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EVALUATING NUTRIENT LIMITATION OF PHYTOPLANKTON GROWTH IN NORTH INLET ESTUARY, SC

by

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DEDICATION

This is dedicated to the many friends and family who provided unlimited support both to get me to the start of this work and to carry me through it. Your constant encouragement, reassurance, and reminders to seek balance have been so important to me, and I would not be where I am today without you.

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ABSTRACT

The biolimiting nutrients nitrogen (N) and phosphorus (P) are vital components of molecules essential to life. In marine systems, N:P ratios tend to follow the "Redfield ratio" of 16:1, which is often used to infer nutrient limitation of phytoplankton biomass. Traditionally, estuaries are thought to be N-limited (N:P < 16), but there have been increasing instances of P-limitation (N:P > 16) in coastal waters worldwide. Over the last few decades, nutrient loading in North Inlet Estuary (NIE) has changed such that dissolved inorganic nitrogen (DIN):dissolved inorganic phosphorus (DIP) ratios have increased by ca. 130%. This increase suggests that the estuary may be transitioning to N and P colimitation or primary P-limitation. We hypothesized that P would be the primary limiting nutrient of phytoplankton biomass and community composition in NIE for the summer of 2023. Dissolved inorganic nitrogen (DIN, 20 μmol l^{-1} N), low phosphate (LP, 5 μmol l^{-1}), and high phosphate (HP, 20 μmol l^{-1}) combined DIN+LP, or combined DIN+HP were added to water samples collected at Clambank Landing in NIE on a monthly basis. Changes in phytoplankton biomass (chl *a*) and community composition were measured via High Performance Liquid Chromatography (HPLC) to determine if nutrient additions were indicative of limitation by that nutrient. N was the single or primary limiting nutrient for all bioassays, with potential P co-limitation. Shifting nutrient ratios and limitation status can impact trophodynamics and nutrient mitigation strategies may be employed to avoid cascading effects in estuarine food webs.

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LIST OF ABBREVIATIONS

C ..... Carbon

chl *a* ..... Chlorophyll *a*

CL ..... Clambank Landing

DIN ..... Dissolved Inorganic Nitrogen

DIP ..... Dissolved Inorganic Phosphorus

DON ..... Dissolved Organic Nitrogen

DOP ..... Dissolved Organic Phosphorus

N ..... Nitrogen

NIE ..... North Inlet Estuary

P ..... Phosphorus

PON ..... Particulate Organic Nitrogen

POP ..... Particulate Organic Phosphorus

SRP ..... Soluble Reactive Phosphorus

CHAPTER 1

INTRODUCTION

There is always at least one factor limiting phytoplankton growth, and the rate of nutrient supply is often the central limiting resource, with nutrient limitation occurring in multiple forms and levels (Hecky & Kilham, 1988; Finkel et al., 2010). Nutrient availability can limit growth rate and biomass at the community and individual level, the potential rate of net primary productivity, and even net ecosystem production (Howarth, 1988; Smayda, 1989). Molar Nitrogen:Phosphorus (N:P) ratios are frequently used as a tool for inferring nutrient limitation, and in marine systems tend to follow what is called the "Redfield Ratio" of 16:1, reflecting the average ratio at which these elements are found in phytoplankton (Redfield, 1958). Ambient conditions frequently stray from this ratio, but the Redfield Ratio has been used as a starting point to examine the status of nutrients in a body of water and indicate potential nutrient limitation on phytoplankton growth (Howarth, 1988). Ratios less than 16 may infer N-limitation and ratios greater than 16 can signal P -limitation, though it is important to note this is not a definitive rule as estuaries are dynamic and may defy this pattern (Howarth, 1988; Howarth et al., 2011). Additionally, these ratios describe nutrient concentrations, while limitation is determined by rate of supply. We can also differentiate between primary limitation and co-limitation. Primary nutrient limitation can be seen where introduction of only a single nutrient produces the largest positive response in phytoplankton growth, while the latter

is characterized as showing the largest positive response to the introduction of combination of essential nutrients (Kolzau et al., 2014).

Historically, estuaries have generally been considered N-limited, while freshwater systems are thought to be P-limited (Smith, 1984). This pattern has been attributed to differences in aquatic and marine nitrogen fixation rates, sediment-water column fluxes, rates of other biogeochemical processes such as denitrification, and nutrient sources (Howarth, 1988; Howarth & Marino, 2006). However, over the last few decades, there have been many demonstrations of primary P-limitation in estuaries and coastal systems. For example, the Mediterranean Sea (Krom et al., 1991); the Pearl River estuary and Xiamen Bay, China (Harrison et al., 1990; Yin et al., 2000); Pensacola Bay, Florida, and Gulf of Mexico estuaries (Myers & Iverson, 1981; Murrell et al., 2002); and the Patuxent River Estuary, Maryland (D'Elia et al., 1986), among others, have exhibited P-limitation during at least part of the year. This has been credited to excess N input from various sources, including atmospheric deposition and anthropogenic origins such as agricultural fertilizer runoff (Harrison et al., 1990; Yin et al., 2000). In cases with high N-loading, systems may even shift from N-limitation to P-limitation (Howarth et al., 2011). These shifts in nutrient limitation status can fundamentally change the biogeochemistry and phytoplankton community composition of those systems, with potential cascading effects on trophodynamics.

At the community level, and even the species level, phytoplankton have differences in nutrient use and ratio preferences among groups due to ranging size-based uptake kinetics, growth rates, enzyme-based nutrient acquisition, and unique biochemistry and biochemical pathways (Sterner & Elser, 2002; Litchman & Klausmeier,

2008). A meta-analysis from Hillebrand et al. (2013) found that phytoplankton group was a significant predictor of optimal N:P ratios, with optimal ratios of 14.9:1 for diatoms, 15.2:1 for dinoflagellates, 25.8:1 for cyanobacteria, and 27.0:1 for chlorophytes. However, these stoichiometric ratios can change with growth rate (Hillebrand et al., 2013). Further, a wide range of optimum N:P ratios (7-30) was found for 7 phytoplankton species, demonstrating high variability in stoichiometry (Rhee & Gotham, 1980). Other support comes from Tilman (1977) who showed that even species in the same group can have very different nutrient requirements and demonstrated that 74.3% of variance in the relative abundance of two aquatic diatom species could be explained by the Monod model of competition, which is based on nutrient uptake kinetics. Some have related these varying nutrient requirements with the evolutionary history of plastid lineages, suggesting that endosymbiosis events resulting in different superfamilies are associated with changes in elemental composition and stoichiometry (Quigg et al., 2011).

Thus, variations in limiting nutrients and nutrient inputs, concentrations, and ratios may also lead to changes in phytoplankton community structure. For example, off the shelf of southwest Florida, community composition of phytoplankton varied along a gradient of N- to P-limitation (Heil et al., 2007). Cyanobacteria and dinoflagellates were more common in the N-limited areas, potentially related to N_2-fixation and osmotrophic uptake of organic N, respectively, while diatoms were a larger contributor in P-limited areas (Heil et al., 2007). Bi et al. (2021) also found that C:N:P stoichiometry was significantly correlated with shifts in phytoplankton communities, where greater particulate organic N:particulate organic P (PON:POP) ratios were related to higher contributions of diatoms. Other models have suggested that there can be shifts in the

dominance of algal groups in response to changes in nutrient concentrations in open ocean environments (Litchman et al., 2006). They demonstrated the potential for increasing N:P ratios, resulting from lower P concentrations, to cause declines in coccolithophore biomass in the North Atlantic (Litchman et al., 2006). Different groups may even have different primary limiting nutrients, the availability of which can impact phytoplankton ecology through competition (Mackey et al., 2007). Transitioning primary limiting nutrients may result in changes in community composition as shown in the North Sea off the Dutch coast, where the sudden appearance of *Phaeocystis* blooms and a decrease in dinoflagellate abundance were concurrent with a shift from P- to N-limitation (Riegman, 1995; Alvarez-Fernandez, 2012).

Beyond limiting biomass and impacting phytoplankton community composition, nutrient stoichiometry has food quality implications for higher trophic levels that also have differing nutrient requirements (Glibert et al, 2011). Phytoplankton play a key role in the dissemination of stressor effects, including nutrient enrichment, through estuarine food webs, so alterations in their composition and elemental stoichiometry can lead to shifts in the growth and elemental composition of higher trophic levels as well (Breitburg et al., 1999; Finkel et al., 2010). When grown in varying nutrient conditions, phytoplankton stoichiometry and biochemical composition can change, potentially resulting in poor nutritional quality that may affect secondary production. For example, several studies have demonstrated slower growth rates of copepods that were fed P-limited algae (Malzahn et al., 2010; Malzahn & Boersma, 2012). *Daphnia* that were fed algae with varying carbon (C):P ratios and P use efficiency had slower growth rates with P-limited prey (Lind & Jeyasingh, 2015). Competition between *Daphnia* genotypes, and

thus relative abundance, was also impacted by prey stoichiometry (Lind & Jeyasingh, 2015). Jones and Flynn (2005) also found that manipulating the nutritional status of phytoplankton can lead to changes in the growth of zooplankton grazers. These impacts on zooplankton grazers can cascade higher up in the food web as well, as several fishery declines have had changing plankton regimes described as the probable cause (Beaugrand et al., 2003; Payne et al., 2009). Additionally, Schoo et al. (2014) demonstrated that these effects can reach secondary consumers, finding that lobster (*Homarus gammarus*) larvae had significant reactions when fed copepods that had consumed nutrient limited algae.

Glibert et al. (2011) proposed a model for changes in the San Francisco Bay Delta that provides a noteworthy example of how variation in nutrient stoichiometry could fundamentally change the trophic structure of an estuary. Following changes in nutrient loading that led to increasing dissolved inorganic N:dissolved inorganic P (DIN:DIP), phytoplankton community composition shifted from diatom to dinoflagellate dominance. That transition brought about changes in zooplankton community composition and biogeochemistry, leading to different environmental conditions and a new steady state in the estuary. These changes further promoted a shift in the planktivore to piscivore ratio, as well as nutrient conditions and stress, which resulted in the decline of pelagic fishes (Glibert et al., 2011).

Over the last couple of decades, North Inlet Estuary (NIE) has seen steady changes in nutrient loading (Dunn et al., 2023). Ammonium (NH_4^+) concentrations within the estuary have increased as NH_4^+ is being exported from marsh porewaters at higher rates due to sea level rise (Krask et al., 2022; Dunn et al., 2023). This has led to an increase in DIN:DIP ratios in estuarine waters, often far exceeding Redfield ratios and

reaching values greater than 25 (Krask et al., 2022; Dunn et al., 2023; NOAA National Estuarine Research Reserve System (NERRS)). Historical bioassays in NIE have suggested that N was the primary limiting nutrient for phytoplankton growth (Van Meerssche & Pinckney, 2019; Pinckney et al., 2020). In 2014-15, several nutrient status indices indicated N deficiencies in NIE, while bioassays from the same period showed N and P co-limitation of phytoplankton growth at high N concentrations (Bell et al., 2018). The shift from primary N-limitation to N and P co-limitation, paired with steadily increasing DIN:DIP ratios in the estuary, may signal that the system is transitioning to primary P-limitation.

The purpose of this study was to examine the current status of nutrient limitation on phytoplankton biomass and community composition in NIE, given the increased DIN loading to the estuary and increasing occurrences of much greater than Redfield DIN:DIP ratios. We hypothesized that nutrient loading has been sufficient to increase DIN:DIP ratios and transition the estuary to being primarily P-limited during the "growing season" (May–September). In this case, groups enriched with P should experience the greatest increases in total chl *a* concentration. Additionally, we hypothesized that addition of P to mitigate P-limitation would result in significant alterations in phytoplankton community structure, favoring the growth of diatoms. Nutrient limitation tends to favor smaller phytoplankton groups, so enrichment could shift community composition toward phytoplankton groups with larger species such as diatoms. This project presented a unique opportunity to examine the effects of climate-related changes (i.e., sea level rise and increased N inputs from porewater) on nutrient concentrations and resulting in

changes in phytoplankton growth, biomass, and community structure in an otherwise relatively undisturbed (by local anthropogenic actions) high salinity estuary.

