Formation of Low-Molecular-Weight Dissolved Organic Nitrogen in Predenitrification Biological Nutrient Removal Systems and Its Impact on Eutrophication in Coastal Waters

Heonseop Eom,† Douglas Borgatti,‡ Hans W. Paerl,§ and Chul Park,*†

†Department of Civil and Environmental Engineering, University of Massachusetts, Amherst, Massachusetts 01003, United States
‡Springfield Water and Sewer Commission, Springfield, Massachusetts 01001, United States
§Institute of Marine Sciences, University of North Carolina, Chapel Hill, North Carolina 28577, United States

ABSTRACT: To alleviate eutrophication in coastal waters, reducing nitrogen (N) discharge from wastewater treatment plants (WWTPs) by upgrading conventional activated sludge (CAS) to biological nutrient removal (BNR) processes is commonplace. However, despite numerous upgrades and successful reduction of N discharge from WWTPs, eutrophication problems persist. These unexpected observations raise the possibility that some aspects of BNR yield environmental responses as yet overlooked. Here, we report that one of the most common BNR processes, predenitrification, is prone to the production of low-molecular-weight dissolved organic N (LMW-DON), which is highly bioavailable and stimulates phytoplankton blooms. We found that in predenitrification BNR, LMW-DON is released during the post-aerobic step following the preanoxic step, which does not occur in CAS. Consequently, predenitrification systems produced larger amount of LMW-DON than CAS. In estuarine bioassays, predenitrification BNR effluents produced more phytoplankton biomass than CAS effluents despite lower N concentrations. This was also supported by stronger correlations found between phytoplankton biomass and LMW-DON than other N forms. These findings suggest that WWTPs upgraded to predenitrification BNR reduce inorganic N discharge but introduce larger quantities of potent LMW-DON into coastal systems. We suggest reassessing the N-removal strategy for WWTPs to minimize the eutrophication effects of effluents.

■ INTRODUCTION

Globally, estuarine and coastal environments are experiencing accelerating eutrophication resulting from increasing anthropogenic nutrient inputs. Under these conditions, potentially toxic phytoplankton blooms can negatively impact ecosystem function by disrupting food webs, causing seasonal hypoxia, and threatening resident aquatic plants and animals. In estuaries and coastal oceans, nitrogen (N) is typically the nutrient controlling primary production and phytoplankton bloom formation.1,2 Consequently, substantial efforts have been made to decrease N loads to these environments, and reducing N discharge from wastewater treatment plants (WWTPs) has been a primary target supported by legislation.

To comply with more-stringent N-discharge requirements, WWTPs typically need to upgrade from conventional activated sludge (CAS) to biological nutrient removal (BNR) processes. The former is a basic form of wastewater treatment used to remove organic matter under aerobic conditions, which is often accompanied by nitrification. While there are a variety of ways to perform BNR, single-sludge BNR processes are common because both aerobic nitrification and anoxic denitrification can be achieved by the same sludge biomass, thus significantly reducing the system footprint in comparison to multisludge systems.3,4 Among single-sludge systems, the predenitrification system, which starts with an anoxic reaction followed by an aerobic reaction with internal recirculation, is used most often because of several benefits, including: (1) the ease of retrofitting from existing CAS systems, (2) the elimination or reduction of the need for adding organic carbon for denitrification, and (3) decrease of the production of sludge biomass.3,4 In the predenitrification system, as in any BNR systems, N removal is mainly achieved by the conversion and removal of dissolved inorganic N (DIN) (i.e., nitrification and denitrification).

WWTPs upgraded to BNR have significantly decreased total N loads to their receiving coastal waters. For example, a total maximum daily load (TMDL) was established for the Long Island Sound (LIS) in 1995; the goal was to reduce anthropogenic N loads by 58.5% by 2014 (NYSED and
systems. on phytoplankton growth potential in estuarine and coastal nonpoint sources, should be considered together for this factors, such as changes in climate and input of N from eutrophication and hypoxia continue to plague the LIS. Multiple factors, such as changes in climate and input of N from nonpoint sources, should be considered together for this "paradox" (i.e., decrease in total N input but increase in phytoplankton bloom). However, these unexpected observations also raise the possibility that some aspects of BNR, especially predenitrification BNR, yield environmental responses, as yet overlooked.

