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The Bioavailability of Effluent-derived Organic Nitrogen along an Estuarine Salinity Gradient

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Abstract Total maximum daily loads for nitrogen (N) are currently being established for the Chesapeake Bay watershed. While we know inorganic N is bioavailable in the environment and therefore its input contributes to cultural eutrophication, the bioavailability of organic N is unclear. Using bioassay experiments, we examined the impact of effluent-derived organic nitrogen (EON) from wastewater treatment plants on natural water samples collected along an estuarine/salinity gradient within the lower Chesapeake Bay watershed. All of the inorganic N and between 31% and 96% of the EON was removed during biotic bioassays within the first 2 days. Further, there was substantial abiotic reactivity of effluent N when it was added to natural water samples. Results demonstrate that organic and inorganic N in effluent is removed to support the growth of microbial communities. These are the first results aimed at assessing the reactivity of EON in natural waters along an estuarine/salinity gradient.

Keywords Effluent · Organic nitrogen · Wastewater treatment plants · Chesapeake Bay

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Introduction

Eutrophication has severely impacted estuaries and coastal waterways worldwide (Howarth and Marino 2006; Paerl 1997), including the Chesapeake Bay watershed (Kemp et al. 2005). Total nitrogen (TN) inputs to aquatic systems in the USA have increased over the past 50 years due to human activities (Howarth et al. 2002). In the Chesapeake Bay system, TN loads are thought to have increased by a factor of 6-8 since the 1800s (Boynton et al. 1995). As a result of this nutrient over-enrichment, coastal ecosystems are increasingly experiencing excess algal production (eutrophication), coastal hypoxia, and fish kills (Conley et al. 2009).

Despite years of targeted efforts to reduce nutrient loads to the Chesapeake Bay system, the Bay remains on the impaired waters list (US Environmental Protection Agency). In order to decrease total nutrient loads to the watershed in response to the Chesapeake 2000 Agreement, tributary strategies were developed to limit point and non-point source nutrient discharges to the estuary; in many cases, this resulted in wastewater treatment plants (WWTPs) aiming to achieve TN concentrations as low as 3 mg L^{-1} (Chesapeake Bay Program 2006). Because the voluntary goals laid out in the Chesapeake Bay 2000 Agreement had not been met by 2010, as specified in two consent decrees settling two lawsuits [the American Canoe Association, Inc. and the American Littoral Society v. EPA,

Civil No. 98–979–A (E.D. Va) and Kingman Park Civic Association, et al. v. U.S. EPA, et al., No. 1:98CV00758 (D.D.C.) (Federal Register 2009)], total maximum daily loads are being set for the various Chesapeake Bay subwatersheds. These will require mandatory nutrient reductions from point and non-point sources.

Because point source discharges, such as those coming from WWTPs, are easier to regulate through standard permitting processes, they continue to be targeted for additional load reductions. Load reductions below 3–8 mg N L^{-1} are costly for treatment plants (e.g., Fleischer et al. 2005). Because of this expense, it was recently argued that recalcitrant N, or N that is not biologically available in the environment, should not be counted when establishing permit discharge limits and allowances (Mulholland et al. 2008). While it is widely recognized that all dissolved inorganic N (DIN) is bioavailable to most aquatic microbes (including bacteria and phytoplankton), dissolved organic N (DON) is thought to be less bioavailable, particularly to phytoplankton. However, recent research has shown that a variety of DON compounds are directly bioavailable to natural plankton communities (reviewed in Berman and Bronk 2003; Mulholland and Lomas 2008).

WWTPs release varying amounts of inorganic and organic nitrogen into natural systems depending on the volume and type of plant influent, the processes employed for nutrient removal, and the plant's operating efficiency. More advanced WWTP designs are being employed in nutrient-sensitive watersheds like the Chesapeake Bay. Treatments are designed to remove both nitrogen and organic carbon from wastewater, and do so by employing sequential nitrification and denitrification zones using any one of a range of reactor configurations (Grady et al. 1999). These biological nutrient removal (BNR) systems are very effective in removing dissolved inorganic nitrogen from wastewater (Grady et al. 1999); however, removal of DON using this process is less efficient (Pagilla et al. 2006). Consequently, discharges from enhanced BNR plants that achieve lower TN effluent concentrations have a higher proportion of DON in their final effluent (Pagilla et al. 2008). While DON can be a component of the influent N entering the WWTP, much of the DON in the final treated effluent may be produced by treatment plant microbes themselves (Parkin and McCarty 1981b, 1981c; Pehlivanoglu-Mantas and Sedlak 2008).

DON that is released to the environment can undergo chemical transformation as a result of biotic (e.g., biologically mediated uptake and degradation; Berman and Bronk 2003; Mulholland and Lomas 2008) and abiotic (e.g., photochemistry and salt effects; McCallister et al. 2005; See and Bronk 2005) reactions. For discharges that flow from freshwater into saline estuaries and the ocean, the biotic and abiotic reactivity of N species in treated effluents (including DIN, DON, and colloidal or particulate N) in the proximate receiving waters may be different from its reactivity downstream. As such, assessing the bioavailability and reactivity of effluent-derived organic N (EON) in both the immediate and downstream receiving waters is necessary for determining the impact of EON in the environment and for setting N discharge allowances for WWTPs that will be protective of water quality in receiving waters, as specified in the Clean Water Act.

The bioavailability of bulk, uncharacterized DON entering aquatic systems has been shown to vary depending on its source (e.g., atmospheric, run-off from forested, agricultural, and urban/suburban lands). Bioassay experiments showed that DON bioavailability varied depending on its terrestrial or atmospheric source and that photochemical and microbial degradation may play a role in DON bioavailability in rivers and bays across the USA (Seitzinger and Sanders 1999; Seitzinger et al. 2002; Wiegner and Seitzinger 2001; Wiegner et al. 2006). Research has also been conducted to determine the bioavailability of effluent N and phosphorus (P) to freshwater and marine phytoplankton (Dunstan and Menzel 1971; Lindehoff et al. 2009; Middlebrooks et al. 1971; Oswald and Golueke 1967) but effluents tested consisted mainly of DIN in the form of ammonium (NH_4^+) or nitrate (NO_3^-). The bioavailability of effluent-derived DON in the environment has only been assessed to date using inoculums from the WWTP biota (Parkin and McCarty 1981a) or in the freshwater green alga, *Selenastrum capricornutum* (Pehlivanoglu and Sedlak 2004; Urgun-Demirtas et al. 2008). Many existing BNR plants discharge into watersheds that drain into estuarine or marine environments where microbial diversity is high and varies along the salinity gradient. Assessing EON bioavailability in estuarine and coastal receiving waters such as the Chesapeake Bay watershed is complicated by the fact that estuaries have salinity gradients along which physical, chemical, and biological properties vary. Generally, as material discharged into freshwater moves through an increasingly saline estuary, the microbial community changes and primary productivity becomes increasingly limited by N (versus P in freshwater) (Fisher et al. 1999). These N-limited estuarine and oceanic microbes are capable of using a broad spectrum of inorganic and organic compounds (Berman and Bronk 2003; Mulholland and Lomas 2008).

