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Investigation of characteristics of effluent DON derived from conventional activated sludge (CAS) and predenitrification biological removal (BNR): In terms of proteins and humic substances

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ABSTRACT

Upgrading wastewater treatment plants (WWTPs) from conventional activated sludge (CAS) to predenitrification biological nutrient removal (BNR) results in improved removal of dissolved inorganic nitrogen (DIN) from wastewater. However, changes in dissolved organic nitrogen (DON) with these WWTP upgrades and their potential impacts on receiving waters have been little researched. In this study, we investigated characteristics of effluent DON derived from CAS and predenitrification BNR, paying special attention to proteins and humic substances. Through a lab-scale reactor study and analysis of full-scale WWTP effluents, we found that in predenitrification BNR effluent, proteins are much more dominant than humic substances, whereas in CAS effluent, proteins and humic substances are similarly abundant. In terms of molecular weight, the majority of proteins were present in the effluent's low molecular weight (LMW) fraction (<1 kDa), while humic substances were found mostly in the effluent's high molecular weight (HMW) fraction (0.45 μm–1 kDa). Determination of dissolved organic carbon (DOC)/DON ratios in effluents supports that proteins (and LMW-DON) were most likely microbial-derived organic N produced during treatment processing, whereas humic substances (and HMW-DON) more likely originated outside of treatment systems. Bioassay tests demonstrated that effluent DON derived from predenitrification BNR was more bioavailable than that derived from CAS. We also found that LMW-DON and proteins were highly bioavailable DON compared to HMW-DON and humic substances. The results of this study suggest that upgrading CAS to predenitrification BNR makes effluent DON to become more conducive to phytoplankton blooms in receiving waters.

1. Introduction

Numerous urban waters, including lakes, rivers, estuaries, and coasts, have been experiencing accelerating eutrophication (Fry et al., 2011; Kabenge et al., 2016; Paerl et al., 2018), which creates multiple problems in aquatic environments such as excessive phytoplankton stimulation, depletion of dissolved oxygen, and disruption of ecosystems' trophodynamic and biogeochemical functions (Pinckney et al., 2001; Whitall et al., 2007; Wurtsbaugh et al., 2019). Among various natural and anthropogenic causes, human-induced nutrient enrichment is a key driver for eutrophication in urban waters (Pinckney et al., 2001; Paerl et al., 2018; Wurtsbaugh et al., 2019). Since many urban waterbodies exist under nitrogen (N)-limited conditions and are thus sensitive to N enrichment, N management has served as the principal means to control eutrophication in such waterbodies (Nixon, 1995; Cloern, 2001; Pehlivanoglu and Sedlak, 2004; Mischler et al., 2014). In particular, significant efforts have been made to cut down N loads from wastewater treatment plants (WWTPs) because wastewater-derived N accounts for a substantial N source to urban waters (Pehlivanoglu-Mantas and Sedlak, 2006; Filippino et al., 2011; Liu et al., 2012).

Upgrading wastewater treatment systems from conventional activated sludge (CAS) to biological nutrient removal (BNR) is a universal trend to decrease N discharges from WWTPs (Eom et al., 2017). BNR lowers the level of effluent N primarily by conversion (nitrification) and removal (denitrification) of dissolved inorganic N (DIN) (Rittmann and McCarty, 2012). Consequently, as WWTPs are upgraded from CAS to BNR, the principal N form in effluents changes from DIN to dissolved organic N (DON) (Pehlivanoglu and Sedlak, 2004; Westgate and Park,

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2010). In addition, these WWTP upgrades can also lead to changes in the characteristics of effluent DON (effluent-derived DON) because CAS and BNR rely on different microbial treatment processing. The literature reports that DON exhibits varying bioavailability depending on its chemical composition and characteristics (Pehlivanoglu-Mantas and Sedlak, 2006; Liu et al., 2012). Hence, it is expected that effluent DON derived from CAS and BNR, which potentially have different properties, can result in dissimilar phytoplankton stimulation in receiving waters. However, in spite of the environmental significance, this issue (the differences in effluent DON and their possible subsequent effects on receiving waters) has been little researched. Only a few studies have investigated dissimilarity of effluent DON derived from CAS and BNR with a special focus on their molecular weight distribution (Czerwionka et al., 2012; Huo et al., 2013; Eom et al., 2017). For example, Eom et al. (2017) reported that effluent DON derived from predenitrification BNR, the most common type of BNR that employs pre-anoxic and post-aerobic treatments, showed greater levels of low molecular weight DON (LMW-DON; less than 1 kDa) than that derived from CAS. The authors attributed this to the release of LMW-DON when treatment conditions were shifted from anoxic to oxic during predenitrification BNR processing. This postoxic release of DON has been likewise observed by other investigators who have studied the dynamics of N in lab-scale and full-scale predenitrification BNR systems (Czerwionka et al., 2012; Huo et al., 2013).

In the present study, we particularly investigated proteins and humic substances in CAS and predenitrification BNR effluents. Proteins and humic substances represent major constituents of effluent DON (Liu et al., 2012; Vakondios et al., 2014); however, the literature reports that they originate from different sources and exhibit dissimilar bioavailability (Bronk et al., 2007; Liu et al., 2012; Maizel and Remucal, 2017; Hu et al., 2018). For example, proteins in effluents are considered microorganism-derived organic N produced during biological treatment processing and are highly bioavailable (Liu et al., 2012; Yang et al. (2017); Hu et al., 2018, 2020). In contrast, humic substances in effluents are largely influent-derived recalcitrant organic N originating from drinking water sources or introduced from residential and industrial sources (Leenheer and Croué, 2003; Hu et al., 2018, 2020); unlike proteins, humic substances tend to resist biodegradation and show lower bioavailability due to their structural complexity (Drewes and Tox, 2000; Liu et al., 2012; Yang et al. (2017); Hu et al., 2018, 2020). Therefore, investigation of proteins and humic substances in wastewater effluents can enhance understanding the biochemical characteristics of effluent DON and estimating their possible impacts on receiving waters.

For the present study, we operated lab-scale CAS and predenitrification BNR treating identical wastewater under controlled conditions. We determined the total quantity and molecular wight distribution of proteins and humic substances in effluents from these lab-scale CAS and predenitrification BNR systems. We also analyzed dissolved organic matter (DOM) in effluents from lab-scale reactors based on DOC to DON ratios in different molecular weight fractions. During this study, a local full-scale WWTP was upgraded from CAS to predenitrification BNR to comply with a new N permit, providing us the opportunity to investigate changes in effluent DON resulting from the WWTP upgrade in a field full-scale system. Levels of proteins and humic substances in effluents from a full-scale WWTP were compared before and after the upgrade. Through bioassay tests, we further investigated bioavailability of effluent proteins and humic substances. Based on the results, we discuss possible impacts of changes in effluent DON resulting from upgrading CAS to predenitrification BNR on ecosystems and environments of receiving waters.

