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Comparison of N_2O Emissions and Gene Abundances between Wastewater Nitrogen Removal Systems

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Abstract

Biological nitrogen removal (BNR) systems are increasingly used in the United States in both centralized wastewater treatment plants (WWTPs) and decentralized advanced onsite wastewater treatment systems (OWTS) to reduce N discharged in wastewater effluent. However, the potential for BNR systems to be sources of nitrous oxide (N_2O), a potent greenhouse gas, needs to be evaluated to assess their environmental impact. We quantified and compared N_2O emissions from BNR systems at a WWTP (Field's Point, Providence, RI) and three types of advanced OWTS (Orenco Advantex AX 20, SeptiTech Series D, and Bio-Microbics MicroFAST) in nine Rhode Island residences (n = 3 per type) using cavity ring-down spectroscopy. We also used quantitative polymerase chain reaction to determine the abundance of genes from nitrifying (*amoA*) and denitrifying (*nosZ*) microorganisms that may be producing N_2O in these systems. Nitrous oxide fluxes ranged from -4×10^{-3} to 3×10^{-1} μmol N_2O m^{-2} s^{-1} and in general followed the order: centralized WWTP > Advantex > SeptiTech > FAST. In contrast, when N_2O emissions were normalized by population served and area of treatment tanks, all systems had overlapping ranges. In general, the emissions of N_2O accounted for a small fraction (<1%) of N removed. There was no significant relationship between the abundance of *nosZ* or *amoA* genes and N_2O emissions. This preliminary analysis highlights the need to evaluate N_2O emissions from wastewater systems as a wider range of technologies are adopted. A better understanding of the mechanisms of N_2O emissions will also allow us to better manage systems to minimize emissions.

Core Ideas

- First direct comparison of N_2O emissions from N removal at a WWTP and advanced OWTS.
- N_2O emissions (mol $area^{-1}$) from OWTS were generally lower relative to BNR at WWTP.
- N_2O emissions normalized per capita and area were similar between WWTP and OWTS.
- N_2O emissions generally represented <1% of N removed.
- N_2O emissions were not related to *amoA* or *nosZ* gene abundance.

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HUMANS substantially modify global nitrogen (N) cycles by industrially fixing N for fertilizer and ultimately releasing reactive N back to the environment through various mechanisms, including wastewater treatment. The continued growth of the human population will lead to further increases in excess reactive N, increasing the need for N remediation (Galloway et al., 2003). In recent years, remediation has focused on upgrading centralized wastewater treatment plants (WWTPs) to include biological nitrogen removal (BNR). Since one in five US homes are serviced by conventional onsite wastewater treatment systems (OWTS) (USEPA, 2013) they can also be large sources of N (Zhu et al., 2008; USEPA, 2015). The use of OWTS can be advantageous relative to centralized WWTPs, as they recharge groundwater supplies, require less infrastructure, and have lower energy costs (USEPA, 2013). To ameliorate N inputs to the environment, conventional OWTS are also being upgraded to advanced OWTS that include BNR.

Although BNR systems at WWTPs and OWTS vary in design, all employ nitrifying (conversion of ammonium to nitrate) and denitrifying (conversion of nitrate to nitrogen gas) bacteria in oxic and anoxic environments, respectively (Howarth et al., 2000). The systems are designed to remove N mainly in the form of N_2 gas, the final product of denitrification. However, in addition to N_2, the BNR process may produce substantial quantities of nitrous oxide (N_2O), a greenhouse gas 265 times more potent than carbon dioxide (CO_2) that can also deplete ozone in the stratosphere (Core Writing Team et al., 2014; Tomaszek and Czarnota, 2015). Nitrous oxide is produced by microbial N transformations including nitrification and denitrification. Nitrification can produce N_2O as a byproduct, and denitrification can be both a source and sink of N_2O (Wrage et al., 2001). Therefore, the abundance of nitrifying and/or denitrifying bacteria is likely a key factor influencing the rates of these N transformations associated with N_2O emissions.

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Abbreviations: *amoA*, ammonia monoxygenase; BNR, biological nitrogen removal; BOD, biochemical oxygen demand; CO_2e, carbon dioxide equivalent; DO, dissolved oxygen; FAST, fixed film-activated sludge treatment; IFAS, integrated fixed film-activated sludge; MG, million gallons; ML, million liters; *nosZ*, nitrous oxide reductase; OWTS, onsite wastewater treatment system(s); PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; scfm, standard cubic feet per minute; SP-D, denitrification sample point; SP-N, nitrification sample point; WWTP, wastewater treatment plant.