Currently, there are no established methods for assessing the bioavailability of EON in the environment. Any assessment of bioavailability of this material to natural microbial communities must be sensitive to the variability in community response due to seasonality, as well as estuarine processes such as tidal forcing and mixing, climatological forcing that affect freshwater flow and temperature, and time-varied exposure to full-spectrum natural light. Because most aquatic microbes are at present

unculturable, the purpose of this study was to determine the bioavailability and reactivity of EON in natural microbial communities collected along a natural estuarine gradient. In this study, we used treated effluents collected from two BNR WWTPs, one that achieves moderate levels of N removal and one that achieves enhanced levels of N removal, in a bioassay experiment to determine the bioavailability of EON along a salinity gradient.

Methods

Effluent Collection and Preparation

Final treated effluent was obtained from a BNR facility in Virginia (E1) and a five-stage Bardenpho BNR plant in North Carolina (E2). The Virginia plant discharges over 100,000 m^3/day into a tributary of the lower Chesapeake Bay (salinity range near outfall is 5–15) about 32 km from its confluence with the Atlantic Ocean (salinity range at the Bay mouth is 25–30) and achieves a moderate level of N removal (TN >200 μmol L^{-1} annually). The North Carolina BNR facility discharges over 60,000 m^3/day of treated effluent into a freshwater creek located about 224 km inland of the Atlantic Ocean and achieves an enhanced level of N removal (TN <200 μmol L^{-1} annually). Upon arrival in the lab, both effluents were concentrated using an ultrafiltration system (Separation Engineering, Inc.) equipped with a polysulfone 1 kDa cartridge to separate both high and low molecular weight fractions. Ultrafiltration was used to remove as much DIN as possible and to concentrate the effluent so that only a small volume would need to be added to the incubations. The addition of small volumes of concentrated effluent was considered desirable to avoid dilution and associated salinity changes in estuarine water samples. The system was cleaned before each sample with 8 L of Alconox detergent (1–2%), sodium hydroxide (0.05 mol L^{-1}), and hydrochloric acid (0.02 mol L^{-1}; Guo and Santschi 1996). Between each cleaning treatment, the system was rinsed with 40 L of de-ionized water (2×20 L). E1 was concentrated approximately fourfold and E2 was concentrated approximately 1.5-fold, and since the cartridge used was 1 kDa, the concentrate consisted of high molecular weight (HMW) material >1 kDa in size. Concentrated effluent was kept frozen until the initiation of the experiments.

Experimental Details

Whole surface water was collected from sites in the Elizabeth River watershed and the Chesapeake Bay with salinities of 5 (low), 16 (middle), and 28 (high) during April 2007. Samples were collected into acid-cleaned 20 L carboys. Ambient nutrient concentrations were measured and algal populations were enumerated in water from each of the sites prior to the initiation of incubation experiments (see below for details on sample collection, storage, and handling). In order to assess the biotic reactivity of EON, whole water from each sampling site was sub-sampled into acid-cleaned, light and dark polyethylene terephthalate glycol bottles (500 mL). Duplicate light and dark treatment bottles were filled for each effluent treatment and controls and for each experimental time point (a total of four time points). Treatment bottles received either 20 mL additions of E1 (final EON addition of 8.5 μmol L^{-1} in incubation bottles) or 40 mL additions of E2 (final EON addition of 5.4 μmol L^{-1} in incubation bottles); control bottles received no effluent additions. Because the DON concentrations in the two effluents were different, additions to treatment bottles were adjusted to achieve approximately the same final EON addition in both the E1 and E2 treatment bottles.

In order to assess abiotic EON reactivity due to salinity alone, additional bottles were filled with the same estuarine water filtered through 0.2 μm sterilized polysulfone cartridge filters. Samples were collected prior to the addition of effluent (T_{amb}) and then immediately after adding effluents to treatment bottles (T0). The remaining treatment and control bottles for the bioassay experiments were transported to a float constructed from neutral density screen and PVC piping for buoyancy and tethered to a dock in the Elizabeth River so that bottles could float below the surface and simulate in situ light intensity, periodicity, and temperature conditions.

Duplicate bottles were sacrificed at each time point for each treatment and control incubation. For whole water incubations, bottles were sacrificed immediately after effluent additions (T0) and after 2 (T1), 4 (T2), and 7 (T3) days. For 0.2 μm abiotic incubations, bottles were sacrificed at just two time points, immediately after the effluent additions (T0) and after 7 days (T3). Samples to estimate dissolved nutrient concentrations were filtered through 0.2 μm sterile polysulfone cartridge filters and the filtrate placed into acid-cleaned polyethylene or sterile cryogenic vials and stored frozen (−20°C) until analysis (see below). Water samples were collected to measure particulate N and carbon (PN and PC, respectively) and chlorophyll *a* (Chl *a*) in each incubation bottle. Samples were filtered onto pre-combusted GF/F filters (450°C for 2 h), placed in sterile centrifuge tubes, and stored frozen (−20°C) until analysis. Samples for picocyanobacteria were preserved with 1% (final concentration) glutaraldehyde, flash frozen in liquid N and then transferred to a −80°C freezer until analysis. Phytoplankton abundance samples were preserved in Lugols and kept in the dark until enumeration. Picocyanobacteria were enumerated using a FACSCalibur flow cytometer (Becton Dickinson Instruments) equipped with a 15 mW air-cooled argon ion laser

tuned for blue excitation (488 nm) and gating upon forward scatter, side scatter, orange fluorescence, and red fluorescence signals. In addition, phytoplankton abundances for five dominant groups were enumerated microscopically for ambient, T0, and T1 samples to determine changes in species composition. The dominant taxa, chlorophytes, cryptomonads, filamentous cyanobacteria, diatoms, and dinoflagellates, were enumerated by concentrating a 10 mL aliquot and examined following an Utermöhl protocol using an inverted microscope at three magnifications: ×150, ×300, and ×600.

