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Composition of inorganic and organic nutrient sources influences phytoplankton community structure in the New River Estuary, North Carolina

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Abstract The New River Estuary, NC, is a nutrient-sensitive, eutrophic water body that is prone to harmful algal blooms. High annual loading from the watershed of varying nutrient forms, including inorganic phosphorus and inorganic and organic nitrogen, may be linked to the persistence of algal blooms in the estuary. In order to evaluate phytoplankton response to nutrient inputs, a series of in situ nutrient addition experiments were carried out during June 2010 to July 2011 on water from an estuarine site known to support algal blooms. Estuarine water was enriched with nutrients consisting of individual and combined sources of dissolved inorganic nitrogen, orthophosphate, urea, and a natural dissolved organic nitrogen (DON) addition derived from upstream New River water. The combined inorganic N and P addition most frequently stimulated phytoplankton biomass production as total chlorophyll *a*. The responses of diagnostic (of major algal groups) photopigments were also evaluated. Significant increases in peridinin (dinoflagellates), chlorophyll *b* (chlorophytes), and myxoxanthophyll (cyanobacteria) were most frequently promoted by additions containing riverine DON. Significant increases in zeaxanthin (cyanobacteria) were more frequently promoted by inorganic nitrogen additions, while increases in fucoxanthin (diatoms) and alloxanthin (cryptophytes) were not promoted consistently by any one nutrient treatment. Evaluating the impact of varying nutrient forms on phytoplankton community dynamics is necessary in order to develop strategies to avoid long-term changes in community structure and larger-scale changes in ecosystem condition.

Keywords Estuary · Eutrophication · North Carolina · Nutrients · Organic nitrogen · Photopigments · Phytoplankton

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Introduction

The abundance and composition of phytoplankton communities is controlled by resources available such as nutrients and light (Cloern 1999; Reynolds 2006). Inorganic nutrient control of phytoplankton abundance and species composition has been widely studied (Harrison and Turpin 1982; Sanders et al. 1987; Fisher et al. 1999). However, the direct effects of a bulk DON mixture on natural phytoplankton communities have been examined in only a few studies (Peierls and Paerl 1997; Seitzinger and Sanders 1999). Here, we used an in situ bioassay approach to examine the regulation of a natural phytoplankton community in a nutrient-sensitive estuary and its

stimulation of growth by inorganic nitrogen and phosphorus, urea, and riverine DON compounds.

Nutrient enrichment in coastal waters is an intensifying problem. Nitrogen (N) is of particular concern, because it is most often the nutrient controlling primary production and promoting eutrophication of these waters (Ryther and Dunstan 1971; Paerl 1988; Boesch et al. 2001; Conley et al. 2009). The microtidal New River Estuary (NRE), North Carolina (NC), is an example of a strongly N-limited system that has experienced negative effects of anthropogenic nutrient enrichment, including toxic algal blooms, hypoxia, fish kills, and benthic habitat degradation (Mallin et al. 1997, 2005; Tomas et al. 2007). The NRE receives external N inputs from a variety of sources, including surface runoff, municipal wastewater, the atmosphere, and groundwater (Mallin et al. 1997; 2005). These sources supply varying forms of N, including nitrate, nitrite, ammonium, and DON compounds.

DON is often the largest pool of fixed nitrogen in most aquatic environments (Bronk 2002). Due to the extremely heterogeneous and cryptic nature of the bulk DON pool, only a small portion of it has been identified and quantified. The DON bulk pool is of both abiotic and biotic origin and is delivered via surface runoff, riverine discharge, groundwater, and atmospheric deposition (Seitzinger and Sanders 1999; Bronk et al. 2007). Biologically produced dissolved combined amino acids and dissolved free amino acids originate from microbial sources (microautotrophs and heterotrophs), zooplankton grazing and excretion activities, and viral release from cell death. Humic acids, which can account for up to 70 % of the DON pool (Bronk et al. 2007), arise from microbial degradation and leaching of plant materials. Another DON compound, urea, is a by-product of biotic metabolism and is of particular interest because of the increasing amounts of urea in fertilizers and feed (Glibert et al. 2006). Moreover, urea loading is a distinct concern in eastern North Carolina watersheds, since it is a waste product from rapidly-proliferating livestock operations in this region.

In the past, DON had been considered to be largely unavailable for phytoplankton growth because of observations of consistently high, invariant concentrations of DON in aquatic systems (Bronk et al. 2007). Furthermore, its role was thought to be purely to support bacterial production and it was not considered directly available to phytoplankton (Bronk et al. 2007). More recent studies, however, have shown that DON compounds may be a source of bioavailable N for phytoplankton. Kudela et al. (2008) showed that multiple coastal Pacific Ocean (California) harmful algal bloom (HAB) species including two dinoflagellates and a raphidophyte exhibited a greater maximal uptake for urea and ammonium over nitrate. Phytoplankton are also able to use humic compounds in rivers and coastal environments (Bronk et al. 2007). Humics may be a source of usable N to some toxic dinoflagellate species, including *Alexandrium tamarense* (Gagnon et al. 2005) and *Alexandrium catenella* (Doblin et al. 2000). Urea uptake and stimulation of phytoplankton biomass has been shown in numerous studies (Antia et al. 1991; Glibert et al. 1991, 2001, 2004; Twomey et al. 2005). Ammonium (NH_4^+) is the most energetically favorable source of inorganic N, and direct evidence of nitrate (NO_3^-) uptake pathways present in phytoplankton cells supports its role as a major available source to algae (Owens and Esaias 1976), but inorganic N forms are not always the exclusive forms of N utilized. Enzymatic activity on the cell surface may enable phytoplankton to access the N associated with organic molecules (Palenik and Morel 1990). Phytoplankton can act as photo-heterotrophs and take up organic molecules via several pathways including active transport, use of proteolytic enzymes, pinocytosis, and phagocytosis (Bronk et al. 2007). Phytoplankton can compete with bacteria for nutrients when they are scarce, and species that can exploit organic nutrient sources may have a competitive advantage (Bronk et al. 2007). Photo-heterotrophic capability may be especially advantageous to phytoplankton that can utilize DON during high activity summer months when nitrate is depleted (Paerl 1991). Therefore, the role of riverine DON in driving phytoplankton production warrants investigation.