Previous studies have documented the magnitude of N_2O emissions relative to N removal rates from various types of BNR systems at centralized WWTPs, with emission factors (percentage of N load released as N_2O) varying by over four orders of magnitude, 0.001 to 25.3% (Tomaszek and Czarnota, 2015). In contrast, there are no published values for N_2O emissions from advanced OWTS designed to remove N. Biological nitrogen removal at both WWTPs and OWTS will become increasingly important as the human population and wastewater production continues to increase. Therefore, the magnitude of N_2O emissions from BNR of both WWTPs and OWTS should be determined to evaluate the effectiveness of these systems in N remediation and their potential impacts on greenhouse gas emissions. In addition, insights regarding the microbial sources of N_2O emissions will help to discern the potential mechanisms by which they may be mitigated through technological and operational changes to wastewater treatment systems while striving to maximize N removal.

We quantified and compared N_2O emissions from BNR at a centralized WWTP and three types of advanced OWTS (Orenco Advantex AX 20, SeptiTech Series D, and Bio-Microbics MicroFAST) in terms of instantaneous emissions, normalized per capita emissions, and emission factors (percentage of N released as N_2O). We also quantified and compared *amoA* (nitrification) and *nosZ* (denitrification) gene abundances and ratios from the same treatment systems to examine potential relationships between abundances of nitrifying and/or denitrifying bacteria and N_2O emissions.

Materials and Methods

Study Sites and Measurement Locations

The wastewater treatment systems we examined were within the Greater Narragansett Bay Watershed in Rhode Island. Field's Point is a full-scale centralized WWTP serving 226,000 people in Providence, RI (Narragansett Bay Commission, 2017). The plant provides primary and secondary treatment for flows up to 291 million L (ML) (77 million gallons [MG]) d^{-1} for combined sewer from domestic and industrial sources. Secondary treatment includes an integrated fixed film-activated sludge (IFAS) system for BNR. The IFAS system consists of 10 identical open air tanks, each with the following four main zones: (i) pre-anoxic (3.4 ML, 0.9 MG), (ii) aerated IFAS (13.6 ML, 3.6 MG), (iii) post-anoxic (5.7 ML, 1.5 MG), and (iv) reaeration (1.5 ML, 0.4 MG) (Supplemental Fig. S1). The aerated IFAS zone provides additional surface area for biofilm growth with the inclusion of perforated high-density polyethylene cylinder media (25-mm diam., 10-mm length). Two N_2O emission measurements and water samples were collected in each of the four zones of one IFAS tank. Water samples were collected from just below the water surface within 3 h of the emission measurements.

We examined three of the most commonly used advanced OWTS technologies for BNR in RI: Orenco Advantex AX20 (recirculating textile media filter), Bio-Microbics MicroFAST (fixed activated sludge treatment unit), and SeptiTech D Series (recirculating trickling filter) (Supplemental Fig. S1). All OWTS were located in Jamestown, RI, with measurements made in three systems per technology (nine systems total). These three technologies are sized and designed to treat wastewater from a three-bedroom dwelling (1703 L d^{-1}, 450 gallons) and, unlike the open-air IFAS system at the WWTP, are closed systems where air is entrained into an aeration compartment or chamber. All systems have an anoxic compartment for denitrification (sample point: SP-D) and an oxic compartment for nitrification (sample point: SP-N). Although all three technologies remove N according to the principles of biological nitrification and denitrification, the specific treatment process varies according to technology. The Orenco Advantex AX20 system is a timed-dosed media filter that uses vertically hanging textile sheets that receive recirculated flow from a processing tank (5678 L, 1500 gallons) that also serves as a denitrification reactor. Nitrification occurs in the nonsubmerged textile filter component (sampled at SP-N), and denitrification in the processing tank (SP-D). Removal of N occurs as wastewater recirculates several times per day between the textile nitrification zone where passive air flow occurs and the denitrification zone.

The Bio-Microbics MicroFAST system is a submerged fixed film-activated sludge treatment system (FAST) that uses a two-compartment tank (5678 L, 1500 gallons) that is not time dosed. The first compartment serves as a primary treatment zone, and the second compartment contains an insert of submerged rigid honeycomb-like block media (FAST insert) where active air entrainment from a continuously operating blower promotes nitrification (sampled at SP-N). Denitrification occurs in the FAST system in the low-oxygen areas of the second compartment (SP-D), adjacent to the block media insert.

The SeptiTech Series D system is a timed-dosed, two-tank trickling filter system. The first tank (3785 L, 1000 gallons) serves as a primary treatment zone and denitrification reactor (SP-D). Flow from this tank enters the trickling filter processor tank (3785 L, 1000 gallons), which contains two pumps involved with N processing: one pump recirculates effluent to the nonsubmerged filter media, where nitrification occurs (sampled at SP-N); the second pump recirculates sludge and nitrified wastewater from the trickling filter processor tank back to the primary tank (SP-D), where further solids processing and denitrification occurs. In all three technologies, effluent from SP-N is the final effluent that is dispersed to the system soil treatment area.

