PHYTOPLANKTON COMMUNITY STRUCTURE RESPONSE TO GROUNDWATER-BORNE NUTRIENTS IN THE INLAND BAYS, DE

by

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ABSTRACT

We conducted a series of experiments, coupling seepage meters directly to phytoplankton enclosure experiments to determine the impacts of groundwater-borne nutrients on biomass and response in community structure of phytoplankton. To assess the impacts of groundwater-borne nutrients, we measured chlorophyll *a* concentrations as a proxy for overall biomass and used genetic sequencing techniques to characterize the phytoplankton community structure. Groundwater carried a high N load to the estuary with NO_3^- up to 295 µmol/L, and NH_4^+ up to 55 µmol/L. As a result treatment mesocosms had NO_3^- concentrations up to 55.5 µmol/L and NH_4^+ up to 4.0 µmol/L, while control mesocosms received filtered seawater, and were thus relatively low in nutrients (24.2 µmol/L NO_3^- and ~2 µmol/L NH_4^+). In June the highest chlorophyll *a* concentrations occurred after 3.5 days, with significant differences between control mesocosms (4.3±0.2 mg/L), groundwater amended mesocosms (7.0±0.6 mg/L), and mesocosms receiving groundwater across the sediment-water interface (10.9±0.2 mg/L). In August, biomass peaked after 3 days and showed larger variation across treatment groups with groundwater amended mesocosms reaching significantly higher values (36.6±2.0 mg/L) than mesocosms receiving groundwater across the sediment-water interface (18.7±4.8 mg/L), which showed significantly different values than both controls and phosphate amended mesocosms (11.9±0.7 and 9.9±0.2 mg/L respectively). Community gene sequence data showed that species assemblage was also impacted by availability of nutrients, with significant differences in community structure for mesocosms receiving nutrients vs control mesocosms in both June and

August experiments. Several harmful algal species also proliferated in high nutrient treatments, including *Cylindrotheca closterium, Karlodinium veneficum, Nitzschia ovalis,* and *Heterocapsa* sp.. Our study demonstrates the importance groundwater-borne nutrients play in structuring the phytoplankton community, and the potential impacts of nutrient loading through groundwater transport. More research is needed to further identify spatial and temporal differences in groundwater-borne nutrient discharge and response of phytoplankton community structure.

Chapter 1

INTRODUCTION

The Inland Bays of Delaware have been subject to the impacts of eutrophication, resulting in ecological degradation (Coyne et al., 2006). Eutrophication occurs mainly as a result of increased nutrient delivery, with anthropogenic nitrogen being the primary driver of these changes in coastal systems (McClelland & Valiela 1998). Coastal waters experiencing eutrophication are subject to adverse impacts including hypoxia, harmful algal blooms (HABs), and loss of habitat and marine life (Gobler & Boneillo, 2003, Howarth 2008). The Inland Bays of Delaware are of particular interest because of the many services they provide to marine species, which have commercial, recreational and ecological importance. Approximately 70% of commercially significant marine species are dependent on estuarine habitats at some point during their life cycle (Peterson et al., 2000).

The correlation between nutrient availability, usually nitrogen (N) and phosphorus (P), and eutrophication is well established (Howarth 2008, Howarth & Marino 2006). Eutrophic conditions develop as a result of increased nutrient loads propelling growth of primary producers. Nutrients emanating from residential and agricultural land-use, locally including intensive poultry operations, ultimately end up in coastal waters (Glibert 2007) where they contribute to the process of eutrophication. Increased N and P loading has also been found to promote the presence of harmful algal blooms (HABs) (Heisler et al. 2008). P is typically the limiting nutrient in

freshwater, while N is usually the limiting nutrient in brackish or saltwater (Howarth & Marino 2006).

Nutrient loading to coastal estuaries occurs through surface flow, atmospheric deposition, and submarine groundwater discharge. Over 80% of the nitrogen, entering Rehoboth Bay is delivered through groundwater and surface water pathways, with 43 to 75% of the N load coming directly from groundwater (Volk et al., 2006). Indian River Bay, another highly eutrophic DIB system has also exhibited similar high N loading via groundwater (Andres 1991, Russoniello 2012). Though atmospheric deposition and surface transport of nitrogen have been studied in depth, the discharge and impacts of nutrient rich groundwater require more attention.

The geological setting is very important in controlling freshwater and nutrient delivery to estuarine systems. Precipitation infiltrates the water table and is transported to estuarine systems through groundwater flow. This is particularly important on the Delmarva Peninsula due to its sandy geology, which allows water to readily enter the water table carrying nutrients from overlying land-use with it. Nutrient loading via disposal of poultry litter and fertilizer addition to crops account for 95% of N inputs to the Delmarva Peninsula (Denver et al., 2004). These nutrients may take days to decades before they are discharged, depending on the geologic characteristics of the subsurface (Puckett et al., 2011). The discharge of fresh nutrient-rich submarine groundwater into estuarine systems contributes to eutrophication while lag times of eventual discharge potentially prolong the effects of previous nutrient additions to the watershed (EPA 2012).

The relative amount of N and P entering estuaries is important in driving eutrophication, but nutrient composition is also important. There is strong evidence

that certain forms of N can promote or inhibit growth of different species (Anderson et al. 2008, Glibert et al. 2007). Previous work suggests that species or groups of phytoplankton may have differential preferences for NO_3^- or NH_4^+ (Zhang et al 2006), for example, evidence suggests diatoms grow faster and dominate when N is in the form of NO_3^-, while dinoflagellates dominate when NH_4^+ is high (Taylor et al. 2006). Field studies have shown that NH_4^+ may make up the majority of N uptake by phytoplankton (York et al., 2007). Other studies have shown that when NH_4^+ is in excess of 4 μmol/L, ammonium inhibition occurs, preventing phytoplankton from taking up nitrate (Dugdale et al. 2008). These biogeochemical interactions are important in shaping the phytoplankton community structure and overall ecosystem impacts of N delivery to such systems; more research is necessary to elucidate many of these concepts in the field.

Stable isotopic approaches have been widely used to determine sources and track transformations of N and other elements (Aravena et al., 1993; McClelland and Valiela, 1998; Kaushal et al., 2011; York et al., 2007). For NO_3^-, the isotopic values of both N ($\delta^{15}N$) and O ($\delta^{18}O$) vary in a consistent way, providing a reliable range of values to NO_3^- from different sources. As a result, measurements of the isotopes of NO_3^- can be used to determine sources of N to water bodies (Böhlke et al., 2009). Isotopic signatures of O in NO_3^- range from -15 to 100‰, with the higher end of the spectrum being associated with atmospheric nitrate (Böhlke et al., 2009; Kendall, 1998), and the lower ranges (-15 to 10‰) being associated with biogenic nitrate (Böhlke, 2002). N isotope values in nitrate have a narrower range (-10 to 30‰) than O isotopes, with nitrate derived from wastewater or manure carrying a $\delta^{15}N$ of 5–15‰ (Böhlke et al., 2009). Synthetic fertilizers typically have similar $\delta^{15}N$ to atmospheric

N_2 gas (0‰) (Böhlke et al., 2009). Nitrate originating from natural soils has a $\delta^{15}N$ range from −5 to 15‰ (Böhlke et al., 2009). Isotopic signatures of phytoplankton reflect the $\delta^{15}N$ of their N source, with some modification due to fractionation (York et al. 2007). For example, phytoplankton that assimilated N from wastewater or manure should have a higher $\delta^{15}N$ value than those assimilating N from inorganic fertilizer. Measurements of N stable isotopes can provide information on both the forms and sources of N stimulating phytoplankton biomass. Stable isotope measurements of C have been used to show shifts in phytoplankton community structure, depending on changes in $\delta^{13}C$ values (Wang et al., 2011), and can be used to provide insight on the proliferation of different algal types.