2. Materials and methods

2.1. Lab-scale CAS and predenitrification BNR reactors

Lab-scale wastewater treatment systems (two CAS and one predenitrification BNR; Fig. S1), seeded with 4 L of identical activated sludge (collected from the aeration basin of an Amherst WWTP, Amherst, MA, US), were operated in sequencing batch mode. All systems (6 L of working volume) had 4 sequencing batch cycles each day and were fed 2 L of identical wastewater, primary effluents from the Amherst WWTP, for one sequencing cycle. Detailed information about carbon (C) and N in influent wastewater is summarized in Tables S1 and S2. In all systems, one sequencing batch cycle (6 h) identically consisted of 10 min influent feeding, 290 min treatment, 50 min settling, and 10 min effluent decanting. However, the treatment conditions in CAS and predenitrification BNR systems were different. In the two CAS systems, the entire 290 min treatment period was maintained under aerobic conditions. In the predenitrification BNR reactor, the initial 2 h operation occurred under anoxic conditions, followed by 2 h 50 min aeration. All aerobic conditions were created by aeration with the same source of house air; the anoxic conditions in predenitrificiation BNR were maintained by purging the reactor with N_2 gas. Solid retention times (SRT) of CAS 1, CAS 2, and predenitrification BNR were 6, 20, and 20 d, respectively. Once the lab-scale reactor operations reached steady states (approximately 2 weeks after starting operations), we analyzed dissolved total nitrogen (DTN), different forms of DIN (NH_4^+, NO_2^-, and NO_3^-), DON, proteins, humic substances, and dissolved organic carbon (DOC) in influents and effluents every week. The number of samples used for the analyses were 18, 15, and 18 for CAS 1, CAS 2, and predenitrification BNR, respectively.

2.2. Upgrade of amherst WWTP from CAS to predenitrification BNR

During the present study, a local full-scale WWTP, the Amherst WWTP, was upgraded from CAS to predenitrification BNR. We investigated changes in C and N in the Amherst WWTP's secondary effluents (which are effluents from the 2nd clarifier and referred to, in this paper, as the Amherst WWTP effluents) before and after the upgrade of its treatment systems. This facility treats approximately 3.5–4 MGD of domestic wastewater, and its treatment processes consist of preliminary treatment (screening and grid removal), 1st clarification, main biological treatment, 2nd clarification, and chlorine disinfection (only for summer). For the main biological treatment, this WWTP has three trains of aeration basins, each consisting of three identical sub-basins. When in CAS mode (until 2011), two of the three aeration trains were operated with 6–8 h HRT and 5–10 SRT. In 2012, trials for retrofitting its system to predenitrification BNR proceeded. Starting in early 2013, the Amherst WWTP was operated as predenitrification BNR. For predenitrification, the first basin was maintained under anoxic conditions, whereas the second and third basins employed intermittent aeration (2.1 h on/1.5 h off). There was internal sludge return from the secondary clarifier to the first basin, which was approximately 25% of the influent flow rate. Predenitrification BNR was operated with 6–8 h HRT and 15–20 d SRT. For analyses of C and N in the Amherst WWTP effluents, we measured DTN, DIN, DON, proteins, humic substances, and DOC. The number of samples used for the analyses was 4 for CAS in 2011 and 7 for predenitrification BNR in 2013.

2.3. Bioassay tests

Bioassay tests were performed by incubating natural receiving water with effluent from either the lab-scale CAS 1 or predenitrification BNR in a 2 L Pyrex glass bottle (Fig. S2). The receiving water was collected from the Connecticut River and passed through a nylon net filter (100 μm pore size) to eliminate large particles but retain phytoplankton and bacteria employed as inoculum in the bioassay tests. The combining ratio of effluent to river water was 1:4. The bioassay bottles were loosely covered with a paper towel, and their contents were continuously mixed using a magnetic stirrer and a stir bar. The bioassay tests were operated in duplicate in a temperature-controlled laboratory and placed in front of a laboratory window. There was no shaded area, providing very similar natural lighting for the bioassays. A control bioassay incubating only river water without effluent was also run. Samples were collected from the bioassays based on the given time interval and visual changes. The collected samples were analyzed immediately for total suspended solids (TSS) and volatile suspended solids (VSS). For determination of N (DTN, DIN, DON, proteins, and humic substances) and P (PO_4^{3-}), samples were filtered using 0.45 µm nitrocellulose and 1 kDa ultra membranes and then frozen at −20 °C until analysis.

2.4. Molecular weight distribution of DON

In the present study, the dissolved phase of effluents was the fraction passing through filters with a pore size of 0.45 µm. DON was determined by the difference between DTN and the sum of DIN (NH_4^+, NO_2^-, and NO_3^-) in 0.45 µm filtrates. DON was further categorized into high molecular weight DON (HMW-DON), which is larger than 1 kDa, and LMW-DON, which is smaller than 1 kDa. To obtain different size fractions of effluents, we conducted ultrafiltration of raw effluent, using an Amicon stirred cell ultrafiltration device (Merck Millipore Corp., Burlington, MA, US) with a 1 kDa cellulose membrane (Merck Millipore Corp., Burlington, MA, US). The quantity of LMW-DON was determined by subtracting the sum of DIN from the amount of total N in the 1 kDa filtrates. HMW-DON was determined based on the difference between DON and LMW-DON.

2.5. Laboratory analysis

Concentrations of DTN and DOC in all samples were determined using a Shimadzu TN/TOC analyzer (Shimadzu North America, Columbia, MD, US). The quantity of DIN (NH_4^+, NO_2^-, and NO_3^-) in effluents was measured by ion chromatography (Metrohm AG, Herisau, Switzerland). Proteins and humic substances in the 0.45 µm and 1 kDa filtrates were determined based on the Lowry method modified by Frølund et al. (1995). Standard molecules for proteins and humic substances were bovine serum albumin (BSA, Fisherbrand Scientific, Pittsburg, PA, US) and humic acid (Sigma-Aldrich, St. Louis, MO, US), respectively. The concentrations of proteins and humic substances were expressed as N (mg N/L) with recognition that 16% and 3.2% of standard proteins and humic substances, respectively, are N. For humic acids, we verified the composition of N based on TOC and TN analysis of the standard molecules. Measurements of TSS and VSS followed the method described in the Standard Methods 2400D and E (APHA, 2005).

2.6. Statistical analysis

All data were analyzed and graphed using Microsoft Office Excel 2016. The statistical significance between the results was evaluated based on *p*-values determined by the *t*-test. A *p*-value less than 0.05 indicated statistical significance.