We made one N_2O emission measurement and collected one water sample from each compartment (SP-D and SP-N) in each of the nine systems per sampling event. The access riser lid to the systems was removed to allow trapped gases to vent for ~15 min before the emission measurement was made. Water samples were collected from the middle of the water column immediately after emission measurements were made.

Nitrous oxide emission measurements and wastewater samples were collected from each system once in June and once in October, resulting in a total of 16 measurements for the WWTP and 36 for the OWTS. Logistical constraints prevented sampling from all sites on the same day. Thus, sampling of all systems took place within 2 wk of each other during each round of measurements.

Nitrous Oxide Emission Measurements

At each study site, N_2O emission measurements were made using a closed chamber connected to a real-time cavity ring down spectroscopy analyzer (Picarro G2508) capable of measuring N_2O approximately every 2 s (detailed in Brannon et al., 2016). At the centralized WWTP, we used a transparent (polypropylene) rectangular floating chamber (height: 0.3 m, width: 0.3 m, length:

0.5 m). At the OWTS sites, an open-bottom polyvinyl chloride cylindrical chamber (i.d.: 0.13 m, length: 0.40 m) was placed on the water so that the bottom was submerged 7.5 cm below the surface. The chamber was kept level and at a constant depth using a stabilizing bar that rested across the top of the access port. The chamber was deployed for between 3 and 10 min at all sites.

Gas emissions from all zones at the centralized WWTP, except the aerated IFAS zone, and both compartments of all OWTS sites were calculated as outlined in Mello et al. (2013) for nonaerated stages. Due to the high aeration rates used in the IFAS zone at the centralized WWTP (~44 m^3 min^{-1} [~1457 standard cubic feet min^{-1}, scfm]), emissions from this zone were calculated using a method for aerated stages that accounts for the effects of air flow (Mello et al., 2013).

The statistical significance of each gas emission was determined following Brannon et al. (2016), with the exception that if the *p*-value of the linear regression of concentration over time was not statistically significant, then the flux was reported as zero. There were four measurements, two each from two different Orenco Advantex systems, that we were not able to calculate the emission value for because the concentration of methane (CH_4), another gas measured by the analyzer, exceeded the upper range of the analyzer and interfered with analysis of the target species (N_2O).

For comparison across systems, N_2O emissions were normalized by population served and area of the treatment tank (mg N_2O $capita^{-1}$ d^{-1}) according to Supplemental Eq. [1] (WWTP) and [2] (OWTS). Also, N_2O emission factors (mass $mass^{-1}$) were computed by normalizing the flux to the quantity of N removed, according to Supplemental Eq. [3] (WWTP) and [4] (OWTS). For the IFAS BNR system at the centralized WWTP, one normalized emission value and one emission factor (mass $mass^{-1}$) was calculated for each date that included the total emissions for the IFAS system (all four zones of all 10 tanks). For the OWTS, one normalized emission value and one emission fraction (mass $mass^{-1}$) was calculated for each house on each date ($n = 6$ per technology).

DNA Extraction

Genomic DNA was extracted from water samples from the WWTP and OWTS. For the centralized WWTP samples, ~50 mL of sample was centrifuged at 3000*g* for 15 min, and the solids were used for DNA extraction using a PowerSoil DNA Isolation Kit (MoBio Laboratories). For the OWTS, ~100 mL of sample was vacuum filtered onto sterile 0.22-μm-pore-size nitrocellulose membrane filters (Millipore Corporation). Nonsterile filters were used for 12 samples, but blanks were included to check for contamination. The filter was used for DNA extraction using a PowerWater DNA Isolation Kit (MoBio Laboratories). The quality and concentration (ng μL^{-1}) of all extracted DNA was determined with a NanoDrop 8000 ultraviolet-visible spectrophotometer (Thermo Fisher Scientific) and stored at −20°C or below until quantitative polymerase chain reaction (qPCR) analysis.

Quantitative Polymerase Chain Reaction

The concentrations of ammonia monooxygenase genes (*amoA*) and nitrous oxide reductase genes (*nosZ*) were quantified by real-time qPCR using the primer sets developed by Geets et al. (2007) and Junier et al. (2009) (Supplemental Table S1). Individual standard curves were prepared for each gene from a sample that presented one clear band of the correct size after PCR amplification and was purified with a QIAquick PCR Purification Kit (Qiagen). The concentration (ng μL^{-1}) of purified products that served as standards was determined using an Invitrogen Qubit 2.0 fluorometer (Thermo Fisher Scientific) and converted to copies per microliter. Tenfold serial dilutions of the purified product were prepared from 10^7 to 10^1 copy numbers μL^{-1}.