CHAPTER 2

METHODS

2.1 STUDY LOCATION

NIE and the nearby Winyah Bay are a National Estuarine Research Reserve (NERR) located in the Pee Dee Region of South Carolina. Water samples were collected at Clambank Landing (CL) near the center of NIE (Fig. 1). NIE is a high salinity estuary within a *Spartina*-dominated salt marsh system that has an area of 32 km^2 with very little development (< 2%) in its watershed. Tides are semidiurnal and >50% of the water volume is exchanged with each tidal cycle (Allen et al., 2014). DIN (NH_4^+, nitrate or NO_3^-, and nitrite or NO_2^-) concentrations in the estuary range from 0.18-17.95 $\mu mol\ l^{-1}$ with DIP (phosphate or PO_4^{3-}) concentrations in the range of 0.02-0.25 $\mu mol\ l^{-1}$, and DIN:DIP ratios from 6 to >100 (NERRS 2023).

2.2 NUTRIENT ENRICHMENT BIOASSAYS

Experimental nutrient addition bioassays were performed monthly during the growing season from May-September 2023. Surface water (0.5 m depth) was collected using a diaphragm pump in the daytime within 2 h of peak high tide. Tissue culture flasks (VWR Tissue Culture Flask, 75 cm2, Surface Treated, Plug seal cap, Sterile; 250 ml each with 35 replicates) were filled for nutrient enrichment bioassays.

There were 6 treatment groups including a control (no nutrient addition) and five nutrient additions, each with 5 replicates per bioassay (Table 2.1). Samples were

incubated for 48 h under ambient temperature and irradiance conditions on water tables adjacent to the estuary (Lewitus et al., 1998). To prevent light inhibition, samples were covered with two layers of neutral density filters (gray fiberglass screen), reducing the irradiance to ca. 40% of solar radiation. Flasks were gently mixed 3-4 times daily during daylight hours.

For analyses, phytoplankton biomass (as chl a in μg l^{-1}) responses were normalized to the control treatment to calculate percent change using the equation:

$$\% \, Change = \left(\frac{B_{nutrient\ treatment} - B_{control}}{B_{control}}\right) \times 100$$

where $B_{nutrient\ treatment}$ is the biomass of the enriched group and $B_{control}$ is the mean biomass of the control group. This was calculated for total biomass as total chl a, as well as abundance of the 5 most common algal groups in my samples and NIE (i.e. cryptophytes, cyanobacteria, diatoms and haptophytes, dinoflagellates, and green algae), for each replicate.

Nutrient limitation category definitions were adapted from Kolzau et al. (2014): Single nutrient limitation occurs when there is an increase in biomass in response to one of the single nutrient additions and it is no different from the combined nutrient treatment. Serial limitation occurs when there is an increase in biomass in response to only one nutrient addition, but the response of the combined nutrient treatment is larger than the single nutrient response. Independent co-limitation occurs when biomass increases for both single nutrient additions and the combined nutrient treatment has a larger response. In this case, the single nutrient addition with the larger response would

be the primary limiting nutrient. Finally, if there is no increase in biomass for any nutrient addition, there is no nutrient limitation.

2.3 PHOTOPIGMENT ANALYSIS

Photopigments (chl *a* and accessory pigments) were analyzed to estimate phytoplankton biomass and community composition, respectively. Water (100-150 ml) was gently vacuum filtered (-50 kPa) onto glass fiber filters (Sterlitech, gf/f, 0.7 μm nominal pore size). Filters were stored at -80 °C until lyophilization for ca. 24 hours at -50 °C, followed by extraction for 24 hours in 1 ml of 90% acetone and 100 μl carotenal (as synthetic carotenoid β-apo-8′-carotenal (internal standard)). The extracts were filtered with a 45 μm nylon syringe filter (VWR), and 400 μl of the extract was combined with 100 μl of 1.0 M ammonium acetate. Extracts (250 μl) were injected into a Shimadzu 2050 high performance liquid chromatograph (HPLC). The stationary phase was a monomeric (Rainin Microsorb, 0.46 × 1.5 cm, 3 μm packing) and a polymeric (Vydac 201TP54, 0.46 × 25 cm, 5 μm packing) reverse-phase C18 column in series. The mobile phase consisted of an 80% methanol/20% 0.5 M ammonium acetate solvent and an 80% methanol/20% acetone solvent (Pinckney et al., 1996). Retention time and absorption spectra were compared with standards to identify pigment peaks (DHI, Denmark).

A chemotaxonomic approach based on photopigment concentrations was used to determine the abundance of phytoplankton groups. This was performed using the *phytoclass* package in R Statistical Software (v4.3.2) (Hayward et al., 2023). Replicates were clustered based on photopigment concentrations. Based on the presence or absence of a pigment for each algal class, minimum and maximum photopigment concentrations derived from Schluter et al. (2006) were utilized as constraints to determine the global

optimum matrix. Simulated annealing is a stochastic approach employed to estimate the global optimum by navigating through numerous local optima. Employing a probability-based acceptance criterion, this analysis was paired with a steepest descent algorithm to find a matrix with the least error. The final output contains the pigment-to-chl *a* ratios and class abundances. HPLC and *phytoclass* results were verified qualitatively via light microscopy. Post-*phytoclass* analysis, diatoms and haptophytes were grouped together as a result of poor differentiation related to diagnostic pigments that are shared between those groups.

2.4 NUTRIENT ANALYSIS

Nutrient concentrations were measured at time zero (T0) and the end of the 48 h incubations. Composite samples were made for each treatment group by combining 50 ml from each replicate. Composites were filtered with a 0.45 µm filter (Nylon VWR, cat. 76308-700) and stored at -20 °C until analysis. A Seal Analytical nutrient AutoAnalyzer3 was used to analyze samples for NO_3^-, PO_4^{3-}, NH_4^+, and NO_2^- following standard colorimetric methods described in Grasshoff et al. (1999).

2.5 STATISTICAL ANALYSIS

A randomized complete blocks design two-way Analysis of Variance (ANOVA) with bioassay as the blocking factor, treatment group as the main effect, and the percent change in total chl *a* from the control as the dependent variable was used to determine nutrient limitation status across the growing season. An REGW test was used for *post hoc* comparisons of means. To determine nutrient limitation status for individual bioassays, we used one-way ANOVAs with treatment group as the factor and percent change in total chl *a* relative to the control as the dependent variable. REGW tests were used for *post hoc*

comparisons of means. Community composition was analyzed using a randomized complete blocks design two-way MANOVA, where bioassay date was the blocking factor, treatment group was the main factor, and the percent change of taxonomic groups were the dependent variables. Discriminant analysis was used to predict treatment group membership based on percent change in abundance of taxonomic groups relative to the control. Randomized complete blocks design two-way ANOVAs with bioassay as the blocking factor, treatment group as the main effect, and the percent change in abundance from the control as the dependent variable were used to determine response of individual taxonomic groups to nutrient enrichment. REGW tests were used for *post hoc* comparisons of means. Statistical analyses were performed with R Statistical Software (v4.3.2).

Table 2.1. Treatment groups for nutrient enrichment bioassays. Groups are referred to by their abbreviations. NO_3^- was added as $NaNO_3$ (crystal, Fisher Scientific cat. S343-500, CAS 7631-99-4), NH_4^+ was added as NH_4Cl (crystal, Macron cat. 3384-12, CAS 12125-02-9), and PO_4^{3-} was added as KH_2PO_4 (monobasic, crystal, J.T. Baker cat. 4008-01, CAS 7778-77-0). Nutrients were dissolved in deionized water prior to addition to bioassays and they were added in excess of *in situ* concentrations to avoid nutrient limitation within the bioassay.

Treatment	Abbreviation	$NaNO_3$ (µmol l^{-1})	NH_4Cl (µmol l^{-1})	KH_2PO_4 (µmol l^{-1})
Control	-	-	-	-
Dissolved Inorganic Nitrogen	DIN	10	10	-
Low Phosphate	LP	-	-	5
High Phosphate	HP	-	-	20
Dissolved Inorganic Nitrogen + Low Phosphate	DIN+LP	10	10	5
Dissolved Inorganic Nitrogen + High Phosphate	DIN+HP	10	10	20

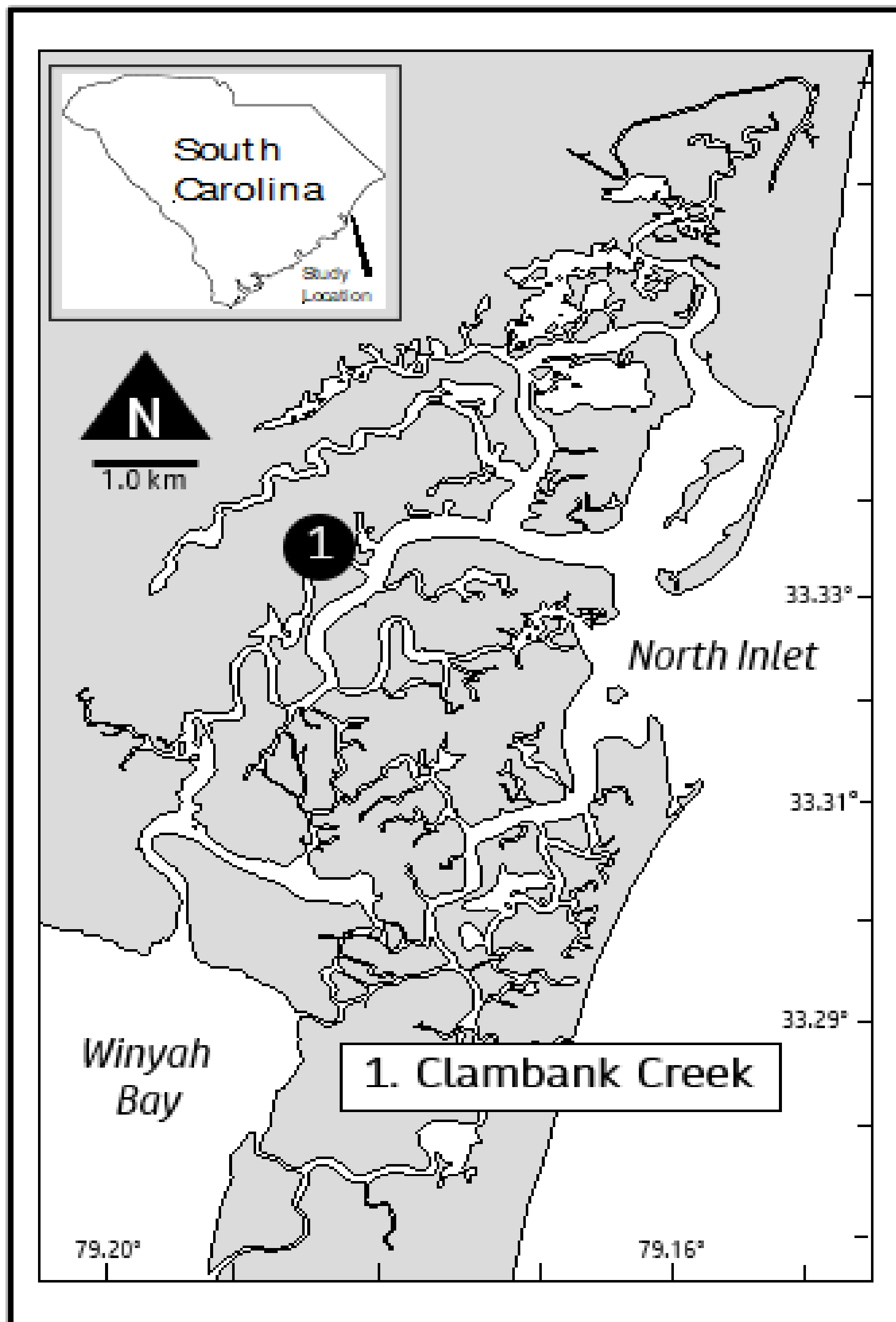

Figure 2.1. North Inlet Estuary, South Carolina and the location for water collections at Clambank Creek (lat. 33°20’02.05” N, long. 79°11’34.62” W).