Chemical Analysis

Nutrient analyses were conducted using standard colorimetric procedures. Analyses of nitrite (NO_2^-) plus nitrate (NO_3^-), and ortho-phosphate (PO_4^{3-}) were performed on a Lachat QuikChem 8500 autoanalyzer using standard colorimetric methods (Parsons et al. 1984). Detection limits were 0.05, 0.10, and 0.10 μmol L^{-1} for NO_2^-, NO_3^-, and PO_4^{3-}, respectively. Urea concentrations were analyzed manually using the colorimetric monoxime method of Price and Harrison (1987); the detection limit was 0.025 μmol N L^{-1}. NH_4^+ concentrations were analyzed manually using the phenol hypochlorite colorimetric method (Koroleff 1983); the detection limit was 0.05 μmol N L^{-1}. TDN was measured after high temperature combustion on a Shimadzu-TOCV (Peltzer et al. 1996) and DON was determined as the difference between TDN and DIN concentrations with errors estimated using propagation of error (Bronk 2002). Chl *a* samples were extracted overnight using 90% acetone and analyzed fluorometrically (Welschmeyer 1994) within 2-7 days of sample collection. PN and PC sample filters were dried (40°C) and pelletized then analyzed on a Europa 20/20 isotope ratio mass spectrometer equipped with an automated N and C analyzer preparation module.

Calculations

In order to compare EON changes over time during the incubations, the initial DON (DON_{T0}) concentration was calculated by adding the concentration of DON (DON_{amb}) in the water sample and the concentration of EON added ($DON_{EON\ addition}$):

$$DON_{T0} = DON_{amb} + DON_{EON\ addition} \quad (1)$$

In order to estimate the percent of EON removed (% EON_R), specific for either E1 or E2 additions during incubations at each time point and salinity, the DON concentration in the control incubation was subtracted from the DON concentration in the treatment bottle of the same salinity and then divided by $DON_{EON\ addition}$. For example, to calculate the $\%EON_R$ at T1 ($\%EON_{RT1}$) in the low salinity incubations, the calculation was as follows:

$$\%EON_{RT1} = 100 - \left[100 \times \frac{(DON_{T1} - DON_{CT1})}{DON_{EON\ addition}}\right] \quad (2)$$

where, DON_{T1} is the concentration of DON at T1 for either E1 or E2 additions and DON_{CT1} is the DON concentration in the whole water control incubation at T1.

Using statistical analysis software, analysis of variance and the least significant difference post hoc test was used to interpret differences between controls and treatment incubations and time intervals for all parameters measured.

Results

The majority of the DIN present in the ambient water collected from each site was NO_2^-+NO_3^- (Table 1). The total concentration of DIN decreased with increasing salinity ranging from 22.4 μmol N L^{-1} at the lowest salinity site down to 1.3 μmol N L^{-1} at the highest salinity site (Table 1). NH_4^+ concentrations were greatest at the middle salinity site where it was 45% of the total DIN concentration. Similar to DIN, DON concentrations in the ambient water samples were greatest at the lowest salinity site (22.7 μmol N L^{-1}) and were lowest in water collected from the site with the highest salinity (11.5 μmol N L^{-1}; Table 1). Ambient PO_4^{3-} concentrations were greatest at the site with the lowest salinity (0.18 μmol L^{-1}) and were similar at middle (0.05 μmol L^{-1}) and high (0.06 μmol L^{-1}) salinity sites (Table 1).

Table 1 Average ambient nutrient and biomass concentrations at the low, middle, and high salinities

Salinity	NH_4^+ (μmolN L^{-1})	NO_3^-+NO_2^- (μmolN L^{-1})	DON (μmolN L^{-1})	PO_4^{3-} (μmolP L^{-1})	Chl *a* (μgL^{-1})	PN (μmolN L^{-1})
Low	0.45 (0.01)	21.9 (0.13)	22.7 (0.42)	0.18 (0.00)	14.6 (1.98)	20.9 (0.58)
Middle	6.44 (0.13)	8.0 (0.01)	14.7 (0.32)	0.05 (0.00)	11.8 (0.45)	14.7 (0.23)
High	0.32 (0.05)	0.99 (0.00)	11.5 (0.06)	0.06 (0.00)	1.5 (0.11)	5.1 (0.03)

Standard deviations are in parentheses

Table 2 Average NH_4^+, NO_2^-+NO_3^-, and DON concentrations of the concentrated HMW fraction of E1 and E2

	NH_4^+ (μmolN L^{-1})	NO_3^-+NO_2^- (μmolN L^{-1})	DON (μmolN L^{-1})	PO_4^{3-} (μmolP L^{-1})
E1 (HMW)	1.38 (0.05)	324.4 (1.2)	220.4 (18.3)	17.9 (0.9)
E2 (HMW)	1.46 (0.08)	111.6 (1.1)	72.9 (4.2)	3.3 (0.1)

Standard deviations are in parentheses

The concentrated HMW fraction of the E1 effluent had three times more TDN than the concentrated E2 effluent (Table 2). For both effluents, TDN consisted of about 40% DON and 60% DIN. The DIN was primarily in the form of NO_3^-; <0.01% of the TDN pool was NH_4^+ (Table 2). Effluent additions increased PO_4^{3-} concentrations by about 0.7 and 0.2 μmol L^{-1} in the E1 and E2 treatment incubations, respectively (Table 2).

Concentrations of DON increased in whole water control, E1, and E2 low and middle salinity incubations (Fig. 1a, b, d, and e) but not in the high salinity incubations (Fig. 1c, f). There were no significant differences in DON concentrations during the time course of whole water samples between light and dark incubations ($p>0.05$; Fig. 1d–f). However, DON removal from the added effluent cannot be assessed on DON concentrations alone, so percent EON removal was calculated from Eq. 2 for each effluent addition in low, middle, and high salinity incubations at T1. Based on the difference between treatment and control incubations, added DON was removed over the course of the bioassay experiment and we calculated that between 51% and 66% of the DON added in E1 and E2 was removed during the first 2 days in the light incubations, while between 31% and 96% of the DON added in E1 and E2 was removed during the first 2 days in the dark incubations (Fig. 2a, b).