Given the important role of coastal and estuarine systems and the negative impacts of eutrophication, more research is necessary to elucidate the biogeochemical interactions leading to degraded water quality. This study uses innovative methods and techniques to provide further insight on how submarine groundwater-borne nutrients may alter community dynamics of phytoplankton in surface water. To do this, we conducted several mesocosm experiments to simulate the enrichment of nutrients via groundwater discharge, and measured the response in phytoplankton community structure and biomass using molecular techniques.

Chapter 2

MATERIALS AND METHODS

Study Site

Guinea Creek is a shallow, poorly-flushed estuary that contributes to Rehoboth Bay, DE (Figure 1). Land-use is dominated by residential development and agriculture, resulting in large nutrient loads to the estuary. Algal blooms, including HAB events, have been recorded sporadically in this system over the past 25 years (Citizen Monitoring Program). Guinea Creek is regularly monitored for nutrients and chlorophyll by Delaware Sea Grant's Citizen Monitoring Program (CMP); nitrate concentrations are 50-70 µmol/L, ammonium concentrations 15-25 µmol/L, and chlorophyll *a* concentrations up to 60 µg/L.

Experimental Setup

We conducted a series of mesocosm experiments to determine the role of groundwater-borne nutrients in structuring phytoplankton community structure and biomass. Using sequenced species data for community structure analysis and chlorophyll *a* as a proxy for biomass, we tested a response based on groundwater-borne nutrient addition versus a control group.

Experiments were conducted in the field with mesocosms suspended in the water column to keep daily and tidal fluctuations in temperature, light, and turbulence as natural as possible. Surface water from Indian River Inlet was filtered, in series, down to 0.2 µm to remove phytoplankton. Ambient water was passed through a 253

µm sieve to remove grazers, but to include the ambient phytoplankton community with which to inoculate the mesocosms. Filtered surface water and ambient water was mixed 4:1 by volume and used to fill all mesocosms to 20 L.

Treatments were run in triplicate with a control group run simultaneously (Figure 2). Treatments included mesocosms amended with nutrients from submarine groundwater pumped from beneath the estuary, enclosures which were attached to open top seepage meters to naturally receive nutrients directly across the sediment-water interface, and mesocosms amended with phosphorus (August experiment only). Open top seepage meter mesocosms were fashioned by attaching clear 50 gallon drum liners to open top seepage meters and deployed in the estuary. Estuarine water was removed, by pumping, from the open top seepage meter treatments and replaced with 20L of experimental water. Care was taken during this set-up process not to disturb the sediment.

June 2015

Groundwater was pumped daily from 50 cm below the sediment estuary interface using a MHE sampler, and 2L was added daily to each of 3 groundwater amended mesocosms. This groundwater was <1ppt salinity and 295 µmol/L NO_3^-, 3.8 µmol/L NH_4^+, 0.2 µmol/L PO_4^{3-}, 79.0 µmol/L SiO_4^{2-}. To keep salinity and volume consistent, 2L deionized water was added to control mesocosms. All mesocosms were sampled twice daily for 5 days.

August 2015

Groundwater was extracted from the same location as the June 2015 experiment and 2 liters were added at the start of the experiment. The groundwater was 2 ppt salinity and 253.2 µmol/L NO_3^-, 16.2 µmol/L NH_4^+, 0.02 µmol/L PO_4^{3-}, 20.3 µmol/L SiO_4^{2-}. Open top seepage meter mesocosms were placed in the same location as the June experiment as well. An additional control group was run with the addition of PO_4^{3-} to test for P limitation. All mesocosms were sampled once daily for 8 days.

Sampling

At each time point, mesocosms were homogenized using a submersible pump prior to collection of a one liter samples. Samples were stored in the dark, on ice until filtered. YSI parameters were also taken and recorded to ensure consistency between ambient conditions of temperature and salinity. Dissolved oxygen was also taken to monitor primary production.

A portion of the water sample was passed through 0.7 µm GFF filters. Filters and filtrate were stored frozen for chlorophyll *a* and isotopic analyses, and nutrient analyses, respectively. Additional sample was passed through 3.0 µm polycarbonate membrane filters to include all phytoplankton; filters were then stored in CTAB+β-ME solution at -80 ºC (Coyne et al., 2001).

Analysis

Analyses included chlorophyll *a*, nutrients (NO_3^-, NH_4^+, PO_4^{3-}, SiO_4^{2-}), N and O stable isotopic ratios of groundwater NO_3^-, N and C stable isotopic ratios of

particles, and terminal-restriction fragment length polymorphism (T-RFLP). Chlorophyll *a* samples were extracted with 90% acetone solution to measure fluorescence (Parsons et al., 1984) and chlorophyll *a* was calculated from fluorescence and used as a proxy for overall biomass of phytoplankton in mesocosms (JGOFS, 1994). Nutrients were analyzed on a Seal AA3 autoanalyzer following manufacturer guidelines (AA3 Protocol).

We prepared $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ samples following the denitrifier method of Sigman et al. (2001) and Casciotti et al. (2002). Isotopic composition of N_2O was analyzed at the Stable Isotope Facility at the University of California at Davis. Values are reported relative to air and V-SMOW standards, for N and O isotopes respectively. Three replicates of two international NO_3^- isotope standards, IAEA-N3 and USGS-34, were included in each set of samples to correct for the cumulative fractionation that occurs over the course of the analysis. The measured $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ were corrected for exchange and fractionation effects using the linear relationship between the known and observed isotopic values of the international standards. We collected particulate matter (seston) from our experiments by passing 200-800 mL of water across a GFF filter. Filters were dried (60°C), packed in tin capsules and analyzed for $\delta^{13}C$ and $\delta^{15}N$ at the Stable Isotope Facility at the University of California at Davis. Isotope values are expressed relative to international standards V-PDB (Vienna PeeDee Belemnite) and Air for carbon and nitrogen, respectively.