Most water quality management practices stress reducing total N inputs to N-sensitive estuaries (Bricker et al. 1999; Boesch et al. 2001). However, this approach does not distinguish between different N forms. In this study, the relationship between the chemical composition of nutrients entering the system, for example, organic and inorganic N forms and inorganic P (orthophosphate, PO_4^{3-}) and changes in phytoplankton biomass and community composition was explored. The following general research question was addressed: How do upstream-derived supplies of

DON, dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphorus (DIP) impact downstream estuarine phytoplankton productivity, biomass, and community composition?

Methods

Site description

The NRE is a relatively small, mid-Atlantic coastal plain estuary located in Onslow County, southeastern North Carolina, USA. Most of the estuary resides within the US Marine Corps Base Camp Lejeune (MCBCL). Jacksonville, a moderate size city (2009 Population: 80,500), is located on the upper part of the estuary on Wilson Bay (Fig. 1). With a surface area of 88 km^2 and a mean depth of 3 m (NOAA 1999), the NRE is a relatively broad and shallow estuary that is made up of a series of lagoons and is confined by barrier islands restricting water exchange with the Atlantic Ocean (Mallin et al. 2005). Flushing time in the NRE varies seasonally with storm and runoff events, ranging from 8 to 187 days, with a mean of 70 days (Ensign et al. 2004). The semi-lagoonal nature of the NRE plays a significant role in its sensitivity to nutrient inputs, since long flushing times allow more time for algal nutrient assimilation, growth, and "internal" nutrient recycling (Kennish and Paerl 2010). Similar to its neighboring semi-lagoonal estuaries to the north—the Neuse River Estuary and Pamlico Sound—the NRE experiences periodic phytoplankton blooms (including harmful species, Tomas et al. 2007) and seasonal periods of bottom water hypoxia (Mallin et al. 2005; Paerl et al. 2007, 2010).

Non-point nutrient sources dominate New River discharge to the estuary (H. W. Paerl et al., unpublished data). The NRE watershed is dominated by agricultural activities, including row crop and concentrated animal feeding operations (CAFOs; Mallin et al. 2005). Downstream, near the entrance to the NRE, there is a history of nutrient inputs from the Jacksonville municipal waste-water treatment plant, which promoted highly eutrophic conditions in the Wilson Bay area (Mallin et al. 1997, 2005). Upstream nutrient inputs associated with burgeoning CAFOs and row crop operations have increased significantly over the past several decades, leading to sustained eutrophication, including exceedances of the State of North Carolina's "acceptable" chlorophyll *a* concentration (40 μg L^{-1}) (NCDENR 2005), and HAB outbreaks (Mallin et al. 2005; Tomas et al. 2007).

Sampling stations

The nutrient enrichment bioassays utilized water from 2 sites: Station "7" which is located in the estuary (34°43′14″N, 77°25′20″W; Fig. 1); Gum Branch, which is located upstream in the river and is United States Geologic Survey (USGS) gaging station #02093000 (34°50′57″N, 77°31′10″W; Fig. 1). Water collected from Gum Branch served as a naturally rich DON source that was added to the Station 7 water to measure its effect on the natural phytoplankton community. Background nutrient concentrations, temperatures, and salinities were measured monthly at both sites from June 2010 to July 2011. Gum Branch nutrients were measured more frequently than monthly during some parts of the year as dictated by the USGS's sampling regime. Surface (0.2 m) water samples were collected and dispensed into 4-L polycarbonate bottles, and brought back to the laboratory for nutrient concentration measurement (method described below). Surface temperatures and salinities were measured using a YSI 6600 sonde. Water temperature for Station 7 ranged from 8.0 to 32.4 °C with an annual mean of 23.6 °C during the study period. Salinity ranged from 2.7 to 24 psu with an annual mean of 15.1 psu. At Gum Branch surface water temperatures ranged from 5.5 to 25.7 °C with an annual mean of 15.6 °C. This site is virtually fresh year-round (psu < 0.25).

Experimental design

The effects of upstream-derived DON and inorganic nutrients on phytoplankton productivity and biomass responses were examined using three nutrient addition bioassays conducted on June 14–22, 2010; September 20–28, 2010; and July 11–19, 2011. Phytoplankton-rich water for each bioassay was collected from near the surface of NRE Station 7 (Fig. 1) and immediately filtered through a 200-μm mesh screen on the sampling boat to remove zooplankton and particulate debris. Filtered water was dispensed into 20-L polypropylene carboys, returned to UNC-CH Institute of Marine Sciences (IMS), and transferred to a set of 4-L