In both control and treatment bottles amended with E1 and E2, there was an immediate drawdown of both the added and the ambient DIN in light bottles such that concentrations were near the limit of analytical detection after 2 days for the lowest salinity incubations and after 4 days for the middle and highest salinity incubations (Fig. 3). DIN and PO_4^{3-} concentrations remained high or increased in dark bottles for both the control and effluent-amended incubations (data not shown). Similar to DIN, the added PO_4^{3-} was depleted rapidly in light bottle incubations in both control and E1 and E2 amended incubations (Fig. 3).

Filtered (0.2 μm) abiotic controls were conducted to determine salinity and matrix (a complex mixture of

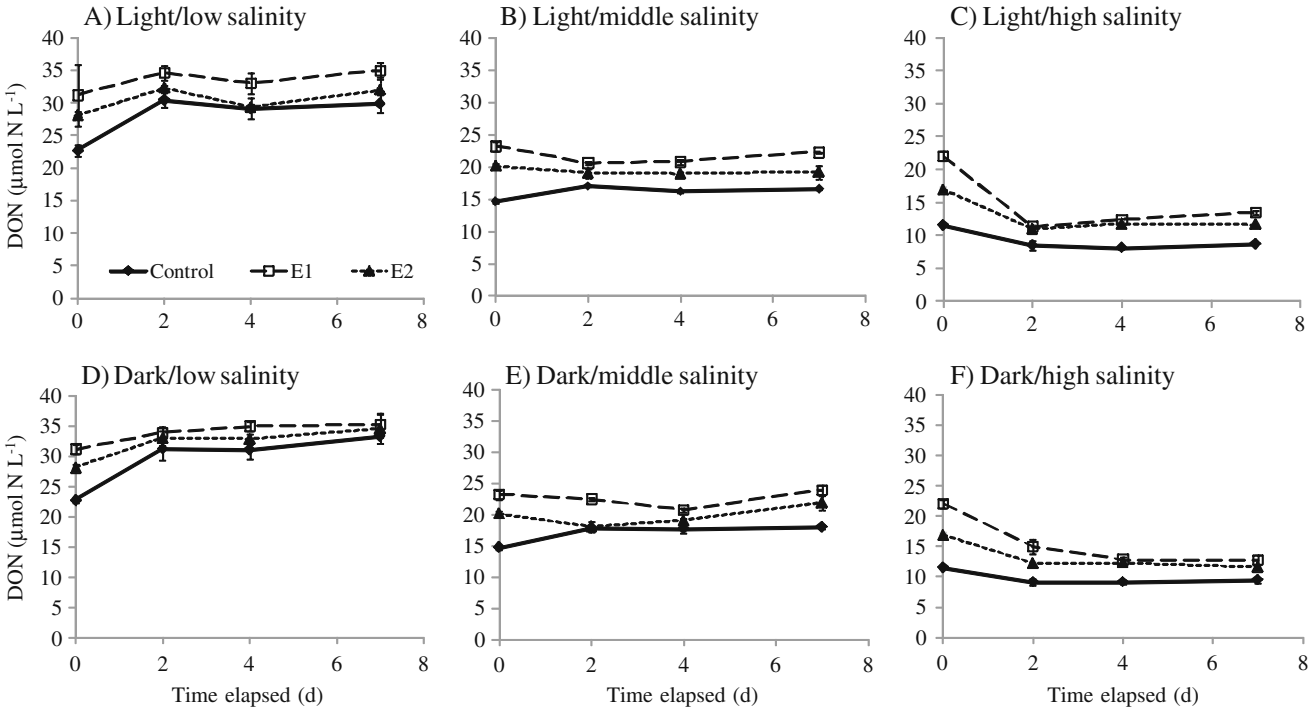

Fig. 1 DON concentrations over time in Control, and E1 and E2 whole water treatment incubations done in light bottles using (**A**) low salinity, (**B**) middle salinity, and (**C**) high salinity water samples or in dark bottles using (**D**) low salinity, (**E**) middle salinity, and (**F**) high salinity water samples

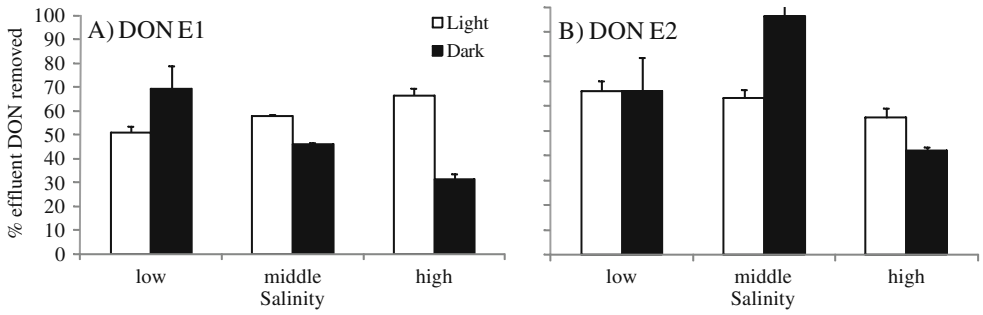

Fig. 2 Percent dissolved organic nitrogen (DON) removal relative to whole water controls after 2 days (T1) for (**A**) E1 and (**B**) E2 whole water treatment incubations

dissolved and particular chemical elements) effects on the added effluent in the low, middle, and high salinity incubations. While the TDN concentrations in abiotic incubations increased as a result of the effluent additions, the changes in the DON and DIN concentrations measured in samples collected immediately after the effluent additions (within ~15 min) differed from those based on the DON and DIN concentrations calculated as the sum of the effluent addition and the ambient concentration in the natural water sample (Eq. 1; Table 3). While total N was conserved, the composition of the added effluent N appeared to instantaneously shift upon addition to the saline matrix such that in the low salinity incubations, DON concentrations were higher and DIN concentrations were lower than calculated, and at higher salinities, DON concentrations were either as calculated or lower and DIN concentrations were as calculated or higher (Table 3). In the low salinity incubations, DON appears to have formed from DIN (50% more) upon the addition of effluent to the ambient water matrix (Table 3). The effect was much less pronounced in the middle salinity abiotic incubations (Table 3). In the high salinity matrix, the effect was different for the two effluents. For E1, DIN, and DON concentrations agreed with values calculated based on the concentrations in the effluents and the saline matrix. For E2, there was production of DIN from DON when concentrated effluent was added to the saline matrix (Table 3).