CHAPTER 3

RESULTS

3.1 TOTAL CHL *A* RESPONSE TO NUTRIENT ENRICHMENT

Nearly all nutrient enrichments resulted in an increase in total chl *a* relative to the control (Fig. 2). The results of an RCB two-way ANOVA suggested differences in percent change of biomass between nutrient enrichment groups ($p < 0.0001$, $F = 63.91$, $n = 100$, $df = 4$), indicative of primary N limitation. The percent change of DIN+HP was significantly greater than DIN ($p = 0.003$), LP ($p < 0.0001$), and HP ($p < 0.0001$). DIN+LP percent change in total chl *a* was also significantly greater than LP ($p < 0.0001$), and HP ($p < 0.0001$), but did not differ from the DIN group. Percent change for the DIN was significantly greater than LP ($p < 0.0001$), and HP ($p < 0.0001$) (Fig. 2).

N was the primary limiting nutrient for the May bioassay (Figure 2). Biomass increased significantly more in response to the DIN, HP, DIN+LP, and DIN+HP treatments than the LP and HP treatments (Table 3.1). The strongest increase was in the combined DIN+HP treatment, followed by the DIN+LP and DIN groups which were not different from each other (Figure 2, Table 3.1). The June bioassay demonstrated serial limitation with N as the primary limiting nutrient, as the response to the combined nutrient treatments was stronger than the DIN treatment, and all N treatments induced a stronger response than P only treatments (Figure 2, Table 3.1). N was the single limiting nutrient for the July bioassay, with the DIN, DIN+LP, and DIN+HP groups showing a

significantly stronger response than the P treatments (Figure 2, Table 3.1). In the September bioassay, N was the primary limiting nutrient, with the DIN group generating the strongest response, followed by the DIN + HP and DIN + LP groups (Figure 2, Table 3.1).

3.2 COMMUNITY COMPOSITION RESPONSE TO NUTRIENT ENRICHMENT

Diatoms and haptophytes comprised the majority (> 60%) of the phytoplankton community across nearly all treatment groups for all bioassays (Figures 3-6). Cyanobacteria were detected at time zero in the May bioassay but were not detected in the control or any treatment groups in May, nor were they detected in the June DIN+LP or DIN+HP groups (Figures 3-4). All algal groups besides cyanobacteria experienced significant changes in biomass between treatment groups (Figures A3-8). Cryptophytes, diatoms/haptophytes, and green algae, which were the most dominant groups, generally had a stronger response to DIN (alone and combined) than either HP or LP (Figures 3-6, A4-5,7).

MANOVA results suggested that community composition changed with nutrient enrichment (Pillai's trace = 0.944, $p < 0.001$) and there was a significant blocking effect (i.e., bioassay date) (Pillai's trace = 22.5, $p < 0.001$). Discriminant analysis also indicated differences in community composition between nutrient enrichment treatments, grouping together DIN enrichments (DIN, DIN+LP, and DIN+HP) separately from P only groups (LP, and HP (Figure 7).

3.3 ENVIRONMENTAL CONDITIONS AND NUTRIENTS

Water quality varied little between bioassays (Table 3.2). Temperature fluctuated, reflecting seasonality with a peak in July. Initial PO_4^{3-} concentrations were below detections limits for all but the May bioassay, where it was 0.19 µmol l^{-1} (Tables 3.3-6). The initial DIN pool was usually dominated by NH_4^+, with NO_3^- below detections limits in the June and July bioassays (Tables 3.3-6). Initial NO_2^- concentrations were relatively consistent between bioassays, at an average of 0.54 µmol l^{-1} (Tables 3.3-6). For all bioassays, final PO_4^{3-} concentrations were highest in the HP or DIN+HP, then LP or DIN+LP treatments (Tables 3.3-6). Final NO_3^- concentrations were at or near 0 µmol l^{-1} for all but the DIN treatments in the May, June, and July bioassays and were highest in September (Tables 3.3-6). NH_4^+ concentrations at 48 h were typically higher in N-amended groups, but there were no clear patterns. Post-incubation NO_2^- concentrations were consistently < 1 µmol l^{-1} throughout the summer and between treatments (Tables 3.3-6). PO_4^{3-} uptake was usually highest in the LP and HP treatments, followed by the DIN+LP and DIN+HP groups (Tables 3.3-6). NO_3^- and NH_4^+ uptake were generally lowest or negative in the control, LP, and HP treatments and higher in the DIN (alone or combined) treatments, except for May when NH_4^+ uptake was always negative (Tables 3.3-6). NO_2^- uptake was low relative to other nutrients during all bioassays (Figures 3.3-6).

Table 3.1. Difference in mean percent change of total chl *a* between treatment groups (described in the first column) and their p-values (determined with REGW tests) for individual bioassays. Non-significant (> 0.05) p-values are noted with "n.s." and significant p-values (< 0.05) are listed and bold.

	May	**June**	**July**	**September**
HP - LP	-20.83, n.s.	-10.47, n.s.	-27.87, n.s.	-27.46, n.s.
DIN – LP	**462.9, < 0.0001**	**610.6, < 0.0001**	**513.3, < 0.0001**	**491.4, < 0.0001**
DIN – HP	**483.7, < 0.0001**	**621.1, < 0.0001**	**541.2, < 0.0001**	**518.9, < 0.0001**
(DIN+LP) – LP	**490.3, < 0.0001**	**1300, < 0.0001**	**444.8, < 0.0001**	**354.9, < 0.0001**
(DIN+LP) – HP	**511.1, < 0.0001**	**1311, < 0.0001**	**472.6, < 0.0001**	**382.4, < 0.0001**
(DIN+LP) – DIN	27.46, n.s.	**689.7, < 0.0001**	-68,55, n.s.	**-136.5, 0.0016**
(DIN+HP) – LP	**676.3, < 0.0001**	**1364, < 0.0001**	**534.8, < 0.0001**	**357.0, < 0.0001**
(DIN+HP) – HP	**697.1, < 0.0001**	**1375, < 0.0001**	**562.6, < 0.0001**	**384.5, < 0.0001**
(DIN+HP) – DIN	**213.4, 0.0127**	**753.8, 0.0272**	**21.46, 0.0272**	**-134.4, 0.0010**
(DIN+HP) – (DIN+LP)	**185.9, 0.0313**	64,10, n.s.	90.00, n.s.	2.091, n.s.

Table 3.2. Water quality parameters at Clambank Landing at T0 (rounded to closest quarter hour). Data taken from NOAA NERR SWMP. No measurements were taken in June due to equipment malfunctions.

	Water Temperature (℃)	Specific Conductivity (mS/cm)	Salinity (psu)	Dissolved Oxygen (mg/L)	Dissolved Oxygen (percent saturation)	Depth (m)	pH	Turbidity (FNU)
May	21.5	50.9	33.5	6	83.1	1.7	7.8	14
July	29	53.67	35.5	4.5	71.7	1.74	7.6	12
September	27.2	54.9	36.3	6.3	96.9	2.34	7.9	14

Table 3.3. Inorganic nutrient concentrations at T0 and post-incubation (48 h) for each treatment group in the May bioassay (± standard deviation). Values represent the mean of 4 subsamples taken from the composite sample. DIN:DIP was calculated as (NO_3^- + NH_4^+ + NO_2^-)/(PO_4^{3-}). Undetected concentrations are represented with zeroes. Uptake rates were calculated as [(T0 concentration – 48 h concentration)/(48 h)]/48 h chl *a* concentration.

	NO_3^- (μmol l^{-1})	PO_4^{3-} (μmol l^{-1})	NH_4^+ (μmol l^{-1})	NO_2^- (μmol l^{-1})	DIN:DIP	NO_3^- Uptake (μmol $μg^{-1}$ chl *a* l^{-1})	PO_4^{3-} Uptake (μmol $μg^{-1}$ chl *a* l^{-1})	NH_4^+ Uptake (μmol $μg^{-1}$ chl *a* l^{-1})	NO_2^- Uptake (μmol $μg^{-1}$ chl *a* l^{-1})
T0	0.63±0.72	0.19±0	0.18±0.36	0.60±0.049	7.25				
Control	0.18±0.21	0.097±0.03	1.41±2.8	0.60±0.025	23.24	2.64×10^{-3}	5.73×10^{-4}	-7.29×10^{-3}	-1.06×10^{-5}
DIN	1.12±0.50	0.12±0.016	15.42±9.0	0.68±0.036	140.18	9.24×10^{-3}	7.07×10^{-5}	-5.10×10^{-3}	-7.29×10^{-5}
LP	0.16±0.24	2.67±0.43	14.47±13	0.80±0.015	5.69	1.89×10^{-3}	1.03×10^{-2}	-5.81×10^{-2}	-7.91×10^{-4}
HP	0.29±0.35	15.43±1.0	8.69±13	0.76±0.024	0.60	1.6×10^{-3}	2.26×10^{-2}	-4.04×10^{-2}	-7.62×10^{-4}
DIN+LP	0	1.21±0.67	7.57±4.1	0.63±0.017	7.70	9.89×10^{-3}	3.71×10^{-3}	-2.43×10^{-3}	-2.16×10^{-5}
DIN+HP	0.12±0.17	12.26±2.9	1.30±1.1	0.64±0.011	0.18	7.56×10^{-3}	5.71×10^{-3}	-6.39×10^{-3}	-2.57×10^{-5}

Table 3.4. Inorganic nutrient concentrations at T0 and post-incubation (48 h) for each treatment group in the June bioassay (± standard deviation). Values represent the mean of 4 subsamples taken from the composite sample. DIN:DIP was calculated as $(NO_3^- + NH_4^+ + NO_2^-)/(PO_4^{3-})$. Undetected concentrations are represented with zeroes. Uptake rates were calculated as [(T0 concentration – 48 h concentration)/(48 h)]/48 h chl *a* concentration. Dashes represent missing data or DIN:DIP ratios and uptake rates that could not be calculated because of concentrations below detection limits.

	NO_3^- (µmol l^{-1})	PO_4^{3-} (µmol l^{-1})	NH_4^+ (µmol l^{-1})	NO_2^- (µmol l^{-1})	DIN:DIP	NO_3^- Uptake (µmol µg^{-1} chl *a* l^{-1})	PO_4^{3-} Uptake (µmol µg^{-1} chl *a* l^{-1})	NH_4^+ Uptake (µmol µg^{-1} chl *a* l^{-1})	NO_2^- Uptake (µmol µg^{-1} chl *a* l^{-1})
T0	0	0	2.78±1.74	0.67±0.019	-				
Control	0.30±0.25	0	1.14±0.99	0.63±0.037	-	-1.87×10^{-3}	-	1.01×10^{-2}	1.98×10^{-4}
DIN	7.44±0.26	0	2.19±1.57	0.71±0.019	-	2.07×10^{-3}	-	8.58×10^{-3}	-3.76×10^{-5}
LP	0.39±0.26	2.44±0.25	1.71±2.36	0.64±0.0069	1.15	-1.62×10^{-3}	1.06×10^{-2}	4.41×10^{-3}	1.18×10^{-4}
HP	0.95±1.89	16.01±1.47	0	0.63±0.024	0.098	-4.19×10^{-3}	1.77×10^{-2}	1.23×10^{-2}	1.42×10^{-4}
DIN+LP	0.018±0.036	1.86±0.16	0	0.65±0.034	0.36	4.24×10^{-3}	1.34×10^{-3}	5.43×10^{-3}	8.33×10^{-6}
DIN+HP	0.71±0.019	14.51±0.78	0	0.66±0.019	0.097	3.78×10^{-3}	5.20×10^{-3}	5.20×10^{-3}	1.45×10^{-6}

Table 3.5. Inorganic nutrient concentrations at T0 and post-incubation (48 h) for each treatment group in the July bioassay (± standard deviation). Values represent the mean of 4 subsamples taken from the composite sample. DIN:DIP was calculated as $(NO_3^- + NH_4^+ + NO_2^-)/(PO_4^{3-})$. Undetected concentrations are represented with zeroes. Uptake rates were calculated as [(T0 concentration – 48 h concentration)/(48 h)]/48 h chl *a* concentration. Dashes represent missing data or DIN:DIP ratios and uptake rates that could not be calculated because of concentrations below detection limits.

