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Phytoplankton community structure response to groundwater-borne nutrients in the Inland Bays, Delaware, USA

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ABSTRACT: To determine the impacts of groundwater-borne nutrients on phytoplankton biomass and community structure, we conducted a series of mesocosm experiments in the Inland Bays of Delaware. Four treatments were tested, including mesocosms coupled directly to submarine groundwater seepage, mesocosms with the addition of pumped submarine groundwater, mesocosms with the addition of phosphate, and control mesocosms with no nutrient addition. 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^{-1} and NH_4^+ up to 55 µmol l^{-1}. As a result, treatment mesocosms had elevated NO_3^- and NH_4^+, while control mesocosms were relatively low in nutrients. In June, the highest chlorophyll *a* concentrations occurred in mesocosms attached to seepage meters after 3.5 d, with significant differences across all treatments. In August, groundwater-amended mesocosms reached the highest biomass concentrations, which peaked after 3 d. There were significant differences across all treatments, except control and phosphate-amended mesocosms which remained unchanged. Community 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. Harmful algal species proliferated in high nutrient treatments, including *Cylindrotheca closterium*, *Karlodinium veneficum*, *Nitzschia* spp., and *Heterocapsa* spp. While the general relationship between nutrient supply and biomass production is well known, we demonstrate the role groundwater-borne nutrients and sediment processes play in shaping community structure in estuarine primary producers and in promoting harmful algal blooms.

KEY WORDS: Phytoplankton community · Nutrient loading · Eutrophication · Groundwater discharge · Harmful algal blooms · Nitrogen assimilation

1. INTRODUCTION

The Inland Bays of Delaware have been subject to the impacts of eutrophication, resulting in ecological degradation and proliferation of opportunistic species (Coyne et al. 2006, Main et al. 2015). 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). Eutrophication in the Inland Bays of Delaware is of particular concern because of the many services these ecosystems provide to mar-

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ine species, which have commercial, recreational and ecological importance (Walch et al. 2016). Approximately 70% of commercially significant marine species are dependent on estuarine habitats at some point during their life cycle (Peterson et al. 2000).

The linkage between nutrient availability, usually nitrogen (N) and phosphorus (P), and eutrophication is well established (Howarth & Marino 2006, Howarth 2008). P is typically the limiting nutrient in freshwater, while N is usually the limiting nutrient in brackish or saltwater (Howarth & Marino 2006). Eutrophic conditions develop as increased nutrient loads relax N and/or P limitation and propel growth of opportunistic primary producers. Nutrients emanating from residential and agricultural land-use (in our study, influenced by poultry operations) ultimately end up in coastal waters (Glibert et al. 2007) where they contribute to the process of eutrophication. Increased N and P loading has also been found to promote the presence of HABs (Heisler et al. 2008).

Nutrient loading to 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 Delaware Inland Bay system, has also exhibited similar high N loading via groundwater (Andres 1991, Russoniello et al. 2016). 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 important in controlling freshwater and nutrient delivery to estuarine systems. A portion of precipitation infiltrates the land surface, passing through the water table and is transported to estuarine systems through groundwater flow. This is particularly important on the Delmarva Peninsula, where sandy geology allows water to readily pass through the water table and transport 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 & Ator 2004). These nutrients may transit the aquifer for days to decades before they are discharged, depending on the distance travelled and subsurface geologic characteristics (Puckett et al. 2011). The discharge of fresh nutrient-rich submarine groundwater into estuarine systems contributes to eutrophication (Bowen et al. 2007, Lecher et al. 2015, Sugimoto et al. 2017) but is difficult to quantify due to natural variability and uncertainty in lag times of eventual discharge, which potentially prolong the effects of previous nutrient additions to the watershed (Laroche et al. 1997, EPA 2012).

The relative magnitude of N and P entering estuaries is important in driving eutrophication, but nutrient speciation is also important. There is strong evidence that different forms of N can promote or inhibit growth of different species (Glibert et al. 2007, Anderson et al. 2008). 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). However, 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^{-1}, ammonium inhibition occurs, strongly suppressing nitrate uptake in phytoplankton (Dugdale et al. 2007). The availability of other nutrients may help to drive biomass and alter community structure. For example, the availability of Si may limit or promote the presence of diatoms (Xu et al. 2008). These biogeochemical interactions are important in shaping the phytoplankton community structure and overall ecosystem impacts of nutrient delivery to such systems; more research is necessary to elucidate many of these mechanisms in the field.

