NUTRIENT AND GRAZING CONTROL OF ESTUARINE PHYTOPLANKTON GROWTH AND COMMUNITY COMPOSITION

A Thesis

by

EMILY K. CIRA

Submitted to the Office of Graduate Studies of Texas A&M University
and Texas A&M University – Corpus Christi
in partial fulfillment of the requirements for the joint degree of

MASTER OF SCIENCE

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ABSTRACT

Nutrient and grazing control of estuarine phytoplankton growth and community composition (July, 2013)

Emily K. Cira, B.A., Boston University

Chair of Advisory Committee: Dr. Michael Wetz

Estuarine phytoplankton growth is often controlled by nitrogen availability. In addition to overall nitrogen loads, nitrogen form (organic vs. inorganic) is an important factor affecting estuarine phytoplankton growth and community composition. Recent studies have shown that in addition to nitrogen availability, trophic cascades and relaxation of grazing pressure may also be important for phytoplankton bloom formation in estuaries.

With a goal of better understanding how nitrogen availability and grazing pressure interact to control estuarine phytoplankton growth and community composition, we examined the individualistic as well as the combined effects of nitrogen (varying availability and form) and grazing pressure on estuarine phytoplankton growth and community composition in the Neuse River Estuary, North Carolina, USA. During each of three sampling events (June 2011, August 2011, March 2012) natural phytoplankton assemblages were manipulated with added nitrogen (as urea or nitrate) and reduced grazing pressure (by filtering out zooplankton grazers). Treatments were incubated for 48 hours in an experimental pond, and subsamples taken daily to assess phytoplankton growth responses to treatments through chlorophyll *a*, diagnostic photopigments and cell enumerations.

The effects of nitrogen additions and reduced grazing pressure varied throughout the events. In June, only nitrogen addition stimulated phytoplankton community growth (chlorophyll *a*), while in August, only grazing reduction had a significant impact on community growth. Neither treatment had a significant effect on community growth in March, as the phytoplankton community faced phosphorus-limitation and decreased grazing pressure associated with cooler winter/spring temperatures. While both treatments did not continuously effect overall phytoplankton growth throughout all experiments, there were always effects seen in some diagnostic photopigments, indicating varying taxa-specific responses to treatments throughout the year, which can be explained by shifts in phytoplankton community composition and environmental factors.

These results demonstrate the importance of both bottom-up (nutrient availability and form) and top-down (grazing) controls in a temperate, eutrophic estuary. Results also hint at the potential for other factors (i.e. light and phosphorus-limitation) to play a role in phytoplankton growth as well. Phytoplankton growth, biomass and community dynamics are relevant indicators of environmental change and this study highlights the need to consider the potential interactive effects of controlling factors for proper management of estuarine ecosystems.

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1. Introduction

1.1 Estuaries

Estuaries are among the most biologically productive ecosystems in the world (Kennish 2002). They are critical habitats for many organisms (Kennish 2002) and are valuable ecosystems to humans (Blaber et al. 2000). About 50% of fisheries landings nationwide are of species known to spend a portion of their life cycle in estuaries, and in regions like the Gulf of Mexico, and mid- and south-Atlantic, estuarine fisheries landings account for 90% or more of total fisheries landings (Lellis-Dibble et al. 2008). Estuaries perform a vast array of ecosystem services, ranging from aesthetic services to disturbance regulation (Costanza et al. 1997). According to a meta-analysis performed by Costanza et al. (1997), per hectare, estuarine ecosystems are the most valuable ecosystems in the world.

Coastal watersheds are a critical source of exogenous nutrients to estuaries via riverine inputs, which enables estuaries to support high primary production (Paerl 1997). Often, high rates of primary production translate into high secondary production (Nixon and Buckley 2002), which is then further transferred to higher trophic levels. However, exceptionally high primary production caused by nutrient loading (i.e. eutrophication) can have deleterious impacts on higher trophic levels (Breitburg et al. 2009) and negatively impact humans (e.g. Kirkpatrick et al. 2004).

1.2 Phytoplankton

Phytoplankton are often the dominant primary producers in estuaries. Phytoplankton growth is tightly linked to changes in their environment, and because of their rapid nutrient uptake and growth-rate potential, phytoplankton are important

indicators of environmental change (Paerl et al. 2010). The recent increased prevalence of phytoplankton blooms in response to anthropogenic change in estuaries exemplifies this (Glibert et al. 2005). Though blooms can be part of natural seasonal cycles, in the past few decades blooms have become larger and more frequent in many estuaries, and often exhibit shifts from benign species to harmful algal bloom (HAB) species, coincident with increased nutrient loads (Anderson et al. 2002). HABs can cause mass disruption to their environment by promoting hypoxia/anoxia, and when composed of toxic species can induce mass mortalities of birds, invertebrates, fish and marine mammals (reviewed in Landsberg 2002) and can initiate human health problems due to direct contact with the toxins or consumption of contaminated organisms (reviewed in Landsberg 2002; Kirkpatrick et al. 2004).

1.3 Anthropogenic impacts

The very thing which makes estuaries so productive, their location at the interface of freshwater and seawater, also places them adjacent to densely populated watersheds, and thus estuaries experience a multitude of impacts from anthropogenic stressors (Kennish 2002). The cumulative effects of climate change (altered freshwater inflow, global warming, extreme weather) and anthropogenic change (nutrient loading, land use change, food web alteration) have had negative impacts on these systems around the world (Anderson et al. 2002; Wetz and Yoskowitz 2013), and continual human modification of natural systems is expected to persist in the near future (Bricker et al. 2008). Understanding the linkages between human activity and the estuarine environment is of the utmost importance for developing effective resource management

strategies. Here I focus on two environmental drivers (nutrient loading, altered trophic structure) that appear to play a particularly important role in estuarine phytoplankton dynamics.

Nutrient Loading

Eutrophication, excessive nutrient and organic matter loading (Nixon 1995), can greatly impact coastal waters. In estuaries, eutrophication is typically synonymous with nitrogen (N) loading because estuarine primary producers are generally N-limited (Seitzinger et al. 2002). There is a strong correlation between total N inputs and phytoplankton production in estuarine waters, and N has been linked to increased phytoplankton biomass in numerous systems (Rudek et al. 1991; Anderson et al. 2002; Glibert et al. 2005; Bricker et al. 2008).

It is not just the quantity of anthropogenic N loads that influences phytoplankton growth and community structure, but also the type of N (Bronk et al. 2007). Most forms of dissolved inorganic nitrogen (DIN) are accessible to the estuarine autotrophic phytoplankton community, while access to dissolved organic nitrogen (DON) is more exclusive. Mixotrophic phytoplankton, because of their ability to augment phototrophy with heterotrophy, are able to utilize the DON pool (Cloern and Dufford 2005), and so are able to thrive in conditions adverse to solely autotrophic phytoplankton (i.e. nutrient- or light-limited; Nygaard and Tobiesen 1993; Legrand et al. 1998; Cloern and Dufford 2005). This implies that they may be more competitive than autotrophic phytoplankton under high levels of DON, which can make up a large portion of allochthonous N inputs to estuaries (Seitzinger and Sanders 1997; Seitzinger et al. 2002). Mixotrophic

phytoplankton include many bloom-forming dinoflagellates, cryptophytes, raphidophytes, and representatives from several other taxa (Burkholder et al. 2008). Many mixotrophs are also HAB species, thus nutrient overloading, especially with DON, can lead to high concentrations of these bloom-forming species and may make an estuary prone to HABs (Burkholder et al. 2008). Additionally, DON is degraded by bacteria, releasing ammonium, which is widely available to the phytoplankton community (reviewed in Berman and Bronk 2003), so DON loading can both directly and indirectly promote phytoplankton growth. With these linkages, it is important to investigate how the quantities and types of nitrogen loaded into these systems impact the phytoplankton community.

One form of DON in particular, urea, is becoming an increasingly large part of organic nitrogen loads into estuarine systems (Glibert et al. 2006). As a major component of fertilizers (Lomas et al. 2001), it is often the most common form of DON in estuaries (Goeyens et al. 1998; Twomey et al. 2005). While there is much variation in phytoplankton species' ability to utilize urea, it can support a large fraction of HAB species' nitrogen demands (Solomon et al. 2010). Notably, areas of the world that are faced with increased N usage and where urea is the major N form used in agricultural applications are also areas with increased frequency and extent of HABs (Glibert et al. 2006).

Unfortunately, the relationship between nutrient loads and phytoplankton abundance is not well understood (Anderson et al. 2002). There are still many unresolved issues surrounding the complexities of organic versus inorganic nutrients (Bradley et al. 2010). While DON can account for a large fraction of the N pool in

aquatic and marine systems, it has traditionally been viewed as unusable by phytoplankton (Berman and Bronk 2003). Only in recent decades have researchers confirmed that DON can help support primary production (e.g. Bronk et al. 2007), but the use of DON is not consistent among phytoplankton species, and varies depending on other chemical factors (such as other nutrient concentrations; Berg et al. 2001). While it is a general rule that phytoplankton prefer ammonium as a N source, DON can account for a majority at N uptake by phytoplankton at times (Bronk et al. 2007). There are still large gaps to fill in understanding the importance of DIN and DON to phytoplankton communities and the role they play in the phytoplankton community structure, creating the need for research on this topic.

Alteration of Trophic Structure

Coastal food web structure has been altered in many systems as a result of overfishing and habitat change (Kiviat 1989; Jackson et al. 2001). Many of the world's commercially important fish and shellfish species rely on estuaries for at least a portion of their life cycle (Blaber et al. 2000), so changes in their population structure impact estuarine systems. Since the 1950s, fisheries landings have shifted from piscivorous fishes to planktivorous fish and invertebrates (Pauly et al. 1998), which can impact estuarine trophic structure. Shellfish harvesting and habitat disruption also negatively impact estuarine shellfish populations and the quality of shellfish habitat (e.g. oyster bars, Rothschild et al. 1994). Trophic cascades caused by loss of important fish or shellfish or species invasions and the subsequent changes in zooplankton grazing pressure have had

noted impacts on phytoplankton community growth and species composition (Alpine and Cloern 1992; Scheffer et al. 2005; Caraco et al 2006; Cerco and Noel 2007).

Trophic cascades can alter zooplankton populations, and create "windows of opportunity" for certain phytoplankton species to bloom (Stoeker et al. 2008). Microzooplankton grazers (20-200 μm) in particular have an active role in controlling phytoplankton populations (Stoecker and Gustafson 2002; Strom et al. 2001; Calbet and Landry 1999). Microzooplankton have more rapid growth rates than mesozooplankton (0.2-2 mm), and have the ability to keep phytoplankton blooms in check (Calbet et al. 2003). Microzooplankton can also be much more abundant in estuaries than larger zooplankton (Day et al. 1989; Buskey 1993; Park and Marshall 2000), making them even more influential as grazers. In the absence of sufficient zooplankton grazing pressure to keep phytoplankton populations controlled, certain phytoplankton can proliferate under conditions such as during nutrient-laden freshwater pulses (Buskey et al. 1997). There is increasing evidence that disruption of the grazing community can play a role in phytoplankton bloom initiation, however the exact role that shifts in food web structure has in determining phytoplankton growth and community structure remains understudied (Buskey 2008).

1.4 PURPOSE/OBJECTIVE

Phytoplankton community composition is a balance between growth rates (impacted by nutrient loads and competition) and grazing mortality (impacted by abundance and selectivity of grazers; Lebret et al. 2012). For a phytoplankton species to bloom, sufficient nutrients must be present, but also grazing pressure must be relaxed

(Smayda 2008). This relationship plays a major role in regulating HAB outbreaks, i.e. timing of a bloom and subsequent collapse (Smayda 2008). Additionally, with preferential grazing, zooplankton may avoid feeding on less-palatable phytoplankton species, thereby allowing certain members of the phytoplankton community (e.g., HABs) to bloom over other species (Buskey 2008). Recent studies have shown that trophic structure alteration, coupled with nutrient loads, may be an important factor in HAB dynamics of estuaries (Stoecker et al. 2008).

Here I present results from a study of the effects of bottom-up (nutrients) and top-down (grazing) environmental controls on estuarine phytoplankton growth and community composition. Knowledge gained from this research will be applicable to other eutrophic, temperate estuaries worldwide, and will help elucidate how anthropogenic nutrient loading and top-down controls interact to structure estuarine phytoplankton growth and community structure on a seasonal basis. Seasonality may also be a critical factor because of potential for shifts in the importance of nutrients and grazing depending on times of year.

Objective 1 of this research is to compare the effects of organic and inorganic nitrogen on the estuarine phytoplankton community. **Objective 2** is to explore the singular and interactive effects of top-down and bottom-up factors on estuarine phytoplankton growth and community structure, as their effects in consort may be different than predicted by each individually (Pitt et al. 2007).