	NO_3^- (µmol l^{-1})	PO_4^{3-} (µmol l^{-1})	NH_4^+ (µmol l^{-1})	NO_2^- (µmol l^{-1})	DIN:DIP	NO_3^- Uptake (µmol µg^{-1} chl *a* l^{-1})	PO_4^{3-} Uptake (µmol µg^{-1} chl *a* l^{-1})	NH_4^+ Uptake (µmol µg^{-1} chl *a* l^{-1})	NO_2^- Uptake (µmol µg^{-1} chl *a* l^{-1})
T0	0	0	-	-	-				
Control	0	0.54±1.08	-	-	0	0	-2.71×10^{-3}	-	-
DIN	2.57±0.59	0	-	-	-	5.83×10^{-3}	-	-	-
LP	0	4.04±0.081	-	-	0	0	3.80×10^{-3}	-	-
HP	0	18.13±0.44	-	-	0	0	9.54×10^{-3}	-	-
DIN+LP	0	2.12±0.23	-	-	0	8.79×10^{-3}	2.53×10^{-3}	-	-
DIN+HP	0	14.91±2.51	-	-	0	7.59×10^{-3}	3.87×10^{-3}	-	-

Table 3.6. Inorganic nutrient concentrations at T0 and post-incubation (48 h) for each treatment group in the September bioassay (± standard deviation). Values represent the mean of 4 subsamples taken from the composite sample. DIN:DIP was calculated as $(NO_3^- + NH_4^+ + NO_2^-)/(PO_4^{3-})$. Undetected concentrations are represented with zeroes. Uptake rates were calculated as [(T0 concentration – 48 h concentration)/(48 h)]/48 h chl *a* concentration. Dashes represent missing data or DIN:DIP ratios and uptake rates that could not be calculated because of concentrations below detection limits.

	NO_3^- (µmol l^{-1})	PO_4^{3-} (µmol l^{-1})	NH_4^+ (µmol l^{-1})	NO_2^- (µmol l^{-1})	DIN:DIP	NO_3^- Uptake (µmol µg^{-1} chl *a* l^{-1})	PO_4^{3-} Uptake (µmol µg^{-1} chl *a* l^{-1})	NH_4^+ Uptake (µmol µg^{-1} chl *a* l^{-1})	NO_2^- Uptake (µmol µg^{-1} chl *a* l^{-1})
T0	1.20±0.29	0	3.30±1.17	0.34±0.21	-				
Control	1.86±0.21	0	7.49±2.27	0.29±0.038	-	-1.94×10^{-3}	-	1.24×10^{-2}	1.52×10^{-4}
DIN	1.64±0.15	0	1.39±0.86	0.33±0.036	-	4.23×10^{-3}	-	5.36×10^{-3}	5.62×10^{-6}
LP	1.61±0.65	1.28±0.20	0.43±1.80	0.29±0.029	1.85	-7.40×10^{-4}	6.72×10^{-3}	5.19×10^{-3}	9.67×10^{-5}
HP	1.96±0.26	12.25±1.47	2.19±1.06	0.36±0.026	0.36	-1.67×10^{-3}	1.67×10^{-2}	2.40×10^{-3}	-2.71×10^{-5}
DIN+LP	1.73±0.46	0	1.46±4.69	0.36±0.06	-	5.38×10^{-3}	2.84×10^{-3}	6.73×10^{-3}	-1.12×10^{-5}
DIN+HP	1.86±0.67	14.09±0.80	7.24±1.62	0.39±0.059	0.66	5.29×10^{-3}	3.35×10^{-3}	3.43×10^{-3}	-2.73×10^{-5}

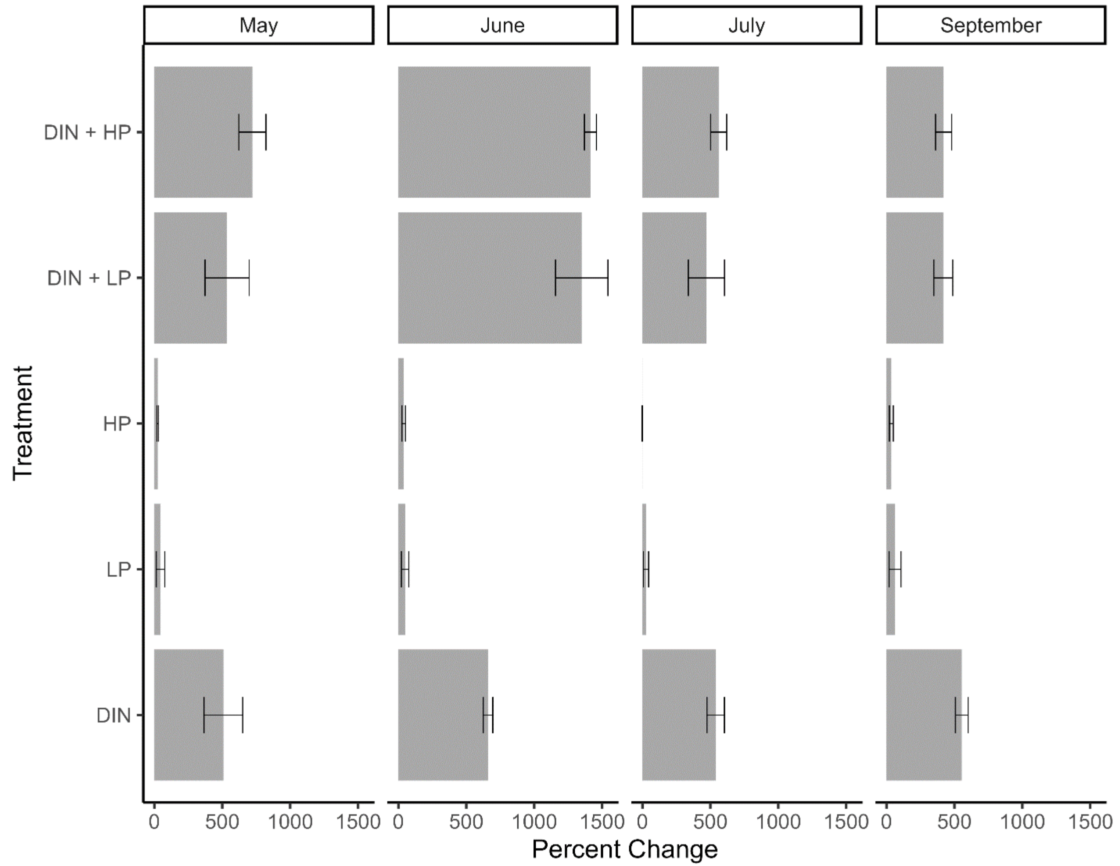

Figure 3.1. Percent change in total chl *a* relative to the control across all bioassays. Error bars represent standard deviation. Values were derived from HPLC analysis.

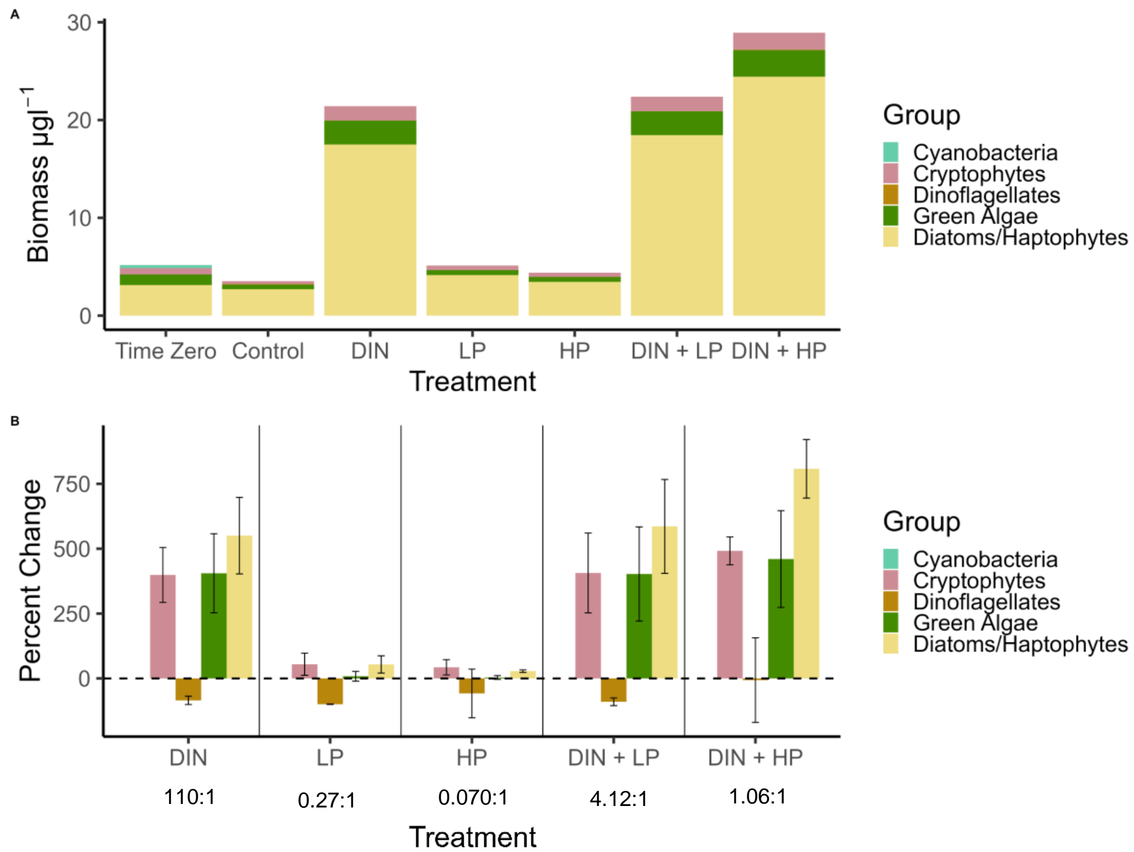

Figure 3.2. Biomass of taxonomic groups (µg chl *a* l^{-1}) for all treatment groups in the May bioassay (A) and percent change relative to control for all taxonomic groups in the May bioassay (B). The black dashed line represents no change from control. Bars above the line represent an increase in biomass relative to the control and bars below the line represent a decrease in biomass relative to the control. Error bars represent standard deviation. Values were derived from analysis with *phytoclass*. Initial DIN:DIP ratios (including nutrient additions) are displayed below treatment group labels.