Differences in the specific sources of nutrients combined with biogeochemical processing during transport result in the delivery of distinct nutrient loads to the estuary. Oxidation/reduction potentials, oxygen availability, ion concentrations, pH, organic matter, microbial community composition and ultimately nutrient composition of submarine groundwater are highly variable both spatially and temporally (Charette & Sholkovitz 2002, Kroeger & Charette 2008, Rogers & Casciotti 2010). Transformations of groundwater-conveyed nutrients occur within surface estuarine sediments colonized by microphytobenthos and associated microorganisms. Thus, substantial seasonality may be expected not only in the rate of groundwater discharge (Michael et al. 2005) but also in the chemical composition of nutrient speciation and loads carried by groundwater. In addition to temporal variation, nutrient speciation often varies greatly on small spatial scales, both vertically (below sediment/estuary interface) as well as laterally (Kroeger & Charette 2008, Liu et al. 2014, Sawyer et al. 2014).

Stable isotopic approaches have been widely used to determine sources and track transformations of N and other elements (Aravena et al. 1993, McClelland

& Valiela 1998, Kaushal et al. 2011). 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 isotopic 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 (Cole et al. 2004). Isotopic signatures of O in NO_3^- range from –15 to 100‰, with the higher end of the spectrum being associated with atmospheric nitrate (Kendall 1998, Cole et al. 2004) and the lower ranges (–15 to 10‰) 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 to 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 would 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.

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. We conducted several mesocosm experiments to simulate the enrichment of nutrients via groundwater discharge, and we measured the response in phytoplankton community structure and biomass using molecular techniques.

2. MATERIALS AND METHODS

2.1. Study area

Rehoboth Bay, DE, is one of many inland bay systems in the Mid-Atlantic region. Guinea Creek (Fig. 1) is a shallow, poorly-circulated estuary contributing to Rehoboth Bay. Land-use is dominated by residential development and agriculture, resulting in large nutrient loads to the estuary. Over the past 25 yr, algal blooms, including HAB events, have been recorded sporadically throughout this system and particularly in Guinea Creek (Walch et al. 2016). Guinea Creek is regularly monitored for nutrients and chlorophyll by Delaware Sea Grant's Citizen Monitoring Program (www.citizen-monitoring.udel.edu); nitrate concentrations are 50 to 70 µmol l^{-1}, ammonium concentrations 15 to 25 µmol l^{-1}, and chlorophyll *a* concentrations up to 60 µg l^{-1}.

2.2. Experimental setup

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

Experiments were conducted in Guinea Creek during June and August of 2015 with mesocosms suspended in the water column to maintain ambient daily and tidal fluctuations in temperature, light, and turbulence. Mesocosms were constructed from clear drum liners supported by PVC frames floating slightly above the water surface to avoid mixing with the estuary but to allow for exchange to the atmosphere.

Relatively low-nutrient surface water was filtered, in series, down to 0.2 µm to remove phytoplankton.

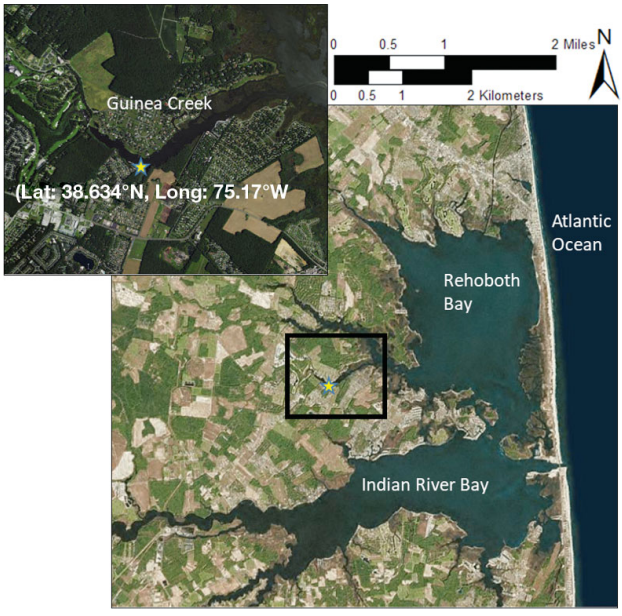

Fig. 1. Study site in relation to surrounding water bodies. Guinea Creek is shown in the map subset (top left). The yellow star represents the location within Guinea Creek where mesocosm experiments were conducted. The approximate coordinates for the study site are shown in the submap

Ambient water from the field site, with a naturally abundant phytoplankton community, was passed through a 253 µm sieve to remove grazers but to include the ambient phytoplankton community with which to inoculate the mesocosms. The filtered surface water and ambient estuarine water were mixed 4:1 by volume and used to fill all mesocosms to 20 l. While treatments were filled with the same water, there is some variability in the initial conditions likely as a result of lag in sample time.

Treatments were run in triplicate including a control group run simultaneously (Fig. 2). Treatments for both experiments included mesocosms amended with nutrients from submarine groundwater pumped from beneath the estuary and enclosures which were attached to open-top seepage meters to naturally receive nutrients directly across the sediment–water interface. It was expected that by adding groundwater pumped from beneath the estuary, we would be simulating a different nutrient addition than open-top seepage meters which received nutrients directly, due to biogeochemical interactions within the sediment. In August, an additional treatment amended with phosphorus was added.