2. METHODS

2.1 Study Site - *Neuse River Estuary, North Carolina*

The Neuse River Estuary (NRE) in eastern North Carolina is part of the Albemarle-Pamlico estuarine system, the second largest estuarine system in the United States (Fig. 1). The NRE is a highly eutrophic system where phytoplankton growth is limited by nitrogen (N) for a large portion of the year (Rudek et al. 1991; Twomey et al. 2005). The slow flushing rates of the NRE (~50 days, but can be over 100 days depending on river flow; Christian et al. 1991) make it especially sensitive to effects of anthropogenic nutrient loading in its drainage basin (Paerl et al. 2007; Rothenburger et al. 2009).

The drainage basin of the NRE has experienced significant population growth in recent decades, and the population is expected to increase by 44% between 2000 and 2020, reaching over 2,000,000 people by 2020 (NCDENR 2009). Additionally, there have been increases in agriculture and confined animal feeding operations (CAFOs) in the NRE watershed (Stow et al. 2001). As of 2008, there are 500 permitted swine CAFOs in the basin, supporting about 2,000,000 swine (summarized in NCDENR 2009). This estuary is currently facing the effects of excessive nutrient loading (Stow et al. 2001), including fish kills (Hall et al. 2008) and algal blooms which have been related to anthropogenic nutrient loads (Paerl et al. 1998).

Fig. 1 Sampling locations. Latitude and longitude of sampling sites are listed in Table 2. Map modified from ModMon and Google Earth.

The NRE is a temperate estuary; surface water temperature ranges from below 5 ºC in the winter to over 30 ºC in the summer months (Christian et al. 1991). As is typical of many temperate rivers, in the Neuse River flow is maximal in spring and minimal in summer and fall (Rudek et al. 1991). Winds also play a role in Neuse River Estuary circulation, primarily through across channel mixing (Luettich et al. 2000).

Associated with the high rainfall runoff periods in winter/spring are pulses of increased nutrient loads, particularly nitrate (Paerl et al. 1998; Christian et al. 1991). Phosphorus (P) is also loosely associated with runoff, but generally follows the trend of low concentrations in winter, and increasing concentrations through summer through fall (Rudek et al. 1991) as it is released from estuarine sediments during frequent hypoxic and anoxic periods in the summer (Paerl et al. 1998). A similar trend holds for ammonium (Paerl et al. 1998). Urea, most often the dominant type of DON in estuaries, does not follow a seasonal trend in the NRE (Twomey et al. 2005), because allochthonous urea is brought into the NRE from the Neuse River, but it also released through the biological processes within the estuary (Twomey et al. 2005).

As with many temperate systems, increases in light and temperature in summer months cause increased productivity during that time of year (Paerl et al 1998). Also similar to other estuaries, phytoplankton blooms tend to form in a distinct zone in the NRE, referred to as the chlorophyll-a maximum (CMAX) which can contain significantly more phytoplankton biomass than surrounding waters (Pennock 1985, Fisher et al. 1988; Valdes-Weaver et al. 2006). Environmental conditions (i.e. river flow and nutrient loads) regulate the formation of a CMAX and its location throughout the estuary (it can shift upstream or downstream along the salinity gradient of the estuary), though in the NRE it usually occurs mid-estuary (Valdes-Weaver et al. 2006). Because upwards of 60% of estuarine primary production is consumed by grazers (Calbet & Landry 2004; Wetz et al. 2011), the CMAX may be an area with disproportionately high transfer of energy up to higher trophic levels. The high density of phytoplankton at the CMAX may also make it a more likely place for harmful algal species to proliferate (e.g., Hall et al. 2008) and for hypoxia/anoxia formation (Paerl et al. 1998).

2.2 Experimental Design

Sample Collection

Experiments were conducted using water collected from the CMAX on June 6, 2011, August 15, 2011, and March 12, 2012 between 0800 and 1230 (Fig. 1). For each event, surface water samples were collected from the CMAX (located with a flow-through chlorophyll-*a* fluorescence sensor onboard the sampling vessel) in 20 L carboys (pre-washed with 10% HCL) and stored under black tarps for transportation to the

University of North Carolina – Chapel Hill's Institute of Marine Sciences (IMS) in Morehead City, North Carolina.

Experimental procedure

Upon return to IMS, while under dim lighting, the collected water was transferred to 4L Cubitainers (Hedwin Co.; ~80% transparent to ambient photosynthetically active radiation, PAR; pre-washed with 10% HCL), in triplicate for each of nine treatments (Table 1). For grazing manipulations, water was filtered while filling Cubitainers through either a 20 µm mesh (to remove micro-, meso- and macrozooplankton grazers) or 153 µm mesh (to remove meso- and macro-zooplankton grazers); whole water samples were not filtered. Additionally, 10 µM-nitrogen as urea or potassium nitrate was added to select treatments (as indicated in Table 1).

For the 48-hour duration of the experiment, Cubitainers were incubated in an outdoor experimental pond at IMS that is flushed with water from Bogue Sound to mimic ambient temperature and light levels. PAR was monitored in the pond for the duration of the experiment with a LI-COR 2pi PAR sensor.

Table 1 Experimental manipulations.

Treatment	Description of experimental manipulations
Whole	Control; Intact community
Whole + urea	Intact community; 10 µM-N added as urea
Whole + nitrate	Intact community; 10 µM-N added as potassium nitrate
< 153 µm	Cells > 153 µm (meso- and macrozooplankton) removed via filtration
< 153 µm + urea	Cells > 153 µm (meso- and macrozooplankton) removed via filtration; 10 µM-N added as urea
< 153 µm + nitrate	Cells > 153 µm (meso- and macrozooplankton) removed via filtration; 10 µM-N added as potassium nitrate
< 20 µm	Cells > 20 µm (micro-, meso- and macrozooplankton) removed via filtration
< 20 µm + urea	Cells > 20 µm (micro-, meso- and macrozooplankton) removed via filtration; 10 µM-N added as urea
< 20 µm + nitrate	Cells > 20 µm (micro-, meso- and macrozooplankton) removed via filtration; 10 µM-N added as potassium nitrate

Initial (hereafter "T0") subsamples were collected immediately after experimental set-up, as described below. At T24 and T48 hours, subsamples (~800 ml) were collected from each Cubitainer for inorganic nutrients, total dissolved N and DON, phytoplankton pigments and phytoplankton/zooplankton abundance. At T0 additional samples were collected for size-fractionated pigment analysis.

In June and August, nitrate concentrations were drawn down to detection limits in control treatments by 24 hours, and by 75% - >90% in N amended treatments, so here I focus on 24 hour growth rates as an indicator of phytoplankton response to N for all months.

Nutrient Analysis

For nutrient analysis, ~ 50 ml of each subsample was filtered through 25 mm combusted GF/F (0.7 µm pore size) filters and immediately frozen in 50 ml polypropylene centrifuge tubes until processed with a Lachat QuickChem 8000 (Lachat Instruments) for NO_3^-, NO_2^-, NH_4^+, PO_4^-, and total dissolved nitrogen (TDN) according to standard colorimetric methods (as stated in Peierls et al. 2003). Method detection limits ranged from 0.27 to 0.36 µg/L for NO_3^-/NO_2^-, from 2.87 to 3.98 µg/L for NH_4^+, from 0.62 to 0.69 µg/L for PO_4^-, and from 25.6 to 36.9 µg/L for TDN. DON concentrations were calculated by subtracting NO_3^-/NO_2^- and NH_4^+ concentrations from those of TDN.

Pigment Analysis

For pigment analysis, 75 to 200 ml of subsample was gently filtered onto 25 mm GF/F filters. These were sonicated with 100% HPLC grade acetone and frozen overnight. Pigment concentrations were quantified by injecting 200 µl of extract into a high performance liquid chromatography (HPLC) photodiode array spectrophotometry system, as described in Paerl et al. (2010). Chlorophyll *a* was used as a proxy for phytoplankton community biomass. Additionally, concentrations were measured for photopigments that are diagnostic of some of the major phytoplankton taxonomic groups in the NRE (Pinckney et al. 1998; see Paerl et al. 2003 for more information on diagnostic pigments). These pigments include: zeaxanthin as a marker for cyanobacteria; fucoxanthin as a marker for flagellates (e.g. raphidophytes, dinoflagellates) and diatoms; peridinin as a marker for dinoflagellates; and alloxanthin as a marker for cryptophytes.

24-hour growth rates were calculated for each pigment and related to growth of the overall community and each functional group according to Equation 1.

$$(\ln (T_X) - \ln (T_0)) / (X-0) = \text{growth } d^{-1} \quad \textbf{(1)}$$

Where: T_X = pigment concentration at ending time

T_0 = initial pigment concentration

X = ending time (in days)

Size-fractionated Pigment Analysis

For size-fractionated chlorophyll *a* analysis at T0, 200 ml of whole water N control samples were filtered through a 20 µm mesh and the filtrate subsequently filtered through a 47 mm GF/F filter. These samples were run for HPLC pigment analysis as described above.

Cell Enumeration

Approximately 60 ml from each subsample were preserved with 2 ml (June; August) or 1.5 ml (March) acid Lugol's solution. Samples were stored in amber glass bottles (June; August) or polyethylene amber bottles (March) until enumerated. For enumeration, bottles were gently inverted and subsamples poured into 5 ml Utermohl chambers and allowed to settle for at least 3 hours. Samples were then analyzed using an Olympus IX71 inverted microscope at 200x. Cells > 5-10 µm were identified down to lowest classification level possible. 24-hour and 48-hour growth rates were calculated for phytoplankton species and groups according to Equation 1.

Statistical Methods

Growth rates from diagnostic photopigments and cell enumeration counts were used for statistical analysis. A response to N addition or grazer removal was first analyzed with Analysis of Variance (ANOVA; α=0.05). For follow-up analyses, Tukey's Honestly Significant Differences (Tukey's HSD) a posteriori comparisons (α=0.05) were used. In one case of an interaction between the N addition and grazer removal variables (the two variables were not acting independently of each other), separate Tukey's HSD unplanned comparisons were conducted for each N and grazer removal treatment. Assumptions of normality and homoscedasticity were checked with the Shapiro-Wilk test (α=0.01; Table 2) and the Brown-Forsythe Levene-type test (α=0.05; Table 3). Statistical analyses were conducted with R version 2.13.1 (R Development Core Team 2011)

Table 2 Shapiro-Wilk normality test (α=0.01) p-values for all growth rates assessed with ANOVA. Peridinin was log-transformed in June to pass normality.

	June 2011	**August 2011**	**March 2012**
Chlorophyll *a*	0.161	0.029	0.394
Zeaxanthin	0.550	0.718	0.011
Alloxanthin	0.966	0.653	NA
Fucoxanthin	0.641	0.040	0.542
Peridinin	0.042*	0.297	0.945
H. rotundata	0.171	NA	NA
K. veneficum	0.164	NA	0.982
Cryptophytes	0.735	0.497	NA
Nostocales	NA	0.246	NA
Euglenoids	NA	0.165	0.846
G. instriatum	NA	NA	0.214
H. triquetra	NA	NA	0.207
M. rubra	NA	NA	0.098

Table 3 Brown-Forsythe Levene-type test (α=0.05) p-values for all growth rates assessed with ANOVA. Peridinin was log-transformed in June to pass normality.

	June 2011	**August 2011**	**March 2012**
Chlorophyll *a*	0.900	0.996	0.991
Zeaxanthin	0.500	0.901	0.511
Alloxanthin	0.819	0.959	NA
Fucoxanthin	0.754	0.985	0.835
Peridinin*	0.615	0.953	0.934
H. rotundata	0.728	NA	NA
K. veneficum	0.497	NA	0.758
Cryptophytes	0.886	0.903	NA
Nostocales	NA	0.992	NA
Euglenoids	NA	0.932	0.947
G. instriatum	NA	NA	0.998
H. triquetra	NA	NA	0.758
M. rubra	NA	NA	0.893

3. RESULTS

3.1 Initial Conditions: temperature, salinity, chlorophyll *a*, nutrients

The initial physical, chemical, and biological conditions associated with the CMAX varied among the three sampling events. Lowest salinity was recorded in August 2011 (hereafter "August"), while lowest surface water temperature was recorded in March 2012 (hereafter "March"; Table 4). Integrated photosynthetically active radiation (PAR) was about 50% higher in summer months than in March (Table 4). Initial chlorophyll *a* concentration was highest in March, when it was about twice as high as in June 2011 (hereafter "June") and August (Table 5). During June and August nearly all of phytoplankton biomass was in the < 20 µm size fraction. Each experimental event was also characterized by different diagnostic photopigment concentrations; there was a dinoflagellate bloom in March, while cyanobacterial abundances (zeaxanthin) were high in June and August, according to the diagnostic pigments (Table 5).