DNA from the 3.0 μm filters was extracted using a combination of chemical and mechanical methods and concentrations were measured on a Nanodrop instrument. Triplicate PCR reactions were completed using Euk-29 forward and 517-

FAM reverse gene primers, following 35 amplification cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C. Replicates were pooled following visualization on 1% agarose gel. Terminal restriction fragment length polymorphism (T-RFLP) was used to characterize phytoplankton assemblage using the fluorescently labeled PCR products with *Hae*III restriction enzyme (Kim et al., 2014). Samples were measured on an ABI Prism 310 genetic analyzer (Applied Biosystems), using Genescan software, and peaks were verified and adjusted as needed using Peakscanner software. T_REX software was used to eliminate noise, and standardize samples before further analysis. Statistical analysis of preliminary community data was completed in Primer v7, where square root transformation was used to downweight and normalize statistical impact of highly dominant T-RFLP fragments. Bray-Curtis similarity values were calculated and added to a non-metric multidimensional scaling (MDS) plot.

A subset of samples were selected for sequencing to determine species assemblage of the phytoplankton community in the mesocosms. PCR primers Euk-7 forward with barcode and 570 reverse were used for amplification which was checked for success on 2% agarose gel. Once all PCR products were checked, samples were pooled together in equal proportions based on molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Purified PCR products were used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed by MR DNA (www.mrdnalab.com) on a MiSeq following manufacturer's guidelines.

Data Analysis

Due to differences in experimental design, data from August and June experiments were analyzed separately. Using R software, data was transformed and then analyzed using associated packages. Differences in overall biomass based on treatment were assessed using one factor ANOVA (α=0.05). Once significance was determined, a post-hoc Tukey-Kramer test was used to test significance between groups.

Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In short, sequence data was joined, barcodes removed, sequences <150bp removed, and sequences with ambiguous base calls removed. Sequences were denoised, and operational taxonomic units (OTUs) were generated, by clustering at 3% divergence (97% similarity). Chimeras were removed, and final OTUs were classified via Blastn against a curated database adopted from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, DeSantis et al 2006, http://rdp.cme.msu.edu).

Following sequence analysis, relative abundance data was analyzed using the package vegan within R software (R Core Team Development 2008, Oksanen et al. 2008). Differences in community composition were analyzed for each mesocosm with a multivariate approach using the non-metric multidimensional scaling (MDS) and adonis procedures. Bray-Curtis similarities were calculated from similarity matrices and used to generate 2D and 3D MDS plots depicting differences in phytoplankton community structure. Observed variation in community composition was tested for significance using the adonis function in the vegan package (Oksanen et al. 2008). The adonis function uses Bray-Curtis similarity measures to perform a permutational

multiple analysis of variance (MANOVA), to assign variation in multivariate data explanatory variables, in this case treatment (differences in nutrient concentrations). 999 permutations were used in these analyses.

Chapter 3

RESULTS

Nutrient Concentration

The goal of this study was to use groundwater to manipulate nutrient concentrations in mesocosms, and assess the impacts on primary producers. Initial NO_3^- concentrations were highest in groundwater amended mesocosms (June: 38±5 and August: 55±12 µmol L-1), while other treatments had NO_3^- concentrations less than half of these (Table 1). NH_4^+ was similar across all treatments ranging from approximately 3 to 4 µmol L-1 in June, and approximately 1.5 to 2.0 µmol L-1 in August. PO_4^{3-} was similar across all groups for both June and August, except for the phosphate amended mesocosms which had the highest concentrations (1.5±0.02 µmol L-1). In June, SiO_4^{2-} concentrations were highest in groundwater amended mesocosms (47±11 µmol L-1), while concentrations in control and open top seepage meter mesocosms were less (27±11 and 29±8 µmol L-1 respectively). SiO_4^{2-} concentrations in August, were similarly high in groundwater amended mesocosms (70±5 µmol L-1), but highest in open top seepage meters (80±10 µmol L-1).

Groundwater

Groundwater samples from multiple locations and depths were collected to determine the amount of nutrients delivered to the estuary and its phytoplankton community. For both the June and August experiments we collected shallow

groundwater (~10 cm below the sediment surface) from underneath the open top seepage meters after the conclusion of our experiments. The lateral distance between these samples was small, ~30 cm between samples. The salinity ranged from 13.6-27.1 ppt, (Table 2), typically lower than estuarine salinity at this site (19-25ppt). Nutrient concentrations in these samples were highly variable, with NO_3^- from 12-150 µmol/L, NH_4^+ from 24-55 µmol/L, PO_4^{3-} from 0.03-2.02 µmol/L, SiO_4^{2-} ranges from 23-167 µmol/L, and $\delta^{15}N$ - NO_3^- from 5.54-19.35 ‰ (Table 2).

Porewater added to groundwater amended mesocosms, was collected from deeper below the sediment-estuary interface, from ~50 cm, and had different nutrient composition. Compared to porewater described above, dosing water had lower salinities (June: 2.0, August: 1.5), higher NO_3^- concentrations (June: 295.29, August; 253.15 µmol L-1), lower NH_4^+ concentrations (June: 3.81, August: 16.20 µmol L-1), comparable PO_4^{3-} concentrations (June: 0.17, August: 0.02µmol L-1), and comparable SiO_4^{2-} concentrations (June: 79.08, August: 20.32µmol L-1).

Biomass

Chlorophyll *a* concentrations were measured throughout both experiments as a proxy for phytoplankton biomass. Phytoplankton biomass was lower in June experiments than August experiments (Figure 3). In June, there were significant differences in chlorophyll *a* by treatment ($F=22.34$; $P<0.001$) with the highest concentrations found after 3.5 days. Open top seepage meter mesocosms had significantly higher chlorophyll *a* concentrations (10.9±0.2 mg/L) than groundwater amended mesocosms (7.0±0.6 mg/L; $P <0.005$) and than control mesocosms (4.3±0.2 mg/L; $P<0.005$; Figure 3). In August, there were significant differences in chlorophyll

a by treatment (F=41.51; P<0.005) with the highest concentrations found after 3 days. Groundwater amended mesocosms had significantly higher concentrations (37±2 mg/L) than open top seepage meter mesocosms (19±5 mg/L; P<0.005), which had significantly higher concentrations than control and phosphate amended mesocosms (11.9±0.7; P<0.005 and 9.9±0.2 mg/L; P<0.005 respectively), which were not statistically different (Figure 4; P=0.35).

Community Structure

Species assemblage was characterized from sequence analysis to determine if treatment or time resulted in differences in community structure. MDS ordination of species assemblage for June and August experiments show distinct community structure across treatment and time (Figure 4). PERMANOVA showed that treatment (differences in nutrient composition) significantly explained 26.5% and 39.9% of the variation in species assemblage in June (Treatment: F=4.133, R=0.265, P < 0.005) and August (Treatment: F=8.276, R=0.399, P < 0.001). In addition, time explained 40.2% and 29.4% of the variation in species assemblage in June (Time: F=12.543, R=0.402, P<0.001) and August (Time: F=18.253, R=0.294, P<0.001). In both experiments community structure was driven by treatment, a result of differences in nutrient composition.