In this study, we report on the potential of the predenitrification BNR process to produce small, yet environmentally significant amounts of low-molecular-weight dissolved organic N (LMW-DON) in their effluents. Moreover, we found that this effluent-derived LMW-DON is highly bioavailable and stimulates phytoplankton growth. These findings suggest that upgrading WWTPs to predenitrification BNR systems may not necessarily reduce eutrophication in downstream estuaries. To discuss our observations, we first present the results from a controlled reactor study demonstrating the fate of DIN and DON in CAS and predenitrification BNR systems. We also show changes of the effluent N in a full-scale WWTP that was converted from CAS to a predenitrification BNR during this study. We present the results from a bioassay study aimed at examining the impacts of effluent N on phytoplankton growth in coastal waters. Based on the results, we discuss the bioavailability of effluent-derived LMW-DON and its influence on phytoplankton growth potential in estuarine and coastal systems.

■ MATERIALS AND METHODS

Operation of Lab-Scale CAS and Pre-denitrification Systems. We operated three lab-scale wastewater treatment systems, two CAS and one pre-denitrification BNR, during 2013–2014. We ran these systems in sequencing batch reactors (4.5 L volume) seeded with the same activated sludge collected from the local WWTP. One batch cycle lasted 6 h, consisting of 10 min feeding, 4 h 50 min treatment with mixing, 50 min settling, and 10 min effluent decanting. The CAS systems were entirely aerobic, whereas the pre-denitrification system included a first anoxic phase (2 h) and subsequent aerobic phase (2 h 50 min). Aeration in both CAS and BNR systems was provided by the same house air unit. The anoxic condition in the pre-denitrification system was created and maintained by sparging with N2 gas. All CAS and BNR systems were fed 6 L per day of identical influent, the primary effluent collected from the local WWTP. The resultant hydraulic retention time (HRT) was 0.75 days. CAS 1 had a 6 day solids retention time (SRT). CAS 2 and BNR systems had the same SRT of 20 days.

During this reactor study, we regularly measured dissolved total nitrogen (DTN) and inorganic N forms in influent and effluent and determined the quantity of DON based on the following equation:

\[
\text{DON} = \text{DTN} - (\text{NH}_4^+ + \text{NO}_3^- + \text{NO}_2^-)
\] (1)

The dissolved or soluble fraction of N was obtained by filtering samples through sterile 0.45 μm nitrocellulose membrane filters. We also analyzed soluble chemical oxygen demand and PO43− during reactor operation. Other routine measurements included total suspended solids (TSS) and volatile suspended solids (VSS) of the influent, mixed liquor, and effluents as well as pH and dissolved oxygen (DO) of the mixed liquor.

Retrofitting of the Local WWTP and Its N Data. During the current study, our local WWTP (Amherst, MA) changed its treatment process from CAS to a pre-denitrification BNR to comply with a new N permit implemented in 2013. We received the three-year (2012–2014) N data from the Amherst WWTP and analyzed them to study the changes in effluent N that occurred before and after upgrade to BNR. This facility treats, on average, 0.18 m3/s wastewater, which is mainly domestic wastewater. The plant has three trains of aeration basins, each composed of three identical sub-basins. Before converting to BNR, the plant typically operated CAS by using two sub-basins at 6–8 h HRT and 5–10 d SRT. The facility retrofitted its treatment process to BNR in early 2013 using pre-denitrification and intermittent-aeration modes with the use of remaining basins. The first basin was kept under anoxic conditions, while the second and the third basins were exposed to intermittent aeration (typically, 2.1 h on and 1.5 h off). There was also a small internal outfall return (i.e., returning sludge to the preanoxic basin prior to secondary clarification), which occurred at approximately 25% of the influent flow rate. The flow rate of return activated sludge was typically 25–50% of the influent flow rate. With BNR operation, the SRT was maintained in the range of 15–20 days. The data we received from the plant showed total N, NH4+, NO3-, total Kjeldahl N, and TSS in primary effluent and secondary effluent three to five times a week.