Chl *a* concentrations in the lowest salinity incubations significantly decreased over time in control incubations, did not change significantly for the E1 treatment, and although there was an initial increase after 2 days, Chl *a* concen-

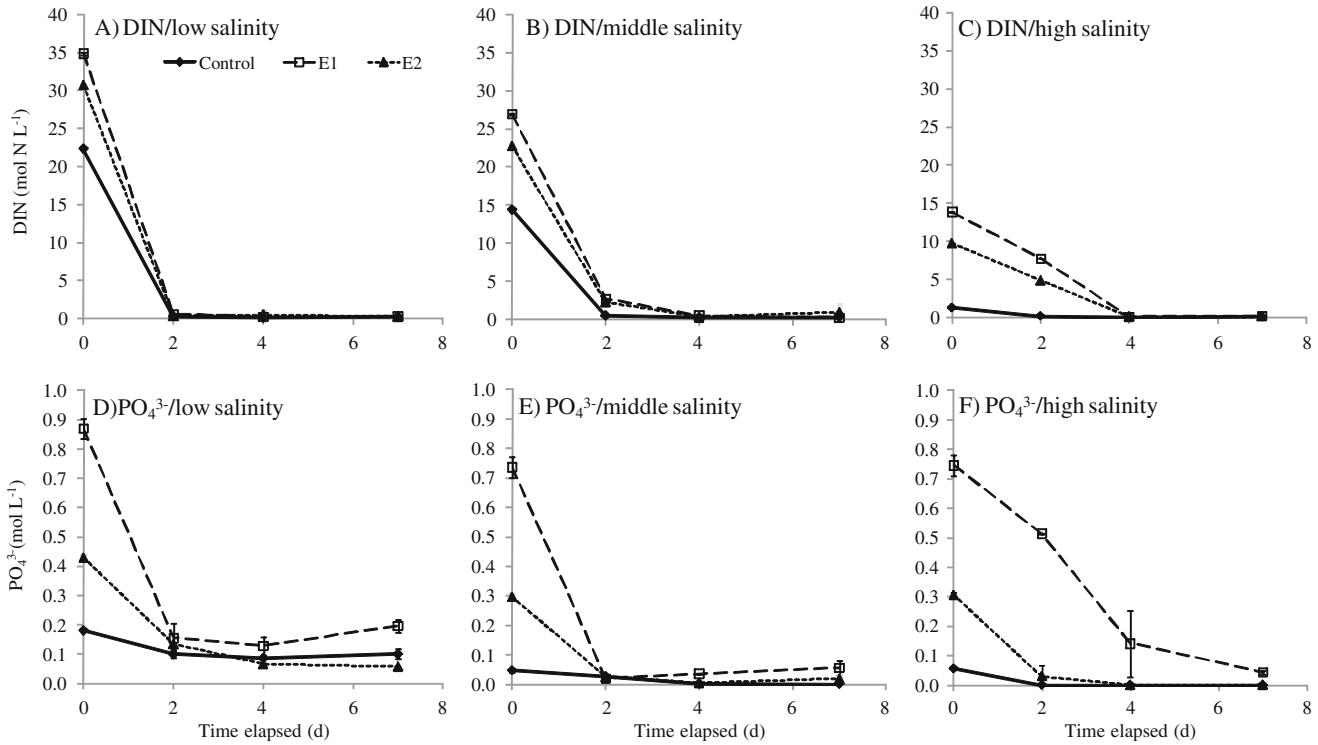

Fig. 3 Nutrient concentrations over time in Control, and E1 and E2 whole water treatment incubations done from light bottle incubations; NO_3^- concentrations in (**A**) low salinity, (**B**) middle salinity, and (**C**) high salinity incubations and PO_4^{3-} concentrations in (**D**) low salinity, (**E**) middle salinity, and (**F**) high salinity incubations

Table 3 Calculated TDN, DON, and DIN: the sum of the ambient total dissolved N in the estuarine water at each salinity and the N addition from the concentrated effluents (E1 and E2) (Eq. 1); the measured TDN, DON, and DIN concentrations just after the addition of effluent at T0 in abiotic (0.2 μm filtered) incubations of low, middle, and high salinity natural water samples

Salinity	Calculated TDN concentration at T0	Measured TDN at T0	Calculated DON concentration at T0	Measured DON at T0	Calculated DIN concentration at T0	Measured DIN at T0
E1						
Low	66.0 (0.8)	62.3 (0.3)	31.1(0.8)	39.3 (0.6)	34.8 (0.1)	22.9 (0.6)
Middle	37.6 (0.8)	49.1 (1.5)	23.2 (0.8)	25.1 (1.6)	26.8 (0.1)	23.9 (0.7)
High	23.3 (0.7)	32.5 (1.4)	22.0 (0.7)	20.0 (3.0)	13.8 (0.1)	12.4 (2.6)
E2						
Low	58.7 (0.8)	52.0 (0.3)	28.1 (0.5)	33.2 (0.3)	30.7 (0.2)	18.8 (0.1)
Middle	34.5 (0.8)	39.4 (0.1)	20.1 (0.4)	20.6 (0.3)	22.8 (0.2)	18.8 (0.2)
High	18.2 (0.7)	27.2 (1.2)	16.9 (0.3)	8.4 (1.2)	9.7 (0.1)	18.8 (0.1)

Concentrations are μmol N L^{-1}. Standard deviations are in parentheses

trations ultimately decreased over the 7-day incubation period for the E2 treatment ($p<0.05$; Fig. 4a). PN concentrations in the lowest salinity incubations increased significantly during the first 2 days in the E1 and E2 treatments ($p<0.05$) but there was no significant change over the course of the 7-day control, E1, or E2 treatment incubations ($p>0.05$; Fig. 4d). In the middle salinity incubations, there were significant increases in Chl *a* and PN concentrations over the first 2 days for all treatments ($p<0.05$), however, the increases were much greater in the E1 (231%) and E2 (272%) additions compared to the control (59%; Fig. 4b, e). In the highest salinity incubations, there was a significant increase in Chl *a* and PN concentrations over the first 2 days for both the E1 and E2 treatments ($p<0.05$), but there was no significant change in the control incubations over the same time period ($p>0.05$; Fig. 4f). In E1 and E2 treatment incubations, Chl *a* concentrations were significantly greater at day 7 than at T0 concentrations, and PN concentrations were significantly greater at day 7 than at T0 concentrations for the E1 incubations only ($p<0.05$; Fig. 4c, f). In the dark incubation bottles, there was either no change or a general decreasing trend of Chl *a* and PN concentrations over the course of the bioassay (data not shown).