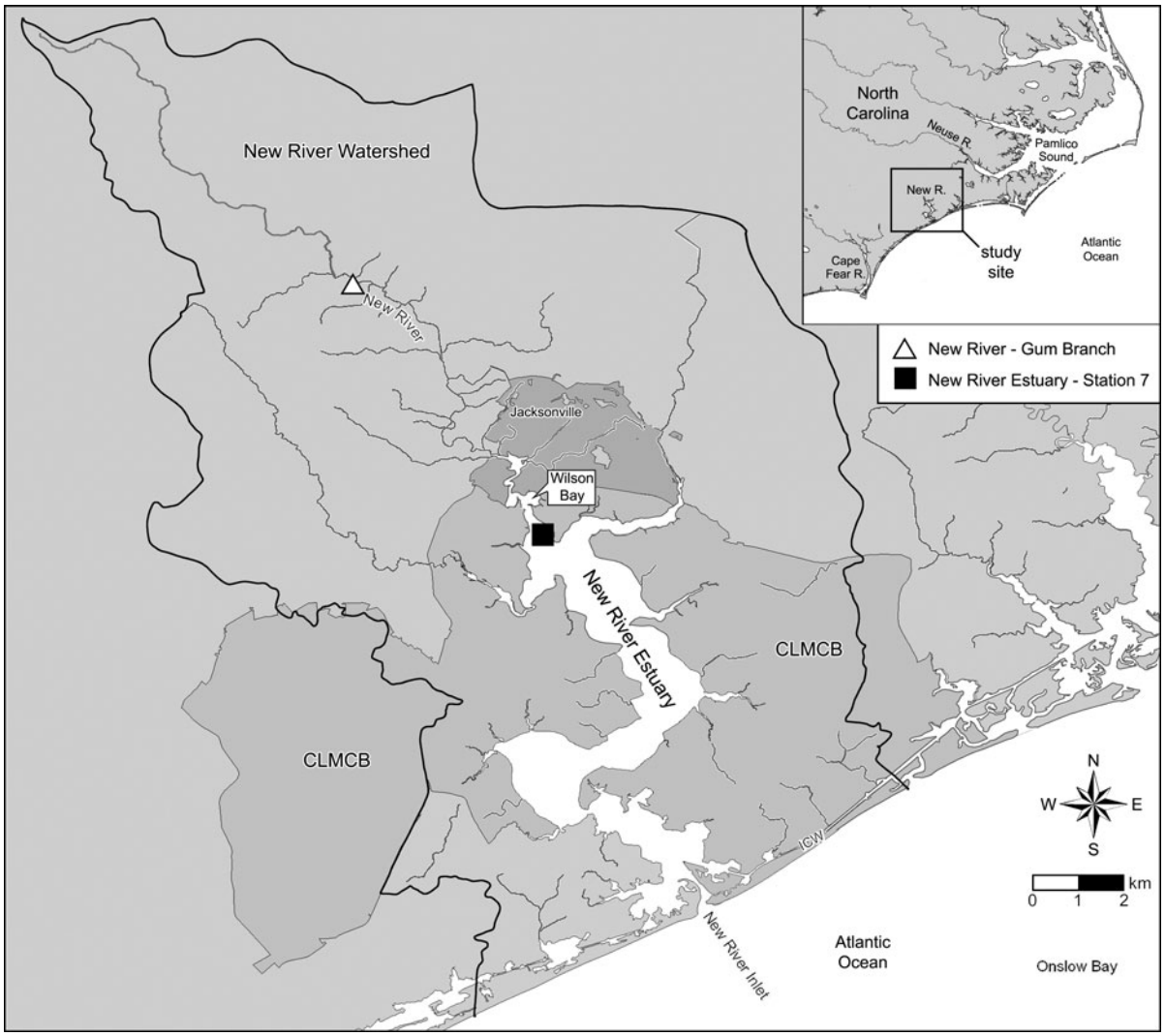
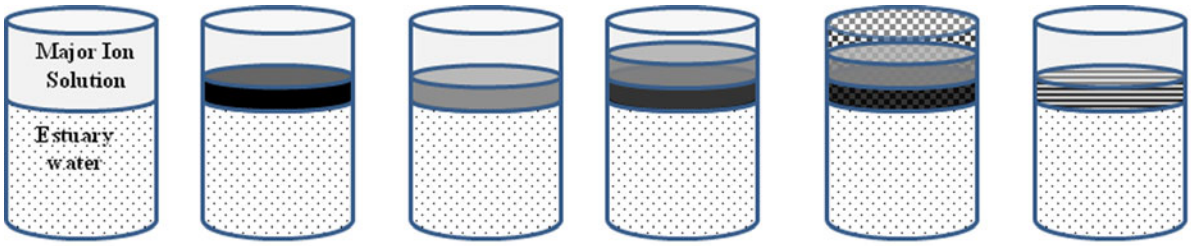


Fig. 1 Map of the New River Estuary, NC, showing its watershed and sampling locations for this study and the surrounding area comprising the Camp Lejeune Marine Corps Base (CLMCB). *Square symbol* marks Station 7, the sample site for the estuary water. *White triangle* is the USGS gaging and sampling station at Gum Branch

transparent polyethylene Cubitainers (Hedwin Inc.). Cubitainers are 80 % transparent to photosynthetically active radiation (PAR, 400–700 nm). Water was also collected from Gum Branch on the initial day of each experiment and brought back to the laboratory for immediate nutrient analysis (described below). Nutrients were added to the Cubitainers in replicates of four for each treatment type (Fig. 2). The first treatment (ORG RIVER) consisted of a 0.8 L addition of DON-rich Gum Branch water that had been filtered through 25-mm diameter Whatman glass fiber (GF/F) filters (0.7-μm pore size). Nutrient treatments were added to the remaining Cubitainers dissolved in 0.8 L of major ion solution (MIS; Paerl and Bowles 1987) in order to replicate the ionic properties of the Gum Branch filtrate addition (Fig. 2). Equivalent amounts of the inorganic nutrients measured in the Gum Branch filtrate were added as other treatments to the Cubitainers as follows: DIN (ammonium and nitrate); DIP (orthophosphate); DIN + DIP (nitrate, ammonium, orthophosphate); and urea (Fig. 2). For the urea treatment, urea was added at the same molar N concentration as was present in the DIN treatment in order to compare the responses per amount of N in



Control	+DIN	+DIP	+DIN + DIP	+ORG RIVER	+UREA
no nutrients added	addition of dissolved inorganic N equivalent to that in ORG RIVER	addition of dissolved inorganic P equivalent to that in ORG RIVER	addition of dissolved inorganic N and P equivalent to that in ORG RIVER	addition of upstream river water filtrate	addition of urea

Fig. 2 Diagram of bioassay treatments with each cylinder representing a Cubitainer from each treatment. Treatments consisted of 3.2 L of estuary water from Station 7 with a 0.8 L addition of either: MIS solution (control); filtered Gum Branch (New River) water (ORG RIVER); MIS solution containing dissolved inorganic nutrients equivalent to those in the Gum Branch water (DIN, DIP, DIN + DIP); or MIS solution containing dissolved urea with the same total N concentration as the Gum Branch water (Urea)

respective compounds. The control addition consisted of 0.8 L of MIS without any dissolved nutrients. Bioassays were incubated under natural light and temperature conditions by suspending the Cubitainers in the experimental ponds at IMS under a layer of neutral density screening ($\sim$60 % of surface irradiance) to avoid photoinhibition. Cubitainers were incubated for 8 days and subsampled for phytoplankton growth parameters on days 0 (T0), 2 (T2), 4 (T4), and 8 (T8).