Bioassay. A total of four sets of bioassays were performed on effluents from our lab-scale systems: March and September 2013 and March and December 2014. The 2013 sets had only CAS 1 and BNR effluent bioassays. The 2014 sets included bioassay on CAS 1, CAS 2, and BNR effluents. On the starting day of bioassays, we collected the coastal water off of the White Sand Beach in Old Lyme, CT, where the Connecticut River drains into LIS. These water samples were transported to the laboratory within 2 h of collection. A nylon net filter (100 μm in pore size) was used to remove large particles but retain phytoplankton and bacteria as inoculum of the bioassay. The mixing ratios of effluent to LIS water in bioassays were 1:4 in 2013 and 1:10 in 2014. Each bioassay treatment, in duplicate, was conducted in a 2 L Pyrex bottle at ambient temperature in a temperature-controlled laboratory (20 ± 1 °C). The bottles were placed in front of laboratory windows (each window was 2.0 m in length and 1.9 m in height) and exposed to natural daytime and nighttime cycles; the light intensity varied during bioassay, e.g., noon in December showed photosynthetically active radiation (PAR) values in the range of 40–230 μmol m−2 s−1. The control bioassay was also run using only LIS water, without the addition of effluents. Bioassays were completed between two and 3 weeks of incubation. We conducted routine sampling during bioassay based on the given time interval as well as visual observation of bioassay experiments. Collected samples...
were immediately used to measure biomass generation by measuring chlorophyll a (chl a), TSS, and VSS. For the analysis of N and P, samples were filtered through 0.45 μm filters and 1 kDa ultrafilters (see below) and kept frozen at −20 °C until analysis.

Size Fractionation of Organic N. DON was classified into two groups on the basis of its molecular weight: high-molecular-weight DON (HMW-DON, >1 kDa) and low-molecular-weight DON (LMW-DON, <1 kDa). To fractionate these sizes, ultrafiltration was conducted using an Amicon ultrafiltration cell (Millipore Corp.) with a 1 kDa cellulose membrane. Effluent samples were directly filtered by 1 kDa ultrafilters, whereas mixed liquor and bioassay samples were filtered by 0.45 μm filters first and then subjected to ultrafiltration. The 1 kDa filtrate was subjected to total N and inorganic N measurements. Then, LMW-DON was determined based on the difference between total N and inorganic N in 1 kDa filtrate. The amount of HMW-DON was obtained by subtracting LMW-DON from total DON.

Chemical Analysis. Concentrations of DTN were measured by a Shimadzu TOC/TN analyzer (Shimadzu North America, Columbia, MD); the detection limit is 5 μg N/L. Concentrations of DIN species (NH4+−N, NO2−−N, and NO3−−N) and PO4−−P in reactor influents and effluents were determined using a Metrohm ion chromatograph (IC) (Metrohm, Herisau, Switzerland). The detection limits by IC for NH4+−N and PO4−−P were 0.1 mg N/L and 0.02 mg P/L. The detection limit of both NO2−−N and NO3−−N was 0.005 mg N/L. Because DIN species in saline waters cannot be measured by IC due to high salinity, we analyzed DIN in LIS water and bioassay samples using wet-chemical-based methods. NH4+−N in saline waters was determined by the phenol hypochlorite colorimetric method.15 Concentrations of NO2−−N and NO3−−N in saline waters were measured by the methods of Strickland and Parson (1968)16 and Zhang and Fischer (2006),17 respectively. For PO4−−P in saline waters, the method of Strickland and Parson (1968)16 was employed. Detection limits for NH4+−N, NO2−−N, NO3−−N, and PO4−−P by these wet-chemical methods were 0.05, 0.01, 0.5, and 0.01 μM, respectively. TSS and VSS were measured according to Standard Methods (10200H).18 Measurements of chl a followed the spectrophotometric method described in the Standard Methods (10200H).18