Table 4 Initial physical conditions for each experimental event. Note that integrated photosynthetically active radiation (PAR) was taken in the experimental pond during the 48-hours of incubation. Sampling locations can be seen in Fig. 1.

Initial Conditions	**June 2011**	**August 2011**	**March 2012**
Latitude and longitude	N 35˚ 1.764 W 76˚ 58.250	N 35˚ 8.096 W 77˚ 2.850	N 34˚ 50.470 W 76˚ 52.178
Salinity	7.1	4.8	6.4
Water temperature (˚C)	28	29	13
Integrated 48-hour PAR (E/m^2)	97	88	62

Table 5 Photopigment concentrations for each experimental event. Alloxanthin concentrations were below the detection limit (~0.02 µg/l) in March 2012.

Diagnostic Photopigment	**June 2011**	**August 2011**	**March 2012**
Chlorophyll *a* (µg/L)	17.2	15.3	32.8
<20 µm chlorophyll *a* (µg/L)	16.3	13.1	22.8
Alloxanthin (µg/l)	0.66	0.80	below detection limit
Fucoxanthin (µg/l)	0.62	1.49	2.50
Peridinin (µg/l)	2.23	0.62	8.79
Zeaxanthin (µg/l)	3.17	1.04	0.35

The total dissolved nitrogen (TDN) concentration was highest in March (Table 6). The ammonium concentration was relatively high in June and August compared to March (Table 6). The majority of TDN was DON on all dates. The DON concentration was higher in March than in June or August (Table 6). Greatest variations in nutrient concentrations throughout the three events were seen in nitrate + nitrite and phosphorus. The nitrate + nitrite concentration was an order of magnitude lower in June than in the other months (Table 6), and the orthophosphate concentration was an order of magnitude greater in August than in the other months (Table 6). In March, the ratio of DIN to dissolved inorganic phosphorus (DIP) was 19.0, higher than that of June (2.2) or August (1.6) and above the Redfield ratio of 16 (Table 4).

Table 6 Initial chemical conditions for each experiment.

Initial Conditions	**June 2011**	**August 2011**	**March 2012**
Nitrate + nitrite (µg-N/L)	7	163	179
Orthophosphate (µg-P/L)	13	116	10
Ammonium (µg-N/L)	22	26	11
DIN:DIP	2.2	1.6	19.0
Dissolved organic nitrogen (µg-N/L)	295	300	366
Total dissolved nitrogen (µg-N/L)	324	489	556

3.2 June 2011 Results

Nutrients

DON concentrations decreased slightly over the experimental period, but concentrations remained above 250 µg/l at 24 hours (data not shown). Phosphate concentrations also decreased throughout the experiment. Nitrate + nitrite concentrations in nitrate amended treatments dropped to concentrations indistinguishable from control treatment levels within the first 24 hours (data not shown).

***Chlorophyll* a**

In June, chlorophyll *a* growth rates were negative in control treatments (Fig. 2). Compared with controls, chlorophyll *a* growth was positively affected by both N addition and grazer removal (Tables 7 and 8). There was an interaction between these two factors for 24-hour growth rates; while N addition consistently resulted in increased growth, removal of grazers > 20 µm only had a positive effect on growth when in combination with N addition (Fig. 2; Table 8).

Table 7 Analysis of Variance (ANOVA) p-values for diagnostic pigments and major enumerated taxa in June. Significant p-values (< 0.05) are in bold. For chlorophyll *a*, there was an interaction between grazing and N (p = 0.0179). Refer to Table 8 for chlorophyll *a* follow-up analyses. For all other follow-up analyses, refer to Table 9.

ANOVA	Grazing	Nitrogen
Chlorophyll *a*	**0.000**	**0.000**
Zeaxanthin	0.560	**0.000**
Alloxanthin	0.064	**0.000**
Fucoxanthin	0.630	**0.000**
Peridinin	**0.006**	**0.000**
H. rotundata	0.394	**0.000**
K. veneficum	0.559	0.356
Cryptophytes	0.966	**0.001**

Table 8 Tukey's HSD p-values for chlorophyll *a* in June. Due to the interaction between grazing and N treatments, Tukey's HSD was conducted for each N and grazing treatment. Significant p-values (< 0.05) are in bold.

Tukey's HSD: effect of grazing for each N treatment	Control	Urea	Nitrate
< 153 µm vs. < 20 µm	0.622	0.190	**0.000**
Whole water vs. < 20 µm	0.513	**0.006**	**0.001**
Whole water vs. < 153 µm	0.978	0.582	0.121
Tukey's HSD: effect of N at each grazing treatment	Whole water	< 153 µm	< 20 µm
control vs. nitrate	**0.000**	**0.004**	**0.000**
control vs. urea	**0.000**	**0.000**	**0.000**
nitrate vs. urea	0.160	0.063	0.498

Table 9 Tukey's HSD p-values for all follow-up analyses in June. Significant p-values (< 0.05) are in bold.

Tukey's HSD: Nitrogen	Nitrate vs. control	Urea vs. control	Urea vs. nitrate
Zeaxanthin	**0.000**	**0.000**	**0.019**
Alloxanthin	**0.000**	**0.000**	0.121
Fucoxanthin	**0.000**	**0.000**	**0.001**
Peridinin	**0.000**	**0.000**	0.997
H. rotundata	**0.003**	**0.000**	0.665
Cryptophytes	**0.003**	**0.004**	0.983
Tukey's HSD: Grazing	< 153 µm vs. < 20 µm	Whole water vs. < 20 µm	Whole water vs. < 153 µm
Peridinin	**0.007**	**0.030**	0.756

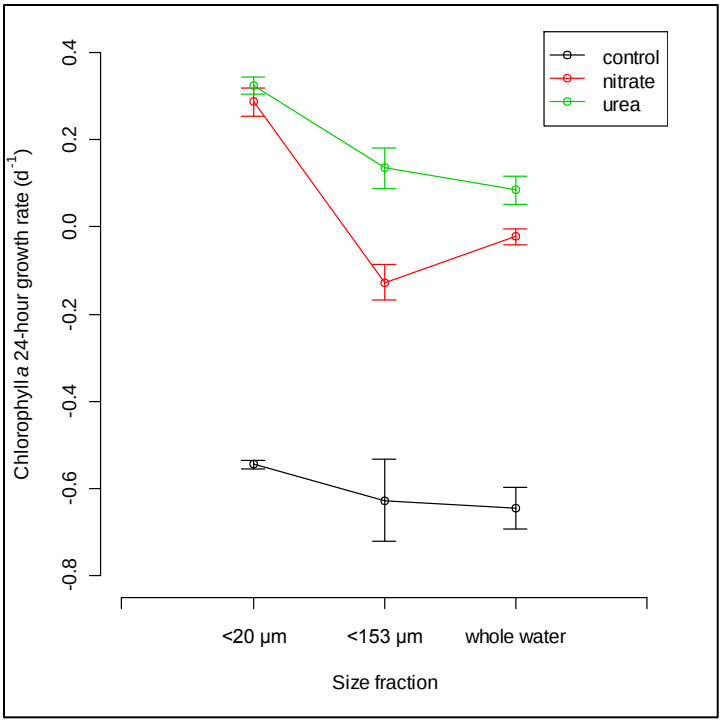

Fig. 2 Chlorophyll *a* 24-hour growth rates in June 2011. Vertical bars represent standard error.

Diagnostic pigments

Nitrogen addition had a widespread effect in June, impacting all assessed diagnostic photopigments; grazing manipulations, however, only had a significant effect on the diagnostic pigment peridinin (Tables 7 and 9). As with chlorophyll *a*, overall pigment growth rates were negative in N control treatments, indicating N limitation. Zeaxanthin (cyanobacteria; Fig. 3) and fucoxanthin (cell counts suggest fucoxanthin was representative of raphidophytes and *Karlodinium veneficum*; Fig. 4) both responded positively to N addition, and to urea more than to nitrate. Alloxanthin (cryptophytes; Fig. 5) and peridinin (dinoflagellates; Fig. 6) both responded positively to N addition, with no difference between N types; peridinin also responded positively to removal of grazers > 20 µm.

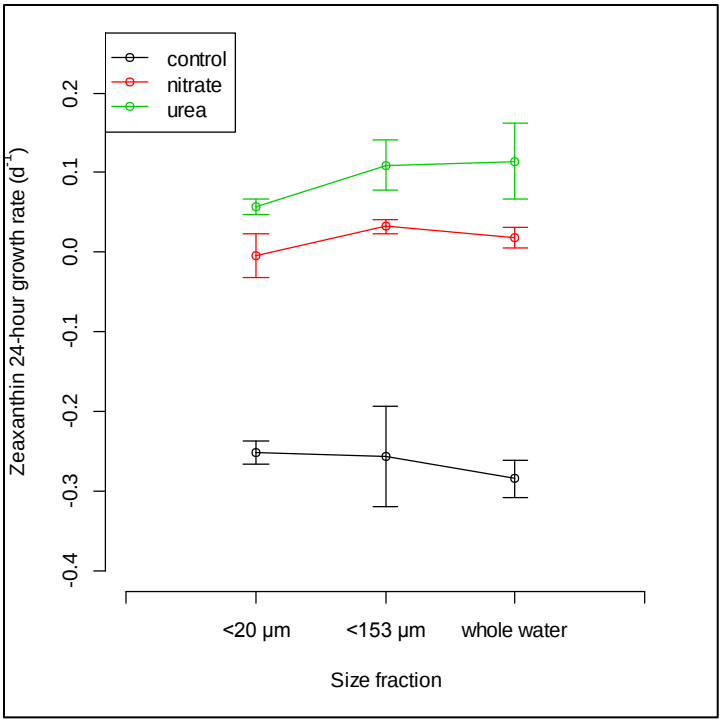

Fig. 3 Zeaxanthin 24-hour growth rates in June 2011.

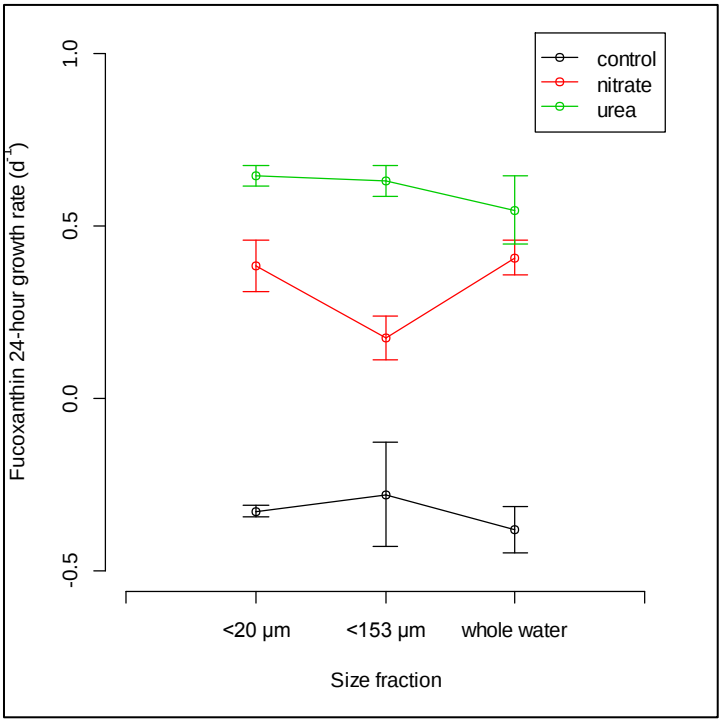

Fig. 4 Fucoxanthin 24-hour growth rates in June 2011.

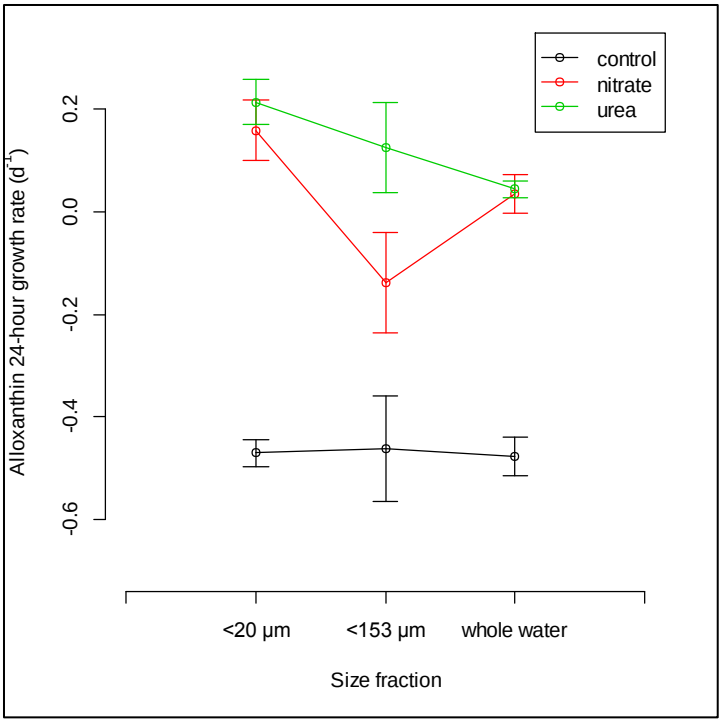

Fig. 5 Alloxanthin 24-hour growth rates in June 2011.