Open-top seepage meter mesocosms were fashioned by attaching clear 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 20 l of experimental water. Care was taken during this process not to disturb the sediment.

June 2015

Groundwater was pumped daily from 50 cm below the sediment/estuary interface using a mini-piezometer sampler, and 2 l was added daily to each of 3 groundwater-amended mesocosms. Addition rates were chosen to match local groundwater discharge rates (Brooks 2018). This groundwater was <1 salinity and contained 295 µmol l^{-1} NO_3^-, 3.8 µmol l^{-1} NH_4^+, 0.2 µmol l^{-1} PO_4^{3-}, and 79.0 µmol l^{-1} SiO_4^{2-}. To keep salinity and volume consistent, 2 l deionized water was added to control mesocosms. All mesocosms were sampled twice daily for 5 d.

August 2015

Groundwater was extracted from the same location as the June 2015 experiment and 2 l were added at the start of the experiment. The groundwater salinity was 2, and the groundwater contained 253.2 µmol l^{-1} NO_3^-, 16.2 µmol l^{-1} NH_4^+, 0.02 µmol l^{-1} PO_4^{3-}, and 20.3 µmol l^{-1} 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 d.

2.3. Sampling

At each sampling time point, mesocosms were homogenized using a submersible pump prior to collection of a 1 l sample. Samples were stored in the dark, on ice until filtered. Environmental data was also collected using a YSI Pro Plus (YSI, Xylem) sonde and recorded for comparison with ambient temperature and salinity.

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 for nutrient analyses, respectively. An additional sample was passed through 3.0 µm polycarbonate membrane filters for DNA analysis of phytoplankton. Filters were then stored in CTAB buffer at –80°C until extraction as described below (Coyne et al. 2001).

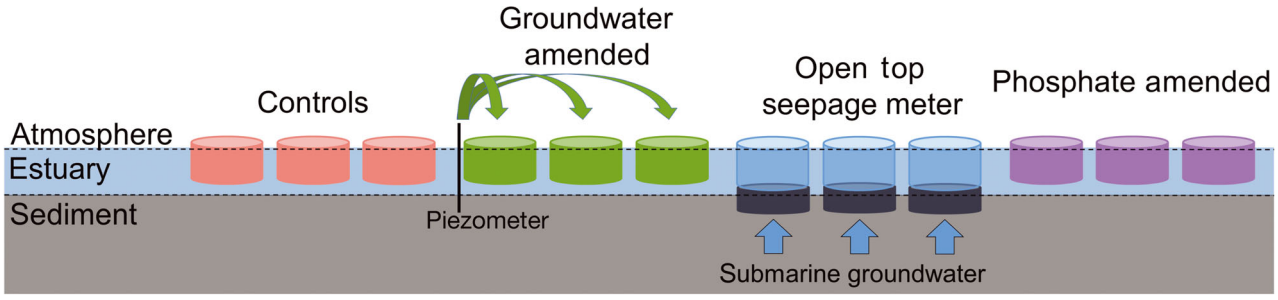

Fig. 2. Experimental design of the mesocosm experiments. Arrows signify addition of groundwater. Green arrows indicate groundwater pumped through a piezometer and added manually into mesocosms; blue arrows indicate natural flow of groundwater into mesocosms across the sediment–water interface

2.4. Sample analysis

Chlorophyll *a* was extracted from filters with 90% acetone, then analyzed fluorometrically (Parsons et al. 1984) and used as a proxy for the overall biomass of phytoplankton in mesocosms (JGOFS 1994). Dissolved nutrients (NO_3^-, NH_4^+, PO_4^{3-}, and SiO_4^{2-}) were analyzed on a Seal AA3 autoanalyzer following manufacturer guidelines (AA3 Protocol).

$\delta^{15}N$-NO_3^- and $\delta^{18}O$-NO_3^- samples were prepared following the denitrifier method of Sigman et al. (2001) and Casciotti et al. (2002). The isotopic composition of N_2O was analyzed at the Stable Isotope Facility at the University of California, Davis. Values are reported relative to air and V-SMOW standards for N and O isotopes, respectively. Three replicates of 2 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 analysis. The measured $\delta^{15}N$-NO_3^- and $\delta^{18}O$-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 to 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, 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 as described by Coyne et al. (2001), and concentrations were measured on a Nanodrop spectrophotometer. DNA was amplified in triplicate PCR reactions of 20 µl consisting of 50 ng of sample DNA, 2.5 mM MgCl2, 1X Taq polymerase buffer (Sigma), 0.2 mM dNTPs, 0.5 units Jump-Start Taq Polymerase (Sigma), 1X BSA, and 0.5 µM forward and reverse primers, Euk29F (5′-GTC TCA AAG ATT AAG CCA TGC-3′) and Euk 517R (5′ GGA CCA GAC TTG CCC TC-3′) (Coyne et al. 2005). The reaction consisted 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. PCR products were fragmented with *Hae*III restriction enzyme (Kim et al. 2014), and terminal restriction fragment length polymorphism (T-RFLP) was analyzed on an ABI Prism 310 Genetic Analyzer (ThermoFisher) using Genescan® software. T-RFLP patterns were examined using Peakscanner® software. T-REX was used to eliminate noise and standardize samples before further analysis (Culman et al. 2009). Statistical analysis of preliminary community data was completed in PRIMER7, where square root transformation was used to down-weight and normalize statistical impact of highly dominant T-RFLP fragments. Bray-Curtis similarity values were calculated and visualized on a non-metric multidimensional scaling (nMDS) plot.