3. Results

3.1. Levels and molecular weight distributions of DOC and DON in CAS and predenitrification BNR effluents

Amounts of DOC in lab-scale CAS 1, CAS 2, and predenitrification BNR effluents were, on average, 12.8 ± 5.5 mg C/L, 14.0 ± 6.3 mg C/L, and 16.9 ± 4.3 mg C/L, respectively (Table 1). Only CAS 1 and predenitrification BNR showed a statistically significant difference in effluent DOC (p-value<0.02). For the molecular weight distribution of DOC, however, both CAS 1 and CAS 2 effluents were substantially different from predenitrification BNR effluents (Fig. 1A). Particularly, levels of LMW-DOC in CAS and predenitrification BNR effluents varied considerably. For example, LMW-DOC in CAS 1 and CAS 2 effluents were found to be, on average, 3.9 ± 1.9 and 3.7 ± 1.9 mg C/L, respectively, whereas LMW-DOC in predenitrification BNR effluents was, on average, 10.1 ± 2.5 mg C/L (p-value<0.01). Proportions of LMW-DOC to whole DOC in CAS and predenitrification BNR effluents were also notably dissimilar. In CAS 1 and CAS 2 effluents, LMW-DOC constituted only 31.6 ± 12.9% and 26.8 ± 6.0% of the whole DOC, respectively, while in predenitrification BNR effluents, LMW-DOC accounted for 60.6 ± 7.0% of the whole DOC (p-value<0.01).

DON concentrations in lab-scale CAS 1, CAS 2, and predenitrification BNR effluents were, on average, 1.9 ± 0.8 mg N/L, 2.2 ± 1.0 mg N/L, and 3.7 ± 0.8 mg N/L (Table 1), respectively, which fall within the typically reported ranges of DON (0.98 mg N/L-4.90 mg N/L) in secondary treated wastewater effluents (Pehlivanoglu-Mantas and Sedlak, 2006). Differences in effluent DON between two CAS and predenitrification BNR (i.e., CAS 1 vs predenitrification BNR; CAS 2 vs predenitrification BNR) were statistically significant (p-value<0.02). In CAS 1 and CAS 2 effluents, DON only composed of 9.6 ± 3.7% and 5.6 ± 7.7% DTN, respectively, whereas in predenitrification BNR effluents, DON made up of 43.3 ± 12.0% DTN. Abundance of DON in DTN is a

Table 1
Concentrations of DOC and DON, and DOC/DON ratios in effluents from lab-scale reactors. Numbers of samples: CAS1 = 18, CAS 2 = 15, and Predenitrification BNR = 18.

	DOC (mg C/L)	DON (mg N/L)	DOC/DON
CAS 1	12.8 ± 5.5	1.9 ± 0.8	6.7 ± 1.0
CAS 2	14.0 ± 6.3	2.2 ± 1.0	6.3 ± 0.7
Predenit. BNR	16.9 ± 4.3	3.7 ± 0.8	4.5 ± 0.3

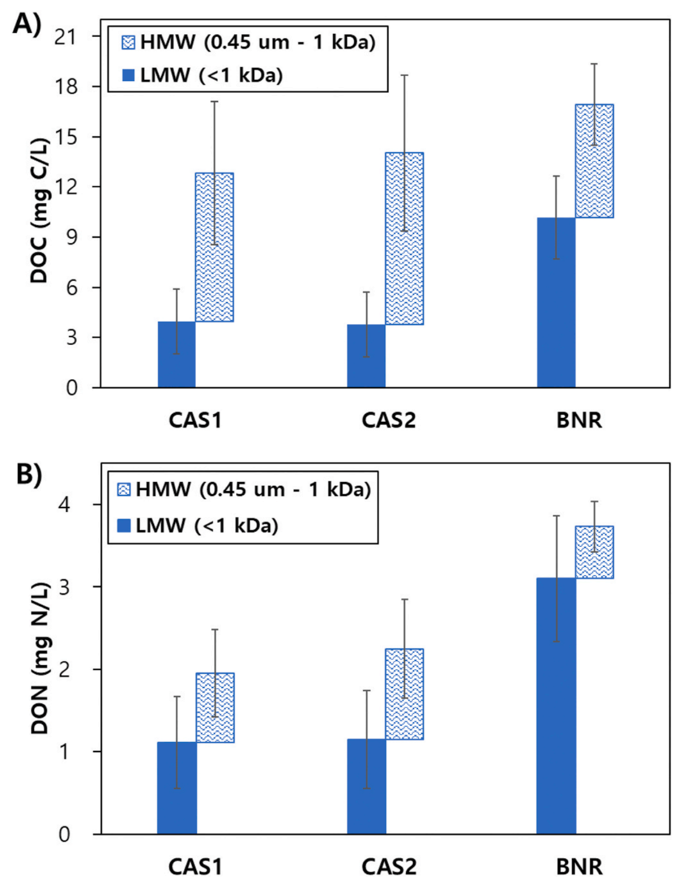

Fig. 1. Molecular weight distributions of DOC and DON in effluents from lab-scale reactors. A) DOC, B) DON. LMW = low molecular weight; effluent passing 1 kDa ultrafilter (i.e., <1 kDa). HMW = high molecular weight; effluent passing 0.45 µm filter but retained by 1 kDa ultrafilter (i.e., 0.45 µm–1 kDa). Number of samples: CAS 1 = 18; CAS 2 = 15; Predenitrification BNR = 18. Error bars represent standard deviations.

well-known characteristic of BNR-treated effluents because BNR removes DIN from raw wastewater (our lab-scale predenitrification BNR showed, on average, 77.4 ± 9.7% of DIN removal efficiency). In terms of molecular weight distribution of DON (Fig. 1B), levels of HMW-DON in CAS and predenitrification BNR effluents were largely similar (0.84 ± 0.53 mg N/L for CAS 1; 1.09 ± 0.60 mg N/L for CAS 2; 0.63 ± 0.31 mg N/L for predenitrification BNR); however, two treatment systems (CAS and predenitrification BNR) resulted in significantly different amounts of effluent LMW-DON. In CAS 1 and CAS 2 effluents, LMW-DON was found to be, on average, 1.11 ± 0.56 mg N/L and 1.15 ± 0.60 mg N/L, constituting 57.4 ± 19.3% and 52.4 ± 13.3% of the whole DON, respectively. However, in predenitrification BNR effluents, levels of LMW-DON were substantially greater, which were, on average, 3.10 ± 0.77 mg N/L, accounting for 82.9 ± 7.3% of the whole DON.

