ml^{-1} and was influenced by an interaction between TC and nutrients. Cells exposed to both TC and nutrients were as much as 50% more abundant than those that received TC or nutrients-only by the end of the experiment (Table 1.6)(Figure 1.5B). Neither TC nor nutrients affected *Scenedesmus* sp. density in the fall pond experiment (Table 1.6)(Figure 1.5C).

Treatment effects on *Navicula* sp. cell density differed between the two stream experiments. An interaction between nutrients and time affected *Navicula* sp. cell density in the summer stream experiment (Table 1.7). From the start of the experiment to day 4, nutrients generally had no effect on *Navicula* sp. density, but appeared to increase *Navicula* sp. density over the remainder of the experiment (Figure 1.6A). In the fall stream experiment, TC rather than nutrients positively influenced *Navicula* sp. density (Table 1.7)(Figure 1.6B). Cell density increased 41-54% in the presence of both TC and nutrients compared to the nutrient-only treatment (Figure 1.6B). In contrast, *Navicula* sp. growth in the pond experiment decreased 15-30% with TC addition relative to the control from days 3-9 (Table 1.7)(Figure 1.6C). Although overall density was elevated relative to the stream experiments (78,000 cells ml^{-1}) in all treatments by the end of the experiment (Figure 1.6C), nutrient addition only trended toward increasing *Navicula* sp. cell density in the fall pond experiment (Table 1.7). Finally, addition of nutrients and TC to the ponds appeared to partially counteract the negative effect of TC (Figure 1.6C).

Density of *M. aeruginosa* decreased from initial to final day across all treatments in both seasons and flow regimes. In the summer streams, nutrients-only addition decreased *M. aeruginosa* and TC-only addition trended toward positively influencing *M. aeruginosa*, but density of this species decreased to 40-350 cells ml^{-1} (77-98% decrease) by the final day across all treatments (Table 1.8)(Figure 1.7A). Neither TC nor nutrients influenced *M. aeruginosa* cell density in the fall streams or ponds (Table 1.8) and all treatments in both the fall streams and ponds were very low (less than 1% of total cells)(Figure 1.7B,C).

DISCUSSION

Tetracycline (TC) and nutrient additions alone vs. in mixture were hypothesized to influence phytoplankton abundance and community composition in different ways. The environmentally relevant concentration of TC tested was expected to influence phytoplankton communities based on demonstrated toxicity of TC to phytoplankton in laboratory tests (Halling-Sorensen 2000; Yang et al. 2013) and previous findings that 0.5 µg L^{-1} TC altered algal species composition by increasing abundance of diatoms relative to cyanobacteria in stream mesocosms (Quinlan et al. 2011). In the present study, TC alone decreased phytoplankton growth (primarily *Navicula* sp.) in the fall pond experiment, potentially due to chloroplast ribosomal toxicity (Brain et al. 2008), but did not influence any of the three species in the summer or fall streams. It is possible that the TC concentration used in this study was too low to consistently have adverse effects on phytoplankton growth, which corroborates results from laboratory toxicity tests demonstrating TC toxicity to phytoplankton in the mg L^{-1} range (Halling-Sorensen 2000; Yang et al. 2013). However, water flow and temperature could have influenced TC toxicity. Phytoplankton abundance was expected to be more susceptible to TC with water flow and colder

temperatures because these conditions inhibit phytoplankton growth (Reynolds 2000; Quinlan et al. 2011; Zhang et al. 2015; Konopka and Brock 1978; James et al. 1989; Kruk and Segura 2012). As expected, TC had no effect on phytoplankton abundance in the summer and negative effects in the fall. However, TC only had adverse effects on phytoplankton abundance under non-flowing conditions, potentially due to nutrient stress following rapid growth in the ponds (Hall et al. 1989; Lurling 2006). Thus, TC effects on stream phytoplankton abundance could be partially dependent on seasonal changes in temperature and flow regime.

Despite the expectation that phytoplankton abundance increases with nutrient addition regardless of water flow or season (Harding 1994; Mallin et al. 2004; Tozzi et al. 2004), nutrients only increased cell density and chlorophyll *a* concentration in the pond experiment. Overall abundance was much greater in the pond compared to the stream experiments in both seasons, suggesting that phytoplankton growth was more limited by water flow than nutrient availability in the streams (Reynolds 2000; Zhang et al. 2015). The addition of nutrients could have relieved nutrient limitation to some extent in the ponds, in which phytoplankton reproduction was not limited by the presence of flowing water (Reynolds 2000; Zhang et al. 2015). However, nutrient addition also had negative effects in the fall streams, reducing phytoplankton abundance in this experiment. A possible explanation for this unexpected result is that the nutrients stimulated growth of aquatic bacteria, which reduced phytoplankton growth due to increased competition for phosphorus (Rhee 1972; Currie and Kalff 1984) and/or micronutrients such as vitamins and trace metals (Grossart 1999). Bacteria were not quantified in the present study, but the potential for nutrients to influence competition between phytoplankton and heterotrophic bacteria merits further study.

Tetracycline-nutrient mixtures affected phytoplankton abundance differently than each individual contaminant. Nutrient addition appeared to counteract the negative effects of TC on *Navicula* sp. abundance in the fall pond experiment, corroborating previous findings that increased nutrient availability reduces contaminant toxicity to phytoplankton (Tubea et al. 1981; Qian et al. 2013; Ozhan and Bargu 2014). In contrast, added nutrients did not seem to counteract or reduce the appearance of TC toxicity in the streams. Instead, TC seemed to reverse adverse effects of nutrients. For example, both total and *Scenedesmus* sp. cell density were highest in the mixture treatment in the fall stream experiment. It is possible that TC removed bacteria from the streams, alleviating competition with phytoplankton (Rhee 1972; Currie and Kalff 1984: Grossart 1999). Another possibility is that the low concentrations of TC were mineralized, resulting in degradation products beneficial to phytoplankton growth (Wong 2000). Exposure to low concentrations of the herbicides 2,4-D and glyphosate stimulated growth of the green alga *Scenedesmus quadricauda*, potentially due to release of beneficial degradation products (Wong et al. 2000). Lastly, low concentrations of TC and nutrients could have caused adaptation of chloroplasts to photosynthetic stress, thereby stimulating phytoplankton growth (Hatfield et al. 1989; Tang et al. 1997). Tang et al. (1997) proposed this mechanism could have been responsible for growth stimulation of the green alga *Chlamydomonas* sp. and the diatom *Synedra acus* after exposure to low concentrations of atrazine. Phytoplankton physiological responses should be investigated to elucidate the mechanism for increased cell density following exposure to TC-nutrient mixtures.

Phytoplankton community composition responded to TC and nutrient addition and changes in temperature/flow conditions, likely due to differences in abiotic requirements and TC sensitivity across taxa (Harding 1994; Wilson et al. 2004; Quinlan et al. 2011). I expected

Navicula sp. to be more tolerant of TC exposure than *Scenedesmus* sp. and *M. aeruginosa* because diatoms are generally more resistant to antibiotics than green algae and cyanobacteria (Halling-Sorensen 2000; Quinlan et al. 2011; Yang et al. 2013), but *Navicula* sp. was the only taxon negatively influenced by TC in the present study. It is possible that *Navicula* sp. are not as tolerant to TC as other diatom taxa. Guo et al. (2016) found that a *Navicula* sp. was more sensitive than both the cyanobacteria *Anabaena flos-aquae* and the green algae *Pseudokirchneriella subcapitata* to trimethoprim, another broad-spectrum antibiotic. Nutrient-only addition stimulated *Navicula* sp. more than the other taxa, consistent with the ability of diatoms to outcompete other algal groups in nutrient replete conditions (Harding 1994; Piehler et al. 2004; Tozzie et al. 2004). In addition to treatment effects, more general seasonal changes in species composition in the present study were also consistent with phytoplankton succession in rivers. Increased irradiance and temperature in the summer allowed *Scenedesmus* sp. to outcompete diatoms while cooler fall temperatures favored diatom abundance (Swale 1969; Leland 2003). In addition, *M. aeruginosa* is usually more successful in warmer water (Lehman et al. 2013; Zhai et al. 2013). Consistent with expectations, *M. aeruginosa* comprised a larger part of the community in summer than fall in all treatments. However, *M. aeruginosa* density was still only up to 4% on average in the summer streams, which could be related to the presence of flow. For example, green algae became more prevalent when flow was added to an artificial lake normally dominated by cyanobacteria (Zhang et al. 2015).

The mixture of TC and nutrients influenced phytoplankton community composition differently compared to the individual contaminants. In the summer, the proportion of *Navicula* sp. in the mixture treatment was greater than the proportions in both the TC- and nutrient-only treatments, which suggests that the addition of both contaminants increased *Navicula* sp. relative

to the other taxa. A different pattern occurred in the fall stream experiment; fewer *Navicula* sp. were present in the mixture than the TC-only treatment. It is possible that temperature changes between seasons increased phytoplankton nutrient uptake (Lomas and Glibert 1999; Staehr and Sand-Jensen 2006), resulting in nutrient availability becoming more important than TC additions in controlling species composition in the colder season. Riedel et al. (2003) also found that seasonal changes in temperature and nutrient availability altered the response of the diatom *Dactyliosolen fragilissimus* to trace metals mixed with nutrient additions. In contrast to both stream experiments, species composition in the fall ponds did not change with TC-nutrient mixtures. Therefore, the effects of TC-nutrient mixtures on phytoplankton species composition are complex and may change depending on seasonal changes in temperature as well as flow regime.

This study demonstrated that mixtures of TC and nutrients influence phytoplankton communities differently than either contaminant alone at environmentally relevant concentrations. TC and nutrients individually had positive, negative, or neutral effects depending on the season and flow regime. TC slowed *Navicula* sp. growth in the fall season under non-flowing conditions, but did not affect abundance in the summer streams or fall ponds. Furthermore, nutrient addition only increased phytoplankton growth in the fall ponds. TC-nutrient mixtures increased %*Navicula* sp. in the summer streams, stimulated *Scenedesmus* sp. growth in the fall streams, and appeared to partially counteract the effects of both TC and nutrients in the fall ponds. Therefore, phytoplankton response to TC, nutrients, and TC-nutrient mixtures are complex and likely dependent on seasonal changes in temperature and flow regime. These findings underscore the need for testing contaminant-nutrient mixtures on communities under environmental conditions to assess risk to aquatic ecosystems.

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Table 1.1: Initial mean water quality measurements ± standard deviation for the summer and fall stream and fall pond experiments.

Experiment	**Variable**	**Treatment**			
		Control	TC	Nutrients	TC + Nutrients
Summer Streams	Temperature (ºC)	29.5 ± 0.3	29.4 ± 0.3	29.6 ±0.4	29.3 ± 0.4
	Dissolved Oxygen (mg L^{-1})	7.1 ± 0.1	7.1 ± 0.1	7.1 ± 0.1	7.1 ± 0.1
	Specific Conductivity (µS cm^{-1})	257.8 ± 3.7	255.4 ± 4.2	256.4 ± 4.1	259.9 ± 5.6
	pH	8.3 ± 0.03	8.3 ± 0.03	8.3 ± 0.04	8.3 ± 0.04
Fall Streams	Temperature (ºC)	25.0 ± 0.4	25.0 ± 0.4	24.9 ± 0.3	25.0 ± 0.2
	Dissolved Oxygen (mg L^{-1})	7.6 ± 0.1	7.8 ± 0.1	7.6 ± 0.1	7.6 ± 0.0
	Specific Conductivity (µS cm^{-1})	267.5 ± 6.2	265.7 ± 5.0	273.9 ± 4.6	277.0 ± 3.0
	pH	7.9 ± 0.1	7.9 ± 0.05	7.9 ±0.1	7.9 ±0.04
Fall Ponds	Temperature (ºC)	18.2 ± 0.3	17.9 ± 0.4	18.2 ± 0.2	18.2 ± 0.3
	Dissolved Oxygen (mg L^{-1})	8.0 ± 0.3	8.0 ± 0.1	8.3 ± 0.1	8.4 ± 0.1
	Specific Conductivity (µS cm^{-1})	252.0 ± 5.5	248.9 ± 6.7	259.4 ± 7.6	260.0 ± 7.8
	pH	7.7 ± 0.2	7.9 ± 0.1	7.9 ± 0.04	7.9 ± 0.1

Table 1.2: Final mean water quality measurements ± standard deviation and final chlorophyll *a* concentrations ± standard error for the summer and fall stream and fall pond experiments. Significant chlorophyll *a* concentration differences between treatments ($^{\epsilon}$) are according to either two-way ANOVA or the Scheirer-Ray-Hare extension of Kruskal-Wallis non-parametric tests (*).