Taxonomic breakdown by class revealed that over the course of the June experiment, Xanthophyceae (golden-brown algae) became the most abundant in open top seepage meter mesocosms, while Bacillariophyceae (pennate diatoms) were dominant in groundwater amended mesocosms (Figure 5a). By the end of the June experiment, the phytoplankton class makeup in the control mesocosms, which

received no nutrient additions, were most similar to those at the initial timepoint. Taxonomic breakdown by class in August revealed large differences across treatments, except for control and phosphate amended mesocosms which showed similar class structure (Figure 5b). In August, Coscinodiscophyceae (centric diatoms) became dominant in control, phosphate amended, and groundwater amended mesocosms, while open top seepage meter mesocosm species were spread among classes.

Harmful Species

In June, harmful algal species were less abundant than in August (Table 3). There was no significant difference in abundance of harmful species across treatments in June. During the August experiment, several harmful algal species became dominant in mesocosms receiving groundwater additions. *Cylindrotheca closterium*, a bloom forming diatom, had significantly higher abundance in groundwater amended mesocosms (13.9±1.4 % abundance), than controls (4.5±0.1 % abundance; $P<0.1$). The potentially toxic species, *Karlodinium veneficum* also showed significantly higher relative abundances in groundwater amended mesocosms (3.0±1.4 %) than control mesocosms (1.7±1.2 %; $P<0.1$). *Nitzschia ovalis* (potentially toxic) and *Heterocapsa* sp. (bloom forming) showed significantly higher relative abundances in open top seepage meter mesocosms (9.5±13.3 % and 1.6±0.7 % abundances respectively) than control mesocosms (0.2±0.0 %; $P<0.05$ and 0.3±0.1 % ; $P<0.01$).

$\delta^{15}N$ and $\delta^{13}C$ of Phytoplankton

We measured the stable isotopic ratios of N and C of particulate matter during our August experiment to track the source of N assimilated by phytoplankton and the pattern of C isotopes. Initially, the $\delta^{15}N$ of particles in August were similar across all treatments ranging from 12-16 ‰. Through time, differences in $\delta^{15}N$ by treatment were observed (Figure 6). Control and phosphate amended mesocosms had similarly high $\delta^{15}N$ values, while groundwater amended and open top seepage meter mesocosms had lower $\delta^{15}N$ values. These patterns suggest that in mesocosms influenced by groundwater additions (groundwater amended and open top seepage meters), phytoplankton nitrogen isotopic ratios were influenced by the $\delta^{15}N$ of their groundwater nutrient source (Table 2). Initial phytoplankton $\delta^{13}C$ values were similar across all treatments. Carbon isotopic values showed distinct patterns across treatments overtime. Control and phosphate amended mesocosms $\delta^{13}C$ values were consistently similar, and showed small increases. Groundwater amended mesocosms $\delta^{13}C$ values increased twice as much during the same time-span, while open top seepage meter mesocosms showed the reverse trend, decreasing after initial measurements.

Chapter 4

DISCUSSION

Submarine groundwater

The goal of this study was to elucidate the impacts of submarine groundwater-borne nutrients on phytoplankton community structure and biomass. We found that nutrient composition of groundwater varied greatly across small distances (>30cm, at the same depth), including NO_3^- concentrations from 12-150µmol/L and NH_4^+ concentrations varying from 23-55µmol/L. These variations likely result from a combination of differences in redox conditions, groundwater flow paths, and lag-times over the course of groundwater travel from the watershed to the point of measurement in the estuary (Puckett et al., 2011). Characterizing these small scale differences in groundwater nutrient sources is critical for determining overall impacts on estuarine ecosystems and the best ways to manage such systems.

Our isotopic data provided evidence for the direct linkages between nutrients delivered by groundwater and increased biomass of the phytoplankton community. Nitrate concentrations in the groundwater-amended treatments were at least double those in other treatments (Table 1). Biomass of the phytoplankton community reached the highest levels in these treatments (Figure 3B), suggesting that high NO_3^- availability drove phytoplankton biomass. Our isotopic data provided further confirmation of this. The $\delta^{15}N$ of NO_3^- in the groundwater that we added was low, 4.6‰, and in the range previously found for NO_3^- emanating from agricultural land-use in this area (Böhlke et al., 2009). $\delta^{15}N$ values of the particulate matter in all

treatments started around 14‰ (Figure 6). Over the course of the experiment, $\delta^{15}N$ of the particles in treatments receiving groundwater decreased to 8 to 9‰, suggestive of incorporation of a low $\delta^{15}N$ nitrogen source, whereas there was no decrease in $\delta^{15}N$ values in the control or P addition treatments. Our isotopic data suggest that stimulation of the phytoplankton community by high NO_3^- delivered from the watershed via groundwater promoted increased biomass and caused shifts in the community composition.

Phytoplankton

Phytoplankton biomass differed seasonally, with higher biomass in late summer relative to earlier in the year (Figure 3). In both sets of experiments, phytoplankton responded similarly to the influence of groundwater nutrients, regardless of season. Seasonal patterns in phytoplankton biomass may be affected by numerous factors including changes in temperature, light availability, relative availability of N and P, or other factors (Grover and Chrzanowski, 2006). Loading rates of N and P to Rehoboth Bay vary temporally (Volk et al 2012). In our experiments, initial N:P ratios in June were much higher than in August, suggesting the possibility that the availability of P may have limited the increase in biomass in the June experiment (Table 1). In August, we tested the possibility of P limitation of phytoplankton biomass, and found no difference in response of biomass between the control and P addition treatment, refuting the possibility of P limitation in August. These patterns in P dynamics could provide insight to the higher biomass observed in August versus June experiments (Figure 3)

Changes in relative availability of nutrients can result in changes in community structure and rise of HABs (Glibert et al. 2011). For example, there was a shift from dominance of diatoms to dinoflagellates due to increased P loading to Tolo Harbor, Hong Kong, shifting N:P ratios from 20:1 to <10:1 (Heisler et al., 2008; Hodgkiss and Ho, 1997; Hodgkiss, 2001). In this study, shifts in phytoplankton class composition of experimental treatments were greater in August than in June (Figure 5). In June, golden-brown algae (Xanthophyceae) became the dominant class in mesocosms connected to the sediment-estuary interface (Figure 5a). These algae are often associated with freshwater and soil habitats (Pannard et al., 2007); these mesocosms received freshwater across the sediment-water interface throughout these experiments which likely stimulated these algal cells. Overall, we saw the greatest difference in the proliferation of centric diatoms (Coscinoidscophyceae) in the August control and P amended mesocosms. In contrast, pennate diatoms (Bacillarophyceae) increased with the addition of anthropogenic N, which has previously been linked to the potential rise of diatom blooms (specifically Bacillarophyeae; Burkholder et al., 2008), through laboratory experiments (Bates et al., 1998; Cochlan et al., 2008). We also found changes in HAB species abundance, with increased relative HAB species abundance (%) later in summer compared to the spring, including harmful species, potentially resulting from an array of factors including seasonal changes in water temperature, N:P ratios, nutrient fluxes delivered via groundwater and light exposure. When influenced by groundwater-borne nutrients, the majority of HAB species were found at higher abundances (Table 3), suggesting a probable correlation between high NO_3^- groundwater discharge and increase of blooms.