At sampling time points, approximately 500 mL was collected from each Cubitainer in pre-cleaned (0.01N HCl followed by 2 deionized water rinses) polyethylene bottles. In the laboratory, water was partitioned into appropriate volumes for the measurements of (1) nutrient concentrations, (2) phytoplankton biomass (i.e., chlorophyll *a*), (3) primary productivity, and (4) photopigment concentrations to derive the taxonomic composition of the resident phytoplankton community.

Nutrient analysis

Sample water was gently filtered through pre-combusted 25-mm diameter Whatman GF/F filters (0.7-μm pore size). Filtrate was analyzed for total dissolved nitrogen (TDN), $NO_2^- + NO_3^-$ (NO_x), NH_4^+, and PO_4^{3-} using the Lachat/Zellweger Analytics QuickChem 8000 flow injection autoanalyzer, employing standard protocol (Lachat method numbers, 31-107-04-1-C, 31-107-06-1-B, and 31-115-01-3-C, respectively). DON concentrations were calculated by subtracting DIN from TDN.

Phytoplankton biomass (fluorometric chlorophyll a analysis)

Approximately 150 mL of sample water was vacuum-filtered onto 25-mm GF/F filters. Filters were stored at −4 °C until they were sonicated and extracted overnight in 90 % acetone. Extracted chlorophyll samples were analyzed using EPA method 445.0 without acidification on a Turner Designs TD-700 fluorometer.

Primary productivity

Primary productivity measurements were taken using the ^{14}C method according to Paerl (2002). Four light and one dark 76-mL polyethylene bottle per treatment type were filled with sample water and 0.3 mL ^{14}C–$NaHCO_3$ (2.8 μCi, 58 μCi μmol^{-1} specific activity: ICN Radiochemicals). The bottles were incubated in the IMS outdoor pond for approximately 4 h under one layer of neutral density screening, which reduced the incident light by about 20 %. Following incubation, samples were filtered onto 25-mm GF/F filters, which were then fumed for at least 2 h in a plastic container with concentrated HCl in order to remove abiotically precipitated ^{14}C–$NaHCO_3$. Filters were then placed in vials with 5 ml of CytoScint

scintillation cocktail and counted on a Beckman Coulter LS 6500 liquid scintillation counter. Dissolved inorganic carbon (DIC) content of the samples was determined using a Shimadzu Total Organic Carbon Analyzer (TOC-5000A0. Using the method of Paerl (2002), we determined total CO_2 fixed.

Community composition analysis

Major algal taxonomic groups were partitioned and identified using high-performance liquid chromatography (HPLC). We used a Shimadzu model LC-20AB HPLC equipped with a photodiode array spectrophotometric detector (Shimadzu SPD-M20AC) (Pinckney et al. 1996, 1998). Approximately 100-mL aliquots were filtered onto 25-mm diameter Whatman GF/F filters and frozen at −20 °C for 1–3 weeks until extraction. Filters were extracted in 100 % acetone via sonication (Sonics Vibra-Cell VCX130, 130 watts) for 15 s at an amplitude setting of 20 %. Extracts were stored at −20 °C for 1–5 days. Pigment extracts (200 µL) were autoinjected and separated using column configurations described by Pinckney et al. 2001. Pigments were identified and quantified based on their absorption spectra calibrated against pure pigment standards (DHI, Denmark). HPLC results were periodically verified using inverted microscopy of preserved (1 % Lugol's solution) water samples (Utermöhl 1958).

Statistical analysis

Responses to nutrient additions (chlorophyll *a*, ^{14}C fixation, and accessory pigment concentrations) were analyzed using repeated measures analysis of variance (RM-ANOVA) in the JMP statistical software program. A Dunnett's test ($\alpha = 0.05$) was used for post hoc multiple comparisons of means for significant response factors.

Results

Background nutrient concentrations at Gum Branch and Station 7

From June 2010 through July 2011, high nutrient concentrations upstream at Gum Branch (ORG RIVER sample site) were paired with low concentrations downstream at Station 7 (Fig. 3). Nitrate/nitrite

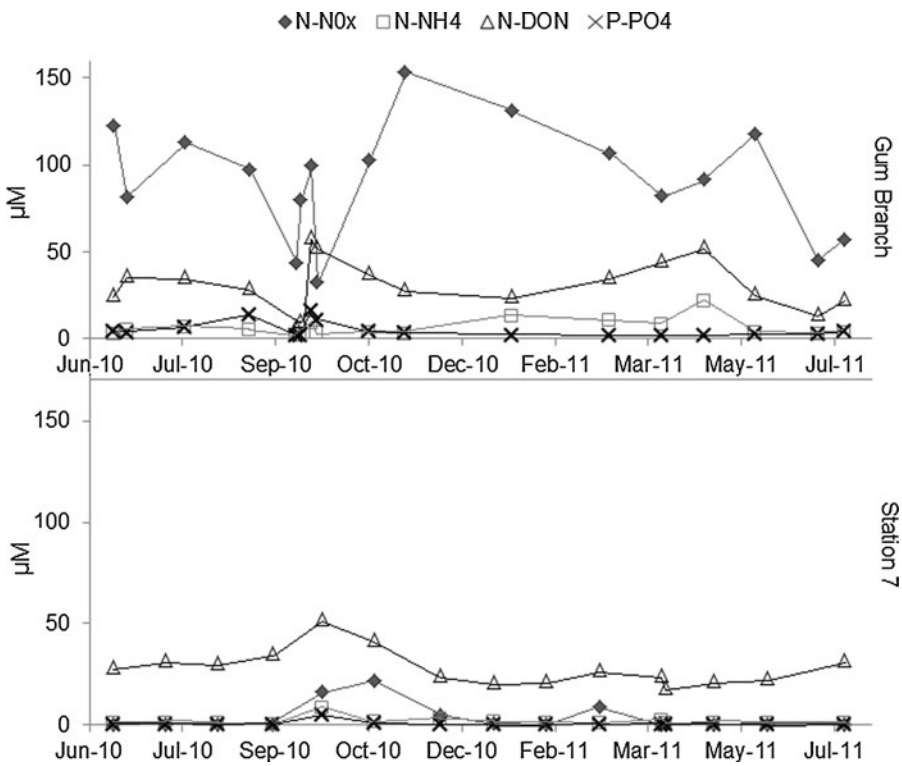


Fig. 3 Surface nutrient concentrations at Gum Branch and Station 7 on the New River measured in June 2010 through July 2011. Estimates for minimum and maximum relative standard deviation (RSD) for analytical replicates were 0.28–1.51 % RSD for NO_x, 0.28–1.95 % RSD for NH_4^+, 0.29–3.76 % RSD for PO_4, and 0.44–2.94 % RSD for TDN. DON was calculated by procedure described in the "Methods"