Experiment	**Variable**	**Treatment**			
		Control	TC	Nutrients	TC + Nutrients
Summer Streams	Temperature (ºC)	32.7 ± 0.5	32.3 ± 0.7	32.8 ± 0.6	32.5 ± 0.9
	Dissolved Oxygen (mg L^{-1})	6.7 ± 0.1	6.8 ± 0.1	6.7 ± 0.1	6.7 ± 0.1
	Specific Conductivity (µS cm^{-1})	275.4 ± 5.7	276.8 ± 7.6	288.3 ± 5.5	290.3 ± 4.1
	pH	8.1 ± 0.05	8.2 ± 0.03	8.1 ± 0.06	8.1 ± 0.04
	Chlorophyll *a* (µg L^{-1})	21.5 ± 1.3	17.9 ± 1.6	18.6 ± 1.2	18.2 ± 1.0
Fall Streams	Temperature (ºC)	24.7 ± 0.5	24.7 ±0.3	24.8 ±0.5	24.9 ± 0.4
	Dissolved Oxygen (mg L^{-1})	8.1 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	8.0 ± 0.1
	Specific Conductivity (µS cm^{-1})	269.9 ± 5.6	267.8 ± 6.8	279.5 ± 10.7	280.2 ± 8.2
	pH	7.9 ± 0.1	7.9 ± 0.1	8.0 ± 0.04	7.9 ± 0.1
	Chlorophyll *a* (µg L^{-1})	4.7 ± 0.5	4.0 ± 0.5	3.7 ± 0.5	4.9 ± 0.8
Fall Ponds	Temperature (ºC)	23.4 ± 0.4	23.1 ± 0.2	23.8 ± 0.5	24.2 ± 0.4
	Dissolved Oxygen (mg L^{-1})	9.4 ± 0.6	9.5 ± 0.5	10.5 ± 0.9	10.6 ± 1.0
	Specific Conductivity (µS cm^{-1})	253.7 ± 6.0	252.0 ± 9.4	265.8 ± 7.4	265.3 ± 8.1
	pH	8.3 ± 0.1	8.3 ± 0.2	8.7 ± 0.1	8.8 ± 0.2
	Chlorophyll *a* (µg L^{-1})*$^{\epsilon}$	30.1 ± 3.7	29.4 ± 3.7	45.4 ± 2.1	40.0 ± 3.5

Table 1.3: Analysis of total cell density (two-way repeated measures ANOVA) from the summer streams, fall streams, and fall ponds.

Experiment	**Factor**	**df (Effect, Total)**	**F Ratio**	**p value**
Summer Streams	Nutrients	1, 28	0.65	0.43
	TC	1, 28	1.15	0.29
	Time	7, 22	23.79	**<0.0001**
	TC*Nutrients	1, 28	0.02	0.91
	Nutrients*Time	7, 28	1.25	0.30
	TC*Time	7, 28	0.72	0.52
	Nutrients*TC*Time	7, 28	1.57	0.20
Fall Streams	Nutrients	1, 28	0.06	0.81
	TC	1, 28	10.98	**0.003**
	Time	3, 84	9.51	**<0.0001**
	TC*Nutrients	1, 28	4.60	**0.04**
	Nutrients*Time	3, 84	0.81	0.49
	TC*Time	3, 84	3.33	**0.02**
	Nutrients*TC*Time	3, 84	2.24	0.09
Fall Ponds	Nutrients	1, 28	1.91	0.18
	TC	1, 28	2.08	0.16
	Time	3, 84	383.67	**<0.0001**
	TC*Nutrients	1, 28	0.07	0.79
	Nutrients*Time	3, 84	2.34	0.08
	TC*Time	3, 84	0.53	0.67
	Nutrients*TC*Time	3, 84	0.47	0.70

Table 1.4: Analysis of final day chlorophyll *a* concentrations (two-way ANOVA or Schierer-Ray-Hare extension of the Kruskal-Wallis test (*)) in the summer streams, fall streams, and fall ponds.

Experiment	**Factor**	**df (Effect, Total)**	**F Ratio**	**p value**
Summer Streams	Nutrients	1, 31	1.37*	>0.05
	TC	1, 31	1.94*	>0.05
	TC*Nutrients	1, 31	0.57*	>0.05
Fall Streams	Nutrients	1, 31	0.00	0.99
	TC	1, 31	0.16	0.69
	TC*Nutrients	1, 31	2.33	0.14
Fall Ponds	Nutrients	1, 31	8.20*	**<0.05**
	TC	1, 31	0.051*	>0.05
	TC*Nutrients	1, 31	0.023*	>0.05

Table 1.5: Permutational multivariate analysis of variance (PERMANOVA) comparisons of phytoplankton community composition across treatments in summer and fall streams and fall ponds.

Experiment	**Factors**	**df (Effect, Total)**	**Pseudo-F**	**p value**
Summer Streams	Nutrients	1, 255	3.71	**0.02**
	TC	1, 255	4.27	**0.03**
	Time	7, 255	29.56	**0.0001**
	TC*Nutrients	1, 255	0.94	0.41
	TC*Time	7, 255	1.83	**0.05**
	Nutrients*Time	7, 255	2.71	**0.001**
	Streams(TC*Nutrients)	28, 255	4.19	**0.0001**
	TC*Nutrients*Time	7,255	0.73	0.71
Fall Streams	Nutrients	1, 127	0.66	0.52
	TC	1, 127	4.31	**0.02**
	Time	3, 127	66.97	**0.0001**
	TC*Nutrients	1, 127	2.30	0.13
	TC*Time	3, 127	0.65	0.61
	Nutrients*Time	3, 127	negative	
	Streams(TC*Nutrients)	28, 127	1.40	0.11
	TC*Nutrients*Time	3, 127	0.86	0.49
Fall Ponds	Nutrients	1, 127	1.20	0.17
	TC	1, 127	1.95	0.30
	Time	3, 127	165.23	**0.0001**
	TC*Nutrients	1, 127	1.46	0.24
	TC*Time	3, 127	0.29	0.83
	Nutrients*Time	3, 127	2.70	**0.05**
	Streams(TC*Nutrients)	28, 127	1.53	0.06
	TC*Nutrients*Time	3, 127	0.65	0.60

Table 1.6: Analysis of *Scenedesmus* sp. cell density (two-way repeated measures ANOVA) from the summer streams, fall streams, and fall ponds.

Experiment	**Factor**	**df (Effect, Total)**	**F Ratio**	**p value**
Summer Streams	Nutrients	1, 28	0.87	0.36
	TC	1, 28	1.31	0.26
	Time	7, 22	57.45	**<0.0001**
	TC*Nutrients	1, 28	0.04	0.85
	Nutrients*Time	7, 28	0.93	0.42
	TC*Time	7, 28	0.85	0.46
	Nutrients*TC*Time	7, 28	1.18	0.32
Fall Streams	Nutrients	1, 28	1.32	0.26
	TC	1, 28	0.52	0.47
	Time	3, 84	19.96	**<0.0001**
	TC*Nutrients	1, 28	5.04	**0.03**
	Nutrients*Time	3, 84	0.14	0.90
	TC*Time	3, 84	1.03	0.37
	Nutrients*TC*Time	3, 84	1.35	0.27
Fall Ponds	Nutrients	1, 28	0.51	0.50
	TC	1, 28	0.07	0.80
	Time	3, 84	64.83	**<0.0001**
	TC*Nutrients	1, 28	0.004	0.95
	Nutrients*Time	3, 84	1.15	0.33
	TC*Time	3, 84	0.73	0.49
	Nutrients*TC*Time	3, 84	0.60	0.59

Table 1.7: Analysis of *Navicula* sp. cell density (two-way repeated measures ANOVA) from the summer streams, fall streams, and fall ponds.

Experiment	**Factor**	**df (Effect, Total)**	**F Ratio**	**p value**
Summer Streams	Nutrients	1, 28	5.85	**0.02**
	TC	1, 28	0.27	0.61
	Time	7, 22	2.69	**<0.0001**
	TC*Nutrients	1, 28	0.01	0.92
	Nutrients*Time	7, 28	3.66	**0.02**
	TC*Time	7, 28	0.51	0.66
	Nutrients*TC*Time	7, 28	0.83	0.49
Fall Streams	Nutrients	1, 28	1.72	0.20
	TC	1, 28	26.71	**<0.0001**
	Time	3, 84	19.26	**<0.0001**
	TC*Nutrients	1, 28	2.68	0.11
	Nutrients*Time	3, 84	1.51	0.22
	TC*Time	3, 84	1.60	0.20
	Nutrients*TC*Time	3, 84	1.46	0.23
Fall Ponds	Nutrients	1, 28	2.98	0.10
	TC	1, 28	4.31	**0.05**
	Time	3, 84	545.92	**<0.0001**
	TC*Nutrients	1, 28	0.005	0.94
	Nutrients*Time	3, 84	0.59	0.62
	TC*Time	3, 84	0.41	0.75
	Nutrients*TC*Time	3, 84	0.59	0.62

Table 1.8: Analysis of *M. aeruginosa* cell density (two-way repeated measures ANOVA) from the summer streams, fall streams, and fall ponds.

Experiment	**Factor**	**df (Effect, Total)**	**F Ratio**	**p value**
Summer Streams	Nutrients	1, 28	8.04	**0.008**
	TC	1, 28	3.34	0.08
	Time	7, 22	98.92	**<0.0001**
	TC*Nutrients	1, 28	0.35	0.56
	Nutrients*Time	7, 28	2.56	**0.04**
	TC*Time	7, 28	1.55	0.19
	Nutrients*TC*Time	7, 28	1.89	0.11
Fall Streams	Nutrients	1, 28	2.19	0.15
	TC	1, 28	1.18	0.29
	Time	3, 84	409.84	**<0.0001**
	TC*Nutrients	1, 28	0.02	0.90
	Nutrients*Time	3, 84	0.39	0.71
	TC*Time	3, 84	1.37	0.26
	Nutrients*TC*Time	3, 84	0.49	0.68
Fall Ponds	Nutrients	1, 28	0.38	0.54
	TC	1, 28	0.86	0.36
	Time	3, 84	57.78	**<0.0001**
	TC*Nutrients	1, 28	3.32	0.08
	Nutrients*Time	3, 84	2.98	0.07
	TC*Time	3, 84	0.16	0.83
	Nutrients*TC*Time	3, 84	0.58	0.55

A) B)

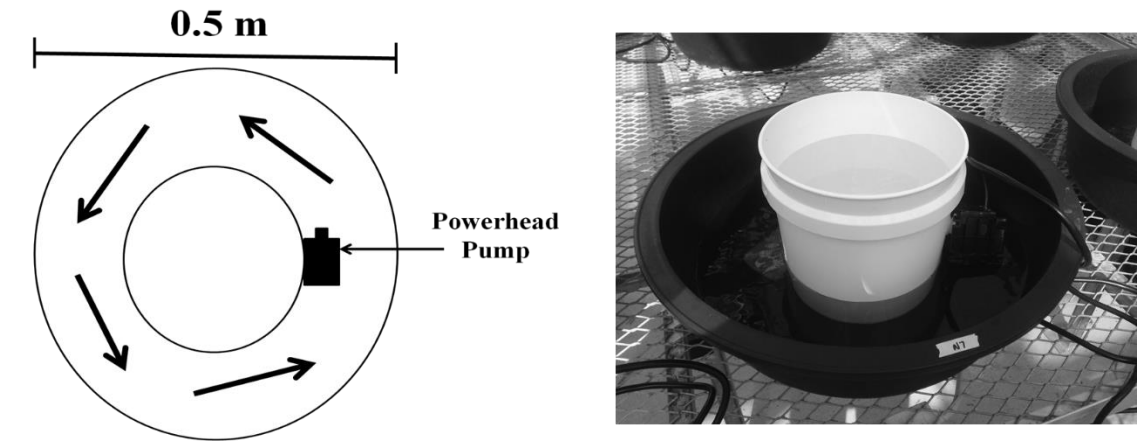

Figure 1.1: A schematic (A) and photograph of an artificial stream and pond unit (B) constructed for this study.