Food web

The proliferation of HABs associated with nutrient loading, as found in this study and others (Heisler et al., 2007), can have broad implications for food web and ecosystem alterations (Howarth, 2007; Howarth et al., 2000; NRC, 2000; Boesch, 2002). Mesocosms receiving groundwater enrichment directly via the sediment (open top seepage meter) or additions of groundwater had increased N and P concentrations and had a higher prevalence of HAB species relative to non-HAB forming species. These HABs include some very well studied harmful species, most notably *Karlodinium venecicum*, a dinoflagellate species known for causing fish kills through the production of karlotoxins (Bachvaroff et al., 2009), and *Cylindrotheca closterium* a diatom species known to produce a mucilage that can suppresses growth of other species (Orsina et al., 2011). In addition to harmful algae blooms, some potentially harmful protozoan species showed increases in abundance under these high nutrient conditions. For example, *Neoparamoeba pemaquidensis*, an amphizoic marine protozoan associated with several diseases in marine organisms including amoebic gill disease (Lee, et al., 2006), increased in abundance from 1.20±0.84 % in control mesocosms to 4.03±1.23 % of the overall population in groundwater amended mesocosms in only three days. Our work has shown that in addition to changes in biomass, nutrient additions can lead to increases in the prevalence of potentially toxic and problematic organisms.

Chapter 5

CONCLUSION

Using a combined isotopic and molecular approach, our work shows that groundwater enriched with N from agricultural practices stimulates phytoplankton biomass, causes shifts in phytoplankton species composition and promotes the proliferation of HABs in the Inland Bays of Delaware. Results from this study are applicable to other coastal regions of similar sandy, porous geology including the Delmarva Peninsula, Cape Cod, Long Island, and portions of New Jersey, in addition to other areas globally with similar geologic settings, particularly those regions with large agricultural or residential impacts, resulting in the accumulation of N and P through groundwater flow.

Our work highlights areas for additional investigation. The variability in nutrient composition of groundwater we found in this study can make studying ecological impacts, and determination of management approaches challenging. These small scale variations must be well-characterized to determine the overall impact of groundwater-borne nutrients at the whole-estuary scale. Additionally, groundwater residence times are long, and may cause a "legacy effect", introducing a lag time to surface water quality improvements from changes in land-use (Puckett et al., 2011). These controls may be critically important in impacts on the biomass and composition of the phytoplankton community, and therefore water quality.

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Appendix A

TABLES

Table 1. Initial mean concentrations ± standard error of nitrate, ammonium, phosphate, silicate, and chlorophyll *a* for June and August experiments. N:P ratio is calculated as $(NO_3^- + NH_4^+)/ PO_4^{3-}$

	NO_3^- (µmol/L)	NH_4^+ (µmol/L)	PO_4^{3-} (µmol/L)	SiO_4^{2-} (µmol/L)	Chl *a* (ng/mL)	N:P ratio
June Experiment						
Control	18.5±2.1	3.2±0.4	0.1±0.0	27.0±10.8	4.3±0.1	308.7±50.4
Groundwater amended	38.0±4.7	4.0±0.4	0.1±0.0	46.5±11.1	4.5±0.2	567.3±11.6
Open Top Seepage Meter	15.3±2.7	3.2±0.4	0.1±0.0	28.6±8.3	8.2±2.9	230.90±21.2
August Experiment						
Control	24.2±2.0	1.5±0.2	0.4±0.0	46.9±2.0	9.5±0.1	74.0±5.0
Groundwater amended	55.5±11.5	1.9±0.4	0.9±0.1	69.9±4.6	11.9±1.6	64.0±10.4
Open Top Seepage Meter	26.7±9.4	1.8±0.3	0.4±0.0	80.1±9.5	15.3±3.1	76.5±26.4
Phosphate Amended	10.4±2.0	1.9±0.3	1.5±0.0	38.7±1.7	8.8±0.3	8.2±1.2

Table 2. Characteristics of groundwater samples including depth of collection, salinity, dissolved oxygen, nutrient concentrations, and NO_3^- stable isotope values, for groundwater used to amend mesocosms, and for groundwater collected from the sediment below open top seepage meter mesocosms.

Experiment	Groundwater Source	Depth (cm)	Salinity (ppt)	DO (mg/L)	NO_3^- (µmol/L)	NH_4^+ (µmol/L)	PO_4^{3-} (µmol/L)	SiO_4^{2-} (µmol/L)	$\delta^{15}N$-NO_3^- (‰)	$\delta^{18}O$-NO_3^-(‰)
June	Open Top Seep 1	11	13.60	2.40	47.99	53.06	0.64	124.83	7.13	4.18
June	Open Top Seep 2	10	20.80	1.90	51.17	54.82	1.29	166.89	19.35	13.40
June	Open Top Seep 3	12	23.10	0.40	150.94	37.23	0.64	86.76	19.18	18.17
August	Open Top Seep 1	12	14.14	2.29	110.08	23.65	0.03	23.06	5.54	3.14
August	Open Top Seep 2	13	27.10	0.23	12.35	25.08	2.02	95.35	12.98	11.73
August	Open Top Seep 3	12	24.68	0.32	33.35	36.25	0.63	110.10	9.93	5.00
June	Amend Source	55	2.03	5.40	295.29	3.81	0.17	79.08	4.39	2.25
August	Amend Source	45	1.50	4.40	253.15	16.20	0.02	20.32	4.62	2.56

Table 3. Mean abundance (%) ± standard error of potentially harmful algal species by treatment for both June and August Experiments. Abundances were calculated from 18s sequence data at a specified time (June=3.5 days and August=3.0 days) and averaged by treatment group. Species data was selected based on their potentially harmful ecological effects as well as their relative abundance in mesocosms. Dunnet's test was used to test the significance of relative abundance of individual species for treatment groups vs. the control group. The Dunnets Key denotes the significance codes.