The average DOC and DON concentrations in effluents from the Amherst WWTP (a field full-scale WWTP) before and after upgrading the system from CAS to predenitrification BNR are presented in Table S3. Overall, the results from the Amherst WWTP are comparable to the findings from the lab-scale reactor study. For example, the Amherst WWTP showed relatively constant DOC concentrations in effluents, regardless of its treatment system (13.8 ± 1.0 mg C/L for CAS; 14.8 ± 1.8 mg C/L for predenitrification BNR). However, molecular weight distributions of effluent DOC were considerably different before and after the upgrade. When the Amherst WWTP was operated as CAS, effluent LMW-DOC was, on average, 3.8 ± 0.9 mg C/L, composing 27.4 ± 4.2% of whole DOC. However, when operated as predenitrification BNR, the average amount of LMW-DOC in effluents was 9.2 ± 1.9 mg C/L, making up 61.5 ± 6.7% of whole DOC. These results support that predenitrification BNR can produce much greater levels of LMW-DOC than CAS. For effluent DON, both total levels and molecular weight distributions were substantially changed by the upgrade. Before the upgrade, the average amount of effluent DON was 1.6 ± 0.1 mg/L, which increased to 2.5 ± 0.4 mg/L after upgrading (this increase is statistically significant; p-value<0.01) even though concentrations of DON in influents (to biological treatment) remained little changed (on average, 6.9 ± 1.0 mg N/L when CAS; 6.3 ± 0.5 mg N/L when predenitrification BNR). Despite the WWTP upgrade not causing a significant change in amounts of HMW-DON, levels of LMW-DON markedly varied before and after the upgrade. When the Amherst WWTP was in CAS mode, the average concentration of effluent LMW-DON was 0.7 ± 0.1 mg N/L (45.6 ± 5.8% of whole DON); after upgrading to predenitrification BNR, the average amount of effluent LMW-DON increased to 1.9 ± 0.3 mg N/L (75.3 ± 5.0% of whole DON), showing that upgrading CAS to predenitrification BNR can lead to a substantial increase in effluent LMW-DON.

3.2. Characterization of effluent DOM derived from CAS and predenitrification BNR based on a DOC to DON ratio

Effluent DOM derived from lab-scale CAS and predenitrification BNR was characterized based on DOC to DON (C/N) ratios in effluents. C/N ratios in CAS 1 and CAS 2 effluents were, on average, 6.7 ± 1.0 and 6.3 ± 0.7, respectively (Table 1). In predenitrification BNR effluents, the C/N ratio was, on average, 4.5 ± 0.3 (Table 1), which was fairly lower than those in both CAS effluents (p-value<0.05). This result suggests that DOM in predenitrification BNR effluents is more N-rich than that in CAS effluents. However, C/N ratios were not different between CAS and predenitrification BNR effluents when compared based on a given molecular weight fraction (Fig. 2). For example, C/N ratios in the HMW fractions of CAS 1, CAS 2, and predenitrification BNR effluents were, on average, 12.2 ± 4.3, 10.5 ± 3.2, 12.0 ± 6.6, respectively, and their differences were insignificant (p-value>0.2). The average C/N ratios in the LMW fractions of CAS 1, CAS 2, and predenitrification BNR effluents were in the range of 3.6 ± 0.8, 3.3 ± 0.3, and 3.3 ± 0.1, respectively, which were substantially smaller than those in the HMW fractions, but differences in the LMW fraction's C/N ratios among the three effluent

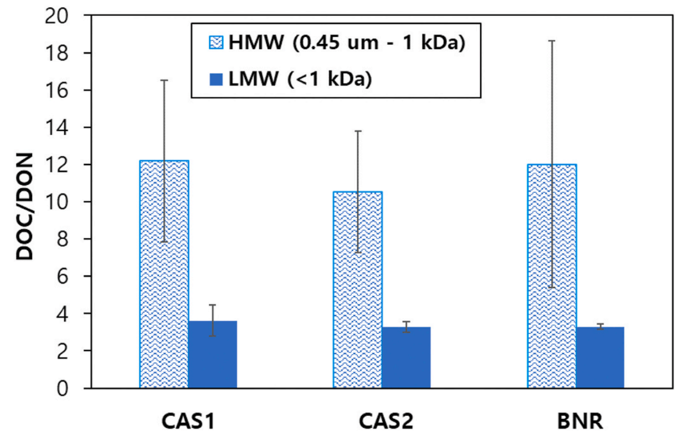

Fig. 2. DOC to DON ratios for HMW and LMW fractions of effluents from lab-scale reactors. Number of samples: CAS 1 = 18; CAS 2 = 15; Predenitrification BNR = 18. Error bars represent standard deviations.

types were, again, insignificant (p-value>0.1). These findings indicate that effluent DOM in the same molecular weight fraction shares a similar nature: HMW-DOM is C-rich, whereas LMW-DOM is N-rich, regardless of whether they are derived from CAS or predenitrification BNR. Fig. 3 also suggests that HMW-DOM and LMW-DOM show different characteristics. We found that LMW-DOC and LMW-DON had a very strong correlation ($R^2 = 0.98$; Fig. 3A), suggesting that effluent LMW-DOM is composed primarily of nitrogenous organic matter. On the other hand, a weaker correlation was observed between HMW-DOC and HMW-DON (R^2 =

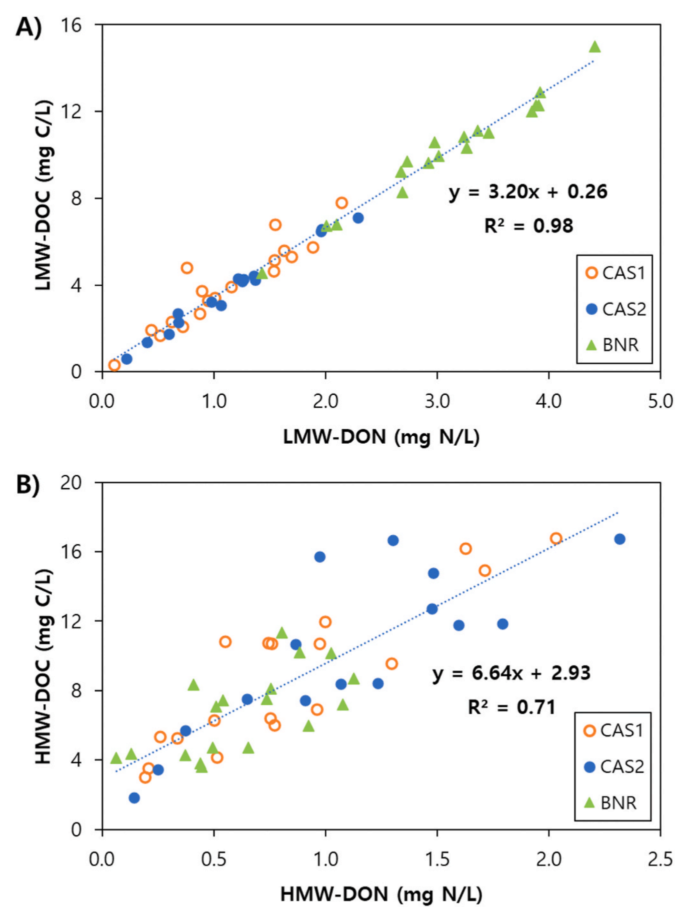

Fig. 3. Relationship between DON and DOC in effluents from lab-scale reactors. A) DOC vs DON in the LMW fraction of effluents (<1 kDa), B) DOC vs DON in the HMW fraction of effluents (0.45 μm–1 kDa).