A subset of samples was selected for 18S rRNA gene sequencing to further analyze species assemblage of the phytoplankton community in the mesocosms. PCR primers Euk-7 forward with barcode (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and 570 reverse (5′-GCT ATT GGA GCT GGA ATT AC-3′) were used for amplification, which was checked for success on a 2% agarose gel. 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 the 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.

2.5. Data analysis

Due to differences in experimental design, data from the August and June experiments were analyzed separately. All statistical analyses were performed in R version 3.5.2 (R Core Team 2013). Differences in overall biomass based on treatment were assessed using 1-factor ANOVA ($\alpha = 0.05$). Once significance was determined, a post-hoc Tukey-Kramer test was used to test significance between groups. Multilevel repeated measures analysis of variance was used to analyze particulate nitrogen data by treatment and time. Following determination of significance, interactions between treatments were investigated using multiple comparisons of means according to a Tukey contrast post-hoc test.

Sequence data were processed using a MR DNA analysis pipeline. In short, sequence data were joined, barcodes were removed, sequences <150 bp removed, and sequences with ambiguous base calls were removed. Sequences were de-noised, 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, https://rdp.cme.msu.edu, DeSantis et al. 2006). Following sequence analysis, relative abundance data were ana-

lyzed using the R package vegan (Oksanen et al. 2008). Differences in community composition were analyzed for each mesocosm with a multivariate approach using the non-metric multidimensional scaling (nMDS) and adonis procedures. Bray-Curtis similarities were calculated from similarity matrices and used to generate 2-D and 3-D nMDS 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 differences in nutrient concentrations; 999 permutations were used in these analyses.

3. RESULTS

3.1. Nutrient concentrations

The objective of this study was to assess how the addition of nutrients from groundwater impact primary producers by manipulating nutrient concentrations in mesocosms. 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 (<1.0 µmol l^{-1}), except for the phosphate-amended mesocosms where concentrations were highest (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 lower (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}).

3.2. Groundwater

Groundwater samples from multiple locations and depths were collected to assess the delivery of nutrients to the estuary and to 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), but geochemical characteristics varied widely. The salinity ranged from 13 to 27 (Table 2), with estuarine salinity ranging from 19 to 25. Nutrient concentrations in these groundwater samples were highly variable, with NO_3^- ranging from 12 to 151 µmol l^{-1}, NH_4^+ from 24.3 to 55.6 µmol l^{-1}, PO_4^{3-} from 0.0320 to 2.02 µmol l^{-1}, SiO_4^{2-} from 23.3 to 167 µmol l^{-1}, and $\delta^{15}N$-NO_3^- from 5.5 to 19.4 ‰ (Table 2).

The porewater we collected to add to groundwater-amended mesocosms was from deeper below the sediment-estuary interface (~50 cm) and differed from the shallower water in its nutrient composition (Table 2). Compared to the shallower porewater described above, the dosing water had lower salinity (June: 2.0, August: 1.5), higher NO_3^- concentrations (June: 295.3, August; 253.2 µmol l^{-1}), lower NH_4^+ concentrations (June: 3.8, August: 16.2 µmol l^{-1}), comparable PO_4^{3-} concentrations (June: 0.17, August: 0.021 µmol l^{-1}), and comparable SiO_4^{2-} concentrations (June: 79.1, August: 20.3 µmol l^{-1}).

Table 1. Initial mean concentrations ± SE 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^{-1})	NH_4^+ (µmol l^{-1})	PO_4^{3-} (µmol l^{-1})	SiO_4^{2-} (µmol l^{-1})	Chl *a* (µg l^{-1})	N:P ratio
June						
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						
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 sampled below	Depth (cm)	Salinity (ppt)	DO (mg l^{-1})	NO_3^- (µmol l^{-1})	NH_4^+ (µmol l^{-1})	PO_4^{3-} (µmol l^{-1})	SiO_4^{2-} (µmol l^{-1})	$\delta^{15}N$-NO_3^- (‰)	$\delta^{18}O$-NO_3^-(‰)
June	Mesocosm 1	11	13.60	2.40	47.99	53.06	0.64	124.83	7.13	4.18
June	Mesocosm 2	10	20.80	1.90	51.17	54.82	1.29	166.89	19.35	13.40
June	Mesocosm 3	12	23.10	0.40	150.94	37.23	0.64	86.76	19.18	18.17
August	Mesocosm 1	12	14.14	2.29	110.08	23.65	0.03	23.06	5.54	3.14
August	Mesocosm 2	13	27.10	0.23	12.35	25.08	2.02	95.35	12.98	11.73
August	Mesocosm 3	12	24.68	0.32	33.35	36.25	0.63	110.10	9.93	5.00
June	Dosing source	55	2.03	5.40	295.29	3.81	0.17	79.08	4.39	2.25
August	Dosing source	45	1.50	4.40	253.15	16.20	0.02	20.32	4.62	2.56