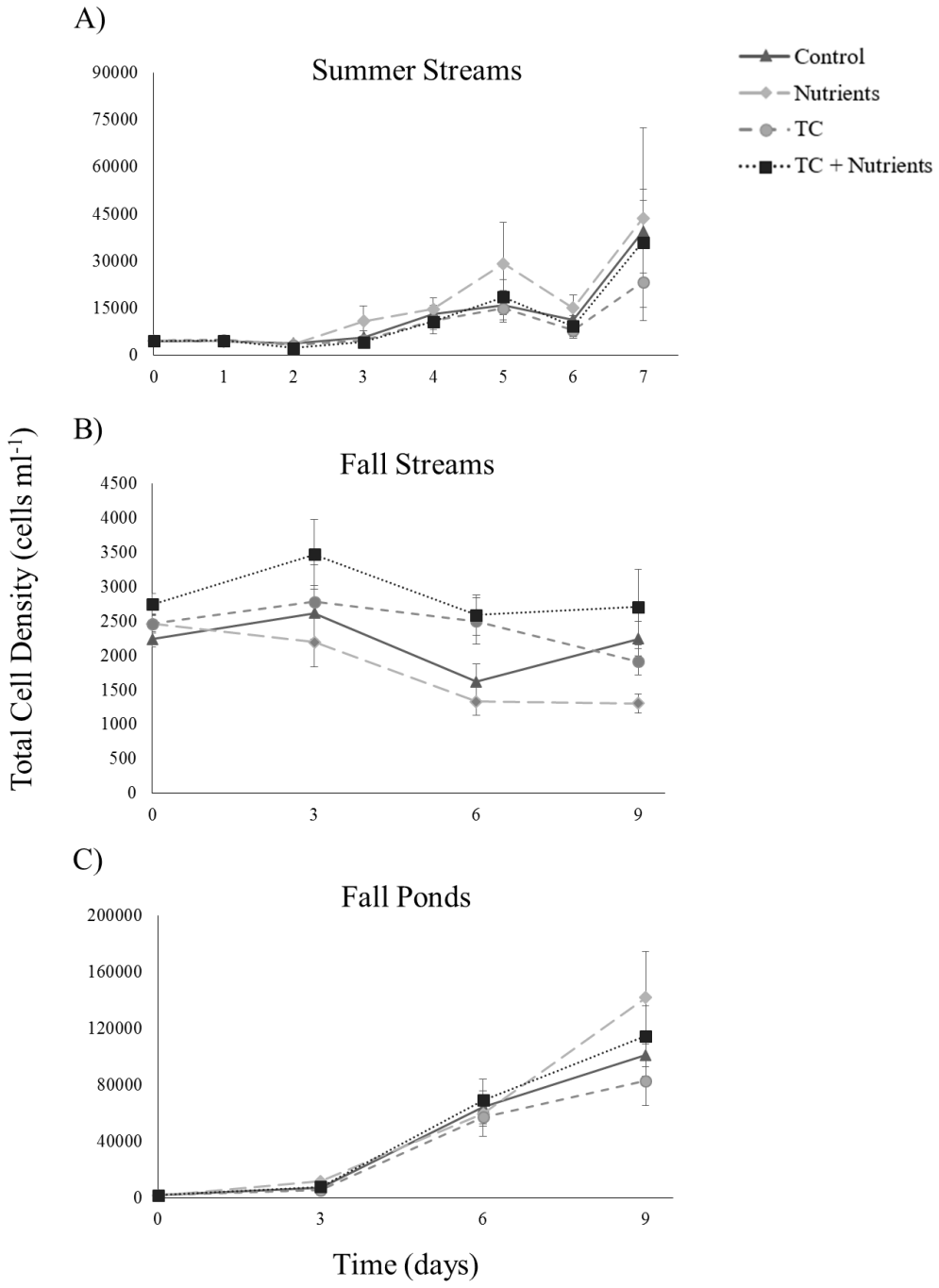

Figure 1.2: Average total cell density in artificial streams and ponds over seven (A) or nine (B,C) days post-exposure to treatments. Error bars are ± one standard error of the mean (SEM) and (n=8).

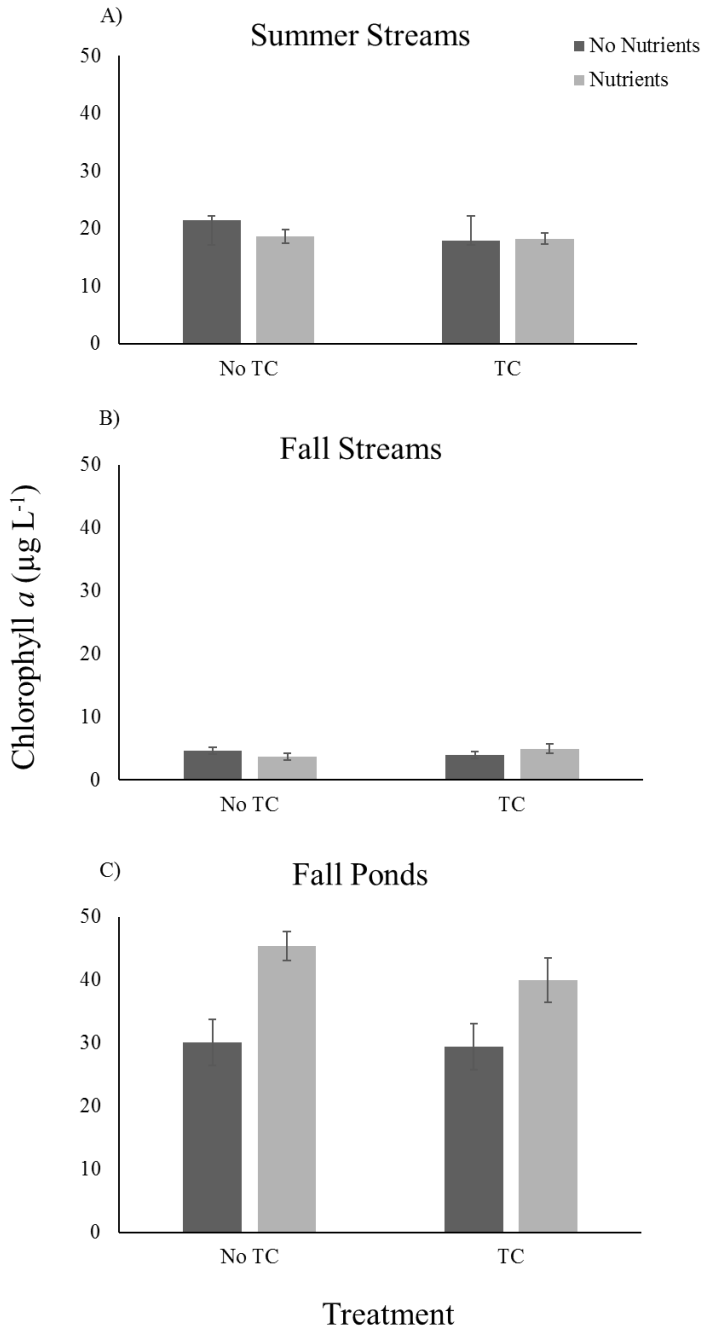

Figure 1.3: Average chlorophyll *a* concentration in artificial streams and ponds after seven (A) or nine (B,C) days post-exposure to treatments. Error bars are ± one standard error of the mean (SEM) and (n=8).

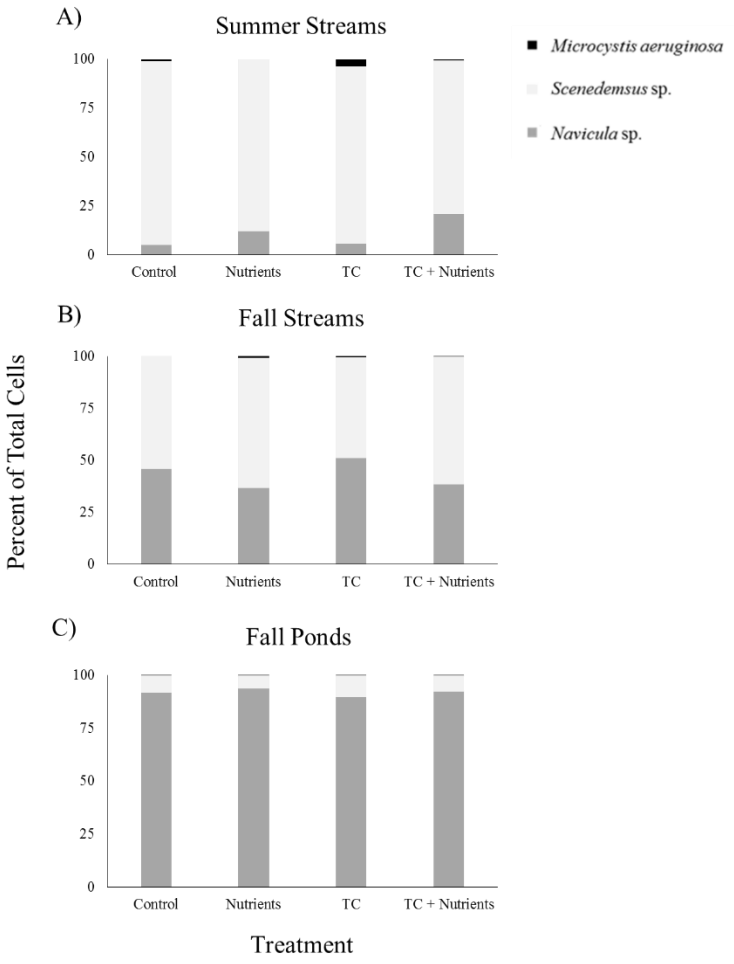

Figure 1.4: Average proportion of cells of each species in artificial streams and ponds on day seven (A) or nine (B,C) following exposure to treatments (n=8).

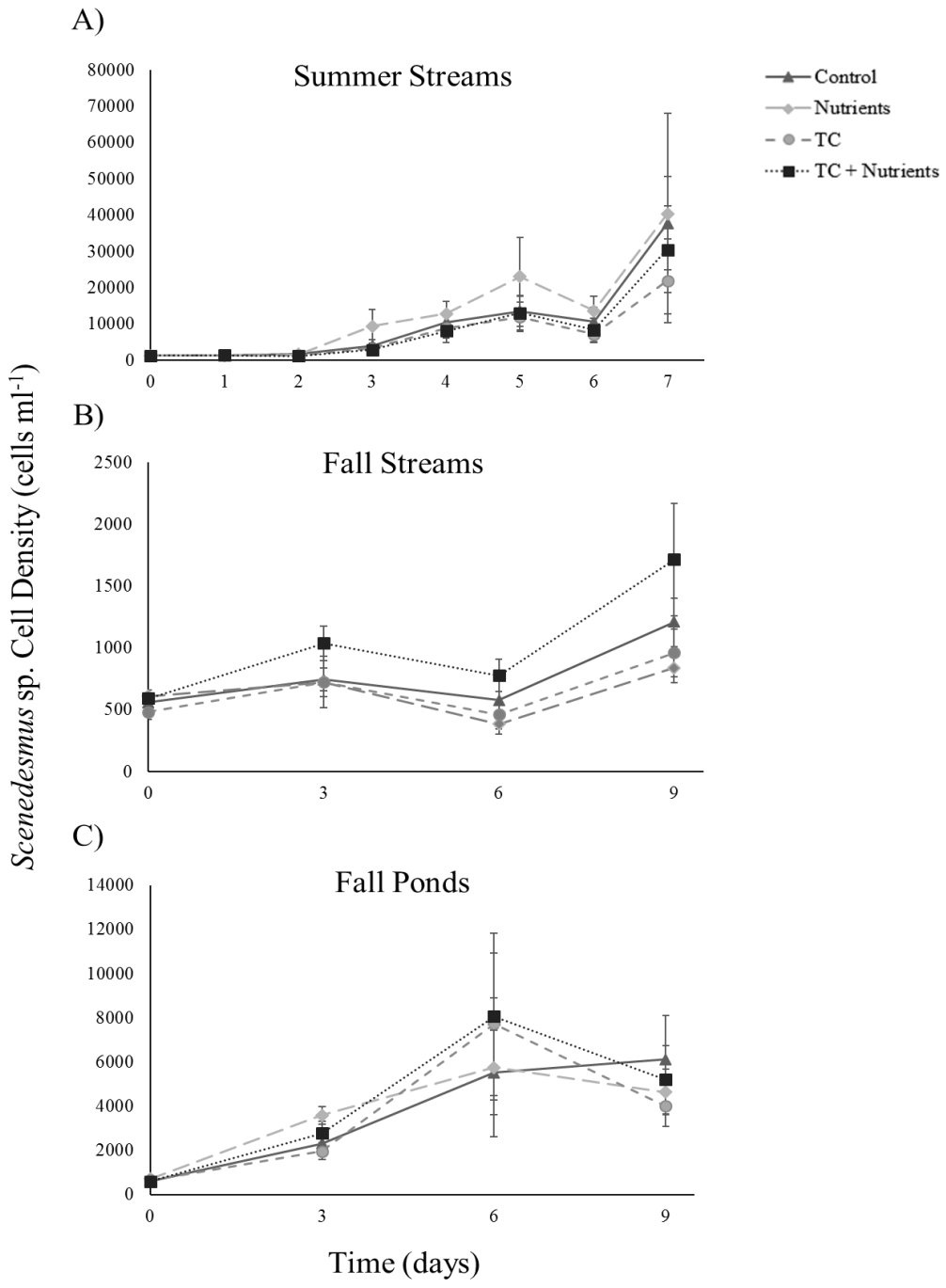

Figure 1.5: Average *Scenedesmus* sp. cell density in artificial stream and ponds over seven (A) or nine (B,C) days post-exposure to treatments. Error bars are ± one SEM and (n=8).