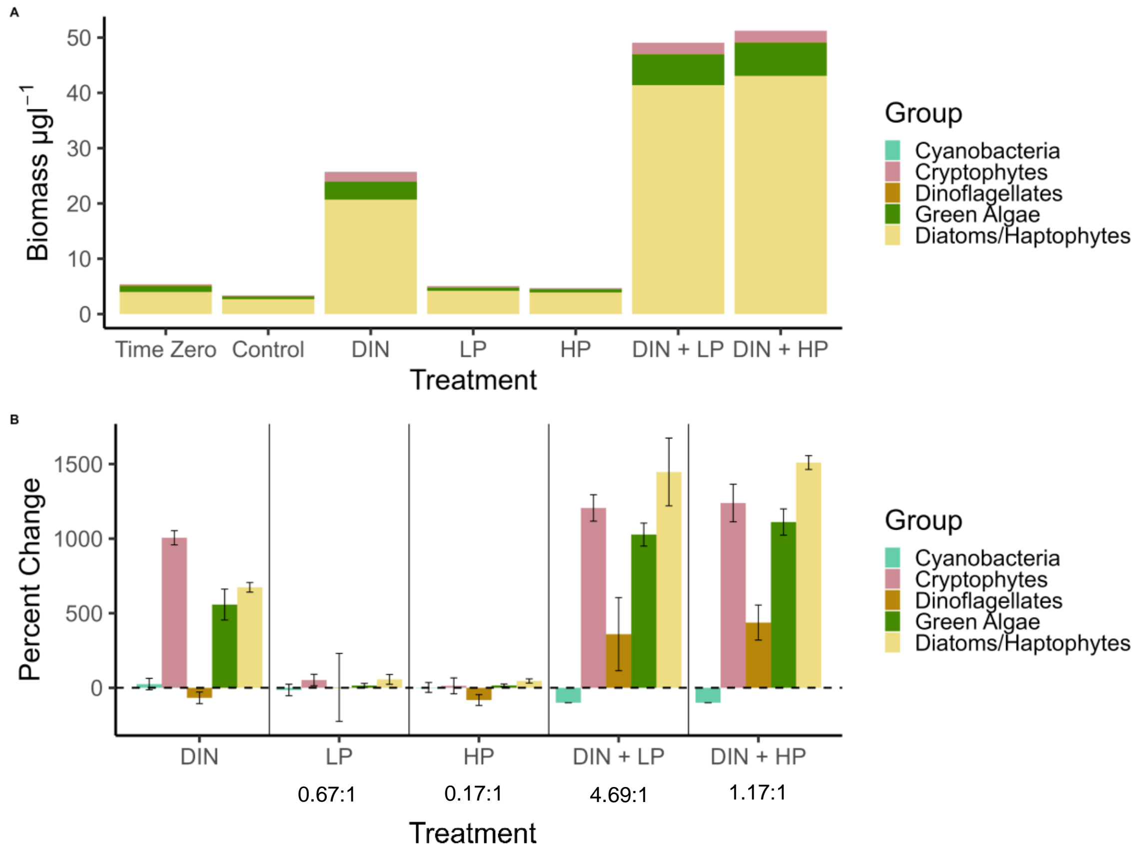

Figure 3.3. Biomass of taxonomic groups (µg chl *a* l^{-1}) for all treatment groups in the June bioassay (A) and percent change relative to control for all taxonomic groups in the June bioassay (B). The black dashed line represents no change from control. Values above the line represent an increase in biomass relative to the control and values below the line represent a decrease in biomass relative to the control. Error bars represent standard deviation. Values were derived from analysis with *phytoclass*. Initial DIN:DIP ratios (including nutrient additions) are displayed below treatment group labels. No PO_4^{3-} was detected initially, so there is no DIN:DIP ratio for the DIN alone treatment.

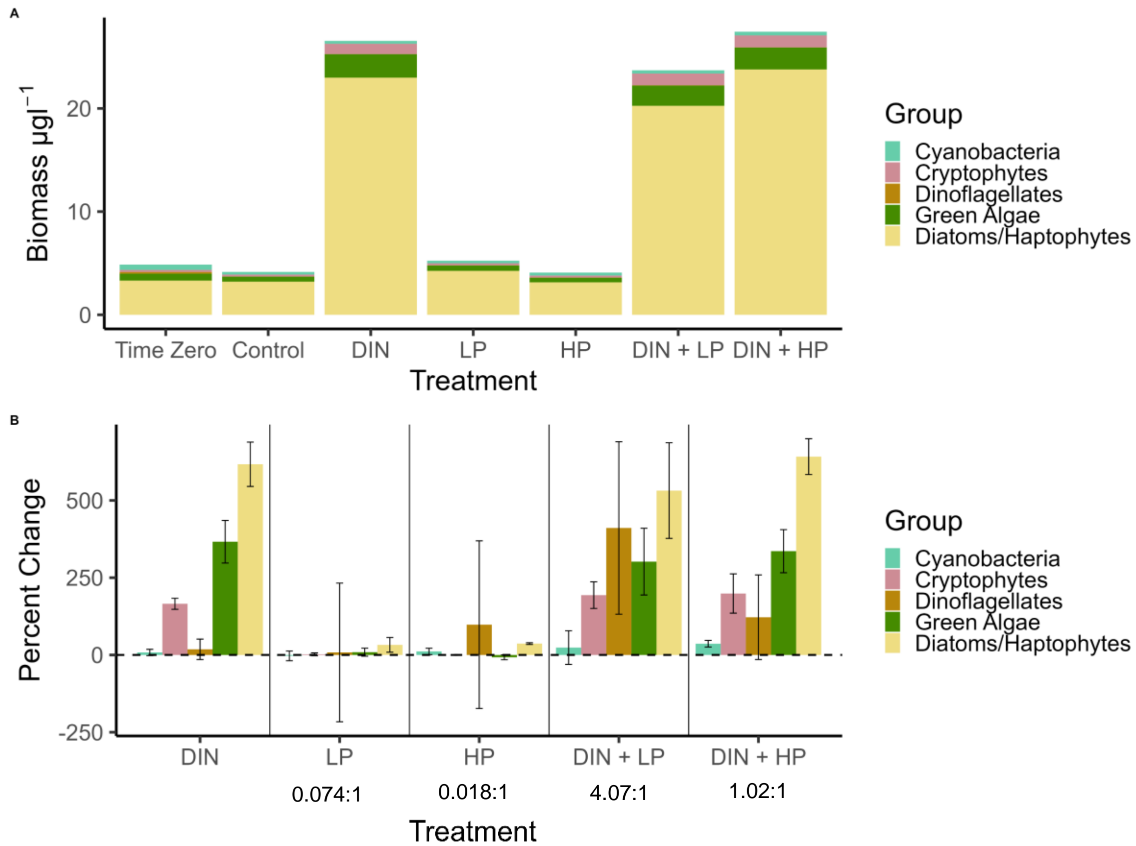

Figure 3.4. Biomass of taxonomic groups (µg chl *a* l^{-1}) for all treatment groups in the July bioassay (A) and percent change relative to control for all taxonomic groups in the July bioassay (B). The black dashed line represents no change from control. Values above the line represent an increase in biomass relative to the control and values below the line represent a decrease in biomass relative to the control. Error bars represent standard deviation. Values were derived from analysis with *phytoclass*. Initial DIN:DIP ratios (including nutrient additions) are displayed below treatment group labels. No PO_4^{3-} was detected initially, so there is no DIN:DIP ratio for the DIN alone treatment.

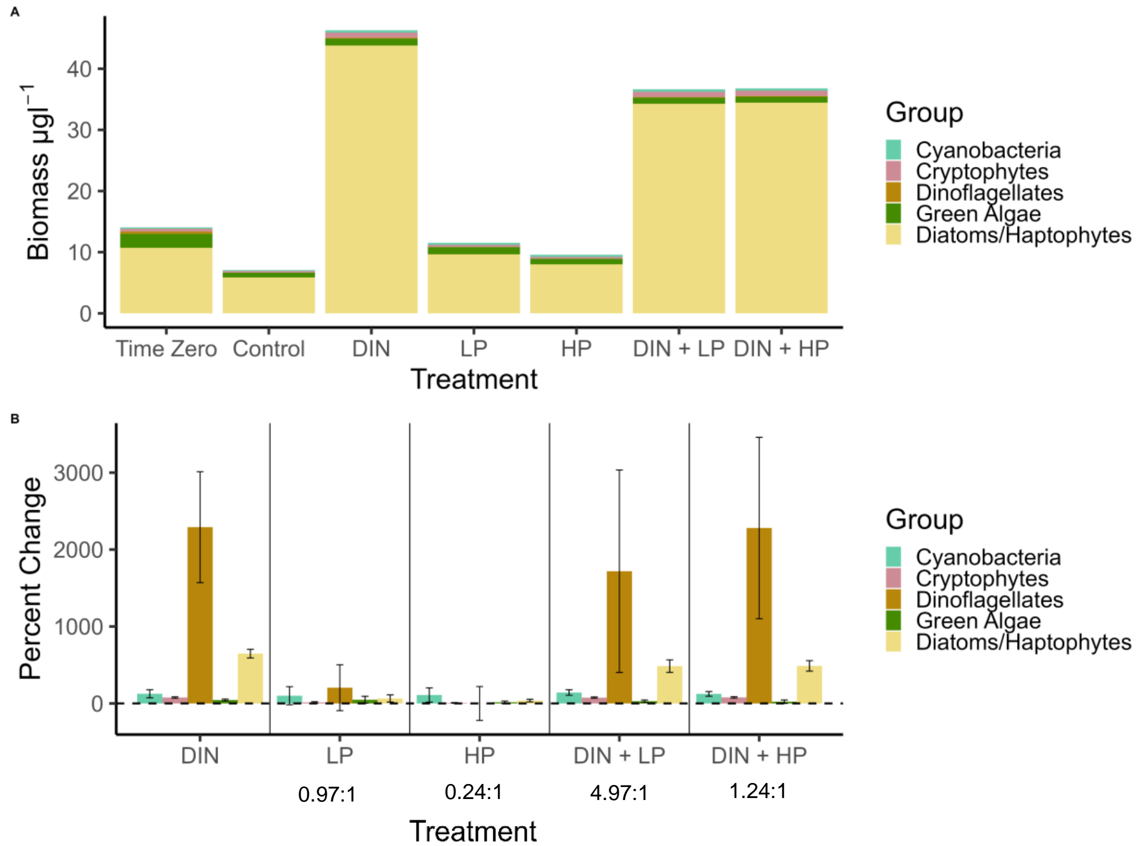

Figure 3.5. Biomass of taxonomic groups (µg chl *a* l^{-1}) for all treatment groups in the September bioassay (A) and percent change relative to control for all taxonomic groups in the September bioassay (B). The black dashed line represents no change from control. Values above the line represent an increase in biomass relative to the control and values below the line represent a decrease in biomass relative to the control. Error bars represent standard deviation. Values were derived from analysis with *phytoclass*. Initial DIN:DIP ratios (including nutrient additions) are displayed below treatment group labels. No PO_4^{3-} was detected initially, so there is no DIN:DIP ratio for the DIN alone treatment.

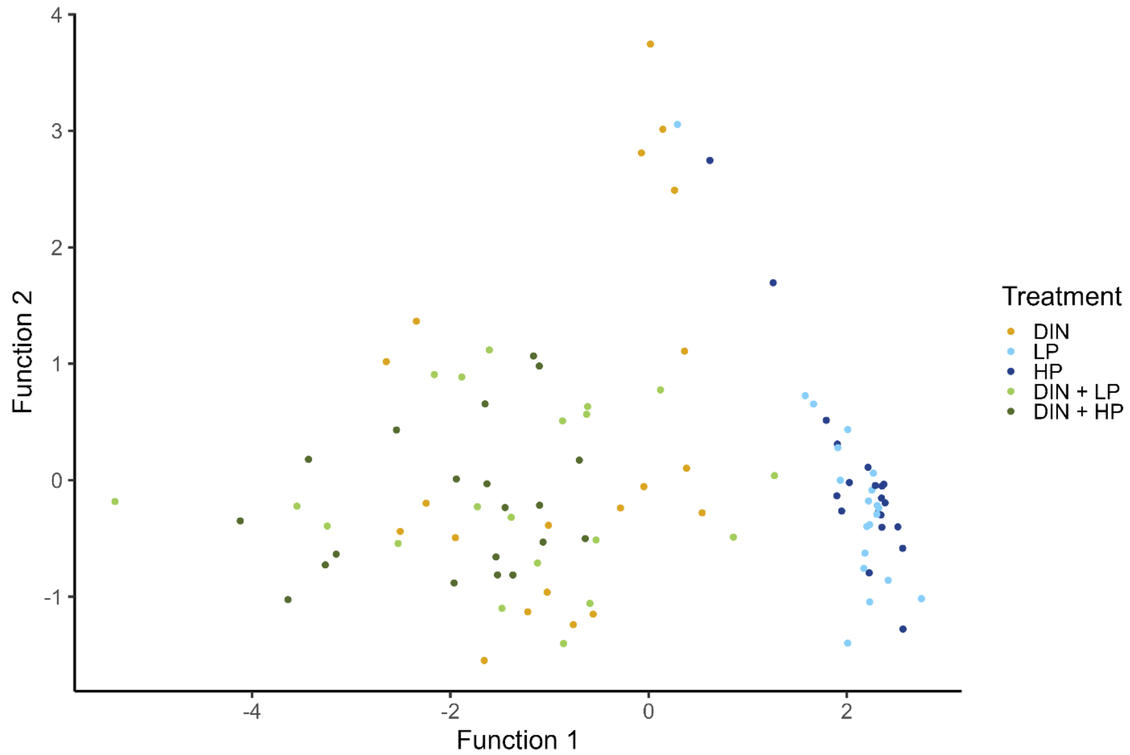

Figure 3.6. Discriminant analysis plot for the percent change from control of taxonomic groups in the phytoplankton community. Points represent replicates from all bioassays. Function 1 explained 98.6% of between-class variance and Function 2 explained 1.31% of between-class variance. Percent classified correctly was 43%.