	Relative Abundances (%)				
June Harmful Species	Control	Open top seepage	Groundwater amended		Ecological effect
Scrippsiella sp.	0.8±0.2	2.6±4.2	0.2±0.3		High Biomass Nuisance
Eutreptiella sp.	0.0±0.0	0.2±0.2	0.0±0.0		High Biomass Nuisance
Gonyaulax spinifera	0.0±0.0	0.2±0.3	0.0±0.0		Potentially Toxic cell
Chaetoceros sp.	1.7±0.6	0.7±0.5	2.8±0.1		High Biomass Nuisance
August Harmful Species	Control	Open top seep	Groundwater amended	Phosphate amended	Ecological Effect
Cylindrotheca closterium	4.5±0.1	4.3±2.8	13.9±1.4 .	4.3±0.5	High Biomass Nuisance
Karlodinium veneficum	1.7±1.2	5.1±2.9	3.0±1.4 .	1.4±0.9	Potentially Toxic cell
Nitzschia ovalis	0.2±0.0	9.5±13.3 *	0.3±0.1	0.1±0.0	Potentially Toxic cell
Heterocapsa sp.	0.3±0.1	1.6±0.7 .	0.7±0.0	0.4±0.0	High Biomass Nuisance

Dunnets Key

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Adjusted p values reported -- single-step method)

Appendix B

FIGURES

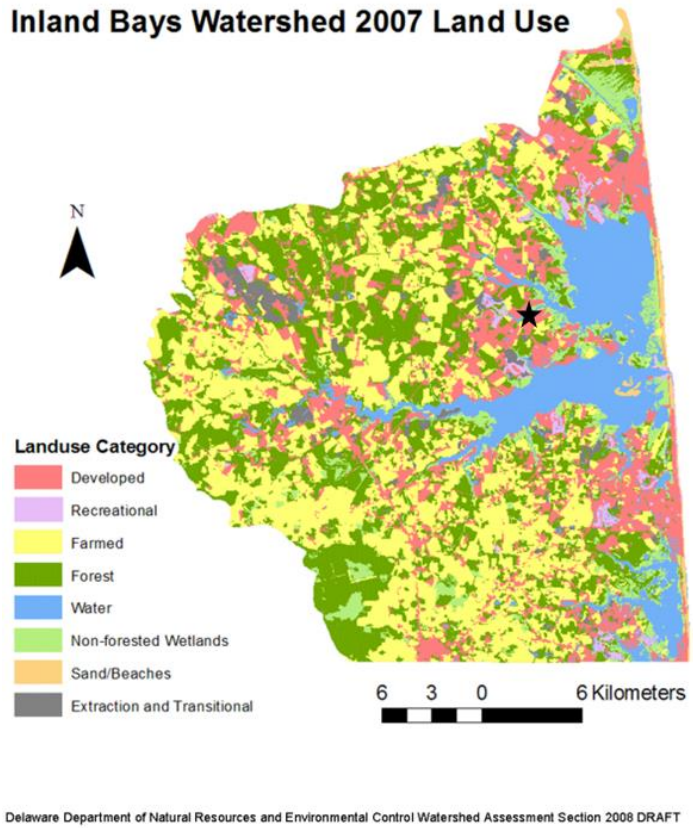

Figure 1. Map of Inland Bays of Delaware, showing land use and study site.

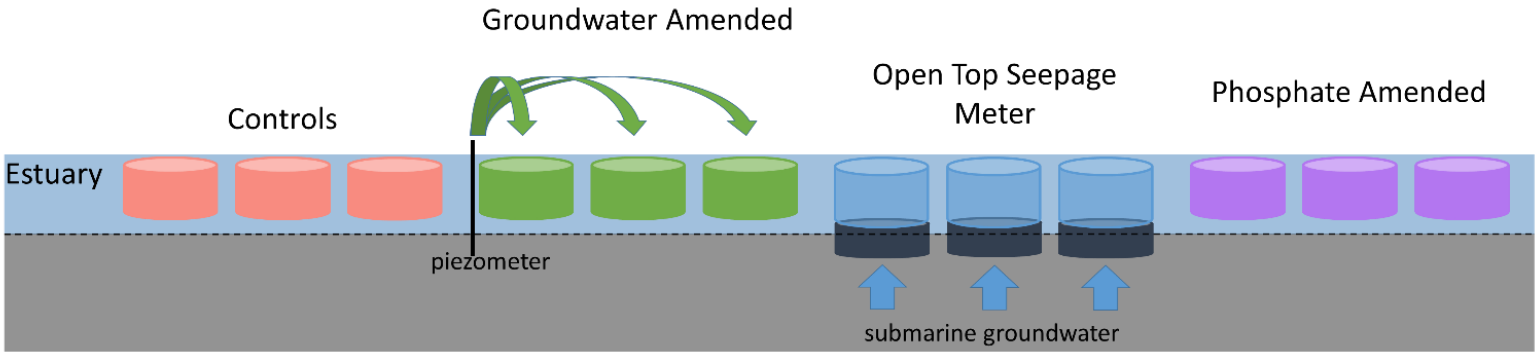

Figure 2. Diagram summarizing experimental design of mesocosm experiments. Arrows signify addition of groundwater. Green arrows indicate groundwater pumped through a piezometer and added manually into mesocosms; Blue arrows indicate natural flow of groundwater into mesocosms across the sediment-water interface.