A diverse phytoplankton assemblage was observed at each of the three sites sampled and included predominantly estuarine species that are normal components of the phytoplankton community (Fig. 5a) that develops season-

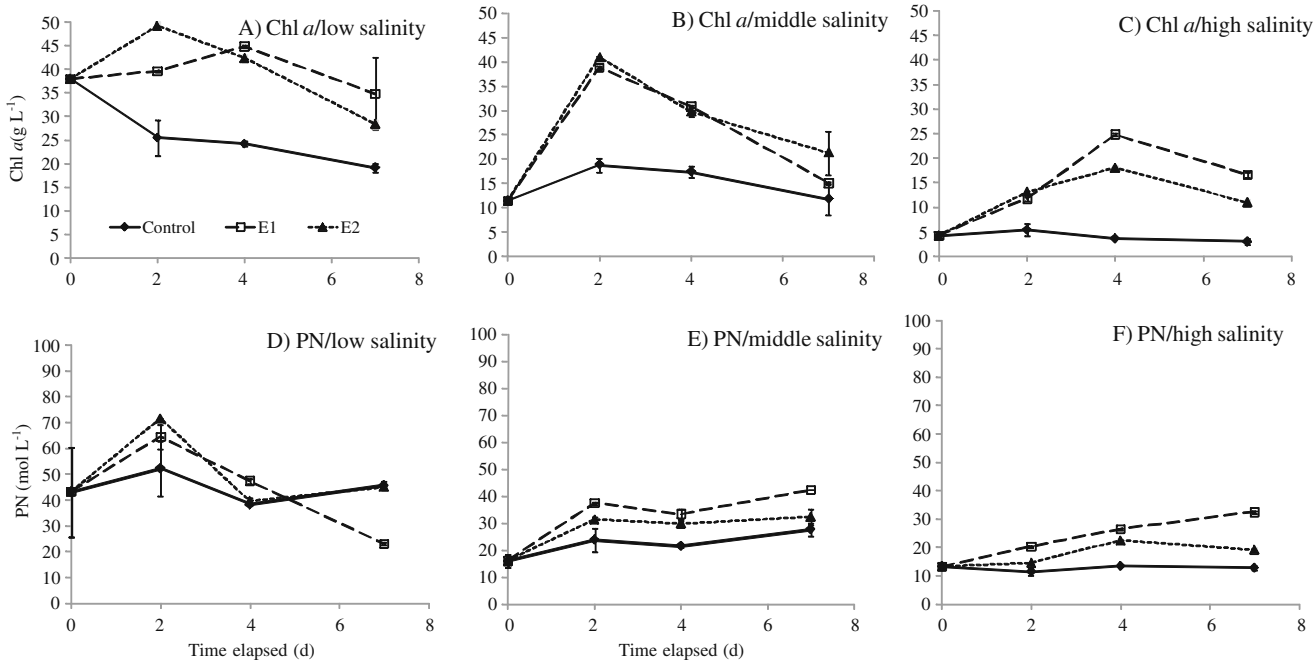

Fig. 4 Chl *a* (**A**, **B**, **C**) and PN (**D**, **E**, **F**) concentrations in Control, and E1 and E2 whole water treatment incubations (*light bottles*) of low salinity (**A**, **D**), middle salinity (**B**, **E**), and high salinity (**C**, **F**) natural water samples

ally in the Virginia tidal tributaries and the Chesapeake Bay (Marshall et al. 2005). The highest Chl *a* and PN concentrations were observed at the lowest salinity site where there was a dinoflagellate bloom comprised of mainly *Prorocentrum minimum* and *Gymnodinium* sp. These two taxa are common bloom-forming species from spring through autumn (Marshall et al. 2005). Diatoms were most abundant at the middle and high salinity sites, while dinoflagellates and diatoms were most abundant at the low salinity sampling site (Fig. 5a).

After 2 days, cell densities of all taxa were higher in the control and the E1 and E2 treatment incubations than initial cell densities in the ambient water samples except diatoms in the low salinity, and dinoflagellates in the high salinity (Fig. 5b–d). In the low salinity incubations, effluent additions stimulated all five taxa relative to the controls with chlorophyte abundance stimulated the most, where cell densities were 20,000 and 9,000 times greater after 2 days relative to controls in E1 and E2 incubations, respectively (Fig. 5b). In the low salinity incubations, E1 stimulated growth more than E2. In the middle salinity incubations, all taxa except dinoflagellates were stimulated in E1 treatment incubations relative to controls (Fig. 5c). However, only diatoms increased and chlorophytes and cyanobacteria decreased in E2 treatment incubations relative to controls (Fig. 5c). In the highest salinity incubations, cell densities did not increase as much over the 2-day period as for the other salinities (Fig. 5d). Compared to control incubations, cryptomonads, cyanobacteria, and dinoflagellates were stimulated by E1 additions. In contrast, cell abundance of all five taxa decreased in the E2 treatment incubation bottles (Fig. 5d). Statistical significance of differences could not be assessed because sufficient volume was not available to make replicate measurements.

Picocyanobacteria were more abundant in natural water samples collected at the low salinity site relative to the middle and high salinity sites. Picocyanobacterial abundance increased significantly in all of the low and middle salinity E1 and E2 treatment incubations over the 7-day incubation period ($p<0.05$; Fig. 6a, b), increasing as much as a factor of 10 in the low salinity E2 treatment incubations (Fig. 6a). In all but the high salinity control incubations, picocyanobacterial abundance increased initially, then decreased after day 4. In the high salinity