3.3. Biomass

Chlorophyll *a* concentrations were measured in both experiments as a proxy for phytoplankton biomass and were lower in June experiments than August experiments (Fig. 3). In June, there were significant differences in chlorophyll *a* by treatment ($F_{2,12}$ = 22.34; $p < 0.001$), with the highest concentrations found at 3.5 d. At 3.5 d, open-top seepage meter mesocosms had significantly higher chlorophyll *a* concentrations (10.9 ± 0.2 µg l^{-1}) than groundwater-amended (7.0 ± 0.6 µg l^{-1}; $p < 0.005$) and control mesocosms (4.3 ± 0.2 µg l^{-1}; $p < 0.005$; Fig. 3). In August, there were significant differences in chlorophyll *a* by treatment ($F_{3,16}$ = 41.51; $p < 0.005$), with the highest concentrations found after 3 d. After 3 d, groundwater-amended mesocosms had significantly higher chlorophyll *a* concentrations (37 ± 2 µg l^{-1}) than open-top seepage meter mesocosms (19 ± 5 µg l^{-1}; $p < 0.005$). Both treatment groups that received groundwater had significantly higher concentrations than the control and phosphate-amended mesocosms (11.9 ± 0.7 mg l^{-1}; $p < 0.005$ and 9.9 ± 0.2 µg l^{-1}; $p < 0.005$, respectively), which were not statistically different (Fig. 3; $p = 0.35$).

3.4. Harmful species

Harmful algal species made up a smaller percentage of total abundance in June than in August (Table 3). During the August experiment, several harmful algal species increased in abundance in mesocosms receiving groundwater additions. *Cylindrotheca closterium*, a bloom-forming diatom, had significantly higher abundance in groundwater-amended mesocosms (13.9 ± 1.4% abundance) compared to controls (4.5 ± 0.1% abundance; $p < 0.05$). The potentially toxic dinoflagellate species *Karlodinium veneficum* also showed significantly higher relative abundances in groundwater-amended mesocosms (3.0 ± 1.4%) than control mesocosms (1.7 ± 1.2%; $p < 0.05$). *Nitzschia* spp., a group of diatoms with several potentially toxic

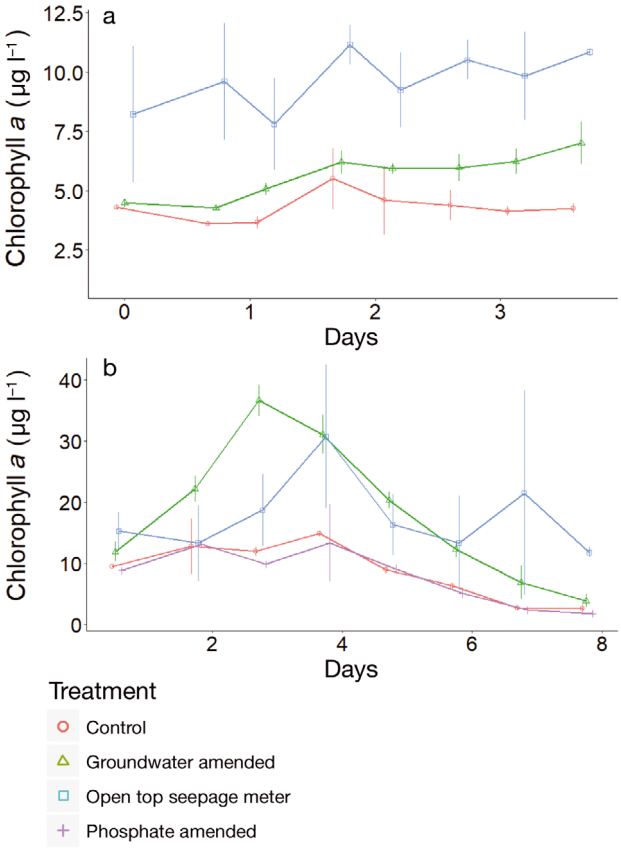

Fig. 3. Chlorophyll *a* concentrations over time, mean ± SE, during (a) June and (b) August, by treatment groups