Statistical Analysis. Data were graphed using Microsoft Office Excel 2013 and Sigma Plot 10. To examine the statistical significance between the results, p values were calculated based on the unpaired t test with unequal variance using the method reported in the study of Welch (1947).19

RESULTS AND DISCUSSION

Increase of DON in Predenitrification BNR Compared to CAS. DON comprises a larger fraction of effluent N in BNR systems compared to CAS systems.20−22 However, little has been documented about whether BNR generates larger amounts of DON than CAS and whether its DON is different than that in CAS. Motivated by the lack of such information and to study the effect of WWTP upgrade on phytoplankton bloom in receiving waters, we first generated effluents from controlled CAS and BNR systems treating the same wastewater in the laboratory.

Figure 1 presents average concentrations of total and different forms of N in effluents of the three lab reactors. CAS 1 and CAS 2 systems showed almost same DTN concentrations, while their ammonium and nitrate concentrations were significantly different, reflecting the different SRTs used in each reactor. As expected, CAS 2 with a longer SRT showed much lower NH4+−N (p value <0.01) but higher NO3−−N (p value <0.05) than CAS 1. Unlike DIN, the difference of effluent DON concentrations in the two CAS systems was insignificant (CAS 1:2.1 ± 1.2 mg N/L vs CAS 2:2.2 ± 1.0 mg N/L).

The predenitrification BNR system showed much less effluent N than the two CAS systems. In BNR effluent, DIN was, on average, 12.5 ± 4.4 mg N/L, and DON was, on average, 3.8 ± 0.9 mg N/L. Although BNR effluent contained much less DTN and DIN, it contained 81% (p value <0.03) and 52% (p value <0.03) more DON than CAS 1 and CAS 2 effluents, respectively. We note that not only concentration but also composition of DON in BNR and CAS effluents was different. We found that greater than 80% of DON in predenitrification BNR effluents resided as LMW-DON, similar to the value reported for a field BNR effluent,23 whereas in CAS effluents, it was only about 50%. These results showed that under controlled reactor operation conditions, the predenitrification BNR system produced not only larger amounts of DON but also higher fractions of LMW-DON in comparison to CAS.

During this study, we also gained the opportunity to evaluate the effluent N data provided by the Amherst WWTP that changed its treatment process from CAS to a predenitrification BNR in 2013. Figure 2 shows average concentrations of effluent total N consisting of DIN and total organic N in 2012, when the facility was operated in CAS, and 2013 and 2014, when the plant was operated in BNR using a predenitrification process. As the facility changed its process, effluent total N decreased mainly due to the decrease of DIN. However, total organic N in the effluent increased from 1.0 ± 0.2 mg/L in 2012 to 1.6 ± 0.3 mg/L in 2013 and to 1.8 ± 0.3 mg/L in 2014, which is a significant increase (p value <0.05); total organic N in influents was not different across the three years (12.5 ± 2.2, 12.3 ± 2.9, and 12.1 ± 3.3 mg N/L in 2012, 2013, and 2014, respectively). The yearly average effluent TSS values in 2012, 2013, and 2014 were 3.5 ± 2.8, 3.2 ± 1.7, and 2.2 ± 1.0 mg/L, respectively (the difference of TSS for 2012 versus 2014 and 2013 versus 2014 was significant, with a p value of <0.05). This strongly indicates...
that the increase in effluent total organic N in 2013 and 2014 was primarily caused by an increase of organic N in the nonparticulate fraction (or basically DON). Assuming that N accounts for 12% of activated sludge and effluent TSS, it can also be inferred that the differences between CAS and BNR effluents were greater for DON than for total organic N.