0.71; Fig. 3B), implying that effluent HMW-DOM is comprised of diverse organic substances having different chemical characteristics.

In the Amherst WWTP effluents, C/N ratios changed after the upgrade (Table S3). In CAS mode, the C/N ratio was, on average, 8.7 ± 0.2; however, in predenitrification BNR mode, it was, on average, 6.0 ± 0.2 (p-value<0.01), supporting that effluent DOM derived from predenitrification BNR is more N-rich than that derived from CAS. Similar to the results from the lab-scale reactor study, we found that C/N ratios in the Amherst WWTP effluents are also comparable at a given molecular weight fraction. For example, when the facility was in CAS and predenitrification BNR modes, the average C/N ratios in the effluents' HMW fraction were 11.6 ± 1.7 and 9.8 ± 2.7, respectively, which are statistically similar (p-value>0.1). Moreover, C/N ratios in the CAS and predenitrification BNR effluents' LMW fraction were, on average, 5.3 ± 1.0 and 4.9 ± 0.5, respectively, which are also comparable (p-value>0.1) but significantly lower than those in the HMW fraction. These results confirm that the characteristics of effluent DOM are determined not only by the treatment system but also by the molecular weight.

3.3. Proteins and humic substances in CAS and predenitrification BNR effluents

Average amounts of dissolved proteins and humic substances in lab-scale reactor effluents are presented in Fig. 4. In CAS 1, CAS 2, and predenitrification BNR effluents, dissolved proteins were, on a N basis, 0.37 ± 0.16 mg N/L and 0.46 ± 0.20 mg N/L, and 1.06 ± 0.24 mg N/L, respectively. The proteins in predenitrification BNR effluents were approximately 3 and 4 times greater than those in CAS 1 and CAS 2 effluents, respectively (p-value<0.01). In contrast to the disparity of proteins, levels of dissolved humic substances were comparable among the three effluent types: on a N basis, 0.37 ± 0.15 mg N/L for CAS 1; 0.43 ± 0.20 mg N/L for CAS 2; 0.29 ± 0.10 mg N/L for predenitrification BNR (p-value>0.05).

Fig. 4 further shows that compositions of proteins and humic substances in effluent DON were significantly dissimilar depending on the treatment system. In CAS 1 and CAS 2 effluents, both proteins and humic substances accounted for approximately 19 %–22% of DON. In predenitrification BNR effluents, however, proteins constituted 28% of DON while humic substances made up only 7% of DON. These results reveal that in CAS effluent DON, proteins and humic substances were similarly abundant, while in predenitrification BNR effluent DON, proteins were much more abundant (4 times) than humic substances. However, in spite of the dissimilar compositions of proteins and humic substances in CAS and predenitrification BNR effluent DON, the sums of proteins and humic substances similarly accounted for 35 %–39% of their effluent DON in all three effluent types. This value approximates the finding by Pehlivanoglu-Mantas and Sedlak (2008) who reported that dissolved amino acids (proteinaceous organic N) and humic substances comprise 20 %–30% of effluent DON. Moreover, the sums of proteins and humic substances correlated positively with both effluent DOC and effluent DON (Fig. 5). In particular, correlation with effluent DON was very strong ($R^2 = 0.92$), suggesting that proteins and humic substances in wastewater effluents can serve as a surrogate for effluent DON, which was likewise reported by Westgate and Park (2010).

Fig. 6 compares molecular weight distributions of proteins and humic substances among the three effluent types. Overall, their patterns are fairly comparable. For example, in all three effluent types, on average, 83 %–87% of proteins were present in the LMW fraction and 74 %–76% of humic substances were found in the HMW fraction. This result suggests that effluent protein and humic substances are based on different molecular fractions. In addition, we found that in all effluent types, on average, 28 %–32% of LMW-DON was protein-DON and 31 %–35% of HMW-DON was humic substance-DON, demonstrating that LMW-DON is protein-rich and HMW-DON is humic substance-rich, regardless of the source of effluents. Fig. 7 illustrates correlations between proteins or humic substances and effluent DON at a given molecular weight (i.e., HMW-DON or LMW-DON). Proteins had a strong correlation with LMW-DON but not with HMW-DON (Fig. 7A and C). On the other hand, humic substances highly correlated with HMW-DON but not with LMW-DON (Fig. 7B and D). These findings clearly show strong association between proteins and LMW-DON and between humic substances and HMW-DON.

In the Amherst WWTP, compositions of proteins and humic substances in effluent DON were appreciably different depending on the

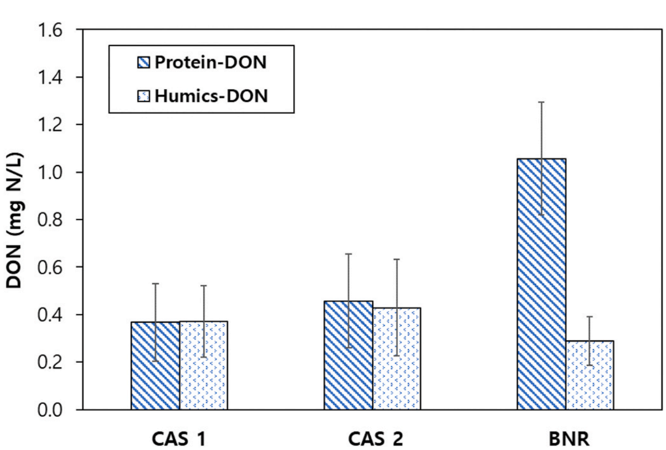

Fig. 4. Levels of proteins and humic substances in effluents from lab-scale reactors. Number of samples: CAS 1 = 18; CAsS 2 = 15; Predenitrification BNR = 18. Error bars represent standard deviations.