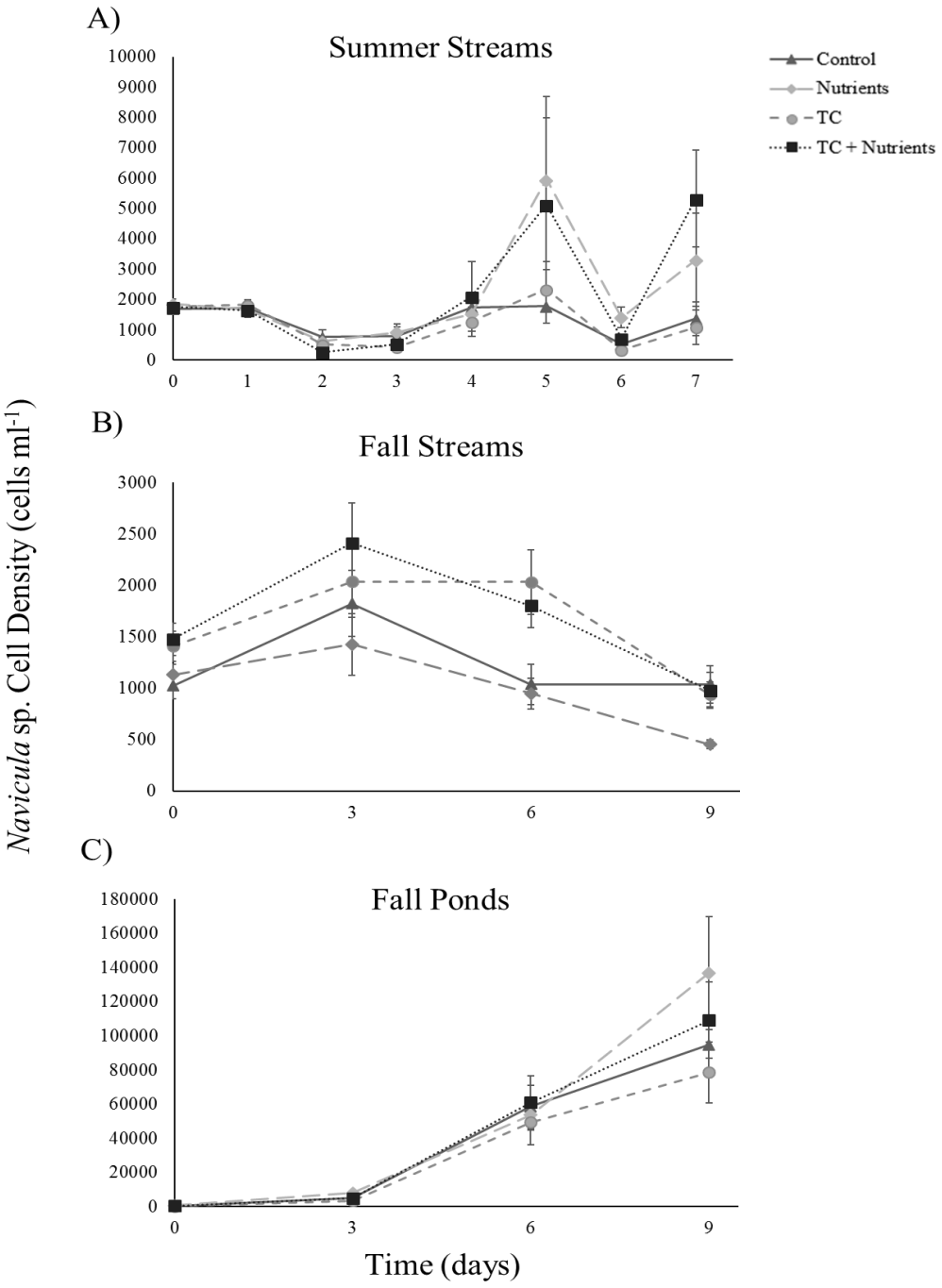

Figure 1.6: Average *Navicula* sp. cell density in artificial stream and ponds over seven (A) or nine (B,C) days post-exposure to treatments. Error bars are ± one SEM and (n=8).

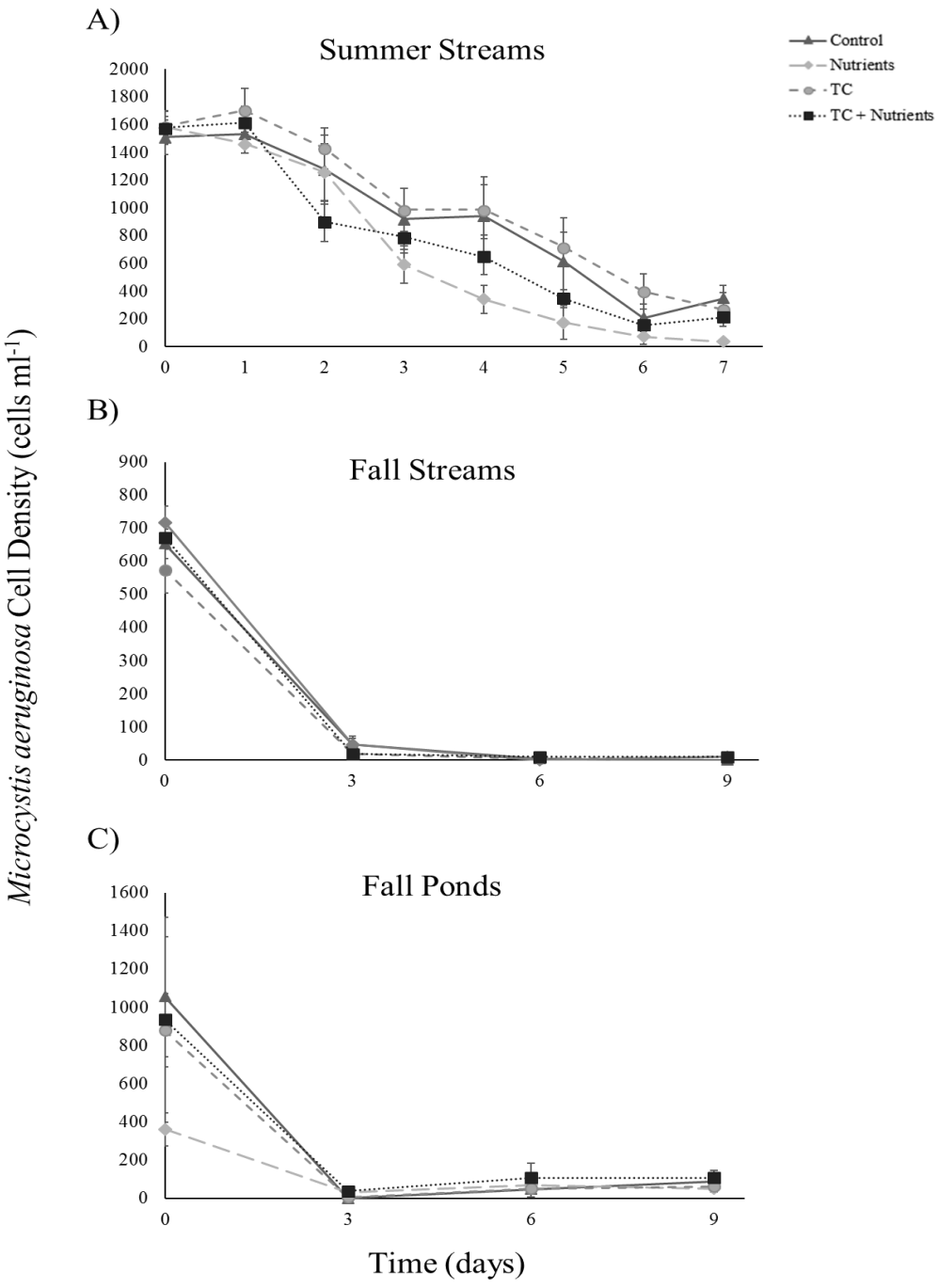

Figure 1.7: Average *M. aeruginosa* cell density in artificial stream and ponds over seven (A) or nine (B,C) days post-exposure to treatments. Error bars are ± one SEM and (n=8).

CHAPTER 2

LAND USE AND ENVIRONMENTAL VARIABLES INFLUENCE ANTIBIOTIC-RESISTANT BACTERIA ABUNDANCE AND PHYTOPLANKTON ASSEMBLAGES IN SOUTHEASTERN COASTAL PLAIN STREAMS

INTRODUCTION

The global presence of antibiotic-resistant bacteria (ARB) in aquatic systems is both a public health and environmental issue (Aminov 2009; Martinez et al. 2009; Allen et al. 2010). Harmful resistant bacteria can either be directly transmitted to humans, or their antibiotic resistance transferred to bacterial species through horizontal gene transfer to form new antibiotic-resistant strains (Kruse and Sorum 1994; Vaz-Moreira et al. 2014). Wastewater effluent and agricultural runoff are also sources of antibiotic pollution whose presence in the environment may select for the evolution of antibiotic resistance (Cattoir et al. 2008; Martinez et al. 2009). Replacement of natural bacterial populations with resistant strains may also disrupt competitive dynamics within microbial communities (Rhee 1972; Currie and Kalff 1984; Drakare 2002). Therefore, it is essential to establish factors that contribute to the formation and persistence of ARB populations in aquatic environments.

The presence of ARB in aquatic environments also has the potential to influence microscopic algal (phytoplankton) populations and species composition. Bacteria compete with phytoplankton including cyanobacteria, chlorophytes, and diatoms for nutrients and micronutrients such as nitrogen, phosphorus, vitamins, and trace metals with the potential to reduce phytoplankton growth (Rhee 1972; Currie and Kalff 1984; Grossart 1999). Resistant-bacteria often outcompete natural bacterial communities (Aminov and Mackie 2007; Martinez et al. 2009), thus replacement of natural bacteria populations with ARB could alter competitive dynamics between phytoplankton and aquatic bacteria. Moreover, antibiotics that accompany

ARB inputs influence phytoplankton abundance via direct toxicity (Halling-Sorensen 2000; Yang et al. 2013) and species composition as a result of differential sensitivity across taxa (Wilson et al. 2004; Quinlan et al. 2011). Despite the potential for ARB presence to influence phytoplankton communities, relationships between ARB and phytoplankton are largely understudied.

Pathways for ARB into aquatic systems include municipal wastewater treatment plant (WWTP) discharge and runoff from both agricultural and undeveloped terrestrial environments (Kim et al. 2007, 2010; Lima-Bittencourt et al. 2007; Storteboom et al. 2010). Resistant bacteria are not typically targeted for removal during WWTP treatment (Schwartz et al. 2003; Guo et al. 2014; Ribeiro et al. 2014). Furthermore, WWTPs create conditions that select for ARB proliferation such as warm temperatures, consistent exposure to dilute concentrations of antibiotics (Mach and Grimes 1982; Kim et al. 2007, 2010; Rizzo et al. 2013) and chlorination of wastewater prior to discharge (Murray et al. 1984; Huang et al. 2011). Antibiotic usage on livestock leads to ARB in surface runoff from liquid manure-fertilized agricultural fields and livestock waste lagoons (Storteboom et al. 2010; Heuer et al. 2011; Maal-Bared et al. 2013). Resistant *E. coli* abundance ranges from <10% in agriculturally influenced streams in France (Servais and Passerat 2009) to 25% of *E. coli* in runoff from an experimental cattle feedlot in Iowa (Jahne et al. 2015). Resistance was also reported in remote areas with little anthropogenic influence, including a remote river island in Alaska (Allen et al. 2009) and a national park in Brazil (Lima-Bittencourt et al. 2007). Thus, the relationship between land use and ARB abundance in surface waters requires further elucidation.