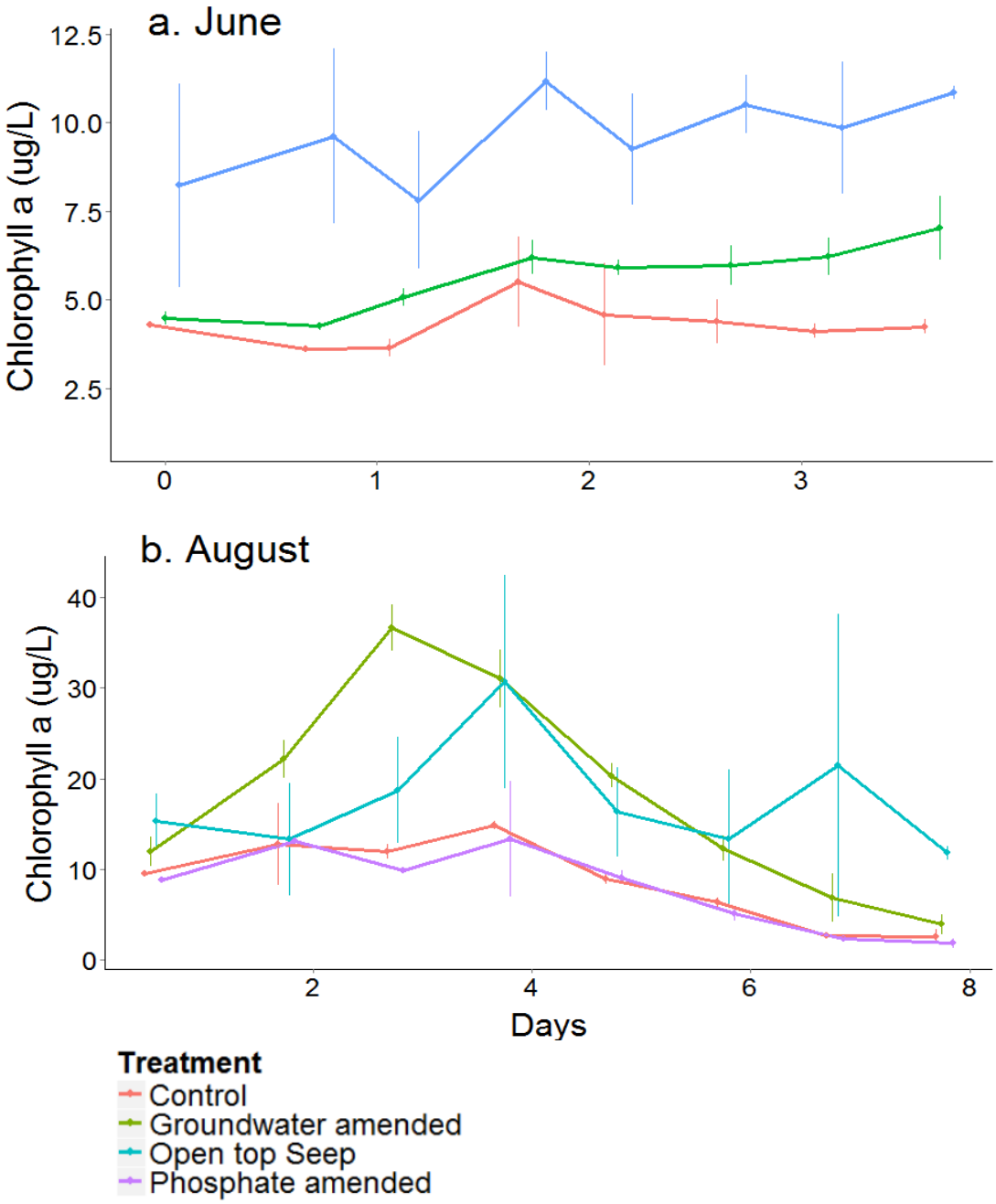

Figure 3. Chlorophyll *a* concentrations over time, mean ± standard error, during June (3a, top) and August (3b, bottom), by treatment groups.

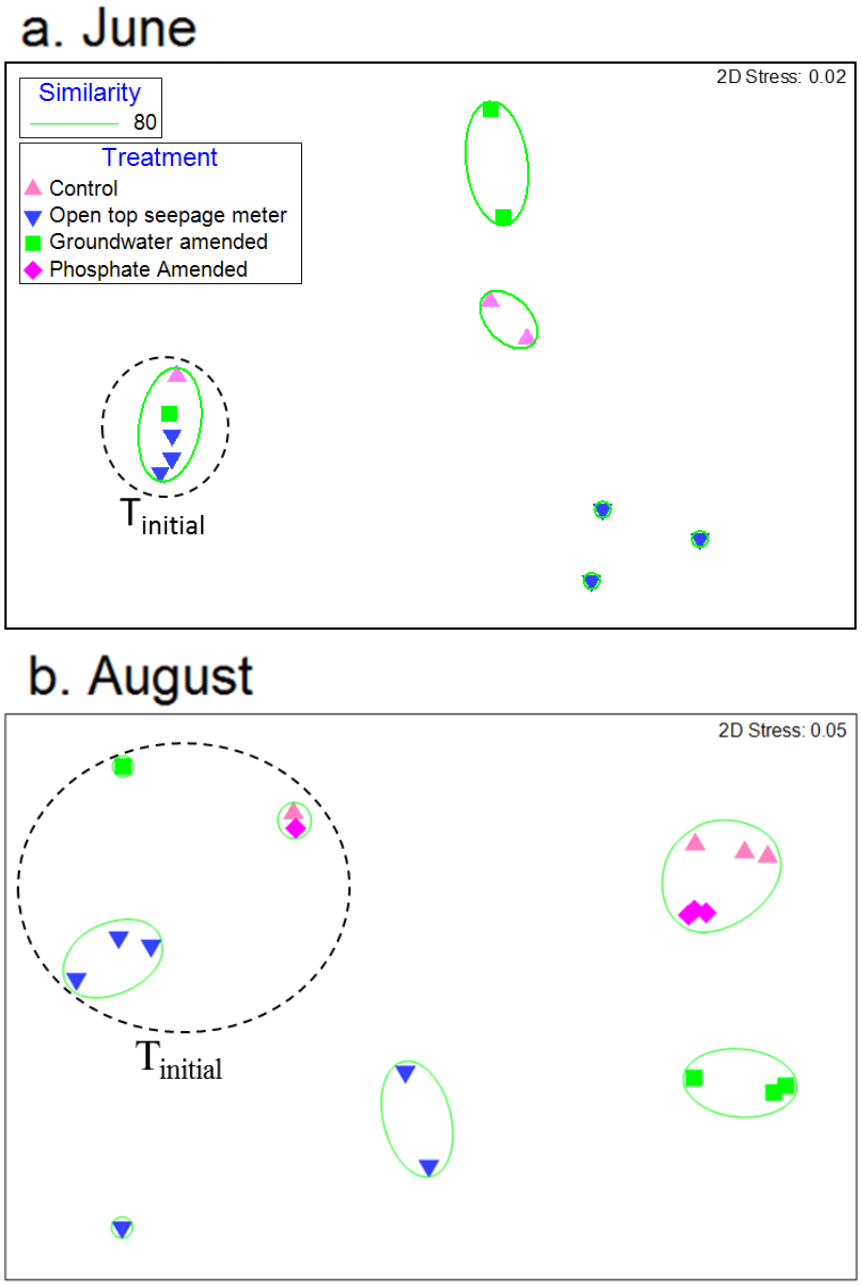

Figure 4. Non-metric multidimensional scaling (MDS) ordination of Bray-Curtis similarities using square root transformed species assemblage data from June (4a top) and August (4b bottom) mesocosm experiments. Similarity clusters are shown by the solid circle, and group samples based on their percent similarity in community structure. Dashed circles differentiate measurements at initial and final timepoints. Symbols are used to differentiate treatment groups.