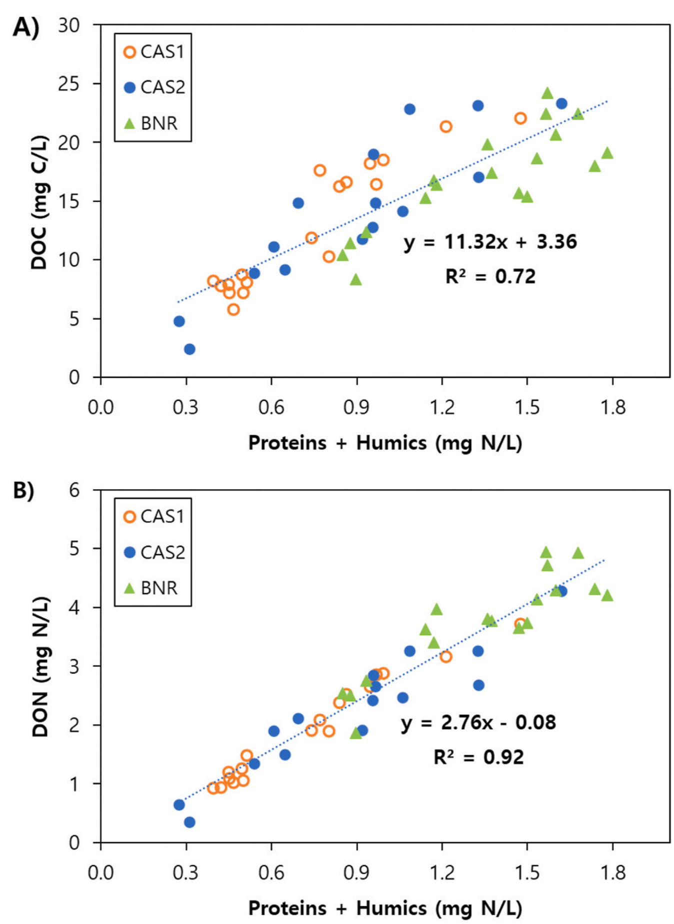

Fig. 5. Relationship between the sum of proteins and humic substances and effluent DOC or DON. A) DOC, B) DON.

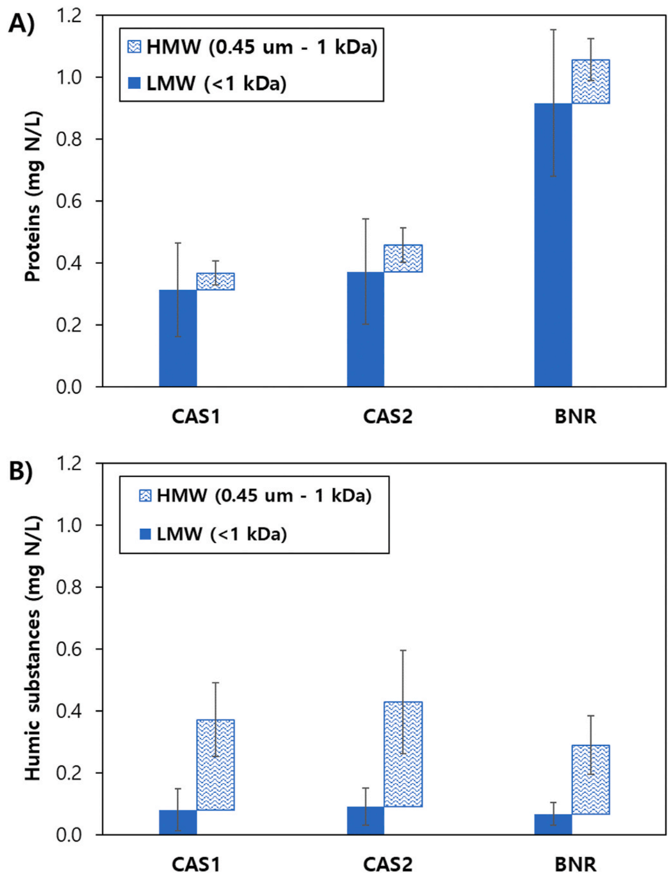

Fig. 6. Molecular weight distribution of dissolved proteins and humic substances in effluents from lab-scale reactors. A) Proteins, B) Humic substances. Number of samples: CAS 1 = 18; CAS 2 = 15; Predenitrification BNR = 18. Error bars represent standard deviations.

treatment process. When the Amherst WWTP was in CAS mode, the average amounts of proteins and humic substances in effluents were 0.37 ± 0.03 mg N/L and 0.40 ± 0.02 mg N/L, accounting for 23.6 ± 0.4 and 25.2 ± 1.3% of effluent DON, respectively. However, when it was in predenitrification BNR mode, it produced, on average, 0.72 ± 0.11 mg N/L and 0.23 ± 0.05 mg N/L of proteins and humic substances, constituting 29.3 ± 1.0% and 9.4 ± 0.6% of effluent DON, respectively. These findings correspond to the result from the lab-scale reactor study that proteins are more dominant in predenitrification BNR effluents than in CAS effluents. We were unable to compare molecular weight distributions of proteins and humic substances in the Amherst WWTP effluents before and after the upgrade due to the absence of data in CAS mode. Nevertheless, data in predenitrification BNR mode showed that in the Amherst effluents, on average, 76.6 ± 4.1% of proteins were LMW-proteins and 70.7 ± 6.7% of humic substances were HMW-humic substances, supporting that effluent proteins and humic substances are based on different molecular fractions.

3.4. *Bioavailability of effluent DON*

Results from bioassays incubating river water with effluent from lab-scale CAS 1 or predenitrification BNR are shown in Fig. 8. Patterns of N consumption and microbial biomass generation (VSS) were largely similar between the CAS and predenitrification BNR bioassays. For example, in both bioassays, DIN was consumed mainly during the initial period of operations (until day 10); DON was utilized later (after day 10–18) until productions of VSS reached maximum values (54 mg VSS/L for CAS bioassay; 66 mg VSS/L for predenitrification BNR) at day 18 (summary of bioassays is presented in Table S4). Commonly, in the CAS and predenitrification BNR bioassays, initial DTN was highly consumed (90 %–94%), whereas only 42%-47% of PO_4^{3-} was used, suggesting that the limiting nutrient for microbial stimulation in the bioassays was not P but N. In addition, the control bioassay, which incubated only river water without wastewater effluent, showed an insignificant increase in VSS (3 mg VSS/L), indicating that microbial growth in the bioassays was stimulated by effluents.

Despite DIN being nearly used up (97 %–98%) in both bioassays, consumption of DON in the two bioassays was dissimilar. In the CAS bioassay, initial DON was consumed by 65.0%; however, in the predentrification BNR bioassay, 81.9% of initial DON was used, suggesting that predenitrification BNR-derived DON is more bioavailable than CAS-derived DON. In terms of molecular weight, LMW-DON was consumed more than HMW-DON. In the CAS and predenitrification BNR bioassays, 80 %–92% of initial LMW-DON was used, while consumption of initial HMW-DON was in the range of only 44 %–62%, demonstrating that LMW-DON was much more bioavailable than HMW-DON in the bioassays. Proteins and humic substances also showed dissimilar bioavailability. In the two bioassays, initial proteins were consumed by 78 %–85%, whereas only 28 %–29% of initial humic substances were used, indicating that proteins are more bioavailable organic matters than humic substances. Overall, the bioassay tests demonstrated that N-rich or smaller-sized organic substances such as proteins, LMW-DON, and predenitrification BNR effluent DON are more bioavailable than C-rich or larger-sized organic matters such as humic substances, HMW-DON, and CAS effluent DON.