The real-time PCR quantification was performed on a Lightcycler 480 (Roche Diagnostics) with SYBR Green I Master (Roche Diagnostics). All standards and samples were analyzed in triplicate, and at least one triplicate negative control containing no template DNA was analyzed in each qPCR run to detect contamination. For both genes, a total reaction volume of 20 μL was used, which contained 5 μL DNA template, 0.5 μL of each primer, 10 μL of the SYBR Green I Master, and 4 μL of water. The thermocycler settings for *nosZ* were as follows: 94°C for 10 min, 45 cycles at 94°C for 10 s, 61°C for 15 s, and 72°C for 20 s. The thermocycler settings for *amoA* were as follows: 94°C for 10 min, 45 cycles at 94°C for 10 s, 54°C for 10 s, and 72°C for 14 s. Amplification efficiencies for both genes ranged from 78 to 100%. A melt curve was analyzed for every run, and the qPCR product for one of each triplicate was examined on a 1% (w/v) ethidium bromide-stained agarose gel to confirm the amplification of a single product for both genes. In addition to concentration (copies μL^{-1}), the abundance of each gene (copies ng^{-1} nucleic acid) was calculated using the qPCR results and the total concentration of DNA.

Wastewater Properties

For WWTP samples, a subset of the water sample used for qPCR analysis was filtered (0.45-μm pore size) and the filtrate used to determine the concentration of ammonium (NH_4) using the phenolhypochlorite method (Solorzano, 1969) and nitrate (NO_3^-) using the dimethylphenol method (Hach Company, 2015). The pH (Seven Go Duo Pro, Metler Toledo) and dissolved oxygen (DO; LDO Probe, HACH Model 57900-00) of wastewater were measured at the surface within 2 h of the emission measurements. The water temperature was continuously measured in the IFAS zone only with a LDO probe (HACH Model 57900-00). The average water temperature during the time of the flux measurements is reported in Supplemental Table S2.

For the OWTS samples, a Hanna Instruments HI9828 Multiparameter Meter was used to determine wastewater pH, DO, and temperature in the field in each compartment. A subset of the sample used for qPCR analysis was used to determine the concentration of NH_4, NO_3^-, and 5-d biochemical oxygen demand (BOD_5) as described in Lancellotti et al. (2016).

Statistical Analysis

We used linear regressions to examine relationships between N_2O emissions and gene abundances and *amoA*/*nosZ* ratios, between N_2O emissions and the wastewater properties, and gene abundances and *amoA*/*nosZ* ratios and the wastewater properties. Two separate regressions were performed: one for nitrification zones (aerated IFAS and reaeration zones for the WWTP and SP-N for the OWTS) and one for denitrification zones (pre-anoxic and post-anoxic zones for the WWTP and SP-D for the OWTS). Gene concentrations below the detection limit of 10 copies μL^{-1} were assigned a value of zero. Wastewater

properties below the detection limit were assigned a value of zero. All data were checked for normality and transformed when necessary. All statistical analyses were performed using JMP 13 (SAS Institute, 2016).

Results and Discussion

Nitrous Oxide Emissions

The largest N_2O emissions at the WWTP were from the aerated IFAS zone and the post-anoxic zone, whereas emissions from the pre-anoxic and reaeration zones were relatively low (Fig. 1A). The emissions of N_2O from the WWTP represented between 0.02 and 0.04% of N removed, which is in the lower end of the range (0.001–25.3%) reported by studies from other types of BNR systems at WWTPs (Tomaszek and Czarnota, 2015).

Our study is the first to measure N_2O emissions from advanced OWTS designed for N removal. The Advantex systems had the highest N_2O emissions of the three OWTS (Fig. 1A), and emissions were similar between SP-D (denitrification) and SP-N (nitrification) for all OWTS systems (Fig. 1A). Similar to the WWTP, the N_2O emissions from the SeptiTech and FAST OWTS represented a relatively small percentage of the N removed (0.0–4.4%). In contrast, the N_2O emissions from the Advantex systems represented a much higher percentage of the N removed (0.05–21.00%). Although 79.0 to 99.5% of the removed N was presumably lost as N_2, conditions within the Advantex treatment train appear to favor more N_2O production compared with the other systems. For example, the Advantex systems had the lowest pH (6.4) (Supplemental Table S2). Previous studies have demonstrated that *nosZ* is sensitive to low pH (<6.5) resulting in reduced conversion of N_2O to N_2 (Law et al., 2012). Differences in the magnitude of N_2O emissions between the OWTS may also be explained by differences in the process configurations including, but not limited to, interaction of the oxic and anoxic zones, stirring methods, and aeration methods. Future studies will be needed to determine the mechanism responsible for the differences in N_2O emissions between systems.