Table 3. Mean relative abundance (%) ± SE of potentially harmful algal species by treatment for both June and August Experiments. Relative abundances were calculated from 18S rRNA gene sequence OTU data at a specified time (June: 3.5 d and August: 3.0 d) and averaged by treatment group. Species data were 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. *p < 0.1, **p < 0.05, ***p < 0.01 (adjusted p-values reported; single-step method)

Harmful species	Control	Open-top seepage	Groundwater-amended	Phosphate-amended	Ecological effect
June					
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					
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 sp.	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

species (Bowers et al. 2018) and *Heterocapsa* spp., a group of dinoflagellates commonly associated with HAB events (Litaker et al. 2002) showed significantly higher relative abundances in open-top seepage meter mesocosms (9.5 ± 13.3% and 1.6 ± 0.7% abundance, respectively) compared to control mesocosms (0.2 ± 0.0%; $p < 0.05$ and 0.3 ± 0.1% ; $p < 0.01$, respectively). There was no statistically significant difference in abundance of harmful species across treatments in June, although it is worth noting that there was a loss of replicates caused by wildlife.

3.5. Community structure

Species assemblages were characterized from sequence analysis to determine if treatment or time resulted in differences in community structure. MDS ordination of species assemblages for both June and August experiments show distinct community structure across treatments and time (Fig. 4). PERMANOVA showed that treatment (differences in nutrient composition) explained 26.5 and 39.9% of the variation in species assemblage in June (Treatment: $F(2,11) = 4.133$, $R = 0.265$, $p < 0.005$) and August (Treatment: $F(3,17) = 8.276$, $R = 0.399$, $p < 0.001$), respectively. In addition, time explained 40.2 and 29.4% of the variation in species assemblage in June (Time: $F(1,11) = 12.543$, $R = 0.402$, $p < 0.001$) and August (Time: $F(1,17) = 18.253$, $R = 0.294$, $p < 0.001$), respectively. In both experiments, community structure was driven over time by treatment, a result of differences in nutrient composition.

3.6. $\delta^{15}N$ and $\delta^{13}C$ of phytoplankton

We measured the stable isotopic ratios of N and C in particulate matter during our August experiment to track the source of N assimilated by phytoplankton and the pattern of C isotopes (Fig. 5). Multilevel repeated measures analysis of variance showed significant differences in $\delta^{15}N$ by treatment and time. Initially, the $\delta^{15}N$ of particles ranged from 13 to 16 ‰ in the enclosed mesocosms and 10 to 12‰ in the mesocosms that were open to the sediment. Through time, differences in $\delta^{15}N$ by treatment were observed, except in control and phosphate-amended mesocosms ($b = 0.4883$, $p = 0.8056$). Particles in the control and phosphate-amended mesocosms maintained a similarly high $\delta^{15}N$ value, while particles in the groundwater-amended and open-top seepage meter mesocosms had lower $\delta^{15}N$ values ($b = -3.0700$, $p < 0.001$, and $b = -4.7422$, $p < 0.001$, respectively) compared to control mesocosms. Groundwater-amended mesocosms maintained a higher $\delta^{15}N$ value than open-top seepage meters ($b = -1.6722$, $p = 0.0179$). 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 (~4.6‰; Table 2). Initial phytoplankton $\delta^{13}C$ values were similar across all treatments. Carbon isotopic values showed distinct patterns across treatments over time. Control and phosphate-amended mesocosms $\delta^{13}C$ values were consistently similar and showed small increases. Groundwater-amended mesocosms $\delta^{13}C$ values in-