CHAPTER 4

DISCUSSION

4.1 NUTRIENT LIMITATION OF PHYTOPLANKTON GROWTH

Our hypothesis was that P would be the primary limiting nutrient for phytoplankton biomass in NIE, meaning that the largest response would be in the LP and HP conditions, with little response to DIN (alone) enrichment. This was not supported; rather, evidence suggests that N was the single or primary limiting nutrient during the 2023 growing season despite the high N:P ratios, as the strongest responses were to DIN or combined additions (Figure 3.1). This is in line with previous investigations that suggested N limitation or co-limitation in NIE (Bell et al., 2018; Van Meerssche & Pinckney, 2019; Pinckney et al., 2020). N limitation in estuaries is a very well documented occurrence, not only historically in NIE, but in other systems as well (Pederson, 1995; Gobler et al., 2006; Cira et al., 2016).

The limiting nutrient is determined by the rate of supply of each nutrient, and phytoplankton have a few strategies for surviving through low P supply and concentrations or high N:P ratios. These are possibly being employed by phytoplankton in NIE, minimizing evidence of P-limitation related to low P concentrations and high DIN:DIP ratios (Glibert & Burkholder, 2011; Glibert, 2017). Some phytoplankton can use alternatives for P in molecules such as lipids, reducing their P requirement (Van Mooy et al., 2009). Others can modulate P demands via reductions in the concentrations

of P-rich cellular components including nucleic acids, ribonucleic acides in particular, and more minor contributors to the P pool such as adenosine triphosphate and glucose phosphate coenzymes (Geider & La Roche, 2002; Bertilsson et al., 2003). Many phytoplankton use high affinity P-transport systems to enhance their ability to assimilate P in environments with low concentrations (Harke et al., 2009; Cáceres et al., 2019). Some can use organic P via the hydrolyzation of refractory phosphonates and phosphomonoesters using enzymes such as phosphonatases and alkaline phosphatases (Dyhrman et al., 2006; Dyhrman & Ruttenberg, 2006; Harke et al., 2009). Phosphomonoesters and phosphodiesters are particularly prevalent in the DOP pool in NIE, which comprises a significant portion of the P pool during some parts of the year (Bell et al., 2018, 2020). Many phytoplankton can also store nutrients (including P, often as organic P compounds) in internal pools during times of high nutrient availability, for later use when the nutrient is scarce (Perry, 1976; Anderson et al., 1991; Geider & La Roche, 2002; Lin et al., 2016). These strategies are often utilized by cyanobacteria and dinoflagellates (Glibert & Burkholder, 2011). NIE is dominated by diatoms, as demonstrated in this study, so these strategies may also be similarly employed by diatoms (Perry, 1976; Diaz et al., 2008; Fuentes et al., 2013; Lin et al., 2013; Lin et al., 2016). More work should be done to evaluate the extent to which phytoplankton are relying on DOP as a source of inorganic P to support primary production in NIE.

Several other studies have found evidence for primary N-limitation or N and P co-limitation in systems with high N:P ratios indicative of P-limitation. This was demonstrated in Pamlico Sound, NC, where a DIN:DIP of 46:1 (greater than Redfield and suggestive of P-limitation) was measured, but bioassay results indicated stimulation

of phytoplankton by N, indicating N-limitation (Piehler et al., 2004). They believed this was due to luxury PO_4^{3-} consumption and storage or use of organic P (Piehler et al., 2004). There is another example in the Mediterranean, which has historically been characterized as P-limited with excess N but was found to be N and P co-limited for phytoplankton (Thingstad et al., 2005). They related this unexpected result to competition with bacterial communities that had better access to excess N available in the system as organic compounds (Thingstad et al. 2005). In the sub-tropical North Atlantic, Moore et al. (2008) found that N was the primary limiting nutrient, despite nutrient ratios greater than 16:1 that were associated with high rates of N_2-fixation increasing bioavailable N and reducing PO_4^{3-} concentrations. They attributed this to phytoplankton plasticity with regards to optimal nutrient ratios, and preferential remineralization of P (Moore et al., 2008). The extent to which any of these mechanisms are occurring in NIE needs to be further evaluated.

This study only examined the nutrient limitation status of phytoplankton during one season, the summer of 2023. Nutrient loading changes seasonally, so it is possible that nutrient limitation status may exhibit seasonality as well. Seasonal transitions between primary P-, primary N-, and N and P co-limitations have been demonstrated in several other systems including the Chesapeake Bay, Neuse River Estuary, Bothnian Sea, and Archipelago Sea (Rudek et al., 1991; Fisher et al., 1992; Tamminen & Anderson, 2007). There is also evidence that estuaries can transition between light and nutrient limitation seasonally depending on temporal cycles of turbidity and nutrient concentrations, like the Delaware Bay that shifted between winter-time light-limitation and spring P-limitation (Pennock & Sharp, 1994). Seasonal variation in nutrient

concentrations and stoichiometry in NIE has been demonstrated by several groups. For instance, Buzzelli et al. (2004) found peak PO_4^{3-} and NH_4^+ concentrations in the summer and peak DON concentrations in late summer at Oyster Landing. They found no significant seasonal patterns for NO_3^-/NO_2^- concentrations but noted that summer NO_3^- concentrations were the lowest (Buzzelli et al., 2004). More recently at the same site, Bell et al. (2018) found DIN and soluble reactive phosphorus (SRP) maxima in the summer and minima in the winter, with DON and DOP spring and summer maxima and fall minima. They also found a DIN:SRP maximum in the spring and minimum in the fall and DON:DOP maxima in winter with minima in the summer (Bell et al., 2018). It is possible that we missed temporal patterns in nutrient limitation, so future nutrient enrichment bioassays should be performed year-round to better understand how phytoplankton biomass is impacted across different seasons.

When nutrient loading is sufficient and absolute concentrations are high, there is potential for the system to shift from nutrient-dominated competition to competition based on light availability (Brauer et al., 2012). For example, light limitation was shown in the Tagus estuary in Portugal, which has nutrient concentrations high enough to sustain growth, but still sees low phytoplankton biomass resulting from insufficient light related to turbidity (Gameiro et al., 2011). This is a particularly viable regime shift in environments like estuaries where turbidity can be high from riverine or tidally driven sediment input and resuspension, increasing the likelihood of light limitation (Cloern, 1987). This has been demonstrated in the Changjiang Estuary, where there was temporal variation in the primary limiting factor being either light or nutrients, depending on seasonal nutrient concentrations and turbidity (Zhu et al., 2009). The increase in nutrient

loading over the last several decades may have pushed NIE further towards light availability as the primary control over phytoplankton growth. Future studies should investigate the roles of light and nutrient limitation in the regulation of phytoplankton growth and biomass in NIE.

4.2 COMMUNITY COMPOSITION

Diatoms and haptophytes made up the majority of the phytoplankton community and typically showed the strongest responses to nutrient addition, particularly N additions (Figures 3.2-5). Though not the nutrient we expected, this does support our idea that enrichment to mitigate the limiting nutrient would favor diatom growth. Diatoms are known to be dominant in NIE, and prior nutrient enrichment bioassays in the estuary also demonstrated a stronger response by diatoms to N enrichment, NH_4^+ in particular, compared to other cyanobacterial and algal groups (Lewitus et al., 1998). In the Neuse River Estuary, NC, Cira et al. (2016) also demonstrated greater increases in fucoxanthin, a diagnostic pigment for diatoms and haptophytes, compared to other photopigments in response to urea and NO_3^- enrichment in one bioassay. However, the other enrichment bioassays they performed indicated similar responses to N enrichment for pigments diagnostic of other phytoplankton groups as well (Cira et al., 2016). Bioassays in another NC estuary, Pamlico Sound, showed diatoms increased in relative concentration with addition of the limiting nutrient, N, as well (Piehler et al., 2004). Diatoms grow rapidly in nutrient replete conditions due to characteristics including size-based uptake kinetics, excess nutrient storage, and lower carbon requirements than other groups (Perry, 1976; Margalef, 1978; Droop, 1983; Diaz et al., 2008; Lin et al., 2016; Inomura et al., 2023).

Diatoms are also good competitors for N, enabling them to have a stronger response than other algal groups during N enrichment (Litchman et al., 2006).

During the September bioassay, dinoflagellates appeared to have a stronger response to nutrient enrichment than diatoms (Figure 3.5). However, dinoflagellates were low in biomass, nearing detection limits, and with abundance that low it is hard to make any strong conclusions about their response. Green algae and cryptophytes followed similar patterns of response to N as diatoms but were not as abundant (Figures 3.2-5). This aligns with increases in the diagnostic pigments chlorophyll *b* (green algae) and alloxanthin (cryptophytes) found by Cira et al. (2016). These strong responses to N enrichment by several groups (diatoms/haptophytes, green algae, and cryptophytes) are likely the driver of changes in community composition between treatment conditions as demonstrated by the discriminant analysis plot (Figure 3.6).

4.3 CONCLUSION

As primary producers at the base of marine food webs, phytoplankton play a critical role in maintaining ecosystem health and functionality. They also facilitate the cycling of nutrients, so understanding the interactions between nutrients and phytoplankton growth has broad implications for evaluating the state of these systems, especially considering changing environmental conditions. This is particularly important in South Carolina, where other lacustrine ecosystems are seeing similar changes in nutrient ratios (e.g., Lake Wateree, A. Bourbonnais, personal communication). Given their position as the interface between ocean, river, and terrestrial systems, estuaries provide critical habitat and ecosystem services, as well as economic and recreational services. However, this also means they face a myriad of overlapping climate change

related challenges, from pollution to ocean warming, and many more (Scavia et al., 2002). Even relatively undeveloped watersheds, such as that of NIE, are facing increasing levels of environmental changes like sea level rise and eutrophication (Krask et al., 2022; Dunn et al., 2023). Knowledge of the consequences of climate change and anthropogenic influence on biota in these systems is vital in determining best management practices to mitigate any negative effects that have potential ecosystem-wide impacts.

Despite high DIN:DIP ratios in NIE, N was the primary limiting nutrient for the 2023 growing season. Diatoms and haptophytes were dominant in the phytoplankton community and showed the strongest response to enrichment by the limiting nutrient, as predicted. While the Redfield ratio is often used to infer nutrient limitation status, high inter- and intraspecific variability and plasticity in the nutrient requirements of phytoplankton can lead to groups and systems straying from 16 as the transition from limitation by one nutrient to the other. Rather, a more accurate critical point marking a shift from N- to P-limitation may actually be higher than the Redfield ratio, somewhere in the range of 20-50 (Geider & La Roche, 2002). Results from this study support a higher breaking point for nutrient limitation and suggest that molar ratios and stoichiometry should be used carefully when analyzing nutrient limitation. They may not always be reflective of the true limitation status within the system (Domingues et al., 2023). Rather, ratios should be used in conjunction with experimental methods, such as enrichment bioassays, that provide more context and a potentially more accurate evaluation of nutrient limitation.

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APPENDIX A

MAXIMAL QUANTUM YIELD OF PHOTOSYNTHESIS

Pulse-Amplitude Modulated (PAM) fluorometry was used to evaluate the Maximal Quantum Yield of Photosystem II (PSII) of phytoplankton for each replicate of each treatment (Schreiber, 2004). The output is given as the ratio F_V/F_M, which can be used as a measure of photosynthetic performance (Maxwell & Johnson, 2000). One-way ANOVAs with treatment group as the factor and F_v/F_m as the dependent variable were performed for each of the June, July, and September bioassays.