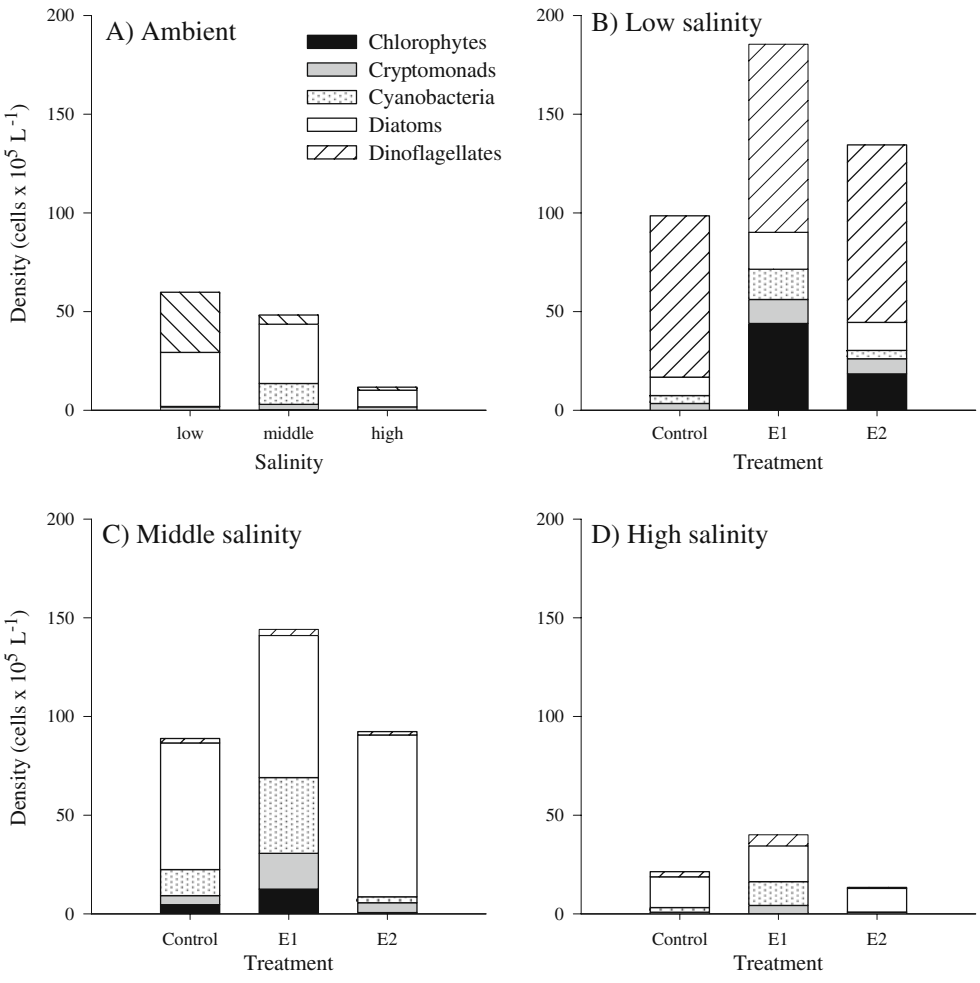

Fig. 5 Abundance (cells×10^5 L^{-1}) of major phytoplankton taxa in (**A**) ambient samples from low, middle and high salinity sites, and after 2 days for (**B**) low salinity, (**C**) middle salinity, and (**D**) high salinity Control, E1, and E2 whole water treatment incubations

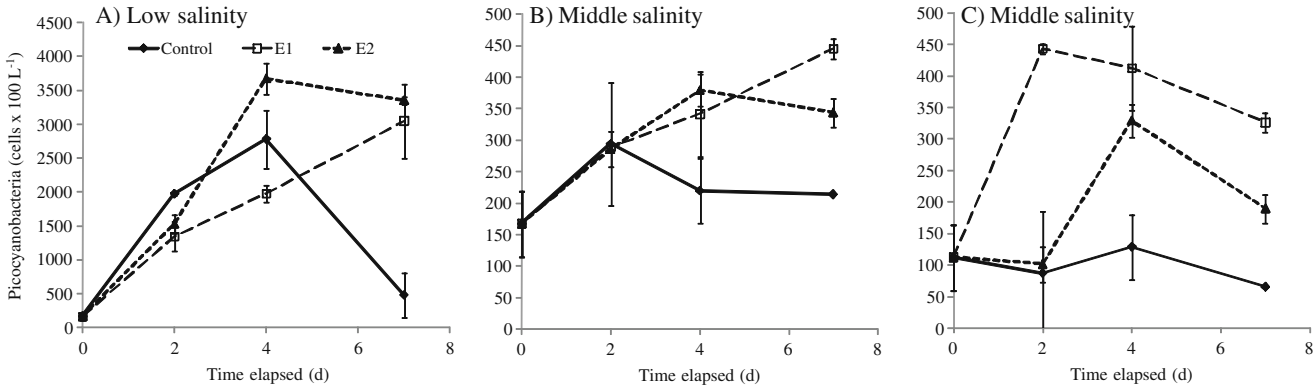

Fig. 6 Picocyanobacteria concentrations in Control, and E1 and E2 whole water treatment incubations (light bottles) of (**A**) low salinity, (**B**) middle salinity, and (**C**) high salinity natural water samples. Note the difference in scale between the low salinity and the middle and high salinities

incubations, picocyanobacterial abundance was significantly higher in E1 treatment incubations than in control incubations after 7 days ($p<0.05$). As for Chl *a*, picocyanobacterial abundance either stayed the same or decreased relative to initial concentrations in the dark bottle incubations (data not shown).

Discussion

This study presents the first results from bioassay experiments aimed at assessing the reactivity of effluent-derived DON in natural waters along an estuarine salinity gradient. Results indicate that 100% of the DIN and between 31% and 96% of the EON added to light and dark environmental samples was removed within 2 days (Figs. 2 and 3); presumably by resident microbial communities using this N to support the observed increase in biomass. Results from this study are consistent with findings from other studies that show a wide range of DON compounds entering aquatic systems from multiple sources (e.g., run-off, atmosphere) are bioavailable to estuarine and marine plankton communities (Hopkinson et al. 1998; Seitzinger and Sanders 1999; Seitzinger et al. 2002; Wiegner et al. 2006).

Results show that N released from WWTPs was biologically removed to support the growth of microbial communities during incubations. Over the first 2 days, Chl *a*, PN, and picoplankton concentrations increased in all treatment incubations while TDN concentrations decreased. Because phytoplankton community composition and concentrations of dissolved nutrients vary along salinity gradients (Fisher et al. 1999; Fisher et al. 1988; Marshall et al. 2005; Marshall et al. 2006; Table 1), it was expected that the response to concentrated effluent additions containing both DIN and DON would vary depending upon the dominant species present at each salinity and the ambient nutrient regime in the natural water samples. In these experiments, we found that after 2 days, all taxa increased in low salinity incubations in response to effluent additions, all taxa but dinoflagellates increased in the middle salinity incubations amended with effluent, and cyanobacteria increased in the high salinity E1 incubations relative to control incubations. A study examining the effect of atmospheric deposition of DON on phytoplankton communities showed similar results, where diatoms and dinoflagellates increased in response to DON additions suggesting that these species are better able to utilize organic matter to support their growth than other taxa (Seitzinger and Sanders 1999). Picocyanobacteria, cyanobacteria, and other phytoplankton have also been shown to utilize DON when DIN concentrations are depleted (Berman and Bronk 2003; Paerl 1991). Indeed, in this study, picocyanobacteria increased throughout the 7-day incubation, long after DIN was depleted (Figs. 3 and 6) and only cyanobacteria cell abundance increased at the highest salinity E1 incubations, where TDN concentrations were lowest initially (Table 1).