4. Discussion

This study investigated characteristics of effluent DON derived from CAS and predenitrification BNR, with particular focus on proteins and humic substances. Through lab-scale reactor study, we found that predenitrification BNR effluent contains significantly greater levels of proteins than CAS effluent (amounts of humic substances in CAS and predenitrification BNR effluents were comparable). Furthermore, the lab-scale reactor study demonstrated that effluent DOM derived from predenitrification BNR is more N-rich than that derived from CAS. Investigations of the Amherst WWTP (a full-scale WWTP) effluents also provided similar results to the lab-scale reactor study: upgrading the WWTP from CAS to predenitrification BNR resulted in an increase in effluent proteins and made effluent DOM more nitrogenous. We attribute these differences between CAS and predenitrification BNR effluents to the characteristics of HMW-DON and LMW-DON. Molecular weight distribution analysis (Figs. 6 and 7) revealed that effluent DON has dissimilar properties depending on its molecular weight (regardless of the treatment system where it originates): HMW-DON is rich in humic substances, whereas LMW-DON is rich in proteins. Accordingly, amounts of HMW-DON and LMW-DON in effluent can determine levels of proteins and humic substances and nature of effluent DOM. Because LMW-DON was more abundant in predenitrification BNR effluent than in CAS effluent (however, the amounts of HMW-DON in the CAS and predenitrification BNR effluents were similar), predenitrification BNR effluent showed a larger amount of proteins and the effluent's DOM had a more N-rich nature, compared to CAS effluent.

The literature suggests that the C/N ratio in DOM can serve as an indicator for its origin (Arheimer et al., 1996; Alberts and Takacs, 1999; Stepanauskas et al., 1999; Westerhoff and Marsh, 2002; Lee et al., 2006). A low C/N ratio with a range of 3–6 usually represents microbial-derived organic matter having proteinaceous characteristics (Stepanauskas et al., 1999; Westerhoff and Marsh, 2002; Lee et al., 2006). In contrast, a high C/N ratio in the range of 15–30 tends to indicate terrestrial material-originated organic matters like humic substances (Lee et al., 2006; Leenheer et al., 2007). As illustrated in Fig. 2, LMW-DOM had an average C/N ratio of 3.4. Moreover, Fig. 7 shows a strong correlation between LMW-DON and proteins in effluents. These findings suggest that LMW-DON is most likely of microbial origin associated with

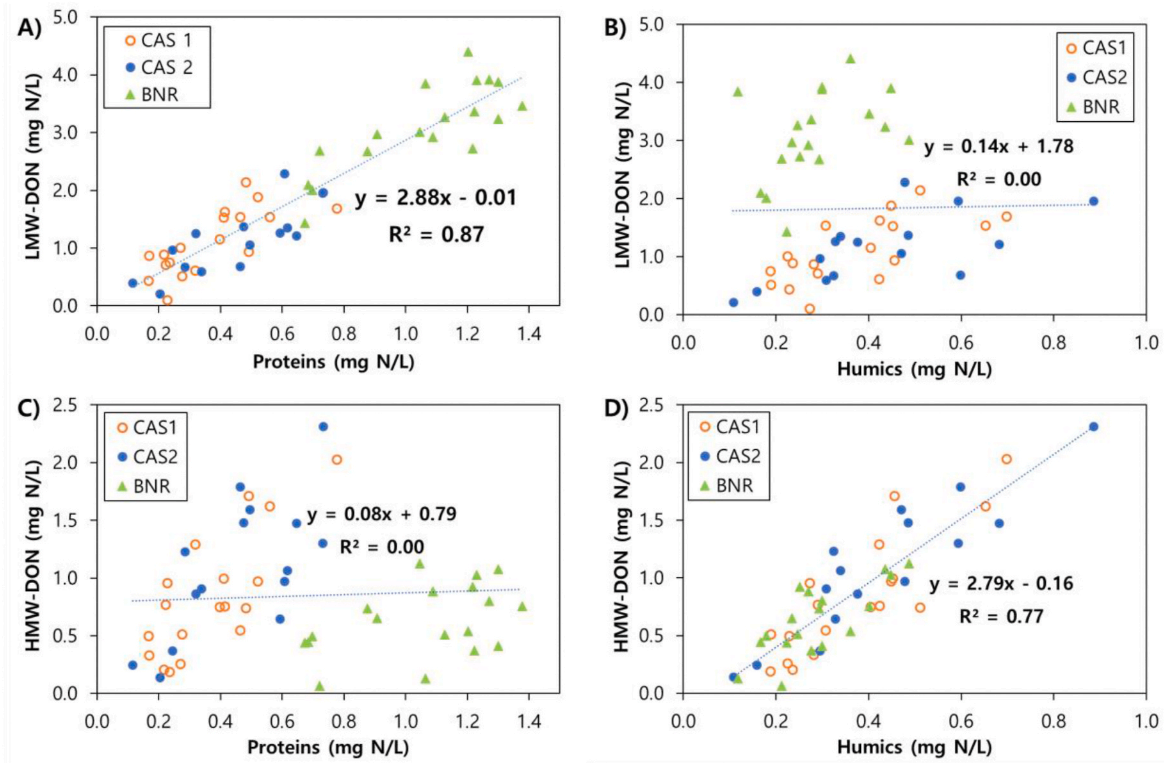

Fig. 7. Relationship between proteins or humic substances and effluent DON at a given molecular weight. A) Proteins vs LMW-DON, B) Humic substances vs LMW-DON, C) Proteins vs HMW-DON, D) Humic substances vs HMW-DON.

treatment processes. On the other hand, in both CAS and predenitrification BNR effluents, the C/N ratio of HMW-DOM was, on average, 11.6 (Fig. 2), and HMW-DON was highly correlated with humic substances (Fig. 7), suggesting that HMW-DON originates most likely from outside the treatment processes (i.e., influent wastewater).

Our earlier study (Eom et al., 2017), which investigated the fate dynamics of DON during CAS and predentrification BNR processing, also supports the above discussion. Eom et al. (2017) showed that influent LMW-DON was readily removed in both CAS and predenitrification BNR. However, significant amounts of LMW-DON were newly produced in predenitrification BNR due to the release of LMW-DON during the postoxic phase following the preanoxic period. This postoxic release causes LMW-DON to be the dominant form of DON in predenitrification BNR effluent. Accordingly, it is reasonable to think that a significant fraction of LMW-DON, which is abundant in predenitrification BNR effluent, is of microbial origin. In contrast, influent HMW-DON persisted in both CAS and predenitrification BNR, particularly in the former, implying that HMW-DON stems primarily from influent wastewater.