Values for F_v/F_m ranged from 0.41to 0.79 (Figure A.1). In the June bioassay, there was a difference in F_v/F_m between treatment groups ($F = 6.4353$, num df = 6, denom df = 28, $p < 0.001$). The DIN + LP group was significantly higher than the time zero measurement ($p < 0.001$), control ($p = 0.001$), DIN ($p = 0.031$), LP ($p = 0.008$), and HP ($p = 0.0002$). F_v/F_m was not significantly different between treatments groups for the July bioassay (Figure A.1B). However, there was a similar pattern to the biomass results, with greater values for the DIN and combined DIN+LP and DIN+HP treatments (Figure A.1A, B). For the September bioassay, there was a significant difference between treatment groups ($F = 20$, num df = 6, denom df = 28, $p = 6.13 \times 10^{-9}$). F_v/F_m was greater in all nutrient additions compared to the control ($p < 0.0001$ for each treatment vs. the control), but there was no difference between nutrient additions (Figure A.1C).

While there were no statistically significant differences in quantum efficiency between treatment groups, the June and July bioassays showed patterns of increase in quantum efficiency that mirrored increases in biomass (Figures 3.1, A.1). The lack of differences between groups could be a sign of balanced growth under nutrient-limited conditions (Moore et al., 2008; Parkhill et al., 2001). It is possible that NIE phytoplankton have acclimated or adapted to ambient nutrient conditions and are able to maintain high quantum efficiency regardless of enrichment. In terrestrial plants, the N content of leaves, of which RUBISCO is a significant contributor, is a strong predictor of photosynthetic capability (Sterner & Elser, 2002). The slight, but insignificant, increases in F_v/F_m in N-enriched treatments could be related to the increased availability of N for RUBISCO, an important photosynthetic enzyme.

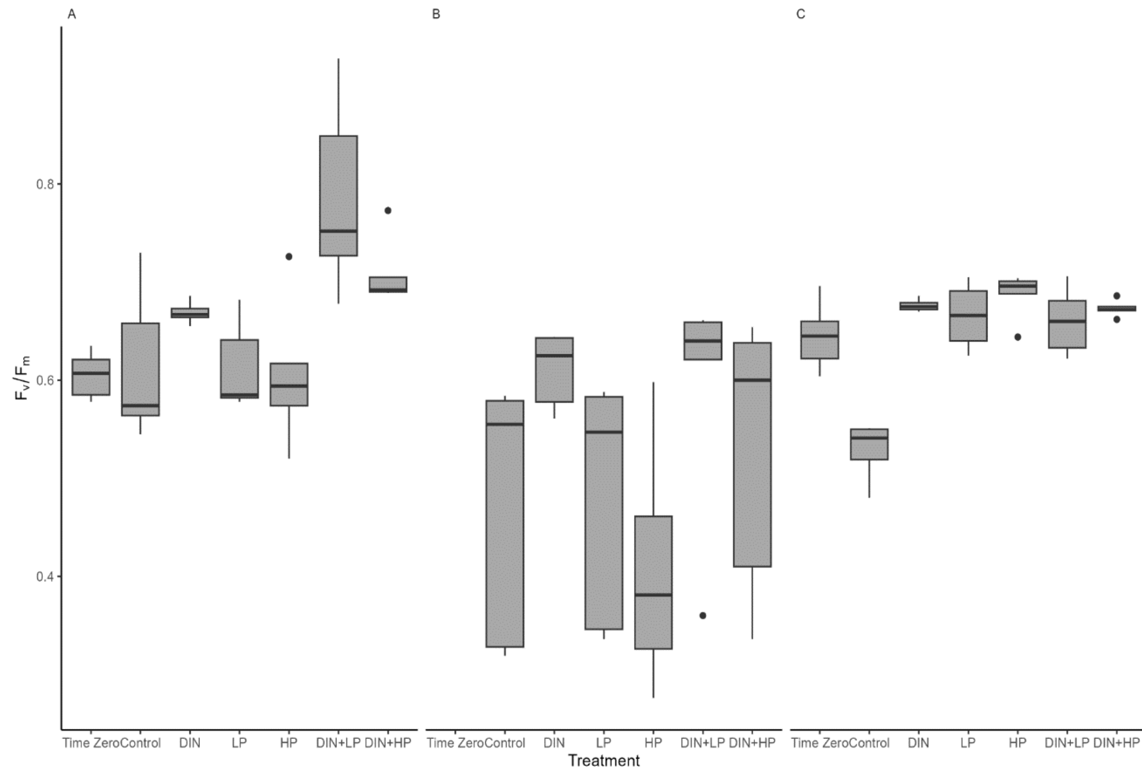

Figure A.1. F_v/F_m values by treatment groups for the June (A), July (B), and September (C) bioassays. Equipment errors resulted in missing data for the July time zero group. The bold line represents the median, the box edges represent the 25th and 75th percentiles, and the extreme ends of the lines represent the smallest and largest values within 1.5 times the interquartile range outside of the 25th and 75th percentiles. Dots represent values that are 1.5-3 times the interquartile ranges.

APPENDIX B

PERCENT CHANGE OF INDIVIDUAL ALGAL GROUPS

Cryptophytes, diatoms and haptophytes, and green algae demonstrated similar patterns of increase in biomass relative to the control in the DIN, DIN+LP, and DIN+HP groups (Figures B.2-3, 5). Cyanobacteria showed no response to any nutrient enrichment (Figure B.1). Dinoflagellates showed little change from the control outside of the September bioassay where they demonstrated a strong response to DIN enrichment (alone or combined).

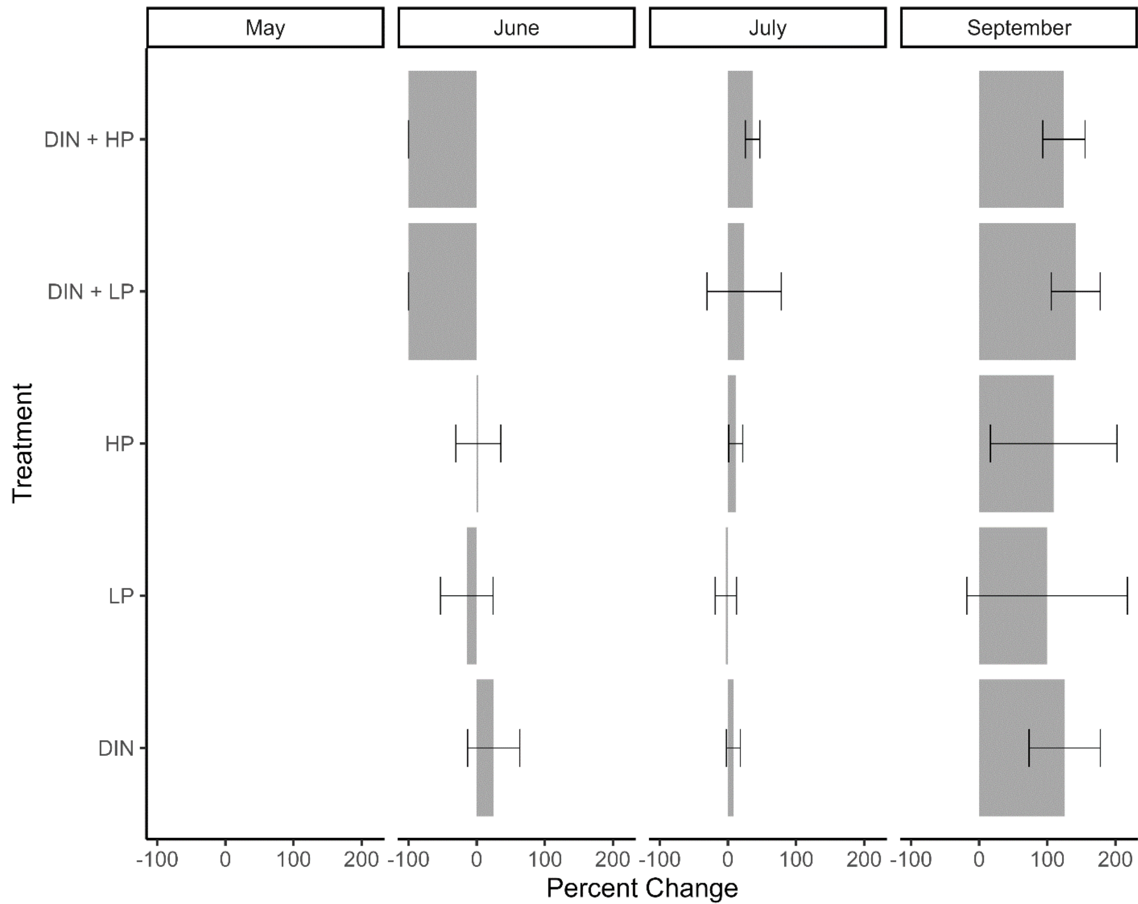

Figure B.1. Percent change in cyanobacteria abundance relative to the control across all bioassays. Error bars represent standard deviation. Cyanobacteria were undetected in the control and nutrient amended groups in the May bioassay. Values were derived from HPLC and *phytoclass* analysis. A two-way ANOVA found no significant differences between treatment groups.

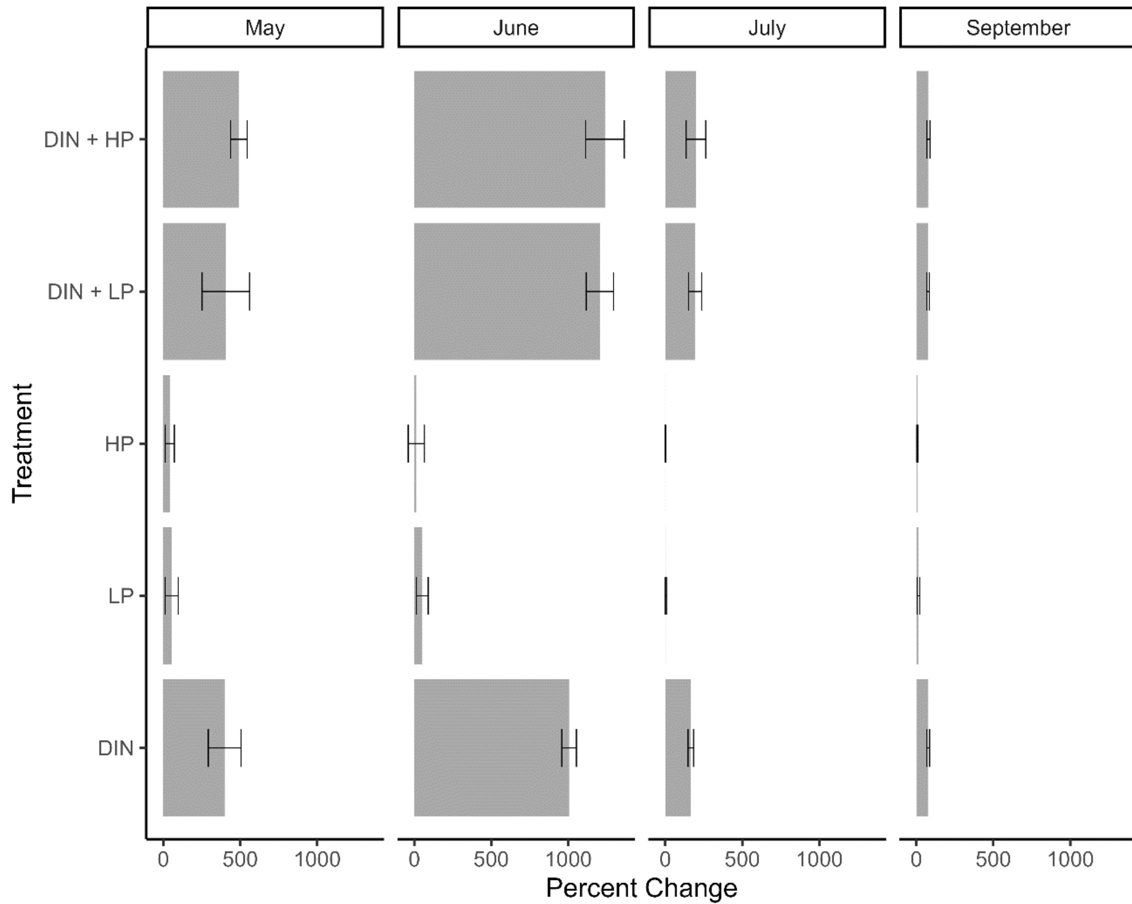

Figure B.2. Percent change in cryptophyte abundance relative to the control across all bioassays. Error bars represent standard deviation. Values were derived from HPLC and *phytoclass* analysis. An RCB two-way ANOVA found significant differences between treatment groups (F = 24.26, df = 4, n = 100, $p < 0.0001$). DIN (alone and combined) groups had a stronger response than P groups but were not different from each other.