($N–NO_x$) concentrations at Gum Branch in ranged from 33 to 154 μM with an annual mean of 91 μM. Ammonium ($N–NH_4^+$) concentrations ranged from 1.5 to 13 μM with an annual mean of 7 μM. Orthophosphate ($P–PO_4^{3-}$) concentrations ranged from 1.6 to 16 μM with a mean of 4.9 μM. N–DON concentrations ranged from 7 to 54 μM with a mean of 27 μM. Station 7 had markedly lower nutrient concentrations, with $N–NO_x$ ranging from below detection limit (BDL) to 21.6 μM with an annual mean of 3 μM. $N–NH_4^+$ ranged from 0.5 to 8 μM with a mean of 1.6 μM. $P–PO_4^{3-}$ ranged from 0.1 to 5 μM with a mean of 0.8 μM and N–DON ranged from 21 to 52.6 μM with a mean of 31 μM.

Bioassay nutrient concentrations

Initial concentrations of nutrients present in the control and treatment Cubitainers varied for each experiment, since background nutrient concentrations at sampling sites varied over time. Mean ambient nutrient concentrations in Cubitainers for each treatment on initial days of experiments are shown in Table 1. Concentrations were measured in control and ORG RIVER treatments, and values were extrapolated to other treatments based on nutrient types that were added. Externally supplied molar inorganic N/P ratios varied for each bioassay. In the control, the external molar N/P ratio was less than 16 for the majority time points except for those of the June 2010 bioassay (Fig. 4).

Primary productivity and phytoplankton biomass

Primary productivity rates in the DIN and DIN + DIP treatments were significantly higher than the controls 50 % of sampling times (Fig. 5). Significant increases were observed in the urea treatment 38 % of the time sampled, the ORG RIVER addition 25 % of the time, and the DIP addition only 12 % of the time (Fig. 5). In the June 2010 bioassay, primary productivity rates were greatest in the DIP treatment with the maximum value observed for all three experiments occurring on day 8 in this treatment type. In September 2010, measurements taken on T2 and T4 (T8 results were omitted due to set-up error) showed significant primary productivity responses to N-containing treatments. Measurements taken in July 2011 showed a similar trend of significant responses in primarily N-containing treatments, but no significant responses in any treatments were observed

Table 1 Mean ambient nutrient concentrations in Cubitainers for each treatment measured on the initial day of experiment

	Treatment					
	Control	DIN	DIP	DIN + DIP	ORG RIVER	Urea
June 2010						
$N–NO_x^-$	BDL	24.1	BDL	24.1	24.1	BDL
$N–NH_4^+$	0.6	1	0.6	1	1	0.6
$P–PO_4^{-3}$	0.1	0.1	0.9	0.9	0.9	0.1
N–Urea	–	–	–	–	–	25.1
N–DON	27	27	27	27	32	27
Sept 2010						
$N–NO_x^-$	BDL	8.6	BDL	8.6	8.6	BDL
$N–NH_4^+$	0.7	1	0.7	1	1	0.7
$P–PO_4^{-3}$	0.2	0.2	0.6	0.6	0.6	0.2
N–Urea	–	–	–	–	–	9.6
N–DON	22.9	22.9	22.9	22.9	24.4	22.9
July 2011						
$N–NO_x^-$	0.1	14.4	0.1	14.4	14.4	0.1
$N–NH_4^+$	0.9	1.7	0.9	1.7	1.7	0.9
$P–PO_4^{-3}$	0.1	0.1	1.1	1.1	1.1	0.1
N–Urea	–	–	–	–	–	16.1
N–DON	20.2	20.2	20.2	20.2	25.9	20.2

Concentrations were measured in control and ORG RIVER and then extrapolated to other treatments based on treatment type.
BDL = below detection limit. Dash indicates value not measured or reported. All concentrations are reported in μM

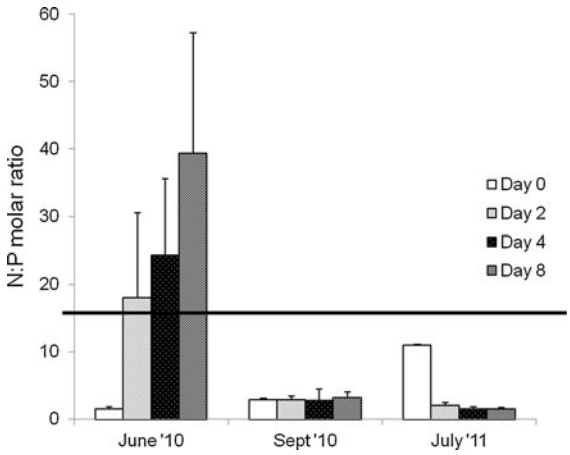


Fig. 4 Dissolved inorganic N/P molar ratios in control Cubitainers on sampling days. Values are the mean ($n = 4$) ± 1 standard deviation (SD). *Horizontal line* indicates N:P = 16

on the last day of this bioassay, indicating that phytoplankton net growth had greatly decreased in all Cubitainers by this point (Fig. 5).