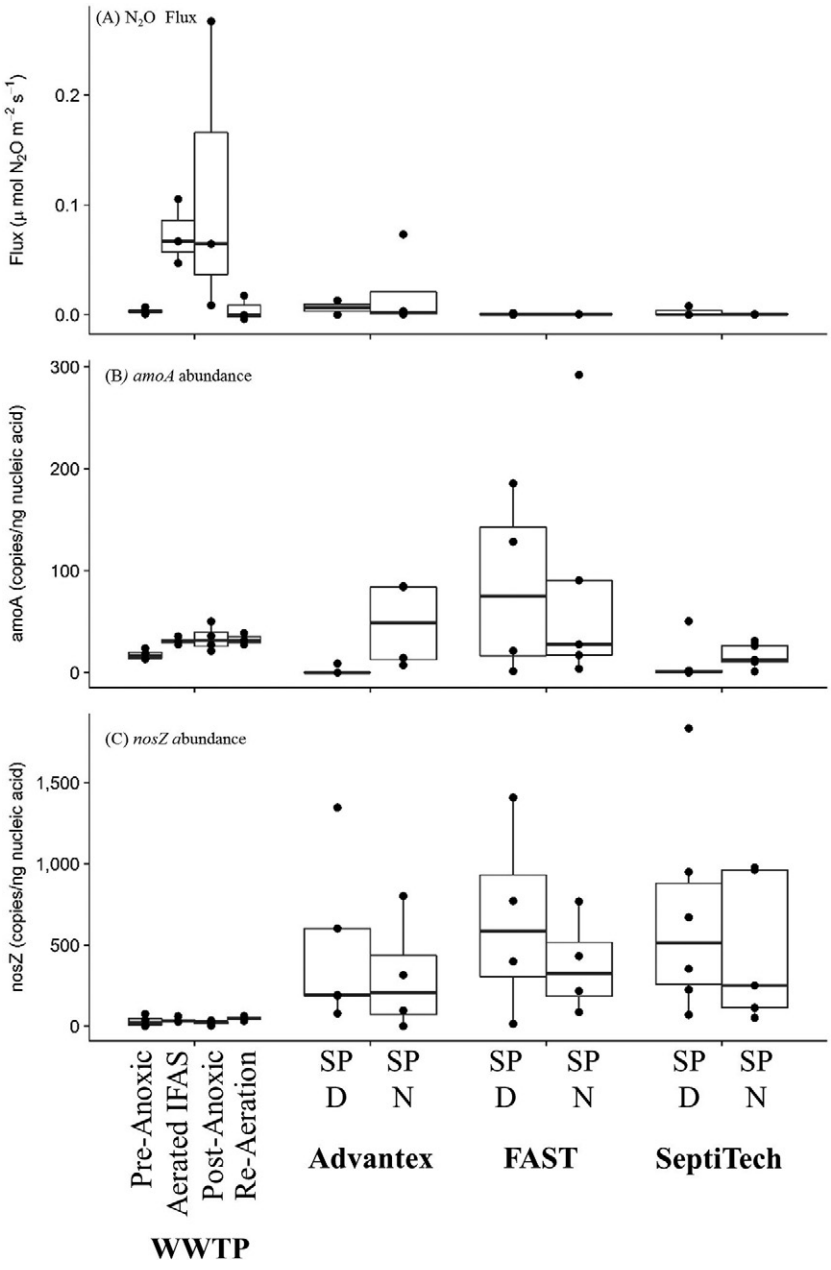

Fig. 1. (A) Nitrous oxide (N_2O) fluxes, (B) *amoA* abundance, and (C) *nosZ* abundance from pre-anoxic, aerated integrated fixed film-activated sludge (IFAS), post-anoxic, and reaeration zones in the wastewater treatment plant (WWTP) and denitrification (SP-D) and nitrification (SP-N) compartments in Advantex, FAST, and SeptiTech (onsite wastewater treatment systems). Solid line in middle of box represents the median, edge of box represents first and third quartile, and whiskers extend 1.5× the inter-quartile range beyond the edge of the box.

The emissions of N_2O from the aerated IFAS and post-anoxic zones at the WWTP were higher than those from all three OWTS (Fig. 1A). In contrast, emissions from the pre-anoxic and reaeration zones at the WWTP were similar in magnitude to those from all three OWTS (Fig. 1A). It is not surprising that the highest N_2O emissions in this study are from the aerated IFAS zone of the WWTP, since it uses high air flow rates (on average 49 m^3 min^{-1} [1638 scfm]) compared with the OWTS (FAST: 0.5–0.8 m^3 min^{-1} [17–25 scfm]; SeptiTech: venturi tube air intake [not quantified]; and Advantex: passive air diffusion). Higher air flow rates at the WWTP may cause higher N_2O emissions due to mechanical stripping of dissolved N_2O. There was not a significant relationship between N_2O and any of the wastewater properties in either the nitrification or denitrification components of these systems (data not shown).