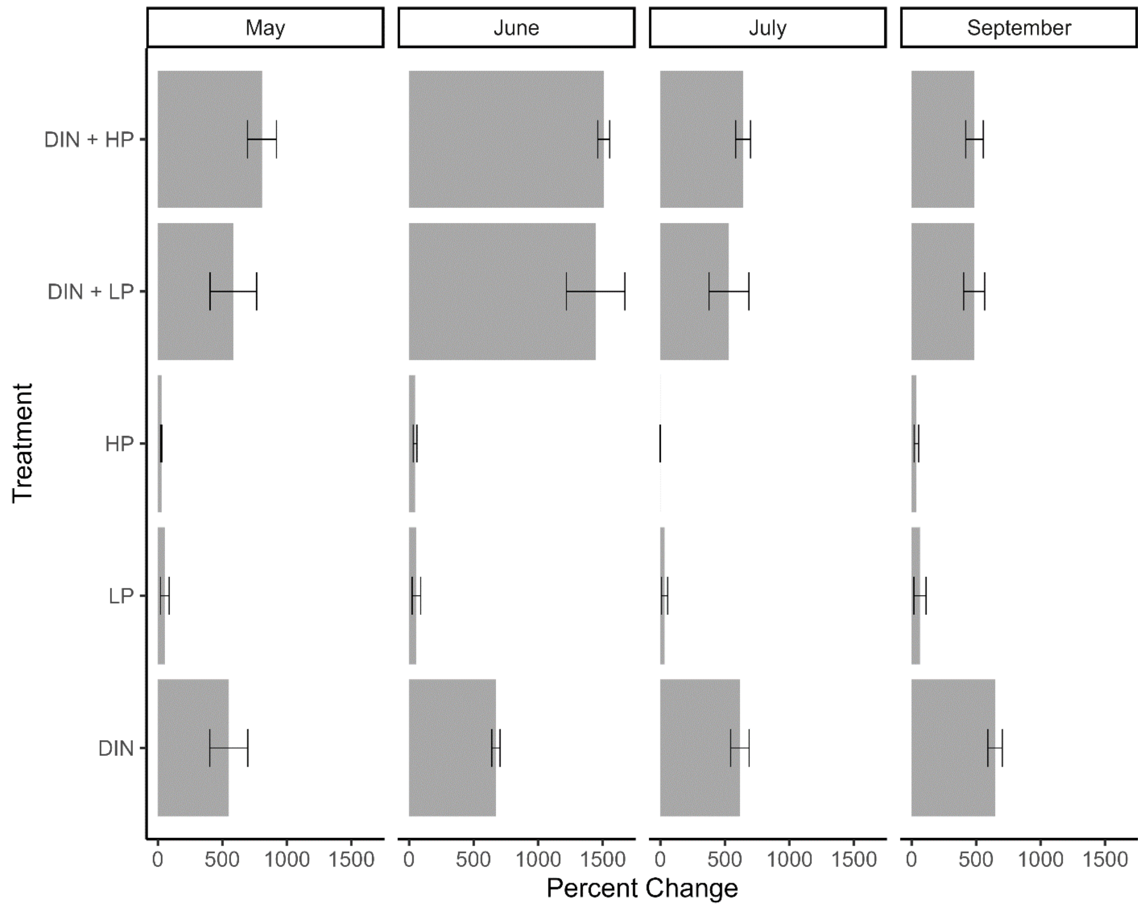

Figure B.3. Percent change in diatom and haptophyte abundance relative to the control across all bioassays. Error bars represent standard deviation. Values were derived from HPLC and *phytoclass* analysis. An RCB two-way ANOVA found significant differences between treatment groups ($F = 68.28$, $df = 4$, $n = 100$, $p < 0.0001$). DIN (alone and combined) groups had a stronger response than the P groups. The DIN + HP group had a stronger response than the DIN alone group.

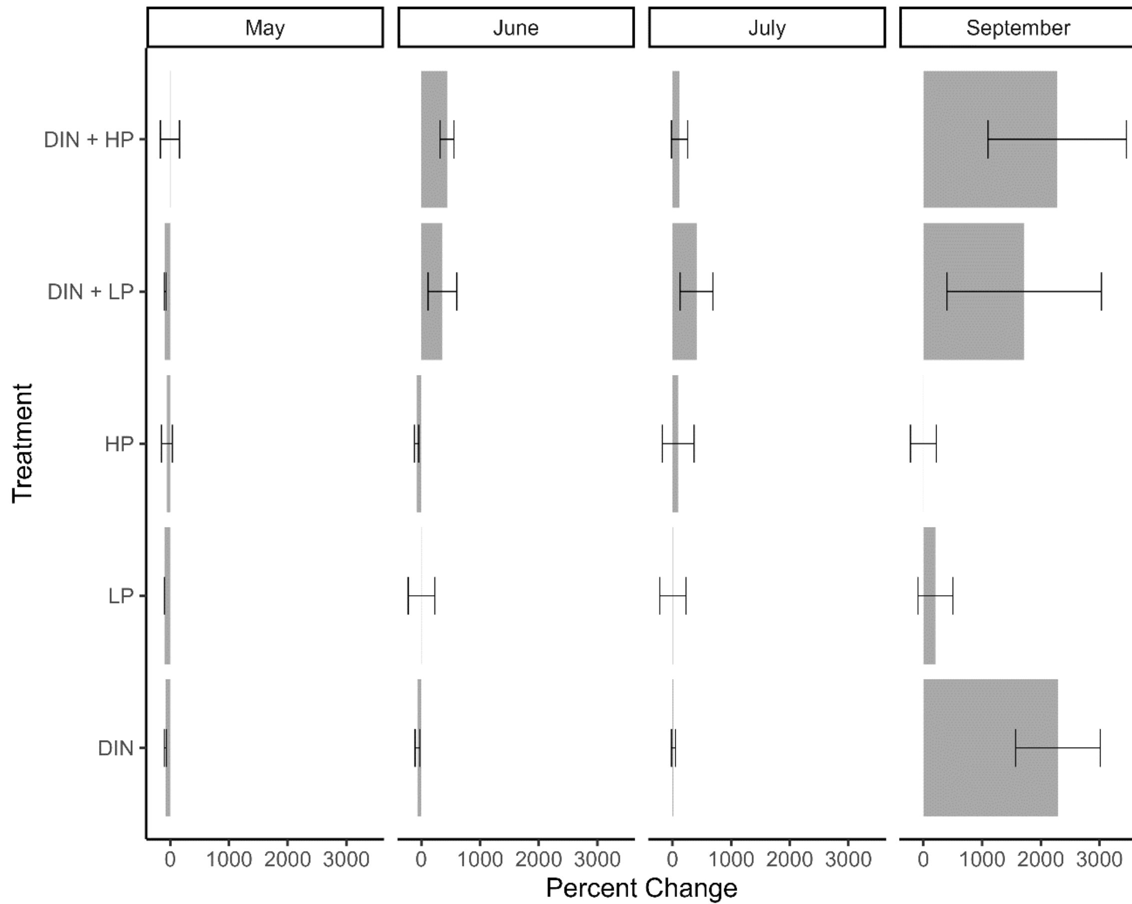

Figure B.4. Percent change in dinoflagellate abundance relative to the control across all bioassays. Error bars represent standard deviation. Values were derived from HPLC and *phytoclass* analysis. An RCB two-way ANOVA found significant differences between treatment groups (F = 6.117, df = 4, n = 100, p = 0.0002). DIN (alone and combined) groups had a stronger response than the HP groups but were not different from each other. Combined DIN groups also had a stronger response than the LP groups.

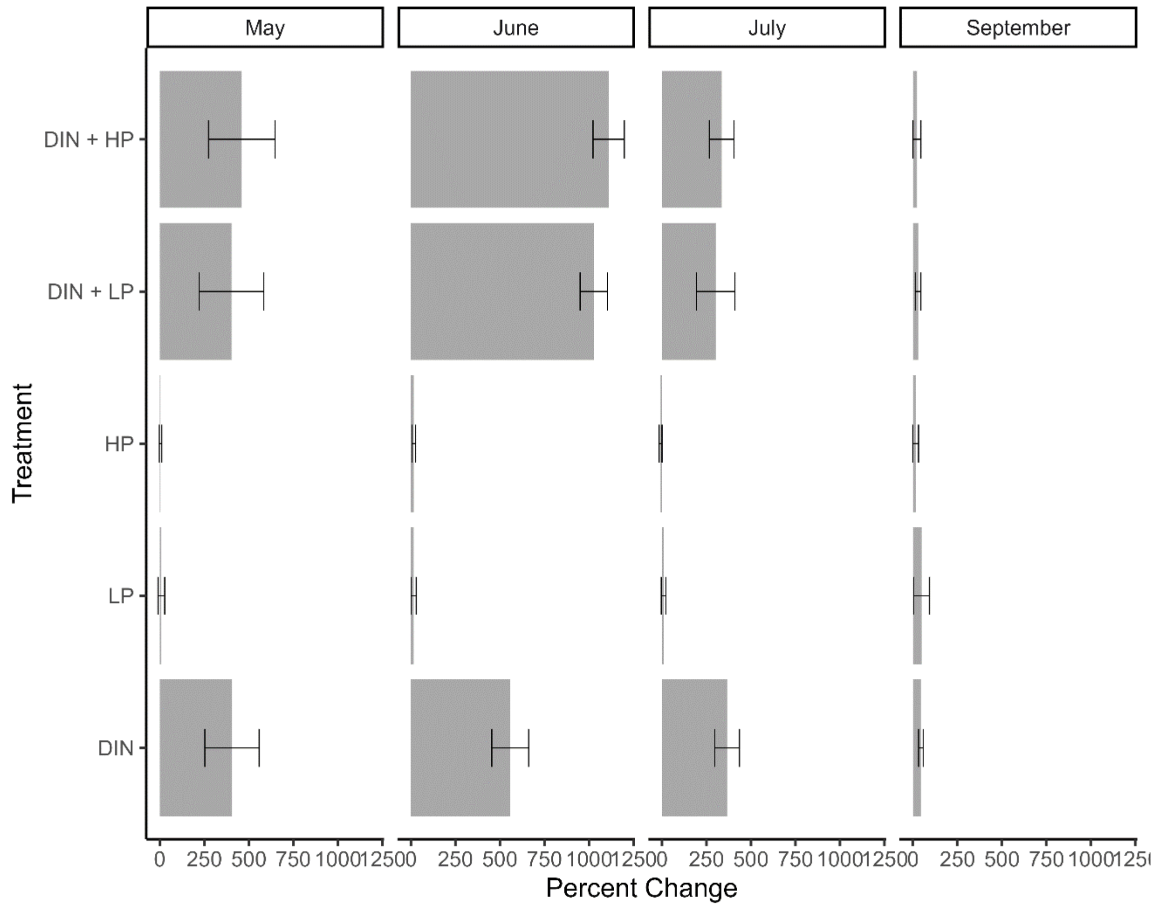

Figure B.5. Percent change in green algae abundance relative to the control across all bioassays. Error bars represent standard deviation. Values were derived from HPLC and *phytoclass* analysis. An RCB two-way ANOVA found significant differences between treatment groups ($F = 25.47$, $df = 4$, $n = 100$, $p < 0.0001$). DIN (alone and combined) groups had a stronger response than P groups but were not different from each other.