Phytoplankton biomass response (measured as chlorophyll *a*) was most often positively stimulated by the treatments containing both N and P additions. Phytoplankton biomass was observed at significantly elevated levels in the DIN + DIP treatment 78 % of the time sampled. The ORG RIVER and urea treatments were the next most stimulatory treatments, with phytoplankton biomass being significantly higher than the control 66 % of the time sampled (Fig. 5). DIN was stimulatory 22 % of the time sampled, and significant responses were observed in the DIP treatment only on two out of the 9 time points: days 4 and 8 of the June experiment. However, phytoplankton biomass values observed on day 8 of the June bioassay in the DIP treatment were the highest of all time points during all three experiments (Fig. 5). In September and July experiments, significant responses were primarily seen in N-containing treatments on T2 and T4 with biomass falling in all treatments on T8. Minimum primary productivity rates and phytoplankton biomasses for all three experiments occurred in the

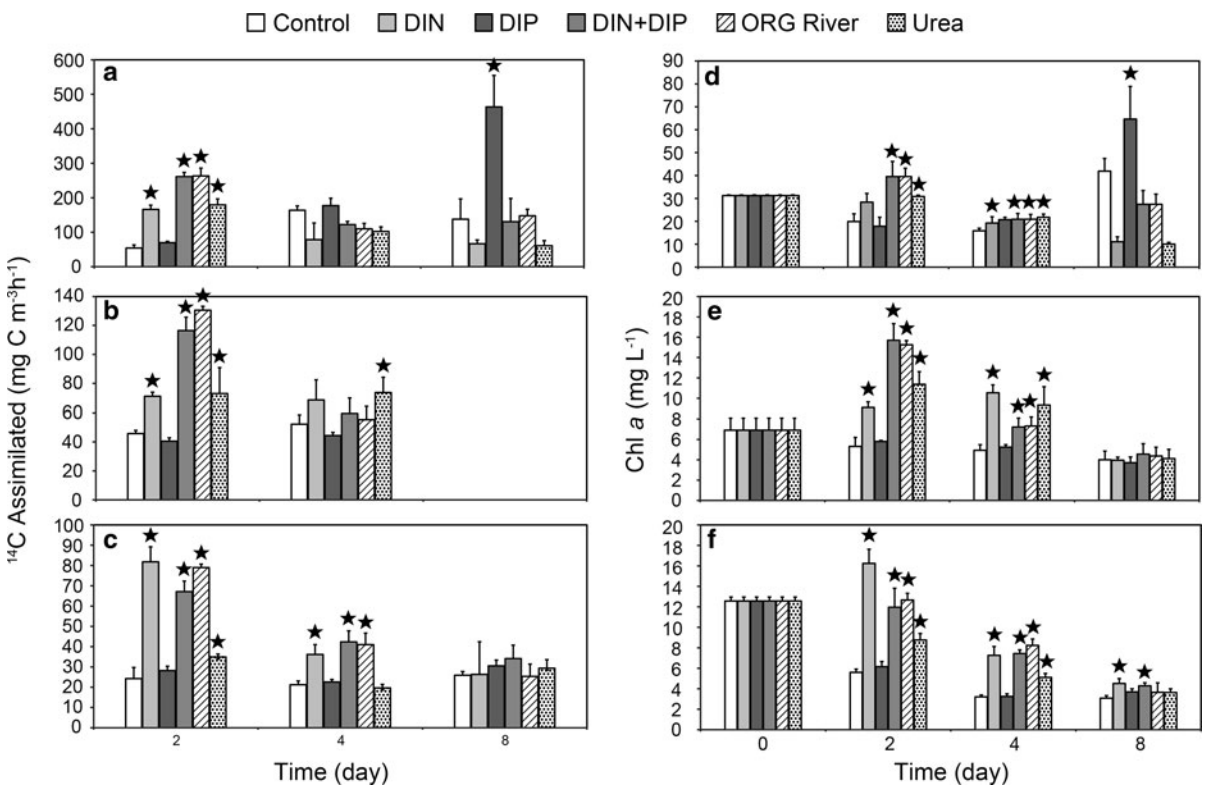


Fig. 5 Primary productivity rates in the control and five treatments in June 2010 (**a**), September 2010 (**b**), and July 2011 (**c**). Chlorophyll *a* in the control and five treatments in June 2010 (**d**), September 2010 (**e**), and July 2011 (**f**). Value representation is the same as Fig. 4. Note changes in *y*-axis scale for graphs, indicating differing ranges in response values. *Indicates response was significantly ($p < 0.05$) greater than the control using Dunnet's post hoc test

control and urea treatments of the July 2011 bioassay on T4 and T8 (Fig. 5).

Photopigment analysis of phytoplankton community structure

High-performance liquid chromatography was used to identify and quantify multiple diagnostic phytoplankton group pigments present in experimental sample water from each sampling time point (Fig. 6). Variations in phytoplankton diagnostic pigments showed shifts in community composition (relative to controls) in some treatments over the course of each bioassay (Fig. 6). In the June 2010 bioassay, the initial pigment composition was dominated by fucoxanthin, zeaxanthin, and peridinin, respectively (indicative of diatoms, cyanobacteria, and dinoflagellates; Jeffrey et al. 1997; Lewitus et al. 2005). These three groups remained dominant in all treatments as well as the control until T4, when the dominant three pigments found in the control and DIP treatments shifted to all cyanobacterial associated pigments (zeaxanthin, myxoxanthophyll, and echinenone; Jeffrey et al. 1997; Paerl et al. 2003). By day 8, these cyanobacterial pigments were dominant in all treatments and peaked at well-above bloom concentrations (>40 μg L^{-1}, NCDENR 2005) in the DIP treatment (Figs. 5, 6). Meanwhile, by day 8, fucoxanthin had diminished to undetectable levels in all but the DIN and urea treatments. Peridinin increased dramatically in the ORG RIVER treatment and then proceeded to drop in all treatments (Fig. 6).

In the September 2010 bioassay, all pigments except for myxoxanthophyll experienced a net decrease from the initial to last day of incubation in all treatments (Fig. 6). The most abundant pigments in the initial water were zeaxanthin, fucoxanthin, and chlorophyll *b,* respectively. Although zeaxanthin (cyanobacteria) decreased in all treatments over time, abundance-wise it remained a dominant pigment relative to the other pigments. Fucoxanthin (diatoms) and chlorophyll *b* (chlorophytes, Jeffrey et al. 1997)