Changes in cell abundance and taxonomic composition of the phytoplankton community in response to effluent additions were likely due to the relative and total amounts of DIN and DON added, the composition of the DON added, and the reactivity of added N in the estuarine water matrix. Overall, more N (and P) was added in the E1 treatments, and therefore this likely contributed to the greater biological response in the E1 treatment. Also, both effluents had more DIN than DON; 100% of DIN was drawn down during the incubations. It is unlikely that the increase in cell density was solely due to DIN, however, because DIN decreased to the limit of detection within 2-4 days while Chl *a*, PN, and picocyanobacteria continued to increase throughout the 7-day incubations, particularly in the high salinity incubations where biomass was lowest initially. In nature, DIN turnover times can be on the order of hours, particularly in the Chesapeake Bay during spring and summer (Fisher et al. 1992; Glibert et al. 1995) and bioassays comparing N drawdown after nitrate versus

concentrated effluent additions have also shown nitrate depletion in incubation bottles within hours (Mulholland et al. unpublished data; Schweitzer et al., unpublished data). After 2 days, DON was likely being produced and consumed by the microbial community at similar rates as there was little change in DON concentrations in incubation bottles after this time (Fig. 1). Similar to DIN and specifically NH_4^+ (Glibert et al. 1995), labile DON compounds can turn over rapidly in natural waters (Fuhrman 1990 [DFAA]; Lomas et al. 2002 [urea]; Mulholland et al., 2009 [peptides]) and a fraction of the DON pool in aquatic systems appears bioavailable to phytoplankton (Berman and Bronk 2003; Bronk 2002). Many larger organic compounds that are present in treated effluents (Pehlivanoglu-Mantas and Sedlak 2006) can also be taken up by microbes after first rendering HMW compounds into low molecular weight compounds through a variety of extracellular enzyme reactions (Berg et al. 2003; Berges and M. R. Mulholland, 2008; Pantoja et al. 1997). However, there is also a large fraction of DON that is degraded over longer timescales or is refractory (Berman and Bronk 2003; Bronk 2002; Kirchman et al. 1993).

Our results indicate that in addition to biotic removal, there is substantial abiotic reactivity of effluent N when it is added to freshwater and saline environments (Table 3). While we observed that a large component of the effluent TDN was removed during biotic incubations, DON was both formed and removed during abiotic and biotic incubation experiments (Fig. 1, Table 3; Mesfioui et al., unpublished data) and it is difficult to determine exactly which components of the TDN pool added were taken up directly by the microbial community. DON can undergo changes when it is added to water of varying salinity as a result of the release of loosely bound humic-N as material moves through a salinity gradient (See 2003; See and Bronk 2005) or via photochemical processes (Bushaw-Newton and Moran 1999; Bushaw et al. 1996; Kieber et al. 1999; Moran and Zepp 1997). In the experiments reported here, effects of photochemical alteration were likely minimal because our incubation bottles were plastic (see "Methods" section). In the lowest salinity incubations, the added concentrated effluent (E1 and E2) immediately reacted such that DIN was removed, adsorbed, or otherwise converted to DON. In the highest salinity abiotic incubations, we saw the reverse, DON appeared to release N to the DIN pool (E2 only). A less pronounced response was observed in the middle salinity abiotic incubations and in the high salinity incubations with E1. Recent findings from abiotic incubations using artificial seawater suggest that NH_4^+ is liberated from EON due to increased salinity (Bronk et al., submitted for publication). The results reported here suggest that the "re-speciation" of N compounds between the DON and DIN fractions was higher than that observed using artificial seawater (Bronk et al., submitted for publication) and suggest that other matrix effects may be important in natural waters where there is already a complex mixture of dissolved and particular chemical elements that interact to affect the reactivity of chemical additions. Others have shown that conformational changes in organic matter, including humics, occur during tidal mixing and bacterial degradation (Baalousha et al. 2006; Boyd and Osburn 2004) but specific matrix effects on EON (other than salinity or photochemical effects) are yet unknown and further investigation into the instantaneous abiotic changes is warranted.

These findings may have wide ranging implications for microbial communities within the Chesapeake Bay and its tributaries, which has already undergone major shifts in response to cultural eutrophication. Large dinoflagellate blooms occur seasonally throughout the Chesapeake Bay watershed and many of these bloom organisms can take up DON (Burkholder et al. 2008; Mulholland et al. 2004; Mulholland et al. 2009) and DON has been implicated as a causative factor of blooms in a variety of systems (Heisler et al. 2008). Cyanobacteria blooms are also common in the summer within the Chesapeake Bay system, particularly in the lower salinity regions (Marshall 1994; Marshall et al. 2006) and often include potentially harmful species (Marshall et al. 2008; Paerl and Piehler 2008). Cyanobacteria also have the capacity to use a variety of N-containing compounds, including DON at a wide range of concentrations, and may therefore, have an advantage during periods when DON inputs are high relative to DIN inputs (Antia et al. 1991; Paerl and Piehler 2008).

This research demonstrates that effluent-derived DON is highly reactive but that reactivity varies among effluents. More work is needed to discern the reasons for differences between treatment plants and whether those differences are driven by the wastewater composition entering the plant, differences in the form of nitrogen remaining in treated effluents due to differences in the treatment process configuration used, or a combination of both. The reactivity of effluent organic N also varies in the environment depending on the salinity, matrix effects, and the biological community present in the receiving waters. The results presented here have important implications for managers and suggest that DON is not recalcitrant and can contribute to eutrophication in the environment. Future experiments should focus on identifying DON fractions present in effluents that are more or less reactive in nature either due to abiotic effects or the activity of microbes in the receiving waters. Using more advanced molecular level chemical analyses will enable us to identify the specific structures in EON that are most chemically reactive. This in turn may advise the development of treatment processes that target and remove environmentally reactive EON. Because EON

can form during microbial processes involved in biological nutrient removal and this microbially produced material is likely to be labile, it may be necessary to add a treatment step that removes this material before effluents are discharged. Finally, it appears that treated effluent inputs can affect the structure of natural plankton communities. A better understanding of how changes in the quality of nutrient inputs to estuaries impacts primary production and the dominant primary producers is important to comprehending future estuarine productivity as we increasingly bias nutrient discharges toward organic nutrient forms.

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