Although N_2O emissions were observed from all systems, a negative N_2O flux (indicating uptake or consumption) was observed on two occasions (two measurements in the WWTP reaeration zone) out of 34 measurements total. Although negative N_2O fluxes have not been reported for BNR systems, they have been observed in soil and aquatic ecosystems (Chapuis-Lardy et al., 2007; Beaulieu et al., 2015; Soued et al., 2016). It is generally assumed that heterotrophic denitrification is responsible for N_2O consumption (Chapuis-Lardy et al., 2007) and that,

in those cases, the N_2O is being reduced fully to N_2. Since NO_3^- is a preferred electron acceptor over N_2O and *nosZ* is sensitive to oxygen, it is likely that N_2O uptake is confined to N-limited systems with low DO (Chapuis-Lardy et al., 2007). However, the two N_2O uptake events in this study did not coincide with excessively low NO_3^- or DO levels in the wastewater. Therefore, the reasons for N_2O uptake are unclear.

We used the total surface area and estimates of the number of individuals served by each system to calculate normalized N_2O emission values, which ranged from 0 to 624 mg N_2O capita^{-1} d^{-1} (Fig. 2). The average for the WWTP was 6.0 mg N_2O capita^{-1} d^{-1}, at the lower end of the range (0.8 to 383.6 mg N_2O capita^{-1} d^{-1}) reported for other types of BNR systems at WWTPs (Ahn et al., 2010). The average N_2O emission from OWTS in this study (60 mg N_2O capita^{-1} d^{-1}) is the first to our knowledge to be reported for any advanced OWTS and is higher than that determined from one conventional OWTS (without BNR) (5 mg N_2O capita^{-1} d^{-1}) (Diaz-Valbuena et al., 2011). Another study measured N_2O emissions from the roof vent (0.013 t CO_2 equivalent [CO_2e] capita^{-1} yr^{-1}), sand filter (6.5×10^{-4} t CO_2e capita^{-1} yr^{-1}), and leach field (2.4×10^{-3} t CO_2e capita^{-1} yr^{-1}) of several OWTS in New York (Truhlar et al., 2016). The N_2O emissions measured in this study (Advantex: 0.08 t CO_2e capita^{-1} yr^{-1}; SeptiTech 7.7×10^{-3} t CO_2e capita^{-1} yr^{-1}; FAST: 1.6×10^{-3} t CO_2e capita^{-1} yr^{-1}) were generally larger than those reported by Truhlar et al. (2016). Our results suggest that advanced OWTS designed for N removal may have higher N_2O emissions than conventional advanced OWTS lacking N removal. The water quality benefits of N removal at both WWTPs and OWTS may therefore come at the cost of increasing N_2O in the atmosphere, which would transfer the N problem from one environment (wastewater) to another (the atmosphere). As more N-reducing advanced OWTS are installed and/or WWTPs are upgraded to include BNR, they may become a larger source of N_2O.

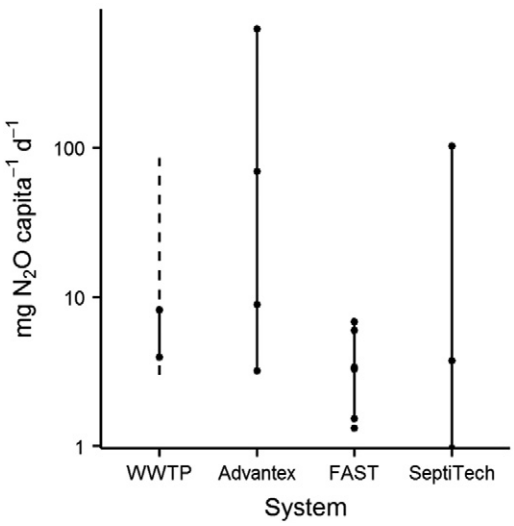

Fig. 2. Range of N_2O emissions (mg N_2O capita^{-1} d^{-1}) for each system as a whole (including all zones and compartments). Dashed line represents previously reported fluxes for the wastewater treatment plant (WWTP) examined in this study. For the WWTP, there is a data point for each day of measurements (n = 2). For the onsite wastewater treatment systems, there is a data point for each house on each date that had significant emissions, Advantex (n = 4), SeptiTech (n = 4), and FAST (n = 6).

Nucleic Acid Concentration

The concentration of nucleic acids (a proxy for the size of the microbial community) in all zones at the WWTP was five times higher than those of the three OWTS (Fig. 3). This is interesting because it does not appear that the WWTP receives larger carbon inputs compared with OWTS. Although the BOD of the influent to the OWTS in this study was not measured, it typically ranges from 145 to 386 mg L^{-1} (Loomis and Kalen, 2014), which is similar to the average BOD of the WWTP influent in this study (200 mg L^{-1}) (Supplemental Table S2). The nucleic acid concentration was generally higher in SP-D (denitrification compartment) compared with SP-N (nitrification compartment) in all three of the OWTS (Fig. 3). This is not surprising because SP-D of these OWTS receive septic tank effluent with high BOD (Supplemental Table S2).