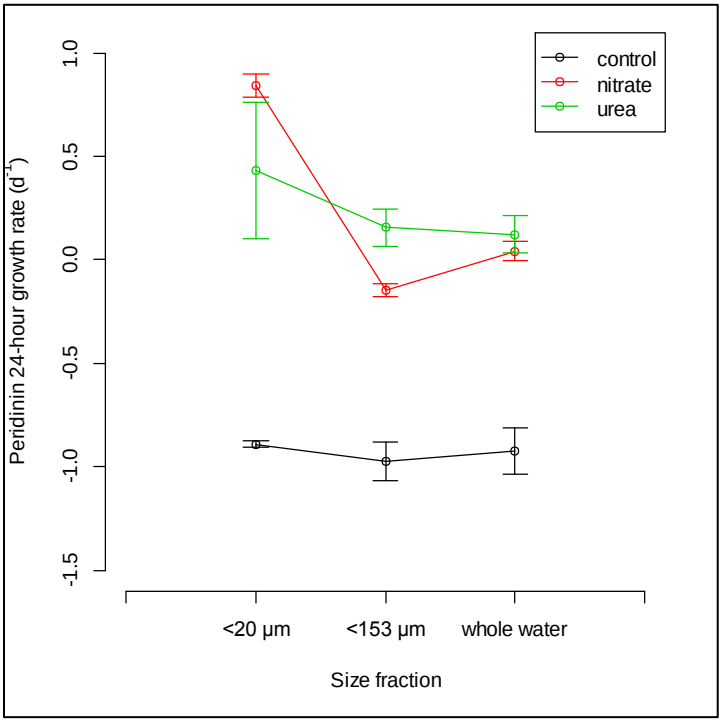

Fig. 6 Peridinin 24-hour growth rates in June 2011.

Enumerated cells

In June, the numerically dominant plankter in the detectable size limit (> ca. 5 µm) was *Heterocapsa rotundata* (~10 µm) at concentrations of ~3200 cells/ml (Table 10). This mixotrophic dinoflagellate responded positively to N addition and exhibited no response to grazer removal (Fig. 7; Tables 7 and 9). A species of note identified in June was the ichthyotoxic mixotrophic dinoflagellate, *Karlodinium veneficum* (~70 cells/ml). Unlike fucoxanthin (diagnostic of *K. veneficum* in June), enumerated *K. veneficum* cells did not respond to treatments, but experienced overall positive growth despite low DIN concentrations in control treatments (Fig. 8). Cryptophytes responded positively to N additions (Fig. 9), mirroring trends of the cryptophyte diagnostic photopigment, alloxanthin.

Table 10 Initial abundances of major taxa identified through microscopy in June 2011.

Taxa	**Abundance (cells/ml)**
Heterocapsa rotundata	3211
Cryptophytes	459
Raphidophytes	186
Chlorophytes	104
Heterotrophic (non-*M. rubra)* ciliates	81
Karlodinium veneficum	66
Myrionecta rubra	49

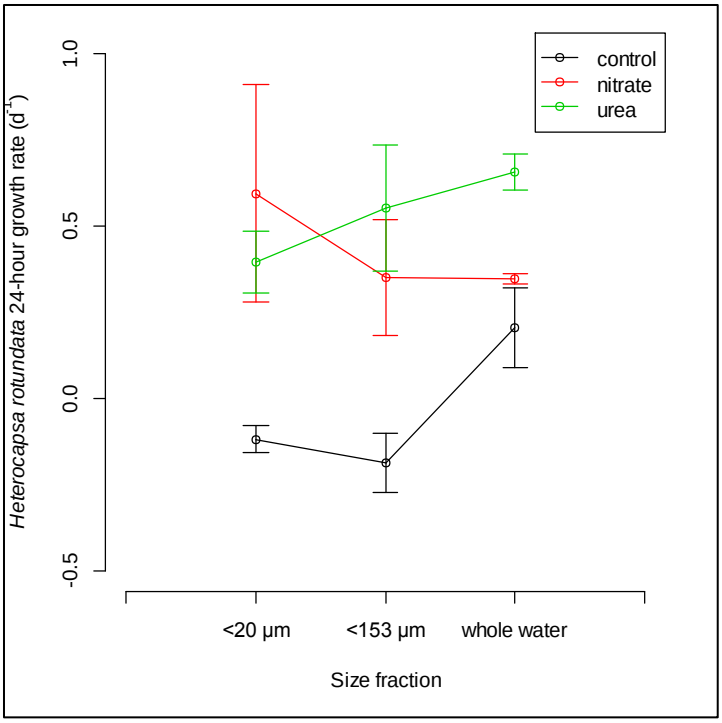

Fig. 7 *Heterocapsa rotundata* 24-hour growth rates in June 2011.

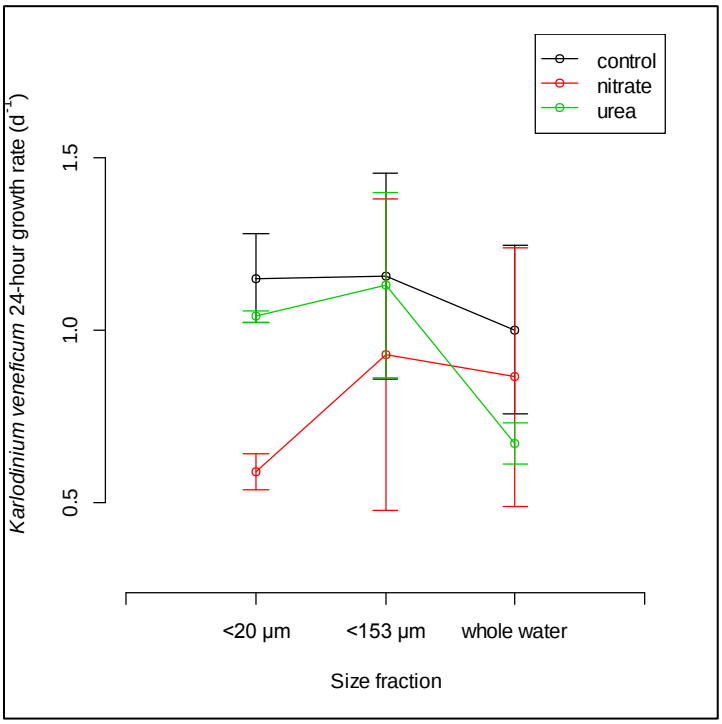

Fig. 8 *Karlodinium veneficum* 24-hour growth rates in June 2011.

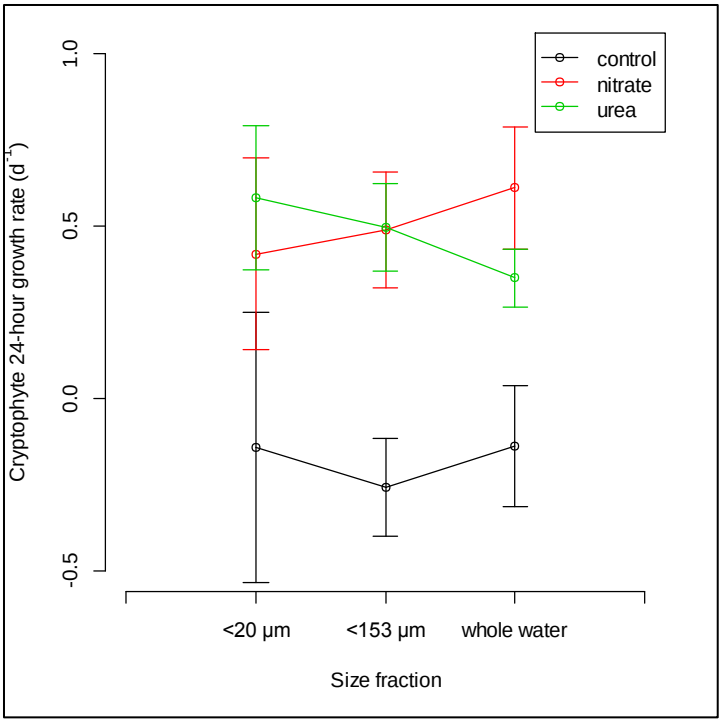

Fig. 9 Cryptophyte 24-hour growth rates in June 2011.

3.3 August 2011 Results

Nutrients

In August, ambient nutrient concentrations were higher than in June. However, as in June, within the first 24 hours, nitrate + nitrite concentrations dropped by over 100 µg/l (data not shown). DON concentrations remained steady through the experiment (data not shown). Phosphate concentrations decreased over time, but were above 75 µg/l at 24 hours (data not shown).

Chlorophyll a

In August, control treatments had positive growth (Fig. 10). No statistically significant effect of N addition was seen, though removal of grazers > 153 µm had a negative effect on chlorophyll *a* growth rates (Fig. 10; Tables 11 and 12).

Table 11 Analysis of Variance (ANOVA) p-values for diagnostic pigments and major enumerated taxa in August. Significant p-values (< 0.05) are in bold. Refer to Table 12 for follow-up analyses.

ANOVA	Grazing	Nitrogen
Chlorophyll *a*	**0.042**	0.093
Zeaxanthin	0.218	**0.002**
Alloxanthin	0.202	0.336
Fucoxanthin	**0.007**	**0.007**
Peridinin	**0.001**	0.618
Cryptophytes	0.563	0.657
Nostocales	**0.001**	0.150
Euglenoids	**0.005**	0.127

Table 12 Tukey's HSD p-values for all follow-up analyses in August. Significant p-values (< 0.05) are in bold.

Tukey's HSD: Grazing	< 153 µm vs. < 20 µm	Whole water vs. < 20 µm	Whole water vs. < 153 µm
Chlorophyll *a*	0.192	0.657	**0.037**
Fucoxanthin	**0.007**	0.590	0.053
Peridinin	0.361	**0.019**	**0.001**
Nostocales	0.403	**0.001**	**0.012**
Euglenoids	0.101	0.453	**0.007**
Tukey's HSD: Nitrogen	Nitrate vs. control	Urea vs. control	Urea vs. nitrate
Zeaxanthin	**0.015**	**0.003**	0.709
Fucoxanthin	**0.029**	**0.008**	0.825

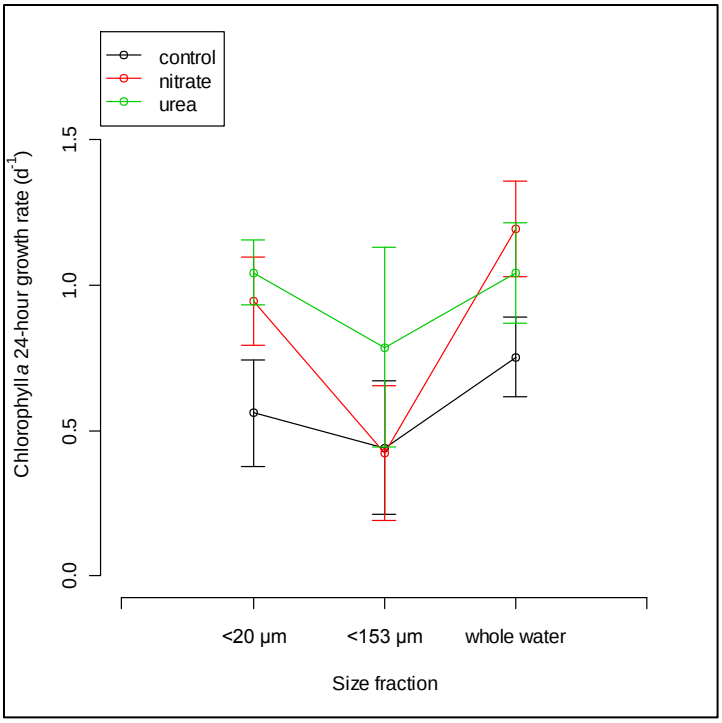

Fig. 10 Chlorophyll *a* 24-hour growth rates in August 2011.

Diagnostic pigments

In August, the response of pigments to N addition and grazer removal was varied (Tables 11 and 12). Zeaxanthin (Fig. 11) and fucoxanthin (cell counts suggest fucoxanthin was representative of raphidophytes and diatoms; Fig. 12) again exhibited similar responses to experimental treatments; both responded positively to N addition but did not respond to grazer removal. Alloxanthin had no significant responses to treatments (Fig. 13). Peridinin did not respond to N, but was the sole diagnostic pigment to respond to grazer manipulations, with a negative response to both levels of grazer removal (Fig. 14).