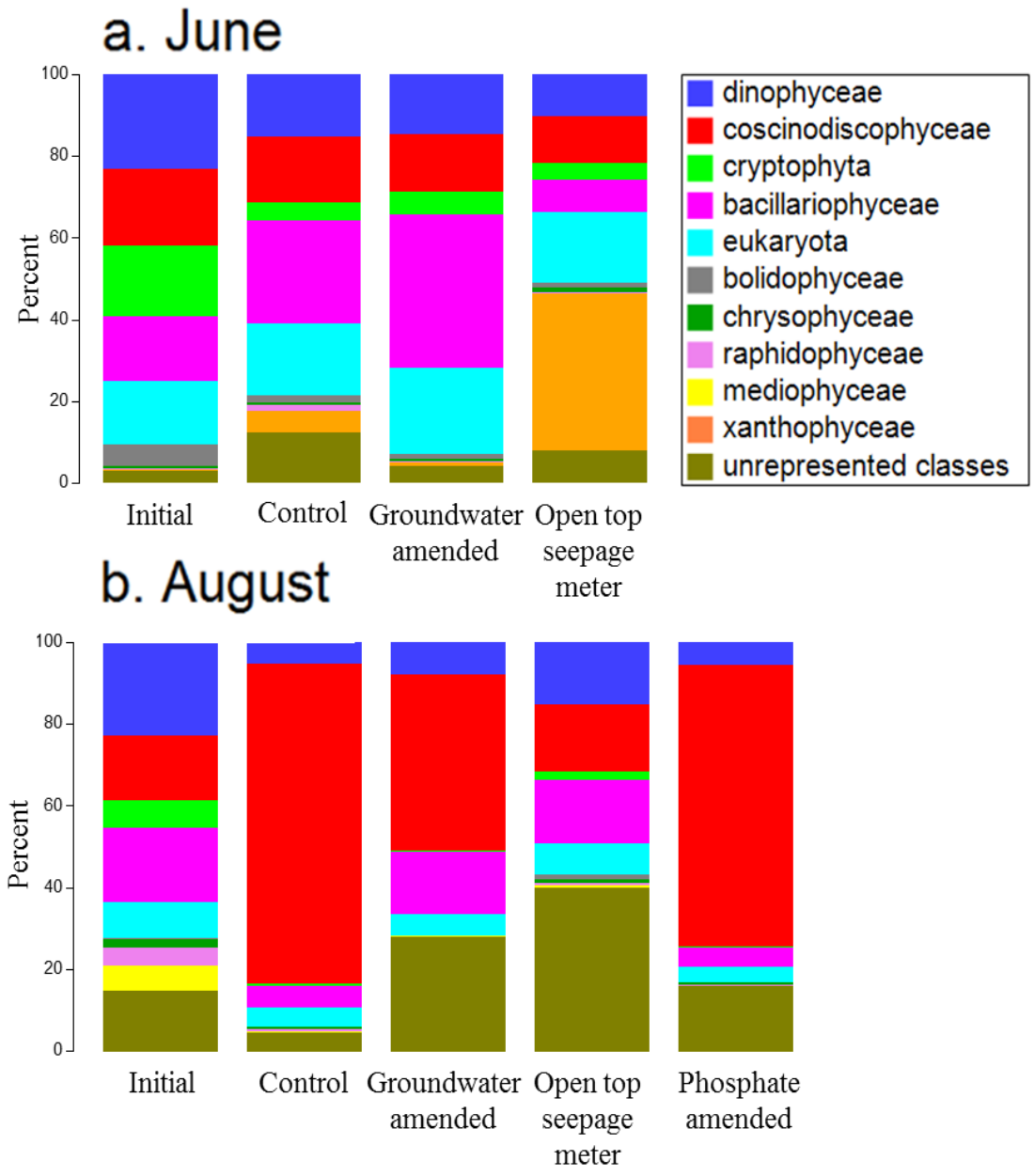

Figure 5. Relative abundance (% of total) of phytoplankton class for averaged initial measurements in June (5a top) and August (5b bottom) experiments, and end time points by treatment group. Classes that made up less than 1% of the population when combined across all treatments or unknown classes are represented by "unrepresented classes".

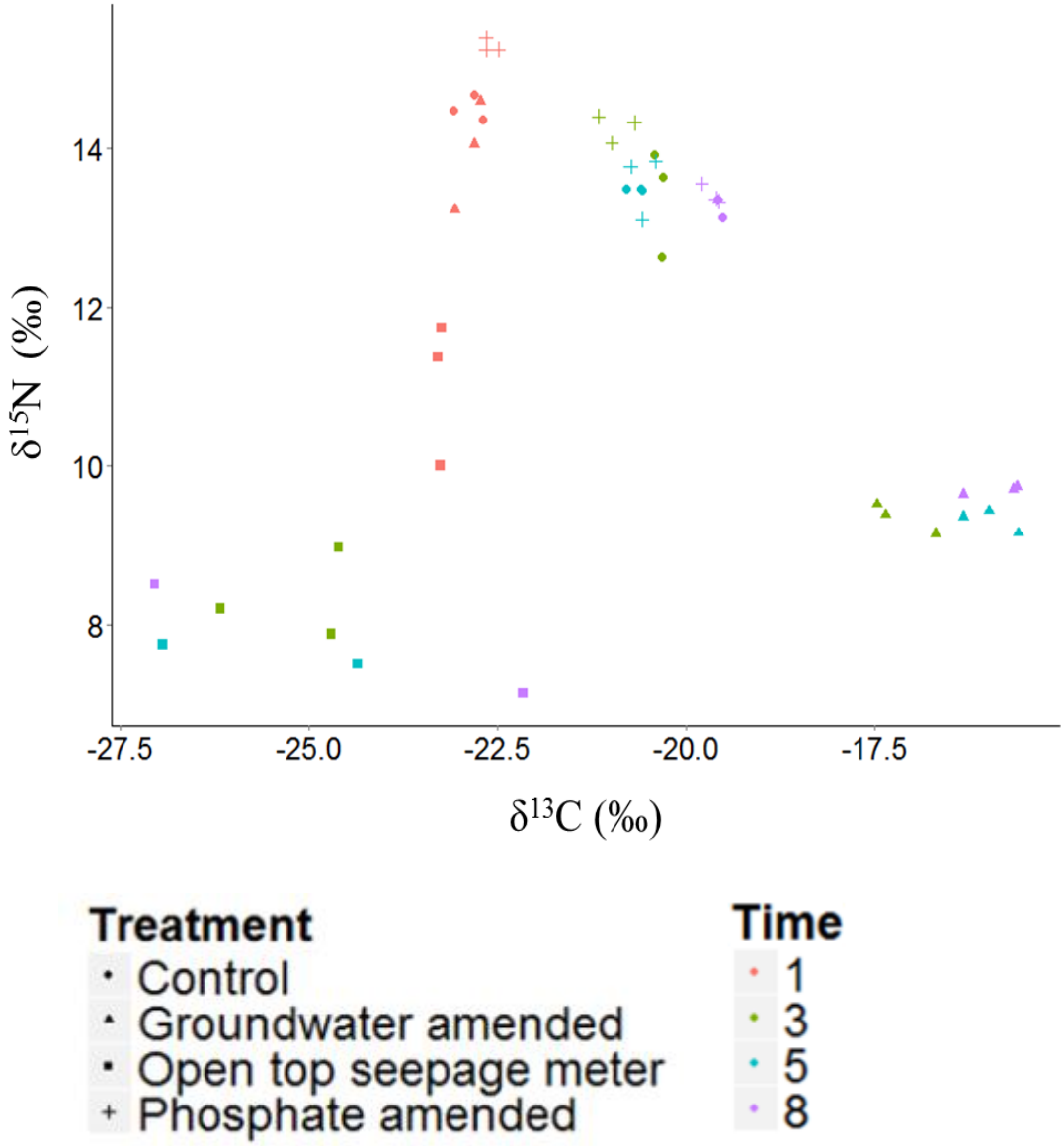

Figure 6. $\delta^{15}N$ versus $\delta^{13}C$ of particulate matter for August experiment. Samples are labeled according to treatment (symbol) and time point in days (color).