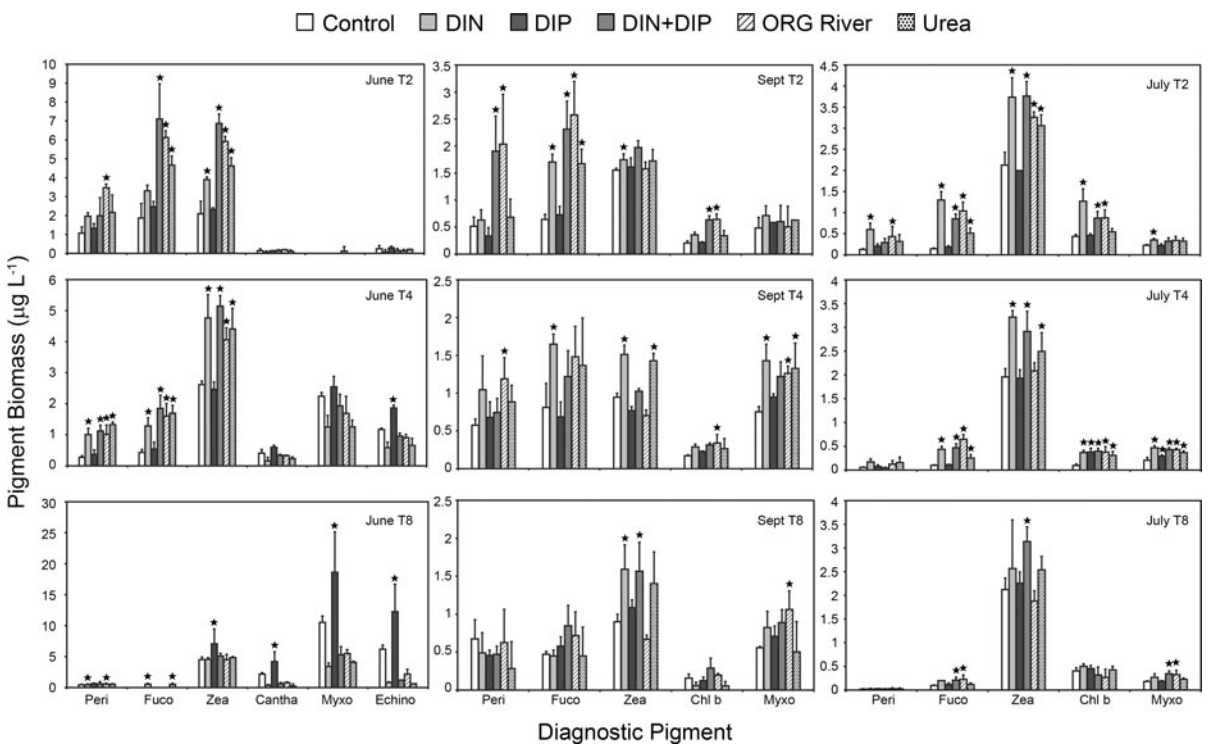


Fig. 6 Biomass of different phytoplankton diagnostic group pigments (deleting pigments whose biomass were <5 % of the total biomass) in the control and five treatments on indicated sub-sampling days. Value representation is the same as Figs. 4 and 5. *Indicates treatment types that yielded pigment responses significantly ($p < 0.05$) greater than the control using Dunnet's post hoc test

increased slightly in the DIN + DIP and ORG RIVER treatments before subsiding in all treatments. Peridinin (dinoflagellates) started off at the lowest concentration of all pigments measured and then increased slightly on day 2, with the largest increase occurring in ORG RIVER treatment. The only pigment that experienced a net increase over the 8-day bioassay was myxoxanthophyll (cyanobacteria), with the largest net increase occurring in ORG RIVER.

The July 2011 experiment exhibited the most consistent community composition trends between treatments (Fig. 6). The two dominant pigments (higher than the other pigments by at least 1 μg L^{-1}) in the initial water were zeaxanthin and chlorophyll *b*. Zeaxanthin (cyanobacteria) had the highest concentration in all treatments and at all time points throughout the experiment. Conversely, chlorophyll *b* (chlorophytes) decreased in all treatments through days 2 and 4 and increased slightly in all treatments on day 8. Fucoxanthin and peridinin decreased stepwise in all treatments over the 8 days, but peridinin decreased less rapidly in DIN and ORG RIVER treatments. Myxoxanthophyll increased from the least prevalent pigment in the initial water to one of the more dominant pigments by day 4 (Fig. 6).

Summary of significant photopigment responses in bioassay treatments

Concentrations of the photopigment peridinin (dinoflagellate biomass) were more frequently stimulated by ORG RIVER than any other nutrient treatment (Fig. 6). Peridinin concentrations were significantly ($p < 0.05$) higher than those of the control in the ORG RIVER treatment 56 % of the time sampled compared to only 22 % of the time in DIN and DIN + DIP, and 11 % of the time in DIP and urea. Chlorophyll *b* and myxoxanthophyll concentrations were more frequently stimulated by ORG RIVER than the other nutrient additions as well. Chlorophyll *b* (chlorophytes) concentration was significantly stimulated in ORG RIVER 56 % of the time compared to 44 % of the time in DIN and DIN + DIP. Myxoxanthophyll (cyanobacteria) was significantly stimulated in ORG RIVER 44 % of the time compared to 33 % of the time in DIN and DIN + DIP. Fucoxanthin (diatoms) showed an equal number of significant responses across treatment types DIN, DIN + DIP, ORG RIVER, and urea (Fig. 6). Alloxanthin, a marker pigment for cryptomonads (Jeffrey et al. 1997), was only present in minor quantities and therefore is not displayed with other dominant pigment groups in Fig. 6. However, this pigment was observed at significantly elevated levels (22 %) of the time sampled in DIN, ORG RIVER, and urea treatments.