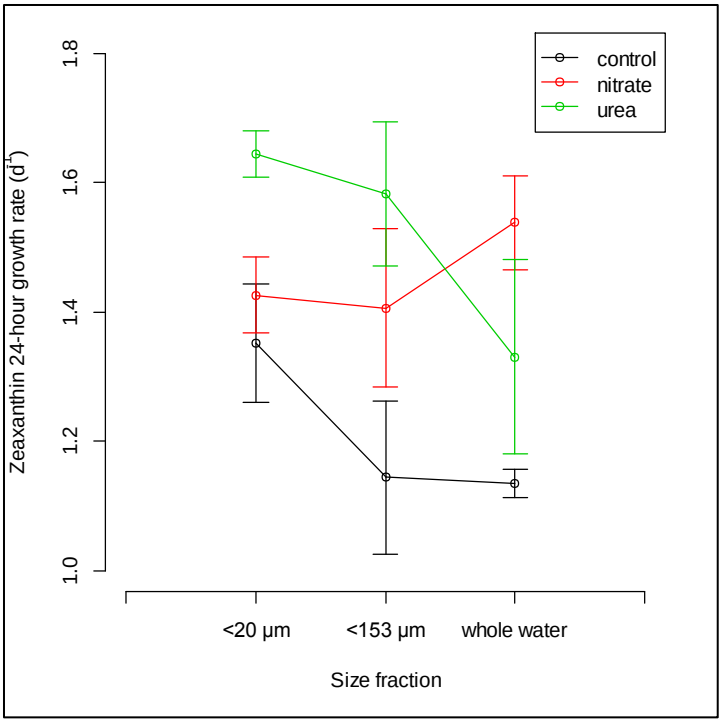

Fig. 11 Zeaxanthin 24-hour growth rates in August 2011.

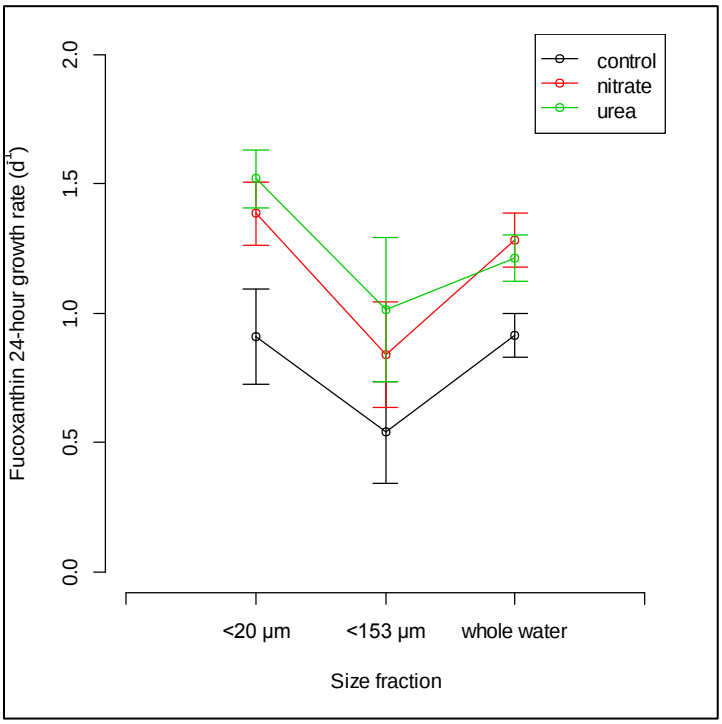

Fig. 12 Fucoxanthin 24-hour growth rates in August 2011.

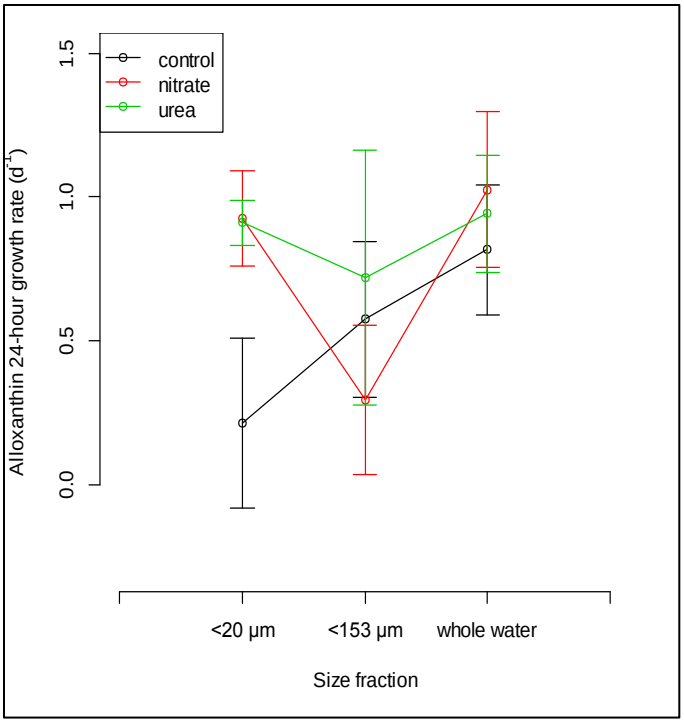

Fig. 13 Alloxanthin 24-hour growth rates in August 2011.

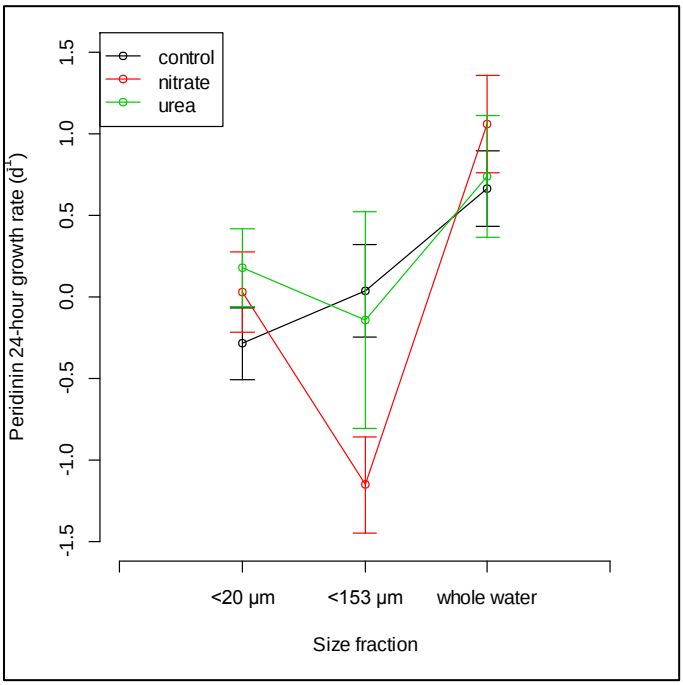

Fig. 14 Peridinin 24-hour growth rates in August 2011.

Enumerated cells

In August, cryptophytes and Nostocales (an order of cyanobacteria) were the most common enumerated phytoplankton taxa of detectable size (> ca. 5 µm) at ~720 cells/ml and ~925 cells/ml, respectively (Table 13). While cryptophytes exhibited no significant responses to treatments (data not shown), Nostocales growth rates responded positively to grazer removal (both of grazers > 20 µm and > 153 µm; Fig. 15; Tables 11 and 12). As with chlorophyll *a*, euglenoids responded negatively to removal of grazers > 153 µm (Fig. 16; Tables 11 and 12).

Table 13 Initial abundances of major taxa identified through microscopy in August 2011.

Taxa	**Abundance (cells/ml)**
Nostocales	925
Cryptophytes	720
Chlorophytes	320
Armored dinoflagellates	226
Euglenoids	203
Diatoms	96
Non-*M. rubra* ciliates	67
Raphidophytes	38
Unarmored dinoflagellates	34
Myrionecta rubra	16

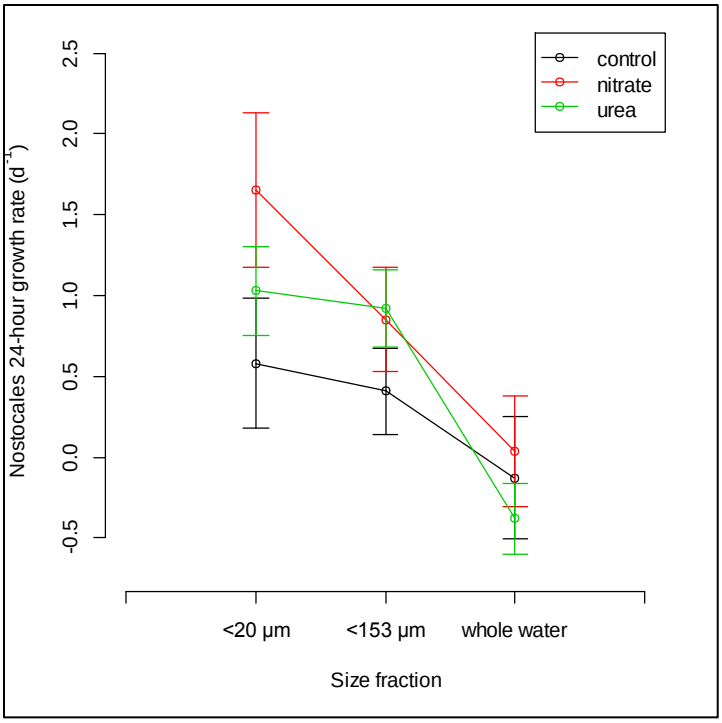

Fig. 15 Nostocales 24-hour growth rates in August 2011.

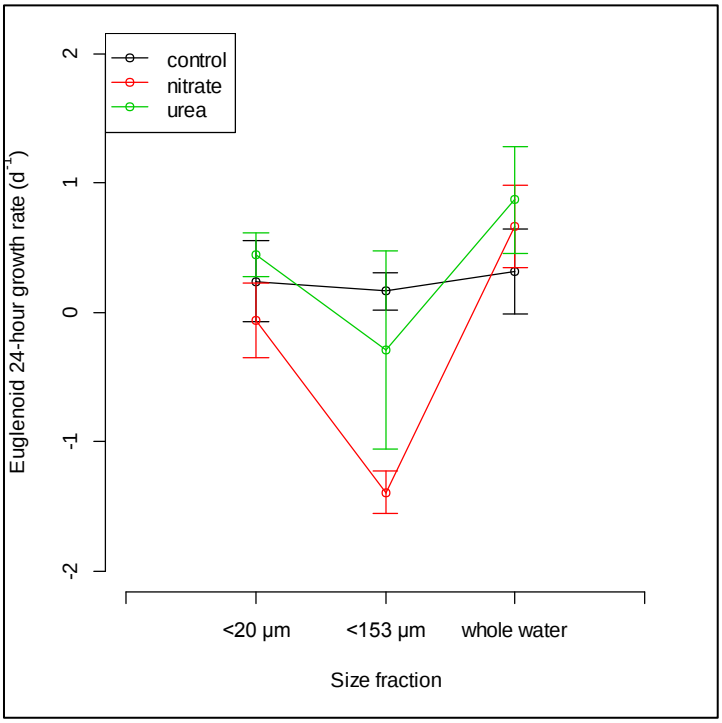

Fig. 16 Euglenoid 24-hour growth rates in August 2011.

3.4 March 2012 Results

Nutrients

Over the experimental period in March, concentrations of nitrate + nitrite, phosphate, ammonium, and urea all decreased. Initially, the DIN:DIP ratio was 19, indicating DIP limitation. Phosphorus concentrations were halved within the first 24 hours (data not shown). DON concentrations also decreased in the first 24 hours (data not shown). Nitrate + nitrite concentrations also decreased, ending at ~150 µg/l in N amended treatments, and at ~ 100 µg/l in N control treatments at 24 hours (data not shown).

***Chlorophyll* a**

In March, chlorophyll *a* growth rates were negative overall. There was no statistically significant effect of N addition or grazer removal on chlorophyll *a* growth rates (Fig. 17; Tables 14 and 15).

Table 14 Analysis of Variance (ANOVA) p-values for diagnostic pigments and major enumerated taxa in March. Significant p-values (< 0.05) are in bold. Refer to Table 15 for follow-up analyses.

ANOVA	Grazing	Nitrogen
Chlorophyll *a*	0.158	0.622
Zeaxanthin	0.090	0.299
Fucoxanthin	**0.001**	**0.000**
Peridinin	**0.000**	0.689
G. instriatum	0.386	0.704
Euglenoids	0.135	**0.036**
K. veneficum	0.055	0.341
H. triquetra	0.577	0.547
M. rubra	0.758	0.971

Table 15 Tukey's HSD p-values for all follow-up analyses in March. Significant p-values (< 0.05) are in bold.