Our lab reactor studies and field effluent analyses therefore suggest that there is a high likelihood that upgrading WWTPs from CAS to pre-denitrification BNR leads to larger amounts of DON, particularly in the form of LMW-DON, in the effluent, although it decreases DTN by reducing DIN. Our findings may also provide one possible explanation for the observation that the level of DON in LIS has actually increased between 1995 and 2009, although total N loads to LIS have significantly decreased mainly due to regional WWTP upgrades.2

**Release of LMW-DON in Pre-denitrification BNR.** To gain insight into the cause of greater amounts of DON, especially LMW-DON, present in BNR effluents compared to CAS effluents, we investigated the fate of DON in lab-reactor operations.

Figure 3 shows changes of DON concentrations over the reaction periods in BNR and CAS systems. The influent contained, on average, 5.5 ± 0.2 mg N/L DON, which was composed of 4.3 ± 0.1 mg N/L of LMW-DON and 1.2 ± 0.1 mg N/L of HMW-DON. In the two CAS reactors, DON decreased continuously as aeration proceeded, and this removal occurred mainly via the removal of LMW-DON. In the predenitrification BNR reactor, DON also decreased from 5.5 ± 0.2 to 3.8 ± 0.4 mg N/L N during the first anoxic phase, again mainly due to the decrease of LMW-DON. However, during the subsequent aerobic phase, DON increased back to 5.1 ± 0.7 mg N/L, which was entirely caused by the release of LMW-DON. These results reveal that the main cause for predenitrification BNR effluents to contain more DON compared to CAS effluents is the increase of LMW-DON under aerobic conditions after preanoxic conditions.

About 40% of HMW-DON was removed during the aerobic phase in BNR and, if this removal was due to its hydrolysis and degradation, could have contributed to the formation of LMW-DON. However, the increased concentration of LMW-DON during the aerobic phase was much greater than the decrease in HMW-DON, indicating that there was a different major source for the LMW-DON release. A sudden shift in the pattern of LMW-DON concentration with the start of the post-aerobic step also suggests that this new release was most likely of biological origin. We speculate at this point that a large part of this LMW-DON is soluble microbial products (SMP), especially utilization-associated products (UAP). Previous works showed that UAP-type of SMP is primarily protein-like or humic-like biopolymer (i.e., organic N) that is also very small in size (such as <1 kDa). UAP is also known to be produced more quickly than other parts of SMP. These characteristics are very similar to those of LMW-DON released during the post-aerobic period in the BNR system. Also, because nitrifying bacteria are known to produce SMP, some part of LMW-DON generated in the aerobic phase of pre-denitrification BNR might have originated from the growth of nitrifying bacteria. Nevertheless, considering the fast degradation rate of UAP, the persistence of LMW-DON...
in the post-aerobic phase in BNR (and in two CAS systems; Figure 3) raises a question regarding its composition and characteristics. Future research should identify LMW-DON increased in the aerobic stage of the predenitrification system (and compare to LMW-DON in the anoxic stage). This will help us understand LMW-DON release phenomenon in predenitrification BNR, which does not occur in the CAS system.

Although information about the concentration of LMW-DON was not directly provided, examples from the literature also support the idea that it is the aerobic tank in which major DON release occurs in predenitrification processes. Czerwionka et al. (2012)\(^\text{27}\) found that most DON removal occurred in anoxic units, whereas DON increased in the aerobic unit during their batch bioreactor operation. Huo et al. (2013)\(^\text{28}\) measured DON in each unit of a full-scale activated sludge WWTP operated with anaerobic, anoxic, and aerobic processes and also showed that major removal of DON occurred in the first two stages, whereas DON was released and increased again in the third aerobic stage.

**Assessing Phytoplankton Growth Supported by CAS and BNR Effluents.** We conducted lab bioassay studies to investigate and compare the impact of CAS and BNR effluents on phytoplankton growth in coastal waters. In our bioassay, we mixed effluents with water from LIS and incubated them under natural light conditions in the laboratory.