In addition to land use, environmental variables such as temperature, precipitation and nutrient availability influence resistant bacteria populations and phytoplankton communities.

Warmer temperatures increase gene transfer between bacterial populations (Williams et al. 1996) with the potential for increased abundance of ARB and formation of new ARB populations. Precipitation positively correlated with antibiotic-resistant *E. coli* isolated from streams in British Colombia, Canada, likely due to increased runoff of ARB from surrounding agricultural land (Maal-Bared et al. 2013). Nutrient concentrations can increase or decrease bacterial resistance to antibiotics, depending on the type of nutrients and antibiotics present. Nitrate additions decreased resistance of *Pseudomonas aeruginosa* to the antibiotics ciprofloxacin and tobramycin (Borriello et al. 2006). In contrast, Maal-Bared et al. (2013) found nitrate+nitrite and ammonium positively correlated with nalidixic acid resistance, and that phosphate positively correlated with ampicillin and TC-resistance in stream *E. coli* isolates. Warmer temperatures and higher nutrient availability also increase phytoplankton abundance and can alter community composition toward more competitive taxa (Rhee and Gotham 1981; Munn et al. 2002; Mallin et al. 2004; Tozzi et al. 2004), which could then influence competitive dynamics between phytoplankton and bacteria (Rhee 1972; Currie and Kalff 1984; Drakare 2002). These studies underscore the importance of investigating environmental effects on ARB abundance, phytoplankton assemblages, and their competitive interactions.

Tetracycline (TC) is one of the most commonly detected antibiotics in wastewater treatment effluent and soil fertilized with liquid manure, likely contributing to TC-resistance in bacteria and altered phytoplankton species composition in adjacent environments (Hamscher et al. 2002; Karthikeyan and Meyer 2006; Quinlan et al. 2011). In surface waters, TC-resistant *E. coli* concentrations typically range from 1-24%, but TC resistance of up to 51% of *E. coli* isolates was reported near a WWTP outflow in Australia (Parveen et al. 1997; Boon and Cattanach 1999; Edge and Hill 2005; Watkinson et al. 2007a,b). TC also affects phytoplankton

taxonomic composition (Wilson et al. 2004; Quinlan et al. 2011). Heterokont species such as diatoms are less sensitive than chlorophytes, cyanophytes, cryptophytes, dinophytes and cyanobacteria to antibiotics in the tetracycline family (Wilson et al. 2004). For example, cyanobacteria-dominated periphyton communities in artificial streams exposed to environmentally relevant tetracycline concentrations became diatom-dominated (Quinlan et al. 2011), suggesting complex relationships between different TC sources, TC-resistant bacteria and phytoplankton communities.

The southeastern coastal plain region in Georgia, USA is an ideal location to study relationships among land use, environmental variables, bacterial resistance and phytoplankton communities. In the Ogeechee River Basin (ORB), agriculture comprises 36% of the land use, and there are four major municipal WWTPs (≥1 million gallons per day) and many small facilities (19 public, 3 industrial/federal, 18 private/industrial facilities) (Ham and Hatzell 1996; GAEPD 2001). In addition to agricultural land and WWTPs, numerous streams within the ORB have watersheds comprised of >75% natural land (USGS 2011). The region also experiences large temperature fluctuations (<0 ºC to >27 ºC) and variable monthly precipitation (4-32 cm) (National Weather Service 2015; USGS 2015). All of these variables have the potential to influence resistant bacterial abundance and phytoplankton abundance/community composition (Rhee and Gotham 1981; Williams et al. 1996; Maal-Bared et al. 2013).

The present study was conducted to test the hypotheses that: 1) land use influences abundance of TC-resistant bacteria in southeastern coastal plain streams; 2) abundance of TC-resistant bacteria is related to temperature, precipitation, and nutrient availability; and 3) phytoplankton abundance and community composition are related to TC-resistant bacterial abundance. I expected streams receiving WWTP inputs and agricultural runoff to have higher

concentrations of TC-resistant bacteria and greater percent TC-resistant bacteria relative to total *E. coli* than reference streams. I also expected TC-resistant bacterial abundance to positively correlate with temperature, precipitation, and nutrient concentrations. Phytoplankton abundance was expected to decrease with increased competition from TC-resistant bacterial strains. Finally, because ARB are positively correlated with nutrient availability, I expected increases in phytoplankton taxa such as diatoms, with increasing ARB abundance. Concentrations of tetracycline-resistant *E. coli* and phytoplankton abundance/community composition were determined from streams influenced by WWTP, agriculture, or reference streams within the ORB once per month for three months and related to environmental variables. Results from this study advance understanding of land use and environmental relationships with ARB populations and phytoplankton communities in surface waters, and will aid in identifying conditions associated with ARB pollution.

METHODS

Sample Sites and Collection

To examine relationships between land use, bacterial abundance, phytoplankton abundance/community composition and environmental variables over time, water samples were collected from streams representing three land-use patterns: 1) WWTP; 2) agriculture; and 3) reference (n=3) within the Ogeechee River Basin (ORB), southeastern GA, USA (Table 2.1; Figure 2.1). Samples from WWTP sites were collected downstream of known discharge locations (10-200 m, depending on access). Agriculture and reference sites were defined by land use characteristics within their sub-watersheds (Table 2.1). Neither agriculture nor reference sites contained WWTP inputs. Land use data were derived from the most recent National Land Cover

Database (USGS 2011) and calculated for each site using ArcGIS 10.1 (Environmental Systems Research Institute, CA, USA). Grab water samples (1 L) were taken once per month for three months (October-December 2016) at each site using sterilized polyethylene bottles and transported in a dark cooler on ice to the laboratory at Georgia Southern University, Statesboro, GA, USA for fecal coliform, *E. coli*, and TC-resistant *E. coli* analyses within 12-24 hours of collection. Samples (100 ml) were also collected in triplicate for analysis of major ions, turbidity, chlorophyll *a*, and phytoplankton cell density. Lastly, a 500 ml water sample was immediately preserved using glutaraldehyde (0.5-1% of total volume; Katano et al. 2009) for subsequent phytoplankton identification. Water temperature, dissolved oxygen, conductivity, specific conductivity, pH, and depth were measured at all sampling locations during each sampling event using a YSI Pro Plus meter (YSI Inc., Yellow Springs, OH, U.S.A.), streamflow was determined using a standard flow meter (Swoffer Instruments, Seattle, WA, U.S.A.), and light attenuation calculated from measurements taken using a LI-250A light meter (LI-COR, Inc., Lincoln, NE, U.S.A.). Precipitation information was obtained from the USGS gauges located nearest each site (waterdata.usgs.gov/) (Table 2.1).

Bacteria and ARB Analysis

Fecal coliform abundance was determined using the colorimetric Colilert-18 method (Dichter 2001). Colilert-18 reagent was dissolved in 100 ml subsamples (n=3) from each site, sealed in a Quanti-Tray, and incubated at 44.5˚C ± 0.2 ˚C for 18-22 hours. After incubation, wells containing yellow solution indicated positive fecal results. The number of positive fecal results was compared to an IDEXX most probable number estimate table to determine the number of colony forming units per 100 ml of water (CFU/100 ml).

Total *E. coli* and TC-resistant *E. coli* abundances were determined following EPA Method 1604 with a modification to assess TC-resistance (EPA 2002). Subsamples of 100, 50, and 10 ml were filtered through cellulose nitrate membrane filters (nominal pore size 0.45 µm) to capture bacterial cells (n=3). Smaller volumes (100, 10, and 1 ml) were filtered for samples suspected to have bacterial concentrations that might result in too many colonies to count accurately (>800 colonies). Filters were placed on either standard MI agar (MI base) for total *E. coli* or MI agar with 16 mg L^{-1} tetracycline hydrochloride added (MI tet) for detection of resistant *E. coli* and incubated at 35 ˚C ± 0.5 ˚C for 22-24 hours to allow for bacterial growth. After incubation, the number of individual colonies on each type of agar was counted.

Phytoplankton Abundance and Taxonomic Composition

Phytoplankton abundance at each site was estimated by measuring pigment concentration (chlorophyll *a*) and cell density. Chlorophyll *a* concentration was determined by filtering 100 ml samples through Whatman GF/F glass fiber filters (nominal pore size 0.7 µm) to collect phytoplankton cells, followed by extraction of pigments from the cells in 90% acetone in the dark for 24 hours at -20°C (n=3). The concentration of the extracted pigments was determined using a Trilogy fluorometer (Turner Designs, CA, U.S.A.) using EPA method 445.0 (Arar and Collins 1997). Phytoplankton cell density was obtained using a BD Accuri C6 flow cytometer (Becton-Dickinson, CA, U.S.A.), which uses lasers to count and distinguish cells based on size and pigment characteristics (Veldhuis and Kraay 2000).

Taxonomic composition of the phytoplankton assemblage at each site was determined visually using a Sedgwick-rafter counting chamber and an inverted light microscope at 400x magnification (EVOS; Life Technologies, CA, USA). The procedure for sample preparation and

counting was modified from LeGresley and McDermott (2010). Each 500 ml sample was settled for a minimum of 24 hours, followed by concentration by decanting liquid to a volume of final 10-100 ml to reach a density of ~10-50 cells per transect. Samples were then homogenized by inversion and 1 ml subsamples pipetted into the Sedgwick-rafter chamber. Phytoplankton were identified and enumerated until 30 transects were scanned or 200 individuals of the most numerous taxon in each sample were recorded. Taxa were separated into the following groups based on trophic similarity for statistical analyses: 1) chlorophyta (chlorophytes and euglenoids); 2) heterokonts (diatoms and chrysophytes); 3) cyanophyta (cyanobacteria); and 4) cryptophyta (cryptophytes)(Yodzis and Winemiller 1999; Wilson et al. 2004). Only samples from October were included in analysis of taxonomic composition because phytoplankton and bacteria abundances were expected to be higher due to warmer water temperature than November or December, thus maximizing potential to evaluate relative abundance of each group.

Water Chemistry

Turbidity was measured immediately upon return to the laboratory using a LaMotte 2020 turbidity meter (LaMotte Company, MD, U.S.A.) according to EPA method 180.1 (EPA 1993). Samples for ion analysis were filtered through Target2 PVDF filters (nominal pore size 0.2 µm) and stored < one month at -4˚C prior to measurement of F^-, Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, and Ca^{2+} concentrations using a Dionex 600DX ion chromatograph (Dionex Corporation, CA, U.S.A.) at the Georgia Southern University Hydrogeochemistry Laboratory.

Data Analysis

Fecal coliform, *E. coli*, ARB and phytoplankton abundance for each month were compared across the three land use categories (WWTP, agriculture, and reference) and time (October, November, and December) using two-way analysis of variance (ANOVA). Assumptions of normality and homogeneity of variances were tested using the Shapiro Wilk *W* test and Levene's test, respectively. Data for % ARB did not meet assumptions of normality or equal variances and were analyzed using the non-parametric analog to two-way ANOVA, the Scheirer-Ray-Hare extension of the Kruskal-Wallis test. Spearman correlations were used to determine relationships between bacterial abundance, phytoplankton abundance, and environmental variables. All univariate statistical analyses were performed using JMP Pro 12.0.0 (SAS Institute Inc., Cary, NC). Significance level was set at ≤ 0.05 for all tests, and p-values of ≤ 0.1 were considered trends.

To examine relationships between relative abundance of the phytoplankton taxonomic groups, land use categories, and environmental variables in October, abundance of each taxonomic group was first converted to proportion of the total abundance. Bray-Curtis resemblance matrices were created for phytoplankton proportions followed by permutational multivariate analysis of variance (PERMANOVA) to test for significant differences in taxonomic composition among land use categories. The relationship between environmental variables and patterns in phytoplankton taxonomic composition was determined using a RELATE procedure and BEST analysis. Lastly, to determine which environmental variables explained changes in taxonomic composition and the proportion of variation explained by each factor, environmental data were first log(x+1) transformed and normalized to account for inherent differences in units and scales across measurement types (Clarke and Gorley 2006),

followed by distance-based linear modeling (DistLM) using step-wise selection. Variables correlated ($p \leq 0.05$) with phytoplankton community data (temperature, PO_4^{3-}, NO_3^-, K^+, Cl^-, and F^-) were included in the DistLM analysis (Table 2.6). Finally, ordination plots were created to visualize relationships between community differences and environmental variables using Distance-based Redundancy Analysis (dbRDA). All multivariate analyses were conducted using PRIMER-E v.7 and the PERMANOVA+ add-on (Plymouth Marine Laboratory, U.K.).