Tukey's HSD: Grazing	< 153 µm vs. < 20 µm	Whole water vs. < 20 µm	Whole water vs. < 153 µm
Fucoxanthin	**0.016**	**0.001**	0.421
Peridinin	**0.001**	**0.000**	0.864
Tukey's HSD: Nitrogen	Nitrate vs. control	Urea vs. control	Urea vs. nitrate
Fucoxanthin	**0.023**	**0.000**	**0.035**
Euglenoids	0.136	**0.034**	0.756

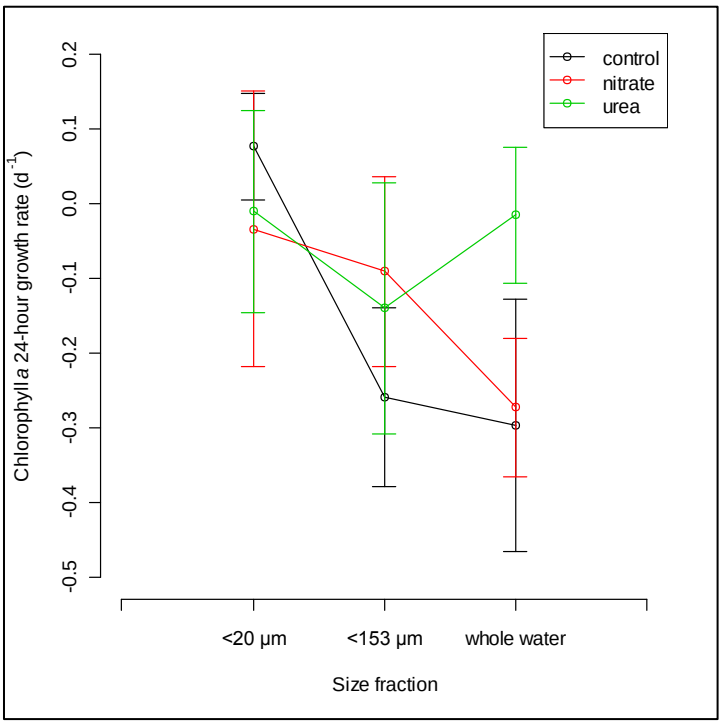

Fig. 17 Chlorophyll *a* 24-hour growth rates in March 2012.

Diagnostic pigments

Alloxanthin concentrations were below the detection limit in March (~0.02 µg/l) Though cryptophytes were present in preserved samples, compared to other months, abundances were low, confirming the low alloxanthin concentrations. The other diagnostic pigments had varied responses to N additions and relaxed grazing pressure in March (Tables 14 and 15). Even though chlorophyll *a* growth rates were negative, both

zeaxanthin and fucoxanthin had positive growth across all treatments. While zeaxathin did not significantly respond to any treatments compared with controls (Fig. 18), fucoxanthin (cell counts suggest fucoxanthin representative of diatoms and *K. veneficum*) did respond to treatments. Fucoxanthin responded positively to N additions, and more so to urea than to nitrate (Fig. 19). It also responded positively to removal of > 20 µm grazers. Peridinin did not respond to N additions, but did respond positively to removal of > 20 µm grazers (Fig. 20).

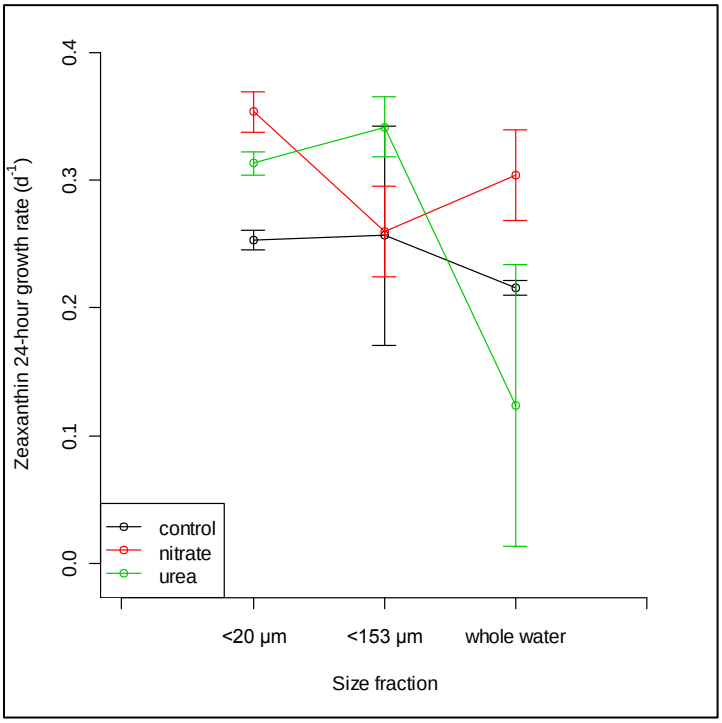

Fig. 18 Zeaxanthin 24-hour growth rates in March 2012.

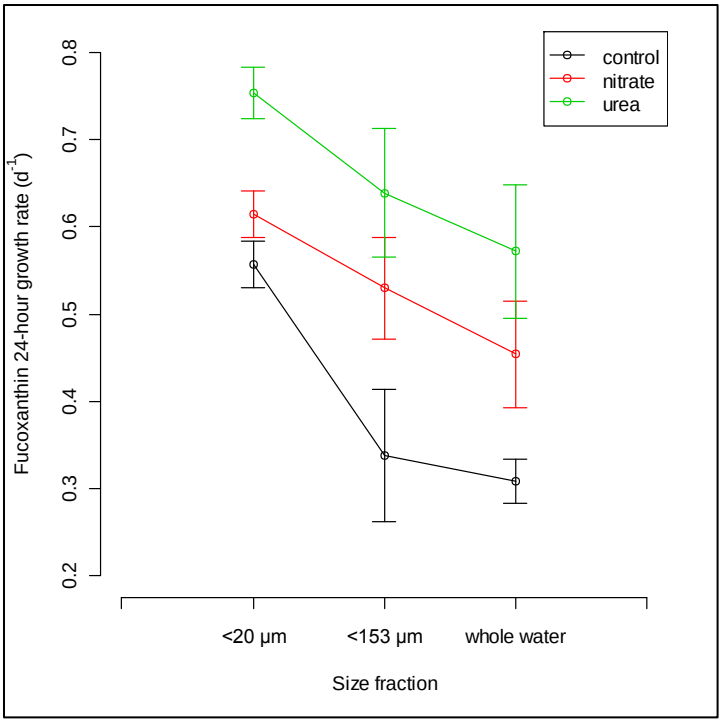

Fig. 19 Fucoxanthin 24-hour growth rates in March 2012.

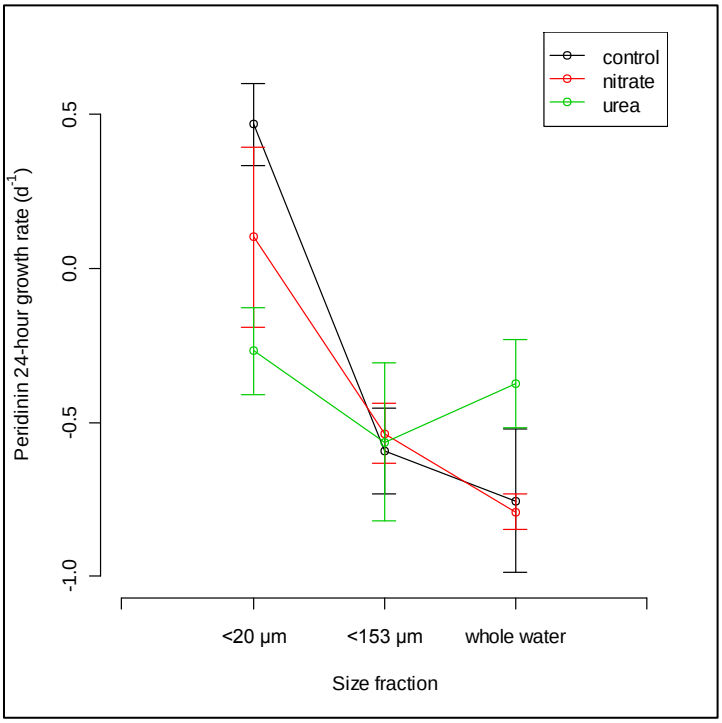

Fig. 20 Peridinin 24-hour growth rates in March 2012.

Enumerated cells

The most abundant phytoplankton species in March of detectable size (> ca. 5 μm) was the dinoflagellate *Gyrodinium instriatum*, with concentrations of ~1600 cells/ml (Table 16). This species experienced negative growth overall, and did not respond to experimental treatments (Fig. 21; Tables 14 and 15). Euglenoids (~30 cells/ml) were the only enumerated group to show significant responses to any treatment in March, and had a positive response to urea (Fig. 22; Tables 14 and 15). Some other enumerated plankters of note include *Karlodinium veneficum* (~50 cells/ml; Fig. 23), *Heterocapsa triquetra* (~120 cells/ml; Fig. 24), both of which had neutral growth for the duration of the experiment, and also cryptophytes (~70 cells/ml), and *Myrionecta rubra* (~30 cells/ml), though none responded significantly to treatments (data not shown).

Table 16 Initial abundances of major taxa identified through microscopy in March 2012.

Taxa	**Abundance (cells/ml)**
Gyrodinium instriatum	1601
Diatoms	158
Heterocapsa triquetra	122
Cryptophytes	67
Non-*M. rubra* ciliates	65
Karlodinium veneficum	53
Euglenoids	29
Myrionecta rubra	29

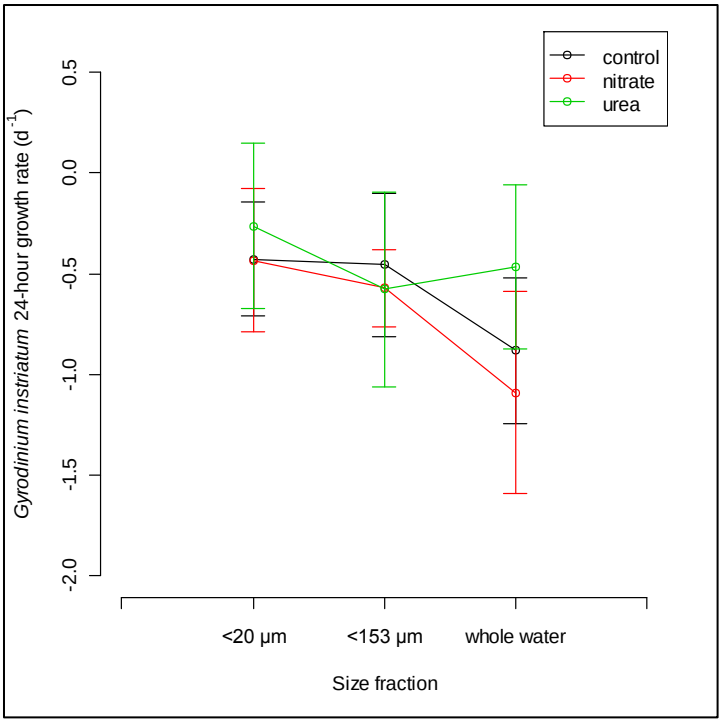

Fig. 21 *Gyrodinium instriatum* 24-hour growth rates in March 2012.

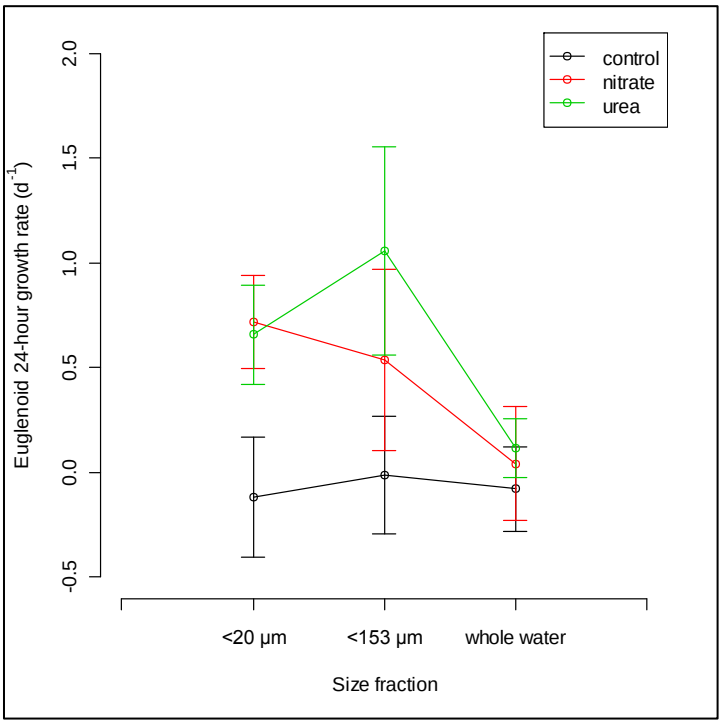

Fig. 22 Euglenoid 24-hour growth rates in March 2012.