Patterns of N consumption and phytoplankton growth were similar for the same type of effluent in four sets of bioassays conducted in different time and seasons (the summary of bioassays is presented in Table S1). Figure 4 shows the results from one bioassay set that was conducted in December 2014. During the initial period of bioassay (until day 5–8), mainly DIN was consumed, regardless of the type of effluent, and this was accompanied by the production of phytoplankton biomass from 0.05 to 0.19 mg/L of chl a (or 11–19 mg/L of VSS; VSS versus chl a (\(R^2 > 0.95\); Figure S1). Removal of LMW-DON occurred more slowly (from day 8–12) than DIN but appeared to lead to greater amounts of biomass, corresponding to the increase from 0.35 to 0.53 mg/L chl a (29–40 mg/L of VSS). The peak biomass concentrations were observed from days 12 or 14. Biomass concentrations started declining after this period, which coincided with the time when N was almost used up. The control bioassay bottles showed insignificant biomass increase (3–5% of experimental sets), indicating that phytoplankton growth in the bioassays was primarily supported by effluents.

In all four sets of bioassays, most of initial DTN (91–96%) was consumed or disappeared, while only 65–71% of PO\(_4\)\(^\text{3−}\) was removed (Table S1), indicating that the limiting nutrient in our bioassay was N, not P. With respect to N removal, nearly all DIN was removed, while DON removal was in the range of 63–91%, depending on the type of effluents. These varying rates of DON removal in bioassays were mainly attributed to the molecular weight distribution of effluent DON. In our bioassays, the average removal rate of LMW-DON (94%) was greater than that of HMW-DON (63%). Therefore, bioassays incubated with effluents containing less HMW-DON but more LMW-DON (i.e., BNR effluents) showed high DON removal; however, bioassays incubated with effluents having more HMW-DON but less LMW-DON (i.e., CAS effluents) exhibited low DON removal (Figure S2).

In terms of phytoplankton biomass production, effluents from the predenitrification system generated larger amounts of biomass than CAS effluents, even though their effluents contained less DTN than their counterparts (Figure 4 and Table S1). These results indicate that N-based biomass productivity (mass of biomass generated per mass of N consumed) was greater for predenitrification BNR effluents (Table S1), which we also observed with effluents from full-scale CAS and BNR facilities in our previous study.\(^\text{29}\) We postulate that this unexpected result was attributed to the composition of N that was available in the bioassays. This is supported by Figure 5 that illustrates correlations between N-based biomass production and ratios of consumed N forms (DIN, DON, HMW-DON, and LMW-DON) to consumed DTN in the four sets of bioassays. As more DIN was consumed, the N-based biomass production, in terms of VSS, decreased (Figure 5A). However, as DON, specifically LMW-DON, was used more, the N-based biomass production increased (Figure 5D) (the biomass production based on chl a, \(R^2 = 0.91\); Figure S3). These results suggest that LMW-DON has a greater potential to stimulate phytoplankton biomass than DIN, and this is likely the reason why predenitrification BNR effluents, containing more LMW-DON but much less DIN,
caused larger increases in phytoplankton biomass than CAS effluents. A previous bioassay study by Urgun-Demirtas et al. (2008) likewise showed a high potential for effluent-derived DON to stimulate phytoplankton growth. This study reported that effluents containing low amounts of DTN but a high DON-to-DTN ratio are a good source of N for phytoplankton growth, resulting in high N-based biomass production.

Implication. N regulations to control eutrophication in water bodies have been developed primarily based on TMDLs, calculations of the maximum amount of a nutrient or pollutant that a water body can receive and still meet water quality standards. For N-based TMDLs managers use the total amount of N as a regulatory parameter with an assumption that all forms of N have the same effect on phytoplankton growth. This has served as the basis for upgrading WWTPs from CAS to BNR, as the latter can certainly decrease total N loads into receiving water systems.