RESULTS

Concentration of *E. coli* and fecal coliforms was influenced by an interaction between land use and month (Table 2.2). The interaction was likely due to higher concentrations of bacteria at reference sites in October compared to the other two months (Figure 2.2). Average *E. coli* concentrations at reference sites exceeded 7500 CFU 100 ml^{-1} (GA EPD threshold for recreational waters = 126 CFU 100 ml^{-1}) in October, twice the average concentration at WWTP and agriculture sites, and then decreased to less than 90 CFU 100 ml^{-1} in November and December (Figure 2.2A). Average *E. coli* concentration at WWTP sites was similar to reference sites in November (97 CFU 100 ml^{-1}), but increased to >1000 CFU 100 ml^{-1} in December (Figure 2.2A). Concentrations of *E. coli* at agriculture sites increased seven-fold compared to WWTP and reference sites in November, (Figure 2.2A). Fecal coliform concentrations generally followed the same patterns as *E. coli* concentrations (Figure 2.2B).

An interaction between land use and month also occurred for TC-resistant *E. coli* (ARB) (Table 2.2). Concentration of ARB appeared to be highest in October, exceeding 300 CFU 100 ml^{-1} at reference and WWTP sites, then decreased to less than 1 CFU 100 ml^{-1} in all land use categories in November (Figure 2.3A). In December, ARB concentrations at reference sites

remained below 1 CFU 100 ml^{-1}, while ARB at WWTP sites increased to >50 CFU 100 ml^{-1} (Figure 2.3A). Average ARB concentration at agriculture sites never exceeded 5 CFU 100 ml^{-1} (Figure 2.3A). Only month influenced the percent of TC-resistant *E. coli* (%ARB) present (Table 2.2). The %ARB was very low in November (<1%) compared to October (at all land use types) and December (at WWTP sites) (Figure 2.3B). There was a trend toward WWTP sites having the highest %ARB compared to the other land use categories (Table 2.2) in October (12%) and December (17%) (Figure 2.3B).

Precipitation positively correlated with all bacterial variables. Temperature, conductivity, and SO_4^{2-} concentration positively correlated with all bacterial variables except *E. coli*, which only correlated positively with precipitation and turbidity (Table 2.3). Only %ARB negatively correlated with light attenuation, positively correlated with PO_4^{3-}, and trended toward a positive correlation with NO_3^- (Table 2.3).

Phytoplankton chlorophyll *a* concentration was affected by land use (Table 2.4). Average chlorophyll *a* concentrations at WWTP and agriculture sites ranged from 1.1 to 3.2 µg L^{-1} in October and December, whereas concentrations at reference sites did not exceed 0.7 µg L^{-1} (Figure 2.3). Phytoplankton cell density and chlorophyll *a* concentration relationships with environmental variables included positive correlations with temperature, conductivity, pH, and nutrient concentrations. However, phytoplankton cell density and chlorophyll *a* concentration negatively correlated with light attenuation and depth (Table 2.5). At the reference sites the water was exceptionally dark in color, and the average light attenuation coefficient exceeded 7 m^{-1}. In contrast, WWTP attenuation coefficients ranged from 1.5-2 m^{-1} and agriculture coefficients from 3.4-3.9 m^{-1} in November and December. Phytoplankton cell density positively correlated with

abundance of fecal coliform, *E. coli*, and %ARB, while chlorophyll *a* concentration trended toward positive correlation with %ARB (Table 2.5).

Although phytoplankton taxonomic composition in October did not differ due to land use (PERMANOVA, pseudo-$F_{2,8} = 1.28$, $p = 0.32$), PO_4^{3-} concentration was the strongest determinant of phytoplankton community composition (BEST analysis, $R_{ho} = 0.67$, $p = 0.072$). Furthermore, 84.1% of variation in the phytoplankton community was explained when temperature, NO_3^-, K^+, Cl^-, and F^- were added into the DistLM analysis (Figure 2.5). Higher concentrations of these explanatory variables and abundance of heterokont species (30-50%) were generally associated with the two large WWTP facilities (Figure 2.5). In contrast, heterokont abundance at the reference sites did not exceed 14% (Appendix 1). Fecal coliform, *E. coli*, ARB, and %ARB did not correlate with patterns in phytoplankton taxonomic composition in October.

DISCUSSION

I hypothesized that land use and environmental variables influence antibiotic-resistant bacteria (ARB) and phytoplankton populations in southeastern coastal plain streams of Georgia, USA, and that ARB abundance is related to phytoplankton abundance and community composition. Wastewater treatment plant (WWTP) and agricultural sites were expected to be sources of fecal coliforms, *E. coli*, total ARB and %ARB (Watkinson et al. 2007a,b; Maal-Bared et al. 2013; Ribeiro et al. 2014), and I generally observed increased ARB abundance and %ARB at WWTPs compared to reference sites. Furthermore, many of the environmental variables that correlated with %ARB, such as temperature, conductivity, and nutrient concentration (PO_4^{3-}, SO_4^{2-}), are generally associated with WWTP inputs (Daniel et al. 2002; Kinouchi et al. 2007; Carey and Migliaccio 2009). The fecal coliform and *E. coli* concentrations at the agricultural

sites were also consistently elevated relative to the Georgia EPD criteria for recreational freshwater (EPD 2012), lending support for agricultural contributions of bacteria to streams (Hagedorn et al. 1999; Hyland et al. 2003; Servais and Passerat 2009; Maal-Bared et al. 2013; Wambugu et al. 2015). Unexpectedly, total ARB and %ARB at agriculture sites was more similar to reference sites than WWTP sites, likely because of high variability in ARB contribution associated with type of agriculture, amount of antibiotic usage, timing/rate of manure application, and watershed characteristics (Kumar et al. 2005). Therefore, future investigation into agricultural ARB contributions to surface water will need to incorporate watershed and agriculture type into the analysis.

While the prediction that reference sites should have the least bacteria of the land use types examined was generally supported, concentration of fecal coliforms, *E. coli*, and ARB in October met or exceeded those at the WWTP sites. One explanation is that the bacteria did not originate at the reference sites, but entered the streams following increased precipitation and terrestrial runoff (Hyland et al. 2003; Sanders et al. 2013). This sampling event was preceded by a hurricane with substantial precipitation (16.7-19.2 cm) (USGS 2017). The strong positive correlation between SO_4^{2-} concentration and the bacterial variables was also indicative of terrestrial runoff; SO_4^{2-} desorbs from soils during storm events resulting in increased concentrations in receiving streams (Rose 2002; Schoonover and Lockaby 2006). Precipitation-driven increases in TC-resistance were also observed in streams in a protected National Park (Serra do Cipó, Brazil) (Lima-Bittencourt et al. 2007). Another possibility is that precipitation triggered resuspension of bacteria from sediments into the water column (Jamieson et al. 2005; Russo et al. 2011). Jamieson et al. (2005) traced *E. coli* populations from sediments in a small alluvial stream and determined that increased resuspension occurred with increasing discharge

following a storm. Regardless of whether the contribution of ARB was primarily from terrestrial runoff or resuspension from sediments, these results suggest that land use and precipitation can influence fecal bacteria and ARB concentrations in ORB streams on both local and regional scales.

Phytoplankton abundance and taxonomic composition were generally unrelated to the presence of resistant bacteria. Phytoplankton abundance was expected to negatively correlate with ARB abundance and %ARB because bacteria can outcompete phytoplankton for essential nutrients (Rhee 1972; Currie and Kalff 1984; Drakare 2002) and resistant-bacteria are often more competitive than natural bacterial communities (Aminov and Mackie 2007; Martinez et al. 2009). In contrast, results from this study showed phytoplankton cell density positively correlated with %ARB, and taxonomic composition did not correlate with any of the bacteria variables. Grossart et al. (1999) found that interactions between natural bacterial populations and marine diatoms (*Cylindrotheca fusi-formis*, *Thalassiosira weissflogii*, and *Nitzschia laevis*) depended on environmental conditions, including amount and ratio of organic to inorganic nutrients. Therefore it is possible that abiotic factors outweigh the negative effects of competition between resistant-bacteria and phytoplankton communities in streams.

Phytoplankton communities were related to environmental variables associated with WWTPs and agricultural sites, particularly light and nutrient availability. The clear water at the WWTP sites relative to reference sites suggested the increased phytoplankton abundance was related to light availability. Light attenuation regulates phytoplankton abundance in blackwater systems such as the ORB where high dissolved organic acid content results in dark color and low light availability (Phlips et al. 2000; Lawrenz et al. 2010). Nutrient concentration is also an important predictor of phytoplankton abundance and species composition in blackwater systems

(Mallin et al. 2004). In this study, naviculoid diatoms comprised 23% of the community from the two largest WWTP sites compared to <4% in the reference streams. Diatoms in particular generally outcompete other algal groups under nutrient replete conditions (Harding 1994; Piehler et al. 2004; Tozzie et al. 2004) and naviculoid diatoms are highly tolerant of pollution associated with wastewater inputs (Palmer 1969; Juttner et al. 1996). Thus, the combination of increased light and nutrient availability could outweigh negative effects of antibiotics and competition with ARB associated with WWTPs in the ORB.

Results from this study indicate that the presence of fecal bacteria and ARB is related to land use in the ORB. Agricultural locations appear to be local sources of fecal bacteria while WWTPs contribute both fecal bacteria and resistant bacteria to adjacent surface waters. Environmental variables associated with WWTP inputs and terrestrial runoff such as nutrient and light availability also positively correlate with bacteria and phytoplankton abundance in ORB streams. These findings also suggest that regional land use affects ARB delivery to streams, particularly after major precipitation events. In addition, changes in light availability and nutrient concentration associated with WWTP and agricultural inputs that promote phytoplankton growth appear to outweigh competitive effects of resistant bacteria and/or adverse effects of antibiotics. These findings demonstrate that WWTP inputs should be targeted for monitoring using water quality measures that include resistant-bacteria populations and phytoplankton communities.

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Table 2.1: Land use characteristics within the sub-watersheds of each stream sampled. All streams were within the Ogeechee River Basin (ORB) (Figure 1). USGS gauges located nearest each site were used to collect precipitation data.

Category	Site	% Natural	% Agriculture	% Developed	USGS Gauge Code
Reference	Ash Branch (AB)	80.4	13.3	6.3	02202600
	Pole Branch (PB)	75.2	19.8	5.3	02202600
	Mill Creek (MC)*	56.1	38.3	5.6	02202190
WWTP	Fort Stewart (FS)	73.2	2.4	24.4	02203518
	Little Lotts Creek (LL)	46.1	25.1	28.8	02202040
	Millen (ML)	68.2	24.4	7.4	02201230
Agriculture	Woodcock Branch (WC)	41.6	49.9	8.1	02202040
	Unnamed Trib. (UT)	50.1	42.9	7.0	02202040
	Big Branch (BB)	50.1	42.9	7.0	02202040

*Due to difficulty finding stream sections without agricultural influence within the ORB, land-use within 1 km of sample spots was also considered for reference sites. While the Mill Creek (MC) sub-watershed only contained 56.1% natural land, it had 85.2% natural land within 1 km of the sampling location for this study.

Table 2.2: The effects of land use and month on bacteria variables. Analyses were either two way ANOVA or non-parametric Kruskal-Wallis with the Scheirer-Ray-Hare extension (*). Numbers in bold type indicate significant differences.

Variable	**Variable**	**F-ratio (or H-statistic*)**	**df**	**p**
Fecal coliforms	Land Use	0.20	2	0.82
	Month	13.62	2	**0.0003**
	Land Use*Month	5.36	4	**0.005**
E. coli	Land Use	0.36	2	0.70
	Month	9.43	2	**0.002**
	Land Use*Month	5.15	4	**0.006**
ARB	Land Use	35.81	2	**<0.0001**
	Month	102.56	2	**<0.0001**
	Land Use*Month	37.47	4	**<0.0001**
%ARB	Land Use	4.82*	2	0.05<p<0.1
	Month	12.29*	2	**<0.05**
	Land Use*Month	3.70*	4	>0.05

Table 2.3: Relationships between bacteria and environmental variables across sampling events (Spearman Correlations).