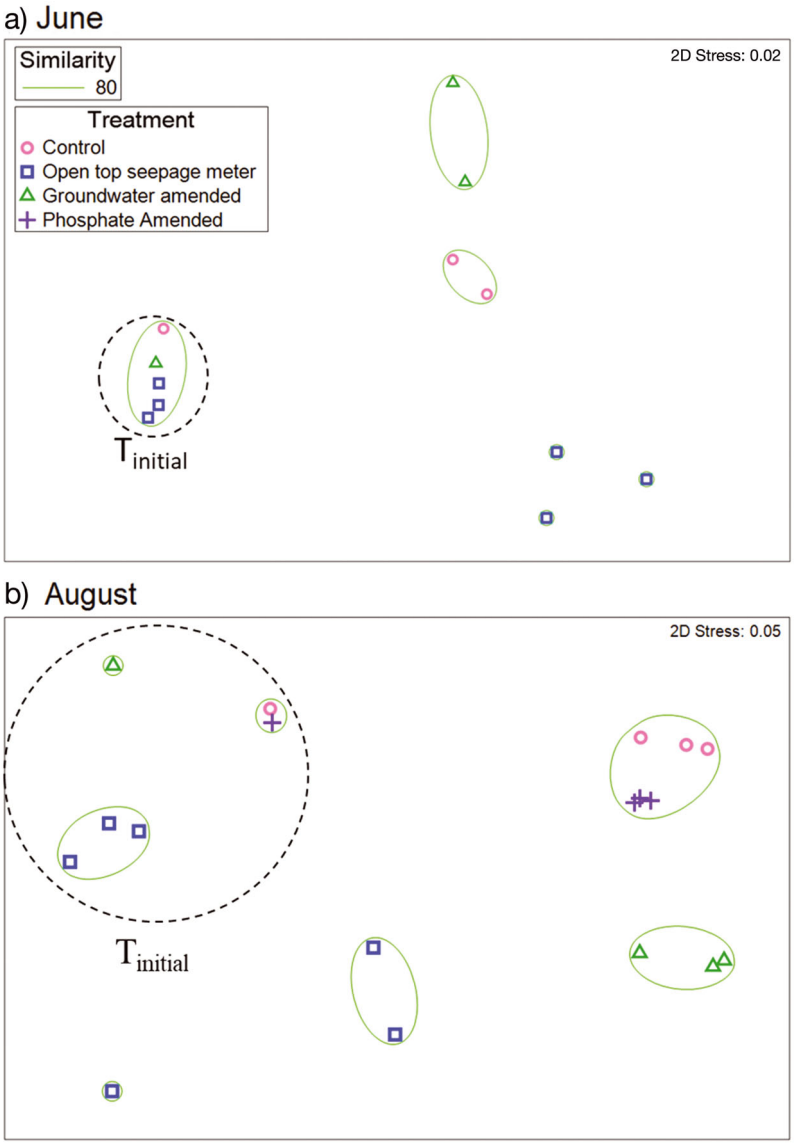

Fig. 4. Non-metric multidimensional scaling (MDS) ordination of Bray-Curtis similarities using square root transformed species assemblage data from (a) June and (b) August mesocosm experiments. Similarity clusters are shown by the solid circle, and group samples based on their percent similarity in community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

creased twice as much during the same time-span, while open-top seepage meter mesocosms showed the reverse trend, decreasing after initial measurements.

4. DISCUSSION

This study demonstrates the importance of submarine groundwater discharge in structuring the phytoplankton community in Delaware's inland bay systems. The addition of groundwater to mesocosms increased available nitrogen and resulted in increased biomass, changes in phytoplankton community structure, and the proliferation of some HAB species. Our isotopic data provided further evidence that these increases in biomass and differences in community structure could be attributed to groundwater-borne nutrients.

4.1. Submarine groundwater

We found that nutrient composition of groundwater varied greatly across small distances and depths, with NO_3^- concentrations from 12 to 150 µmol l^{-1} and NH_4^+ concentrations from 23 to 55 µmol l^{-1}. These variations likely result in part from differences in flow paths from the watershed, but more importantly from biogeochemical processes within shallow estuarine sediments and during mixing with saline groundwater (Kroeger & Charette 2008, Sawyer et al. 2014).

Initial 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 during August (Fig. 3), suggesting that high NO_3^- availability drove phytoplankton biomass. Our isotopic data provided further confirmation of this relationship. 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 except open-top seepage meters started around 14‰ (Fig. 5). Over the course of the experiment, there was a slight decrease in $\delta^{15}N$ values in the control and P addition treatments, whereas $\delta^{15}N$ of the particles in treatments receiving groundwater decreased to 8 to 9‰, indicating incorporation of a low $\delta^{15}N$ nitrogen source. Our isotopic data suggest that stimulation of the phytoplankton community by high NO_3^- delivered from the watershed via groundwater pro-

moted increased biomass and caused shifts in the community composition. In addition, these analyses show that treatments closed off from the sediment experienced an enrichment in $\delta^{13}C$ over time, suggesting inorganic C limitation. In contrast, the open-top seepage meter treatments showed a decrease in $\delta^{13}C$, implying that the terrestrial groundwater source was increasingly important to primary production.

4.2. Phytoplankton

Phytoplankton biomass differed seasonally, with higher biomass in late summer relative to late spring (Fig. 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 & Chrzanowski 2006). Loading rates of N and P to Rehoboth Bay vary temporally (Volk et al. 2012). In our experiments, the initial N:P ratios in June were much higher than in August, suggesting that the availability of P may have contributed to the decreased response of biomass in the June experiment (Table 1). In August, we tested the possibility of P limitation of phytoplankton biomass and found no difference in biomass response between the control and P-addition treatment, invalidating the possibility of P limitation in August. These patterns in P dynamics may have resulted in the higher biomass observed in August versus June experiments (Fig. 3).

These experiments demonstrated that changes in relative availability of nutrients can result in changes in community structure and rise of HABs as discussed by Glibert et al. (2011). We also found changes in HAB species abundance, with increased relative HAB species abundance later in summer compared to the spring, potentially resulting from an array of factors such as 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 compared to control treatments (Table 3), suggesting a probable correlation between high NO_3^- groundwater discharge and increases in blooms.