Discussion

Phytoplankton response to various nutrient additions

Riverine loading is the dominant source of allochthonous nutrients to the NRE (Hall et al. 2011, in preparation). Thus, elevated nutrient loads from upstream are transported downstream to the estuary where the physical conditions including lowered turbidity, relatively long residence time, and periodic vertical stratification are favorable for excessive algal growth. This study demonstrated that nutrient enrichment was likely an important factor promoting phytoplankton growth, but there were substantial differences in terms of the magnitudes and types of phytoplankton community stimulation promoted by individual and combined forms of N and P. Total biomass was most frequently stimulated by DIN + DIP, followed by ORG RIVER, urea, DIN, and DIP, respectively. Total phytoplankton biomass response to nutrient enrichment may have been linked to nutrient limitation, which is indicated by external molar N/P ratio. Molar ratios of N/P less than 16 indicate nitrogen limitation (Redfield 1958). The N/P ratios were consistently less than 16 in the water during the majority of the experiments (Fig. 4), which indicates N-limitation in NRE estuary. This observation agrees with other findings that the NRE is strongly N-limited (Mallin et al. 1997, Hall et al., in preparation). In the present experiment, production of new biomass was more often stimulated by nutrient additions containing additional N and P rather than N alone (Fig. 5). An explanation for the strong response to combined N and P may be that single nutrient enrichments by either N or P alone may quickly induce limitation by the nutrient not supplied (Elser et al. 2007). Phosphorus limitation is generally indicated by N/P ratios greater than 16 (Redfield 1958; Smith 1990; Justić et al. 1995). The molar N/P ratio of the control water was significantly greater than 16 on day 8 of the June 2010

bioassay (Fig. 5). The only treatment that elicited a significant chlorophyll response at this time point was DIP, suggesting P-limited conditions, which was also indicated by the molar ratio of the soluble nutrients. Moreover, high chlorophyll *a* and cyanobacterial pigment concentrations indicated that a cyanobacterial bloom was present at this time (Figs. 5, 6). Microscopic examination (not shown) indicated that the bloom was composed of a heterocystous cyanobacterium (*Anabaenopsis* sp.). Since the phytoplankton in these Cubitainers had been supplied with extra P, *Anabaenopsis* sp. was likely able to grow and supply itself with N through N_2-fixation (a high frequency of heterocysts was observed, indicative of N_2 fixation). Cyanobacteria likely did not thrive in the other Cubitainers due to constraints on P supply. This series of experiments agreed with findings in other studies that urea additions can stimulate phytoplankton growth (Twomey et al. 2005; Glibert et al. 2006). Elevated chlorophyll *a* levels were seen more often in the urea treatment than in the DIN treatment. These results warrant further investigations into urea and its sources in our rivers and estuaries, since urea may be readily hydrolyzed to ammonia and utilized as an N source by phytoplankton (Glibert et al. 2006).

Implications for bloom formation of specific taxa

Phytoplankton groups that were promoted by specific nutrient forms and combinations varied in their response. The most common photopigments observed were peridinin, zeaxanthin, fucoxanthin, myxoxanthophyll, and chlorophyll *b*. Elevated peridinin levels were linked to ORG RIVER additions (Fig. 6). The link between organic N supply and dinoflagellate production has been shown in other studies in recent years (Palenik and Morel 1990; Doblin et al. 2000; Gagnon et al. 2005). Myxoxanthophyll (cyanobacteria) and chlorophyll *b* (chlorophytes) were also more frequently stimulated by ORG RIVER, indicating that phytoplankton from these groups were able to exploit some portion of the DON pool as well. A recent study by Wawrik et al. (2009) demonstrated the ability for a cyanobacterium, *Synechococcus* spp., to actively incorporate N from urea, glutamate, and a mixture of 16 amino acids. In contrast, alloxanthin (cyptophytes) and fucoxanthin (diatoms) did not appear to be significantly stimulated by the dissolved organic N additions. Accordingly, in the same study by Wawrik et al. (2009), diatoms did not incorporate N from amino acids or glutamate, suggesting that members of this phytoplankton group may not exploit N resources in the DON pool.

The possible link between organic N enrichment and enhanced growth of dinoflagellates and cyanobacteria may have significant implications for eutrophication in nutrient-sensitive waters such as the NRE. Dinoflagellates are the dominant HABs in the NRE (Mallin et al. 2005; Tomas et al. 2007), and cyanobacteria have been noted as dominant taxa in the freshwater and brackish regions of North Carolina estuaries, including the NRE (Paerl et al. 2007; Tomas et al. 2007). Blooms of both groups have been linked to the production of toxic metabolites, bottom water oxygen depletion, reduction in water clarity, and successional adverse effects on fish and shellfish habitat (Paerl et al. 1998; NCDENR 2005; Diaz and Rosenberg 2008).

Conclusions and future work

Using three in situ bioassay experiments, we evaluated phytoplankton response to inorganic N and P, a known organic N compound (urea), and a bulk DON mixture derived from New River water. However, individual organic compounds present in the bulk organic mixture were not measured. This makes interpretation of the specific effects of DON enrichment challenging, since organic compounds may go through a series of biochemical transformations before being used by phytoplankton and the role of bacteria may be important in mediating these processes. A somewhat unexpected finding in our study may be attributed to the role of bacteria. We found that total phytoplankton biomass was less frequently stimulated by the ORG RIVER addition than by the DIN + DIP addition despite the two treatments having equal concentrations of inorganic nutrients and ORG RIVER having more TDN (Table 1; Fig. 4). This suggests that inhibition of phytoplankton growth may have been taking place in response to the river water filtrate treatments on some occasions. Competition for inorganic N resources between phytoplankton and bacteria and suppression of phytoplankton growth has been noted by Bronk et al. (2007). Because it was filtered through 0.7-μm pore size GF/F filters, the river filtrate likely still contained some bacteria. The major ion solution, which was used to account for dilution in the

inorganic nutrient additions, was made up of deionized water and assorted minerals and did not contain bacteria. Therefore, all of the additions except for ORG RIVER likely had lower bacterial abundances, which may have translated to less competition pressure on phytoplankton for nutrient resources and greater phytoplankton biomass responses.

In estuaries, where nutrient loading is often dominated by river discharge, nutrients are delivered in various forms and magnitudes, which can impact phytoplankton community structure (Valdes-Weaver et al. 2006; Paerl et al. 2007). Evaluating the potential for these various nutrient forms to promote phytoplankton growth, especially by HAB species, is important in order to develop targeted, nutrient-specific coastal management strategies. Future work evaluating nutrient resources use should continue to use naturally occurring phytoplankton communities rather than axenic cultures since they may not be representative of the ecosystem response. Additionally, as advanced techniques are developed that can characterize more components of the DON pool, these compounds should be delineated in eutrophic environments and evaluated for availability to estuarine primary producers. DON utilization research of this nature could also be illuminated by screening for enzymes responsible for metabolizing organic compounds, which would augment evidence confirming phytoplankton utilization of these compounds.

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