Previous research has demonstrated that the relative enrichment of C and N in organic matter can lead to varying bioavailability of N from organic matter in aquatic environments (Leenheer et al., 2007; Urgun-Demirtas et al., 2008; Liu et al., 2012; Qin et al., 2015). For example, it has been suggested that N-rich organic matter is a preferred source of N by microorganisms (Qin et al., 2015). In contrast, it has been shown that the complex structure in C-rich organic matter makes it difficult for microbes to liberate N and thus it is resistant to biodegradation (Leenheer et al., 2007; Liu et al., 2012). These examples from the literature therefore suggest that protein-DON and humic substance-DON in wastewater effluents would show dissimilar N bioavailability when released to receiving waters. Moreover, we can expect that effluent DON derived from CAS and predenitrification BNR exhibits varying bioavailability in receiving waters because the each DON has a dissimilar composition of proteins and humic substances. This expectation is consistent with the results from our bioassay tests which demonstrated that effluent DON derived from predenitrification BNR is more labile in stimulating microbial growth than that derived from CAS. There are numerous earlier studies reporting high bioavailability of N-rich organic matters. For example, Eom et al. (2017) and Hu et al. (2017) found that LMW-DON in wastewater effluents, protein-abundant DON, is highly bioavailable. Moreover, Yang et al. (2017), Hu et al. (2018), Liao et al. (2019), and Hu et al. (2020) found that microbial-derived proteinaceous DON is more bioavailable than humic substance-like DON.

The present study, as well as the above mentioned literature, suggests that upgrading WWTP from CAS to predenitrification BNR makes effluent DON to be more bioavailable. This implies that lowering N discharge via this WWTP upgrade may also negatively impact receiving water environments by altering the characteristics of effluent DON to be conducive of supporting phytoplankton stimulation. However, despite the coexistence of these positive and negative effects for upgrading CAS to predenitrification BNR, the latter consequence (changes in effluent DON by the WWTP upgrade and their possible adverse impact on receiving waters) has not been particularly embraced in current N management. This is because current N management plans are generally based on achieving total maximum daily loads (TMDLs) which solely target total quantity.

TMDL-based N plans cannot reflect the potential effects on the aquatic environments resulting from changes in the characteristics of N and cannot necessarily mitigate eutrophication in N-sensitive receiving waters. The example of Long Island Sound supports this concern (Suter et al., 2014). Long Island Sound is an urban coastal water that has been experiencing serious seasonal hypoxia for years. To improve the conditions of Long Island Sound, stringent TMDL-based N allowances were introduced, and a large number of WWTPs discharging into Long Island Sound was upgraded from CAS to BNR to lower their N discharges. As a result, total N loading to Long Island Sound decreased by approximately 20% since 2004 (whereas levels of DON in Long Island Sound increased continuously during this period). However, in spite of this significant decrease in N loading, the area affected by hypoxia in Long Island Sound

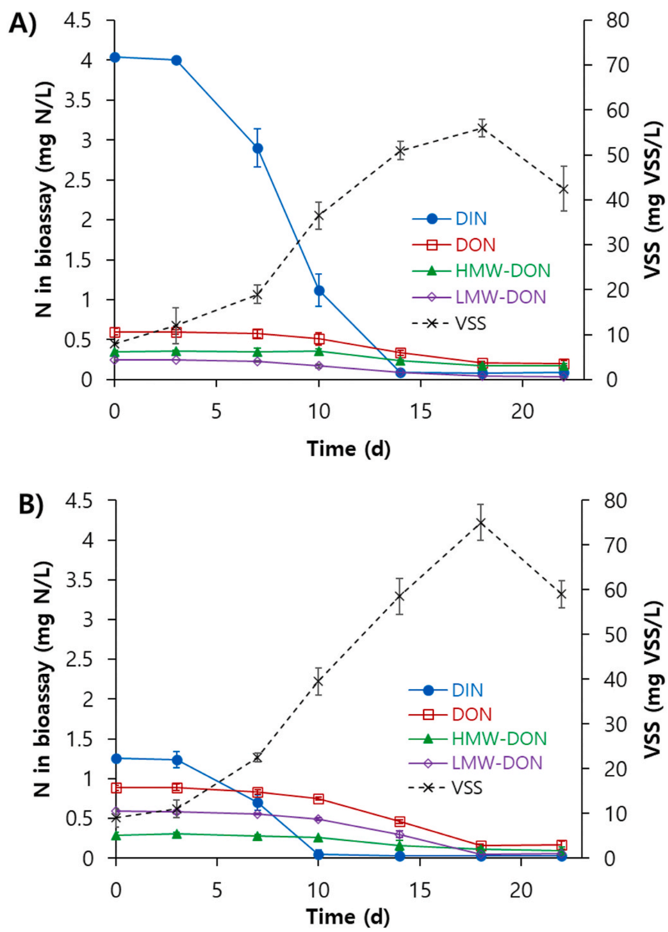

Fig. 8. Consumption of N and generation of VSS during bioassay tests. (A) CAS 1 effluent bioassay, (B) Predenitrification BNR effluent bioassay. Error bars represent the range of the results from the duplicate bioassays.

in 2006 actually expanded from 1991 to 2006 with a longer period of hypoxia. We concede that there are multiple factors causing this unexpected result in Long Island Sound. Nonetheless, we speculate that effluent DON may be one contributor. We believe that more comprehensive consideration based on both quantitative and qualitative assessments is necessary to establish better N management plans and actually mitigate eutrophication in N-sensitive aquatic environments.

5. Conclusions

In this study, we investigated properties of effluent DON derived from CAS and predenitrification BNR with determination of total quantities and molecular weight distributions of proteins and humic substances in effluents. The results showed that predenitrification BNR produced greater levels of proteins than did CAS; however, amounts of humic substances in effluents from the two systems (CAS and predenitrification BNR) were largely similar. Analyses of molecular weight distributions of effluent proteins and humic substances demonstrated that they were based on different molecular weight fractions. Proteins were found mostly in the LMW fraction, whereas humic substances were present mainly in the HMW fraction. C/N ratios in the effluents' HMW and LMW fractions suggest that proteins and LMW-DON are microorganism-derived organic N produced during treatment processing, whereas humic substances and HMW-DON originate outside of treatment systems. Bioassay tests demonstrated that predenitrification BNR effluent DON was highly bioavailable compared to CAS effluent DON. We also found that proteins and LMW-DON were more bioavailable DON than humic substances and HMW-DON. The findings from this study suggest that upgrading WWTPs from CAS to predenitrification BNR changes characteristic of effluent DON, making it more liable and capable of supporting microbial stimulation.

Author statement

Heonseop Eom: Conceptualization, Investigation, Data curation, Writing – original draft. Chul Park: Writing – Reviewing & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.110912.

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