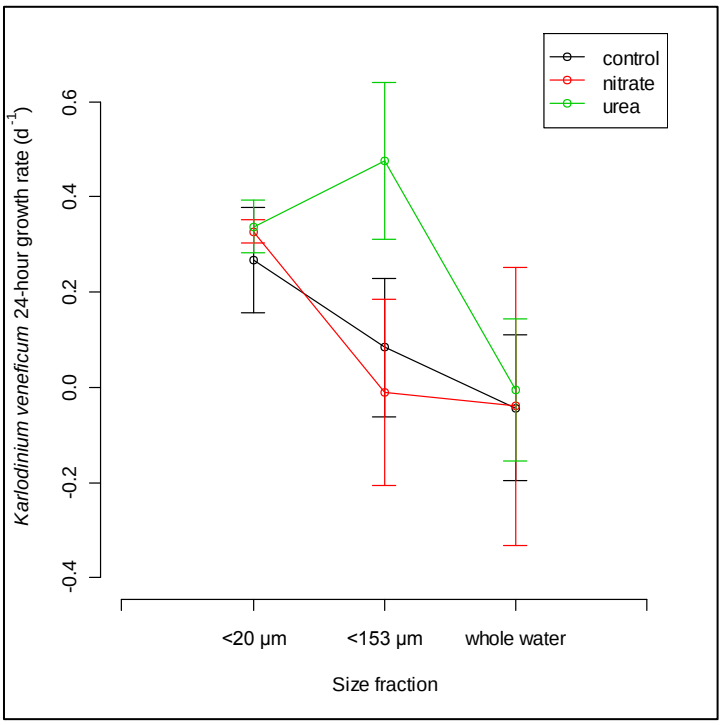

Fig. 23 *Karlodinium veneficum* 24-hour growth rates in March 2012.

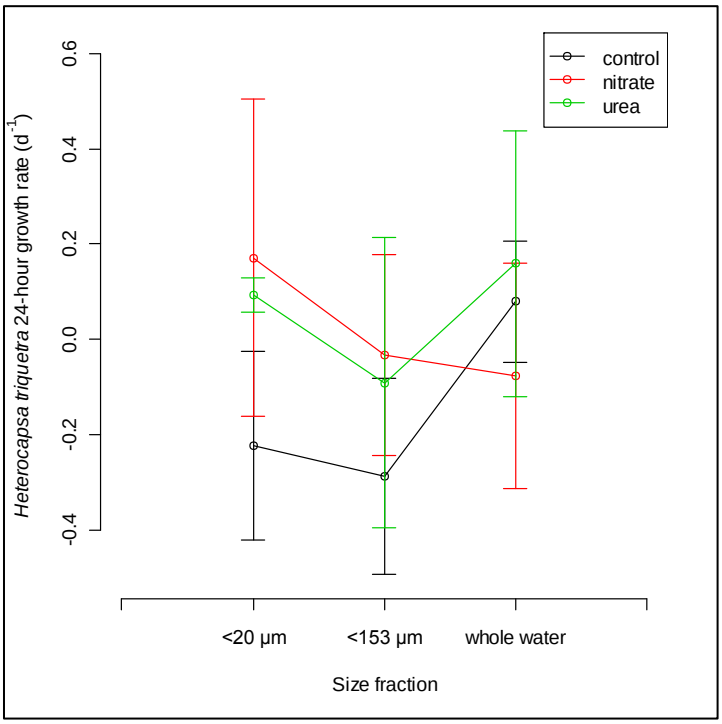

Fig. 24 *Heterocapsa triquetra* 24-hour growth rates in March 2012.

4. DISCUSSION

4.1 June Summary

In June, chlorophyll *a* and all other pigments experienced negative growth without added N, which is indicative of N-limitation and isolation from regenerated nutrients. The NRE commonly experiences N-limitation in summer months when standing phytoplankton biomass is high and freshwater inflow rates are low (Rudek et al. 1991). In addition to the seasonal N-limitation, this experiment imposed further nutrient limitation because the phytoplankton community was isolated from the bottom sediments of the NRE. Regeneration of nutrients from the bottom sediments is an important source of nutrients to the NRE phytoplankton community in the warmer months (e.g. Paerl et al. 1998).

In the summer in the NRE, phytoplankton blooms have been shown to closely follow large N inputs (Pinckney et al. 1998). This trend is supported in this experiment, as chlorophyll *a* growth rates increased in response to the N amendments (Fig. 2), and the phytoplankton community quickly utilized the added N. However, without continual N addition this growth was not sustained, and returned to negative rates after the added N was depleted (data not shown). In addition to impacting chlorophyll *a* growth, N amendments had a widespread effect in June, impacting all assessed diagnostic pigments (Table 17), and though N type was not a significant factor for overall community growth, urea stimulated higher growth than nitrate for some phytoplankton groups. This effect of urea on phytoplankton composition will be elaborated on later.

Table 17 Summary table of responses of photopigments to experimental manipulations during each experiment month. Alloxanthin concentrations were below the detection limit in March.

Pigment	Treatments	June	August	March
Chlorophyll *a*	Nitrogen addition	+		
	Grazer removal	+	-	
Fucoxanthin	Nitrogen addition	+	+	+
	Grazer removal			+
Alloxanthin	Nitrogen addition	+		**NA**
	Grazer removal			**NA**
Zeaxanthin	Nitrogen addition	+	+	
	Grazer removal			
Peridinin	Nitrogen addition	+		
	Grazer removal	+	-	+

In addition to N amendments, changes in grazing pressure also had a significant role in structuring the phytoplankton community in June. Relaxed grazing pressure by grazers > 20 µm was an important factor in chlorophyll *a* growth (when in combination with N addition). The phytoplankton community composition in June may have enabled this; almost 95% of chlorophyll *a* (Table 3) was < 20 µm, which is the optimal prey size range for microzooplankton, the major phytoplankton grazers (e.g. Jonsson 1986; Calbet et al. 2003).

4.2 August summary

Highest chlorophyll *a* growth rates were measured in August, and unlike in the other months, control treatment growth was also positive in August, indicating sufficient ambient nutrients. The high ambient nutrient concentrations in August, combined with warm temperatures, which support high productivity and fast nutrient regeneration (Christian et al. 1991), muted the effects of N additions.

Although N additions did not affect chlorophyll *a* growth rates in August, grazer removal treatments did (Table 17), with reduced growth when meso- and macrozooplankton were removed, suggesting trophic interactions. In August, over 90% of chlorophyll *a* was in the < 20 µm fraction, which would be primarily grazed upon by microzooplankton. Previous work has shown mesozooplankton to be a controlling factor on microzooplankton community structure (Miller et al. 1995). Removal of meso- and macrozooplankton (via 153 µm mesh) would release predation pressure on micrograzers and initiate a trophic cascade, increasing microzooplankton grazing upon phytoplankton. This would result in decreased phytoplankton growth with decreased mesozooplankton abundance. Though not significant, at 24 hours in August, heterotrophic ciliates had higher abundances in treatments with meso- and macrozooplankton removed than in controls (data not shown); this increase was not seen in June (data not shown), indicating that meso- and macrozooplankton had a stronger control on microzooplankton community in August.

4.3 March summary

Ambient chlorophyll *a* concentrations were approximately twice as high in March than in the summer months, and chlorophyll *a* and peridinin growth rates were mostly negative, suggesting a declining dinoflagellate bloom (of *Gyrodinium instriatum*), a typical occurrence in the late winter in the NRE (Pinckney et al. 1998). The key factor moderating the phytoplankton community in March seems to be P-limitation. As can be expected under P-limiting conditions (e.g. Paerl et al 1998), N addition had no effect on community growth (Table 17). Adding to the lack of response to experimental treatments

in March were the environmental conditions typical of late winter/early spring months (low temperatures, low PAR) which are associated with slower plankton growth rates (Mallin et al. 1991). Microzooplankton (heterotrophic ciliate) abundances were low in March, and grazing rates tend to also be lower in the colder months (Mallin and Paerl 1994; Litaker et al. 2002), leading to a limited effect of grazer removal on phytoplankton growth.

4.4 Objective 1: *Compare the effects of DIN and DON on the estuarine phytoplankton community*

Nitrogen type is an important factor in phytoplankton community growth and in growth among phytoplankton groups. Responses of diagnostic photopigments to DIN vs. DON varied throughout the experimental events, indicating that responses to N type are affected by other environmental factors. Even so, while DON was sometimes more stimulatory than DIN among some phytoplankton groups (flagellates, diatoms and cyanobacteria), the opposite (DIN being more stimulatory than DON) never occurred. This effect is supported by other work showing DON to be a preferred N source in some coastal systems (Berg et al. 2001). Urea in particular can account for a major portion of N uptake by phytoplankton in coastal waters (Kudela and Cochlan 2000; Solomon et al. 2010). In the NRE, urea can account for almost 50% of N uptake in the mid-estuary region (Twomey et al. 2005), and in the Chesapeake Bay, at times can account for up to 80% of N uptake (Glibert et al. 1991). Urea loading may favor mixotrophic species by directly providing DON to these species, and also may provide an indirect source of N to a wide variety of phytoplankton species as ammonium after bacterial degradation of urea

(reviewed in Berman and Bronk 2003). Ammonium is a widely used and sometimes preferred N source for phytoplankton (Berg et al. 2001; Boyer and Christian 1994).

While N was limiting in both June and August, with DIN:DIP ratios of 2.2 and 1.6, respectively, it only stimulated community growth in June. The difference between these months is the DIN concentration, suggesting that when ambient DIN concentration is high (as in August) additional N loads of either type (DIN or DON) do not have a strong effect on community productivity. When initial DIN is low, however (as in June), N load and type is important. The effect of N loads having a positive effect on growth is intuitive for N-limited systems, but the effect of N type can be explained in several ways. DON concentrations were not as variable as other nutrients through the months, so the relative abundance of DON compared with DIN was higher in June than in August (even in nitrate addition treatments). According to Twomey et al. (2005), in the NRE, the relative importance of urea increases as its relative abundance increases, so the community in June may have been more suited for uptake of urea than nitrate. Additionally, increased temperatures are associated with higher bacterial productivity in the NRE (Peierls and Paerl 2010), suggesting faster bacterial turnover of urea to ammonium, which would increase community growth in response to urea additions.

Zeaxanthin had a range of responses to N additions throughout the year, which could be due to a variety of physical and chemical factors. The warmer temperatures in the summer months are more conducive for cyanobacterial growth (Paerl 2008), and in the NRE, picoplanktonic cyanobacteria are typically more prevalent in the summer (Gaulke et al. 2010; Wetz et al. 2011). Zeaxanthin did not respond to treatments in March likely because of the cooler temperatures or P-limitation. The standing crop of

cyanobacteria was much lower in March as well (absent from T0 enumeration counts, and zeaxanthin concentration in March was an order of magnitude lower than other months). In June there was higher zeaxanthin growth in response to urea than to nitrate, while in August there was no difference in zeaxanthin growth between N types. Cyanobacteria have been shown to utilize urea and DIN (e.g. Solomon et al. 2010), so the apparent preference for urea is not surprising. This difference could be due to higher DIN concentrations in August and higher nutrient regeneration rates associated with the positive community growth compared to June. Also, in June, when the ambient DIN pool was low, cyanobacteria were competing with other phytoplankton groups for access to nitrate, but had less competition for access to urea, which would promote higher cyanobacterial growth rates with urea additions. There were also variations in cyanobacterial community composition between June and August. The enumerated cyanobacterial community consistently contained Nostocales, though in August, additional taxa (e.g. *Spirulina* spp.) were also present. However, much of the cyanobacterial abundance in both months was likely picoplankton, so further differences in cyanobacterial community composition could not be assessed with cell enumeration.

As with chlorophyll *a*, alloxanthin only responded to N additions in June, but showed no preference for N type. While cryptophytes produce some of the largest blooms in the NRE (in terms of chlorophyll-*a* concentration) they do not seem to follow seasonal trends. They instead followed pulses of nutrient inputs (Pinckney et al. 1998), as was seen in June when alloxanthin growth responded to N additions. Some cryptophytes are mixotrophic in that they can ingest bacteria (Cloern and Dufford 2005), but other work has not shown a clear response of cryptophyte growth to DIN vs. DON

(e.g. Altman and Paerl 2012), so N type may not be as important as overall N quantity for cryptophyte growth.