For WWTP upgrades to BNR, the predenitrification systems have been widely used due to several operational and economic advantages, and this has been a global trend for countries using the activated sludge system for wastewater treatment. Based on controlled reactor and field effluent studies, however, we found that there is a high probability that predenitrification BNR systems generate larger amounts of DON, especially LMW-DON, than CAS, which also showed greater potential to stimulate phytoplankton growth than DIN in coastal waters.

DON used to be considered an inert N source, particularly for phytoplankton. Recent studies, however, have provided convincing evidence that DON can serve as a N source to support phytoplankton growth. Literature indicates that both abiotic and biotic pathways to make DON bioavailable to phytoplankton, which includes the release of inorganic N from humic substances by salinity (i.e., ion exchange) and photocatalytic degradation, enzymatic breakdown of DON, and direct uptake of DON, such as via pinocytosis and phagocytosis.

Furthermore, our bioassay studies revealed that LMW-DON can lead to greater stimulatory effects on phytoplankton biomass than DIN. Thus, the merit of removing DIN by predenitrification, with higher capital and operational costs compared to CAS, could be potentially offset by a small yet potent source of LMW-DON in its effluent and discharge to N-sensitive coastal waters.

Another adverse effect that can be associated with higher DON release is that it can stimulate harmful phytoplankton species in estuarine and coastal systems. It has been reported that some harmful phytoplankton bloom species can utilize DON as a N source and may even prefer it to DIN. For example, dinoflagellates appear to have higher affinity for DON than DIN; some algal species causing red and brown tides such as *Lingulodinium polyedrum* and *Aureococcus anophagefferens* are also known to use DON as key N sources during bloom events. Moreover, there have been studies showing the possible link between escalated anthropogenic DON inputs to coastal area and global proliferation of harmful phytoplankton species. Based on the results of our study, we determined that the substantial increase in DON input to coastal area could also be partly due to upgrades of WWTPs to BNR.

Eutrophication in estuarine and coastal environments is a complex phenomenon affected by many different factors, but of fundamental importance is nutrient, specifically N, over-enrichment. Anthropogenic N sources include nonpoint (e.g., agricultural and stormwater runoff, groundwater, and atmospheric inputs) and point sources (industrial and urban

Figure 5. Correlations between N-based biomass production, based on volatile suspended solids (VSS), and the ratio of consumed N form to consumed dissolved total N (DTN). (A) DIN, (B) DON, (C) HMW-DON, and (D) LMW-DON.
wastewater inputs). We acknowledge that given these diverse sources, coastal eutrophication is not solely be driven by the increased release of LMW-DON from WWTPs after upgrades to BNR. Nevertheless, the significance of our study is the finding that different forms of effluent N show different phytoplankton stimulatory effects (LMW-DON showing greater potential for phytoplankton production than DIN), and the pre-denitrification BNR is vulnerable to releasing larger amounts of LMW-DON than CAS. Consequently, depending on the level of DIN removal and DON generation, upgrading WWTPs from CAS to pre-denitrification BNR cannot ensure alleviating eutrophication in estuarine and coastal receiving waters, especially those downstream of large urban centers, where WWTP effluent constitutes a significant source of N enrichment.

It is expected that LMW-DON generated during pre-denitrification BNR may not be easily removed by post-treatment processes, such as slow-sand filtration or biofiltration at WWTPs, because it is very small and also recalcitrant to the degradation by activated sludge-like microbial community (otherwise, it should have been degraded in the system). The removal of LMW-DON by the addition of multivalent cations that aid bioflocculation by effectively binding proteinaceous materials\(^\text{(45,46)}\) (i.e., organic N)\(^\text{(45,46)}\) may warrant future research. Furthermore, determining if different configurations of BNR would show a similar release of LMW-DON could also be important for future research on this topic. Finally, future research should focus on clarifying the quantitative and qualitative impact of N loads from WWTPs on estuarine and coastal eutrophication. This will aid in improving management of impaired estuaries and coastal oceans with proper responses from upstream wastewater treatment facilities.

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