	Fecal coliforms		***E. coli***		**ARB**		**%ARB**	
Variable	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p
Temperature	**0.34**	**0.005**	0.17	0.12	**0.34**	**0.002**	**0.38**	**0.0006**
Dissolved Oxygen	0.02	0.87	-0.09	0.44	0.005	0.97	0.08	0.49
Conductivity	**0.26**	**0.04**	0.12	0.29	**0.22**	**0.04**	**0.33**	**0.003**
Specific Conductance	0.16	0.19	0.04	0.75	0.13	0.25	**0.26**	**0.02**
pH	0.12	0.35	0.02	0.89	0.04	0.72	0.15	0.18
Turbidity	0.04	0.74	**0.23**	**0.04**	0.07	0.53	0.07	0.56
Precipitation	**0.73**	**<0.0001**	**0.62**	**<0.0001**	**0.77**	**<0.0001**	**0.68**	**<0.0001**
Light Attenuation	-0.001	0.99	0.09	0.49	-0.06	0.62	**-0.25**	**0.05**
F^-	0.16	0.19	0.09	0.40	0.08	0.48	0.16	0.15
Cl^-	-0.06	0.62	-0.09	0.43	-0.07	0.54	0.06	0.58
NO_3^{2-}	0.15	0.24	0.09	0.42	0.08	0.45	0.19	0.09
PO_4^{3-}	0.19	0.13	-0.05	0.64	0.17	0.12	**0.29**	**0.008**
SO_4^{2-}	**0.26**	**0.03**	0.07	0.51	**0.34**	**0.002**	**0.39**	**0.0003**
Na^+	-0.04	0.74	-0.05	0.66	-0.07	0.56	0.03	0.79
NH_4^+	-0.02	0.89	0.12	0.28	-0.008	0.94	0.11	0.31
K^+	0.11	0.39	0.11	0.35	0.08	0.46	0.19	0.09
Mg^{2+}	0.11	0.40	0.11	0.31	-0.02	0.85	0.03	0.77
Ca^{2+}	0.14	0.25	-0.04	0.71	0.13	0.26	**0.27**	**0.01**

Table 2.4: Total phytoplankton cell density and chlorophyll *a* concentration by land use and month (two-way ANOVA).

Variable	**Factor**	**F Ratio**	**df**	**p**
Total Cell Density (cells ml^{-1})	Land Use	0.41	2	0.67
	Month	0.81	2	0.46
	Land Use*Month	0.70	4	0.60
Chlorophyll a ($\mu g\ L^{-1}$)	Land Use	3.54	2	**0.05**
	Month	0.77	2	0.48
	Land Use*Month	1.26	4	0.32

Table 2.5: Relationships between total phytoplankton abundance (cell density and chlorophyll *a*) and environmental/bacterial variables across all months (Spearman Correlations).

	Cell Density		**Chlorophyll *a***	
Variable	Spearman ρ	p	Spearman ρ	p
Temperature	**0.35**	**0.002**	**0.48**	**<0.0001**
Dissolved Oxygen (mg L^{-1})	**0.28**	**0.01**	0.21	0.06
Conductivity	**0.26**	**0.02**	**0.49**	**<0.0001**
Specific Conductance	**0.22**	**0.05**	**0.46**	**<0.0001**
pH	**0.34**	**0.002**	**0.41**	**0.0001**
Turbidity	0.21	0.06	**0.26**	**0.02**
Depth	**-0.41**	**0.0002**	**-0.51**	**<0.0001**
Precipitation (72 hours)	0.06	0.60	-0.006	0.96
Precipitation (12 days)	0.21	0.06	0.02	0.86
Light Attenuation	**-0.35**	**0.004**	**-0.44**	**0.0002**
F^-	**0.54**	**<0.0002**	**0.69**	**<0.0001**
Cl^-	**0.38**	**0.0004**	**0.59**	**<0.0001**
NO_3^{2-}	**0.46**	**<0.0001**	**0.51**	**<0.0001**
PO_4^{3-}	**0.46**	**<0.0001**	**0.43**	**<0.0001**
SO_4^{2-}	**0.25**	**0.03**	**0.23**	**0.04**
Na^+	0.21	0.06	**0.51**	**<0.0001**
NH_4^+	0.09	0.42	0.16	0.15
K^+	**0.51**	**<0.0001**	**0.67**	**<0.0001**
Mg^{2+}	**0.39**	**0.0003**	**0.54**	**<0.0001**
Ca^{2+}	0.17	0.13	**0.38**	**0.0005**
Fecal coliforms	**0.29**	**.021**	0.11	0.39
E. coli	**0.30**	**0.006**	0.13	0.24
ARB	0.19	0.10	0.09	0.43
%ARB	**0.26**	**0.02**	0.21	0.06

Table 2.6: Relationships between environmental variables and phytoplankton taxonomic composition in October from distance-based linear models.

Variable	**SS(trace)**	**Pseudo-F**	**p**	**Prop.**
Temperature	1544.8	6.90	**0.01**	0.50
F^-	1440.6	6.03	**0.02**	0.46
Cl^-	1108.6	3.87	**0.05**	0.36
PO_4^{3-}	1601.8	7.42	**0.01**	0.51
NO_3^-	1523.6	6.71	**0.01**	0.49
K^+	1207.8	4.44	**0.04**	0.39

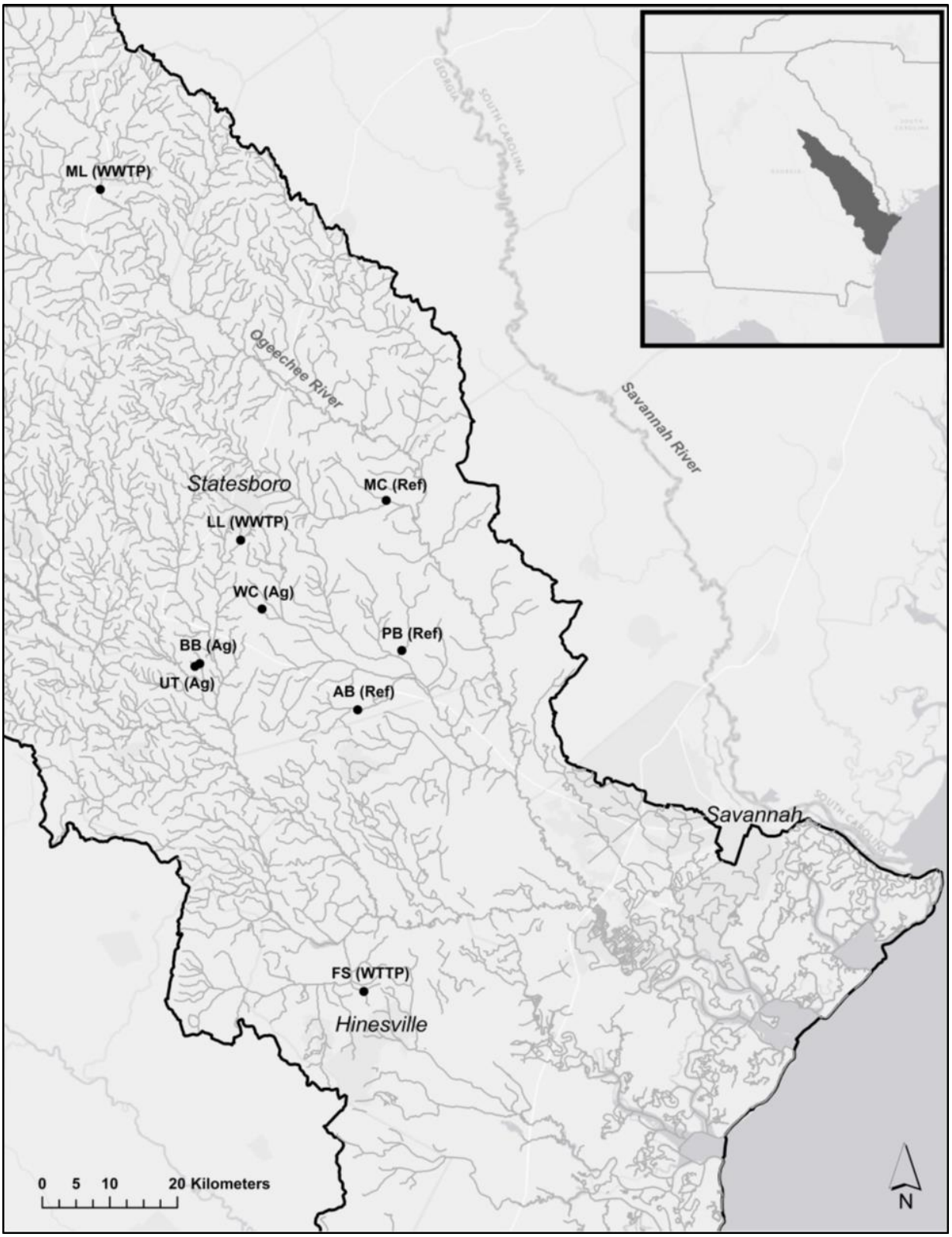

Figure 2.1: Location of sample sites within the Ogeechee River Basin, GA, USA (inset), a major river system located in the southeastern coastal plain.

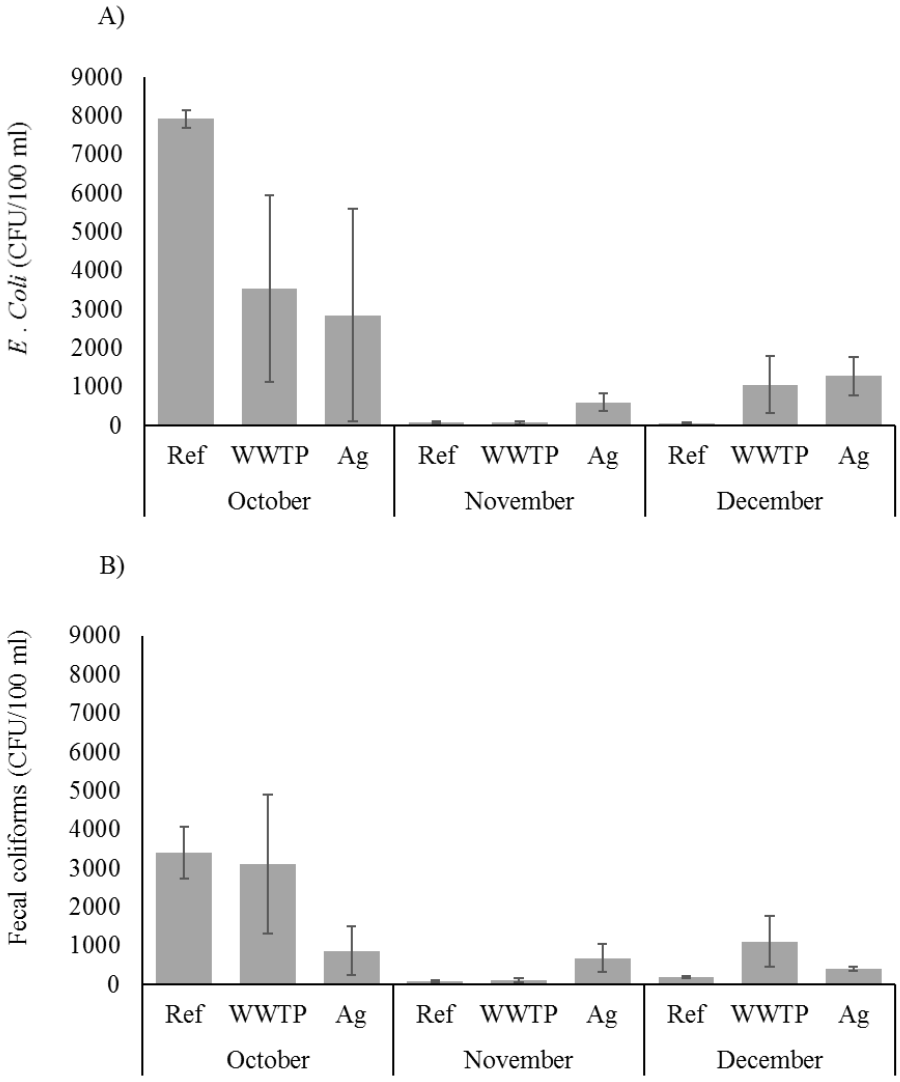

Figure 2.2: Average concentrations of *E. coli* (A) and fecal coliforms (B) from water samples taken at sites with different land use in October, November, and December 2016. Error bars are ± one standard error of the mean (SEM) and (n=3).