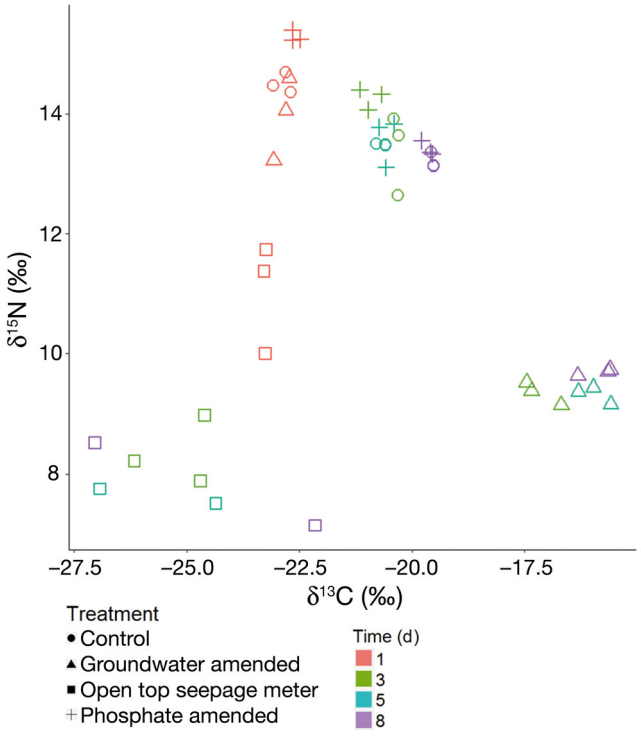

Fig. 5. $\delta^{15}N$ versus $\delta^{13}C$ of particulate matter for August experiment. Samples are labeled according to treatment (symbol) and time point in days (color)

4.3. Food web

The proliferation of HABs associated with nutrient loading, as found in this study and others (Heisler et al. 2008), can have broad implications for food web and ecosystem alterations (Howarth et al. 2000, Boesch 2002, Howarth 2008). Mesocosms receiving groundwater enrichment directly via the sediment (open-top seepage meter) or through groundwater-addition 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 veneficum*, a dinoflagellate 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 suppress growth in other species (Orsini et al. 2002). In addition to harmful algae species, some potentially harmful protozoan species increased in abundance under our high-nutrient conditions. For example, *Neoparamoeba pemaquidensis*, an amphizoic marine protozoan associated with several diseases in marine organisms (e.g. amoebic gill disease; Lee et al. 2006), increased in abundance from 1.20 ± 0.84 % of the overall population in control mesocosms to 4.03 ± 1.23 % in groundwater-amended mesocosms in only 3 d.

5. CONCLUSIONS

Our experimental set-up provided valuable insights regarding the importance of processes at the sediment–water interface, processes which may be obscured in more traditional nutrient addition experiments. In particular, the open-top seepage meter treatment offered a novel approach and an interesting comparison with the groundwater-amended treatment. We added groundwater to the amended treatment at a rate designed to simulate the typical rate of discharge at the study site, to be consistent with the delivery of nutrients and groundwater that the seepage-meter treatments likely received. However, as we have noted, nutrient flux from the sediment to estuary might be different from the flux predicted based on groundwater concentrations and discharge rate, and, at present, it is not possible to directly measure the flux. Ultimately, our experiments tested the response of the phytoplankton community to total sediment nutrient flux vs. the nutrient additions from groundwater additions. In June, chlorophyll concentration was consistently higher in the open-top seepage meter treatment, perhaps indicating that sediment processes augmented the nutrient supply in a way that stimulated algal productivity. For example, open-top seepage meter mesocosms may have received enhanced nutrient inputs due to ongoing remineralization of N and P in the estuarine sediment (Table 2); however, macronutrients may have not been the only contributing factor. In August, the 2 treatments resulted in similar biomass, with both being substantially greater than in June and with the groundwater-amended treatment reaching a greater peak concentration of chlorophyll. We can speculate that benthic algae may have competed with phytoplankton for nutrients, resulting in lower peak phytoplankton biomass.

Our work has shown some of the potential impacts of the transmission of groundwater-borne nutrients in estuarine systems. Nutrients traveling to the estuary via groundwater, which are often elevated in concentration due to surrounding anthropogenic land-uses, led to elevated biomass of primary producers in our simulations. In addition to changes in biomass, the overall phytoplankton community structure changed as a result of groundwater-borne nutrient addition and further varied by the method of nutrient addition (natural groundwater seepage vs. pumped groundwater). Lastly, nutrients originating from groundwater were shown to increase the prevalence of potentially toxic HABs and other problematic organisms. Isotopic analyses indicated that these nutrients were directly tied to changes in community composition as well as increases in biomass. This work elucidates important connections in the process of coastal eutrophication, including the presentation of results that are not only locally pertinent but also regionally relevant as many systems share similar geology and biologic attributes and are under similar threat due to anthropogenic nutrient loading.

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