Nitrifier (*amoA*) and Denitrifier (*nosZ*) Specific Abundance

In general, *amoA* specific abundance was higher at the WWTP than any of the three OWTS technologies, except SP-D of FAST and SP-N of Advantex (Fig. 1B). At the WWTP, the lowest *amoA* abundance was in the pre-anoxic zone, whereas the abundance in the other three zones (aerated IFAS, post-anoxic, and reaeration) was similar in magnitude (Fig. 1B). Of the three OWTS, the highest *amoA* abundance was in FAST systems (Fig. 1B). In addition, there was a trend of higher *amoA* abundance in the SP-N than SP-D in Advantex and SeptiTech systems (recirculating media filter technologies), but not FAST systems (extended aeration technology) (Fig. 1B). There was a significant positive relationship between *amoA* abundance and DO in denitrification zones and compartments ($p < 0.01$, $r^2 = 0.88$). The specific abundance of *amoA* in this study (0–10^2 copies ng^{-1} DNA) was within the range reported from other BNR systems (10^1–10^5 copies ng^{-1} DNA), including an integrated anoxic or oxic reactor (Wang et al., 2014) and conventional activated sludge (Song et al., 2014).

The specific abundance of *nosZ* did not follow the same trends within and between system types as *amoA* abundance (Fig. 1). The specific abundance of *nosZ* was generally higher in all three OWTS than in all four zones of the WWTP (Fig. 1C). At the WWTP, there was higher *nosZ* abundance in the aerated zones (aerated IFAS and reaeration) compared with the anoxic zones (Fig. 1C). It is possible that the high DO levels maintained a supply of oxidized N (as NO_3^-) that supported the growth of denitrifiers (many of which contain *nos*Z). Another study of BNR systems at WWTPs found a similar trend of higher *nosZ* abundance in aerobic zones compared with anoxic zones (Wang et al., 2014). Further, in our study, there was a significant, albeit weak, positive relationship between *nosZ* abundance and nitrate in the nitrification zones and compartments ($p < 0.01$, $r^2 = 0.31$). Some microorganisms can reduce nitrate even in the presence of relatively high DO concentrations (Robertson and Kuenen, 1984; Zhang et al., 2016). Although we do not know if the microorganisms in this study were actively reducing N_2O, we do know that they had the genetic capacity to do so and were relatively abundant in the aerated zones.

The abundance of *nosZ* was similar among the three OWTS (Fig. 1C), which suggests it did not play a strong role in accounting for differences in N_2O emissions from the systems (Fig. 1A).

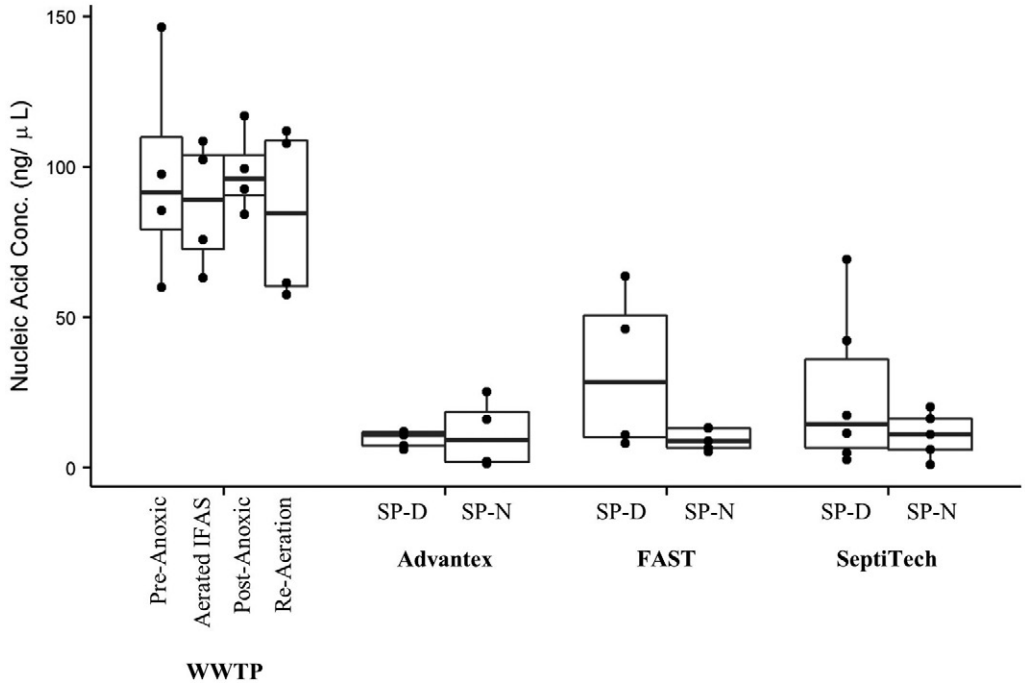

Fig. 3. Nucleic acid concentration from pre-anoxic, aerated integrated fixed film-activated sludge (IFAS), post-anoxic, and reaeration zones in the wastewater treatment plant (WWTP) and denitrification (SP-D) and nitrification (SP-N) compartments in Advantex, FAST, and SeptiTech (onsite wastewater treatment systems). Solid line in middle of box represents the median, edge of box represents first and third quartile, and whiskers extend 1.5× the inter-quartile range beyond the edge of the box.