Peridinin did not have increased growth with urea compared with nitrate for any of the three experiments. This was not expected, as dinoflagellates include many mixotrophic species (Burkholder et al. 2008), and in the nearby New River Estuary, NC, DON additions had a stronger positive effect on peridinin concentrations than DIN (Altman and Paerl 2012). The dinoflagellate community composition did shift throughout the year. June was strongly dominated by *H. rotundata* (~3200 cells/ml) and March by *G. instriatum* (~1600 cells/ml), while August had lower dinoflagellate abundances overall (~260 cells/ml; reflected in changes in peridinin concentrations through experiments). In both June and March, negative peridinin growth rates suggest declining blooms for each species, which can explain the lack of response to N type (March was also P-limited). *H. rotundata* and *G. instriatum* are both phagotrophic (Seong et al. 2006, Uchida et al. 1997), but no evidence of beneficial effects of urea on *H. rotundata* could be found, and *G. instriatum* has not been shown to directly utilize DON (Nagasoe 2010). In August, peridinin concentrations and dinoflagellate abundances may have been too low to capture a significant response to either form of N.

Not only was fucoxanthin the only pigment that consistently responded to N additions for all months, but it also responded more positively to urea than nitrate for two of the three experiments (June and March). While traditionally fucoxanthin is diagnostic of diatoms, in the NRE it can be an indicator for a larger variety of phytoplankton groups throughout the year (N. Hall, personal communication). In June, when fucoxanthin was representative of raphidophytes and *K. veneficum*, there was a more positive response to

urea than to nitrate. Not only are the major raphidophytes in the NRE mixotrophic (*Chattonella* spp. are phagotrophs and *Heterosigma akashiwo* is phagotrophic and directly utilizes urea; Burkholder et al. 2008) but both are potentially toxic species (Rothenberger et al. 2009). The ichthyotoxic *K. veneficum* (Kempton et al. 2002) is also phagocytic and directly utilizes urea (Burkholder et al. 2008). While this explains the increased fucoxanthin growth in response to urea compared to nitrate in June, it also hints that some harmful bloom-forming species may be favored with increased DON loads in the NRE.

In August, fucoxanthin was representative of raphidophytes and diatoms and there was no difference between N types reflected in fucoxanthin growth. In March, fucoxanthin was representative of diatoms and responded more positively to urea than nitrate, indicating uptake of urea or ammonium. As stated above, raphidophytes are mixotrophic, but the nutritional modes of diatoms are less clear. Some research shows urea utilization potential by diatoms (e.g. Lomas and Glibert 1999), but in the nearby New River Estuary, fucoxanthin was not stimulated by DON (Altman and Paerl 2012). , The varying responses of diatoms to N types in this experiment may be due to shifts in community composition and the ambient nutrients (though DIN and DON were high in March, ammonium was at its lowest concentration). Diatom abundance was highest in March, and the most taxa were identified in March as well, consisting of *Leptocylindrus* spp., *Skeletonema* spp., *Chaetoceros* spp., and *Cyclotella* spp., among others. These assemblages are consistent with previous diatom surveys in the NRE, and none are known to be harmful (e.g. Mallin et al. 1991; Rothenburger et al. 2009). Diatom abundance tends to be maximal in spring or summer in the NRE (Mallin et al. 1991;

Pinckney et al 1998). According to Pinckney et al. (1998), shifts in diatom abundance tend to be due to seasonal cycles, rather than sporadic nutrient loads. While the results of this experiment do indicate that diatoms have the potential to respond to ephemeral increases in N (as seen in August and March) and potentially more so to urea than nitrate, the responses were not consistent, which may reflect the potential for diatom species-specific responses to DON.

The results of this research indicate that DON is an important N source to estuarine phytoplankton and that increasing DON loads could be associated with increased overall phytoplankton productivity and bloom potential compared with DIN. This association is supported by other work worldwide, (e.g. Glibert et al. 2006), and has negative implications for systems such as the NRE that are currently facing increased DON loads (Stow et al. 2001). The amount and form of N can be an important determinant of phytoplankton species composition in the NRE (Rothenberger et al. 2009). Though N-limitation may be viewed as a dominant influence controlling the phytoplankton biomass and productivity of the NRE (Pinckney et al 1998; Rudek et al. 1991), as this work shows, N type should be recognized as an equally important factor.

4.2 Objective 2: *Explore how the top-down and bottom-up factors interact to influence the estuarine phytoplankton community.*

As verified in this study, DIN and DON are not the sole factors affecting phytoplankton growth; top-down effects also significantly structure the community. The effects that N loads and trophic interactions have on the phytoplankton community are regulated by other environmental controls, such as the concentrations of other nutrients,

namely P, which follows seasonal trends. The classic trend in the NRE is that of P-limitation in late winter/spring and N-limitation the rest of the year (Rudek et al. 1991). This trend is seen in many other eutrophic, temperate systems including the Chesapeake Bay (Fisher et al. 1992). In the late winter/spring, when river discharge is high, nitrate loading is typically highest (Rizzo and Christian 1996), while in the summer, surface DIN concentrations are typically low (Wetz et al. 2011). In the summer months, regeneration of N and P from the bottom sediments is the primary source of these nutrients to the phytoplankton community (Cowan and Boynton 1996).

Physical environmental controls also impact phytoplankton growth on seasonal scales. There is a relationship between the seasonal shifts in temperature and phytoplankton biomass (Wetz et al. 2011), and phytoplankton community composition in the NRE (Rothenberger et al. 2009; Wetz et al. 2011), though other factors impact phytoplankton assemblages as well, such as light availability. In temperate zones, PAR is dependent on the season (i.e. highest in the summer months, lowest in the winter months; Kuwahara et al. 2000) since it is based on the elevation of sun and day length. In the NRE, most primary production occurs during summer months when PAR is greatest (Mallin et al. 1991).

Phytoplankton grazers exhibit seasonal trends as well. Some suggest decreased grazing rates in winter (Mallin and Paerl 1994), and a shift in the relative importance of grazing and nutrients throughout the year (grazing in summer, nutrients in winter; Lewitus et al. 1998). These shifts occur in temperate systems worldwide (e.g. Tan et al 2004), and have implications for estuarine phytoplankton communities (e.g. allow bloom initiation; Buskey et al. 1997). Microzooplankton grazing can be important in regulation

of HABs (Calbet et al. 2003). Work by Buskey (2008) indicates that grazers are able to keep HAB species and other phytoplankton species under control when the abundance of phytoplankton is low, but once the abundance goes above a threshold level, grazers are no longer able to control the blooming species. The trigger that allows phytoplankton abundances to get above this threshold could be due to increased nutrient loads, or a disruption in grazers allowing for a "window of opportunity" for the phytoplankton species to bloom (Stoecker et al. 2008). The effects of trophic interactions on phytoplankton are particularly important because, while the effects of N loading are generally in the same direction (increased N loads lead to increased primary production), the effects of grazer reduction in this study were widely varied and associated with significant increases and decreases in pigment growth rates. Therefore, it is much more difficult to elucidate and predict trends involving trophic interactions, which is a critical component of effective management practices.

In June, both N additions and reduced grazing pressure affected the phytoplankton community growth rates, and there was a statistical interaction between N loading and reduced grazing pressure on chlorophyll *a* growth. Only in treatments when N was added did a reduction in grazing pressure have a significant effect on phytoplankton growth (Fig. 4). Additionally, treatments with N additions combined with removal of grazers > 20 µm had highest growth. This indicates that when manipulated together, these factors may have more impact on the phytoplankton community than each by itself, especially under the conditions of N-limitation and high grazer activity in the summer months in the NRE. As anthropogenic nutrient loads into the NRE are increasing (Stow et al. 2001),

the results of this research suggest the effects of top-down stressors may play a larger role in shaping phytoplankton communities than previously recognized.

In August, grazing pressure affected chlorophyll *a* growth, while effects of N addition were not seen. The warm temperatures in August promoted higher grazing, while high ambient DIN concentrations muted the effects of N additions. The incidence of trophic cascade in August indicates how sensitive the phytoplankton community is to shifts in the grazing community. Estuaries such as the NRE serve as nursery habitats, and support planktivorous fish and bivalves (e.g. Friedland et al. 1996; Selberg et al. 2001), both of which directly connect with planktonic trophic levels, and can regulate the abundance and composition of zooplankton (Horsted et al. 1988). Disruption to fish or bivalve communities, such as due to harvesting or habitat disruption, in the NRE and other temperate systems could have an abrupt effect on estuarine phytoplankton.

Chlorophyll *a* growth rates in March were not responsive to any treatment. River discharge tends to be highest in the late winter/early spring, as seen during these experiments (USGS river gauge data not shown, USGS 2001). While N concentrations were replete, P concentrations were limiting, indicating why growth was not stimulated by N addition. Grazing manipulations did not have an effect either, due to decreased grazing rates associated with cool winter/spring temperatures. Also, abundances of microzooplankton (as heterotrophic ciliates) were lower in March than in other months, in accordance with other studies suggesting that microzooplankton and mesozooplankton may not be as prevalent in the winter months (Mallin and Paerl 1994; Wetz et al. 2011).

While chlorophyll *a* integrates the overall effects of these two interacting factors, the trends exhibited by chlorophyll *a* are not representative of all assessed phytoplankton

groups. Both zeaxanthin and alloxanthin responded positively to N additions in at least one experiment, but neither responded to grazer manipulations. The absence of a response by both groups is supported by other research. Sellner et al. (1993) found that much of the cyanobacterial production on the upper Potomac River Estuary was not grazed upon, and that micro- and meso-zooplankton grazers had lower grazing rates on cyanobacteria than other phytoplankton assemblages. Cryptophytes can form large blooms in the NRE (Pinckney et al. 1998) and though these blooms may be related to N loads (Pinckney et al. 1998), Mallin and Paerl (1994) did not find an association between zooplankton grazing and cryptophytes abundance. However, Mallin and Paerl (1994) attributed this to a lack of grazer abundance during periods of high cryptophyte abundance, a trend not followed in this experiment.

Peridinin was affected by either top-down or bottom-up manipulations for each of the three experiments. While the response to N addition was only positive (as in June), there were both positive (June, March) and negative (August) responses to grazer removal. Peridinin growth was even influenced by grazers in March, when heterotrophic ciliate abundances were low. Dinoflagellates are a dominant component of the phytoplankton community in the NRE, and a major bloom-forming group, forming both nuisance and harmful blooms throughout the year (e.g. Hall et al. 2008) so it is particularly important to understand how the top-down and bottom-up factors interact over a seasonal scale.

Fucoxanthin was positively affected by grazer reduction (> 20 µm) in March, and consistently responded positively to N additions. In March, fucoxanthin represented diatoms, which tend to be most prevalent in the spring and early summer months in the

NRE (Pinckney et al. 1998). In general diatoms are non-toxic species (pseudo-nitzschia being the major exception), and are also readily grazed organisms (Paerl et al. 2003). Some of the most abundant diatom taxa identified in March, *Cyclotella* spp, and *Skeletonema* spp. are of an ideal size and shape to be grazed upon by zooplankton (Mallin and Paerl 1994). Even when ciliate abundances were low, diatoms may have been preferentially grazed upon, indicating why they responded positively to grazer removal. Preferential feeding is an important factor in structuring the planktonic community, (e.g. Stoecker and Sanders 1985) and is especially important since many harmful species may be avoided by grazers, in favor of more palatable groups like diatoms (Buskey 2008).

As seen in this study, bottom-up and top-down controls upon estuarine phytoplankton do not work independently. Though the relative effect of each factor does vary throughout the seasons, both are continuously affecting a part of the phytoplankton community. This work indicates that there may be more of a relationship between the top-down and bottom-up pressures than previously acknowledged in estuarine phytoplankton dynamics, and most importantly that the interactions between these factors need to be better understood for proper ecosystem-based management of estuaries.

Future work

Bioassays are a useful way to investigate top-down and bottom-up factors of phytoplankton growth in a controlled environment. The use of natural phytoplankton assemblages in this project allows for relevant and valuable information on the phytoplankton community response to multiple anthropogenic factors. The NRE is a

typical eutrophic estuary, and the conclusions from this research are applicable to many temperate estuaries undergoing similar eutrophication and anthropogenic impacts.

The broad scope of this work sets the foundation for more in-depth analysis of the interaction between nutrient loading and grazing pressure over daily, seasonal, and yearly timescales. The combination of bottom-up pressures (e.g. eutrophication) and the top-down pressures (e.g. fisheries exploitation) promote increased phytoplankton biomass, degraded habitat and decreased fish and shellfish biomass (Breitburg et al. 2009). Getting a better grasp of the immediate effects of changes in the top-down and bottom-up stressors on the phytoplankton community, how the phytoplankton community response shifts through the year, and how long-term changes in climate patterns influence these factors is essential for proper management of valuable estuarine systems.

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