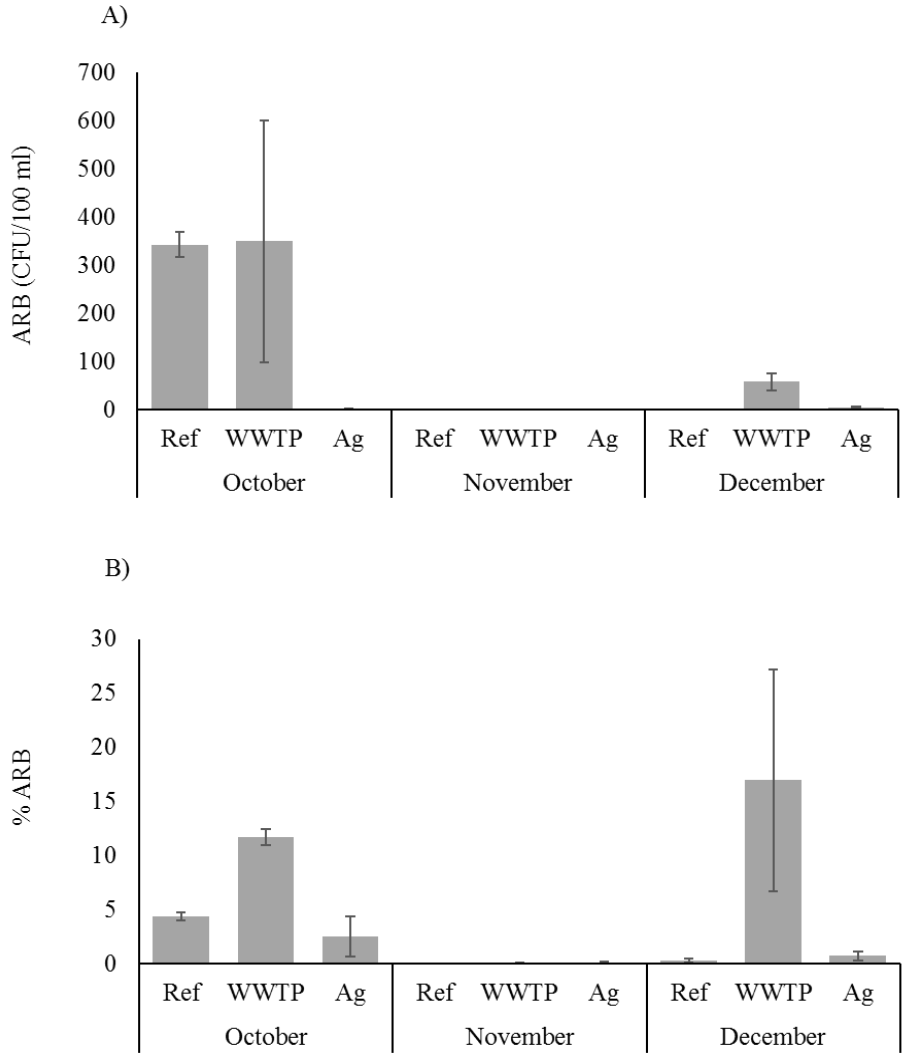

Figure 2.3: Average concentrations of *E. coli* resistant to TC (ARB) (A) and percent ARB relative to total *E. coli* (%ARB) (B) from water samples taken at sites with different land use in October, November, and December 2016. Error bars are ± one SEM and (n=3).

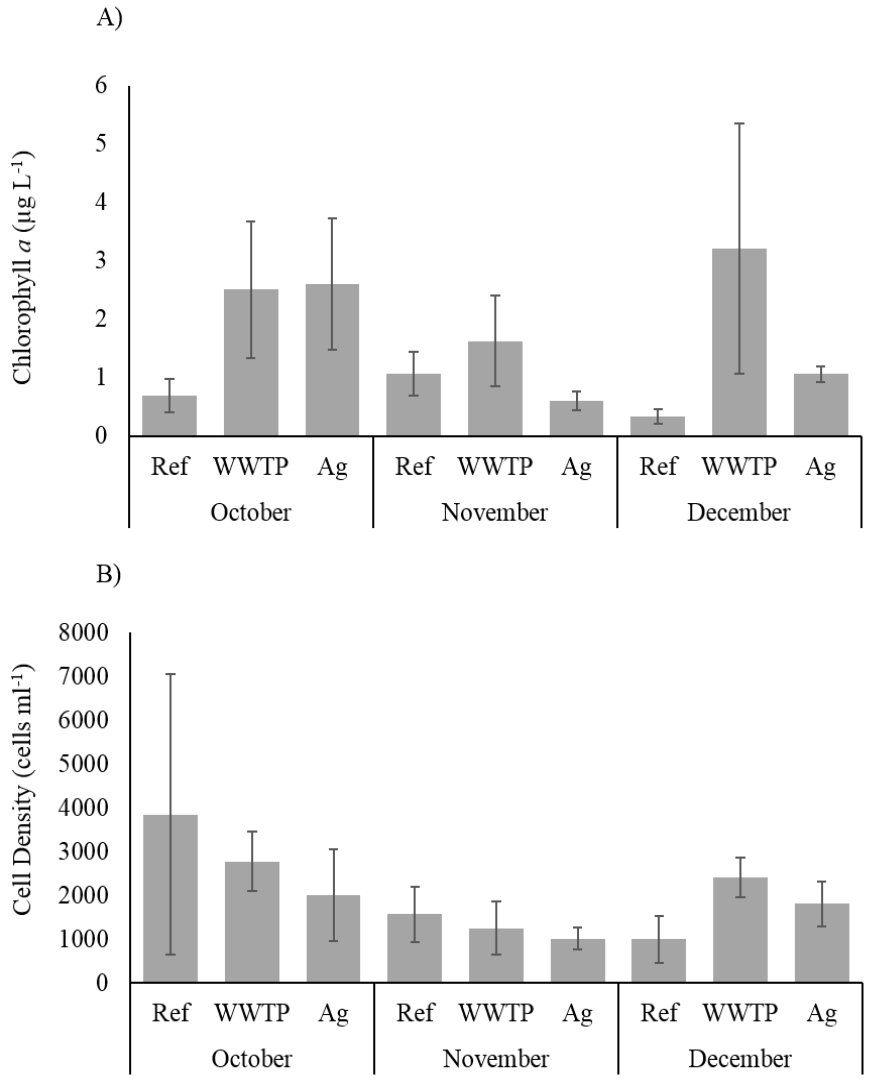

Figure 2.4: Average phytoplankton cell density (A) and chlorophyll *a* concentration (B) from water samples in October, November, and December 2016. Error bars are ± one SEM and (n=3).

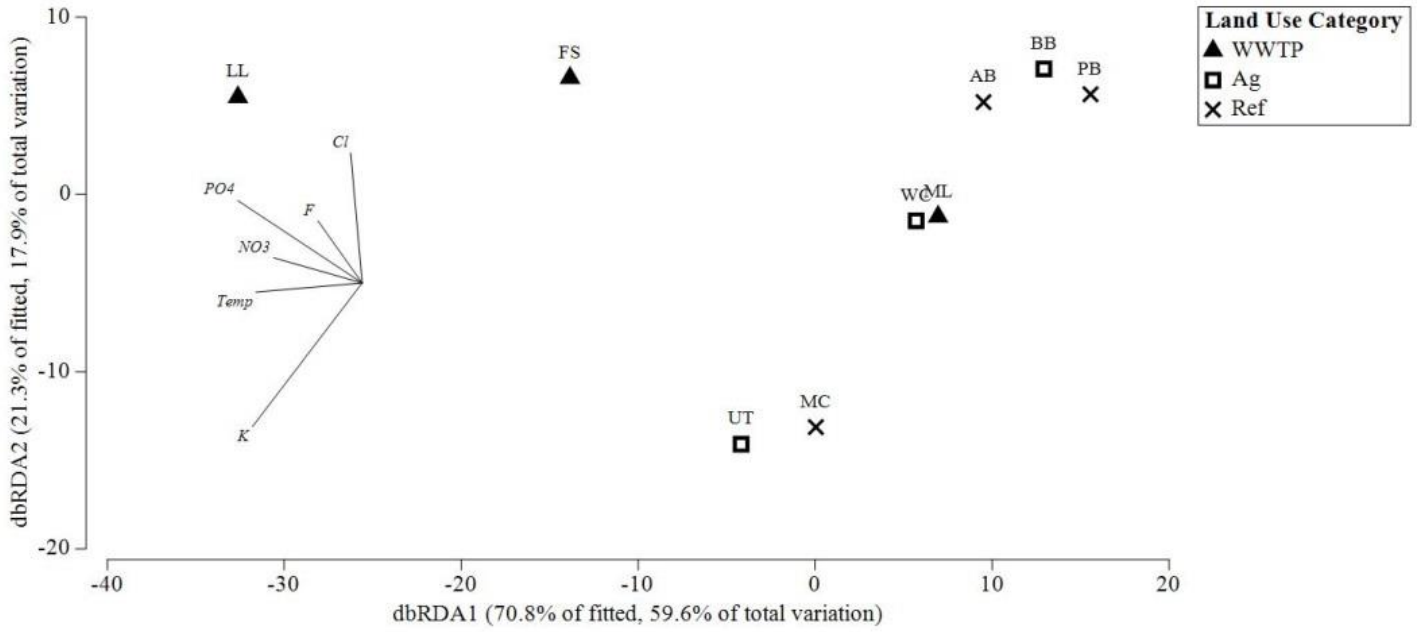

Figure 2.5: Distance-based redundancy analysis plot showing the relationships between phytoplankton taxonomic composition and environmental variables for water samples in October.

CHAPTER 2 APPENDICES

Appendix 1: List of phytoplankton taxa found in water samples from streams within the Ogeechee River Basin in October, 2016.

Taxa	**Group**	**Land Use Presence**
Actinastrum	Chlorophyta	WWTP
Chlamydomonas	Chlorophyta	Ref, WWTP, Ag
Cladophora	Chlorophyta	WWTP
Closterium	Chlorophyta	WWTP
Coelastrum	Chlorophyta	Ref
Cosmarium	Chlorophyta	Ref, WWTP, Ag
Crucigenia	Chlorophyta	Ref, WWTP
Desmidium	Chlorophyta	Ag
Euglenoids	Chlorophyta	WWTP
Haematococcus	Chlorophyta	Ag
Microspora	Chlorophyta	WWTP
Monoraphidium	Chlorophyta	Ref, WWTP, Ag
Phacus	Chlorophyta	Ref, WWTP, Ag
Scenedesmus	Chlorophyta	WWTP, Ag
Staurastrum	Chlorophyta	Ref, WWTP, Ag
Tetraedron	Chlorophyta	Ref, WWTP, Ag
Tribonema	Chlorophyta	Ref
Ulothrix	Chlorophyta	WWTP
Westella	Chlorophyta	Ref, WWTP, Ag
Asterionella	Heterokonta	WWTP
Unk. centric diatoms	Heterokonta	Ref, WWTP, Ag
Cocconeis	Heterokonta	WWTP, Ag
Diploneis	Heterokonta	WWTP, Ag
Eunotia	Heterokonta	Ref, WWTP, Ag
Fragellaria	Heterokonta	Ref, WWTP, Ag
Gyrosigma	Heterokonta	WWTP, Ag
Mallomonas	Heterokonta	Ref, WWTP, Ag
Melosira	Heterokonta	WWTP
Naviculoids	Heterokonta	Ref, WWTP, Ag
Pinnularia	Heterokonta	Ref, WWTP, Ag
Synedra	Heterokonta	Ref, WWTP, Ag
Tabellaria	Heterokonta	Ref
Cryptomonas	Cryptophyta	Ref, WWTP, Ag
Chroomonas	Cryptophyta	Ref, WWTP, Ag
Synura	Cryptophyta	Ag

Anabaena	Cyanophyta	Ref, WWTP, Ag
Chroococcus	Cyanophyta	WWTP
Unk. colony	Cyanophyta	Ref, Ag
Oscillatoria	Cyanophyta	WWTP
Spirulina	Cyanophyta	Ref, WWTP, Ag
Synechococcus	Cyanophyta	Ref, WWTP, Ag

Appendix 2: Taxonomic composition of phytoplankton communities in water samples taken from streams within the ORB in October, 2016.

Land Use	Site	% Chlorophyta	% Cryptophyta	% Heterokonta	% Cyanophyta
Ref	AB	84.9	3.8	7.9	3.4
Ref	MC	64.5	20.9	14.2	0.3
Ref	PB	92.3	2.9	4.1	0.7
WWTP	FS	56.6	2.0	30.9	10.6
WWTP	LL	41.1	7.2	50.6	1.1
WWTP	ML	78.3	8.8	11.4	1.5
Ag	BB	90.5	3.6	5.1	0.8
Ag	WA	65.8	24.1	8.8	1.3
Ag	WC	60.4	4.0	33.8	1.8