As expected, there was a trend of higher *nosZ* abundance in SP-D than SP-N for FAST and SeptiTech systems (Fig. 1C). The specific abundance of *nosZ* ranged from 0 to 10^3 copies ng^{-1} DNA and was larger and more variable than that of *amoA* but was lower than reported from other types of BNR systems at WWTPs (10^4–10^5 copies ng^{-1} DNA) (Song et al., 2014; Wang et al., 2014).

The ratio of *amoA* to *nosZ* was higher in all zones of the WWTP than all three OWTS technologies (Fig. 4). In some instances, the *amoA*/*nosZ* ratio at the WWTP was above one, indicating that there was a higher abundance of *amoA* than *nosZ* (Fig. 4). In contrast, the *amoA/nosZ* ratio for OWTS was only above one once (Fig. 4). The higher *amoA/nosZ* ratio at the WWTP seems to be related to the high N_2O emissions observed there. However, there was not a significant relationship between N_2O emissions and *amoA/nosZ* ratio among either the nitrification or denitrification zones and compartments of all systems (data not shown). The strongest relationship of *amoA/nosZ* was with BOD in nitrification zones and compartments ($p = 0.01$, $r^2 = 0.43$).

Relationships between Gene Abundance and N_2O Emissions

There was no significant relationship between N_2O emissions and *amoA* or *nosZ* abundance or wastewater properties for nitrification or denitrification zones and compartments (data not shown). The lack of statistically significant relationships was not particularly surprising. First, gene abundance indicates

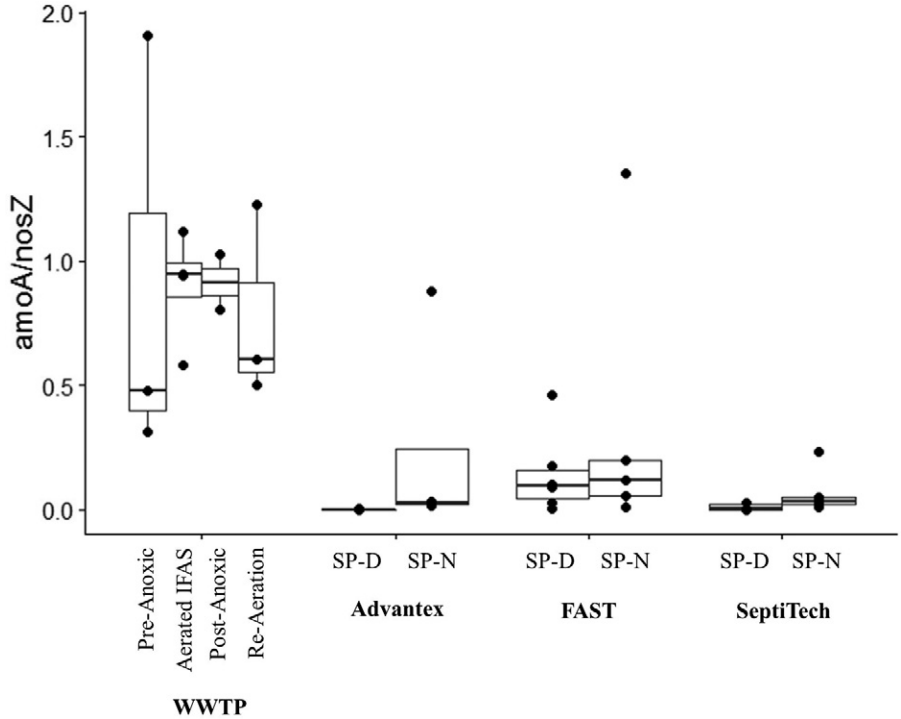

Fig. 4. *amoA/nosz* ratio from pre-anoxic, aerated integrated fixed film-activated sludge (IFAS), post-anoxic, and reaeration zones in the wastewater treatment plant (WWTP) and denitrification (SP-D) and nitrification (SP-N) compartments in Advantex, FAST, and SeptiTech (onsite wastewater treatment systems). Graph excludes one outlier (value = 16) from the post-anoxic zone of WWTP. Solid line in middle of box represents the median, edge of box represents first and third quartile, and whiskers extend 1.5× the inter-quartile range beyond the edge of the box.

population size of specific microbial groups but not gene expression. For example, other studies have found that although abundance of DNA (*amoA* and *nosZ*) did not differ between BNR trains at a WWTP, messenger RNA gene expression did (Song et al., 2014). Further, they found a strong negative relationship between *nosZ* expression and N_2O emissions (Song et al., 2014). Second, we collected water samples from a single depth. The abundance and activity of nitrifiers and denitrifiers may vary with depth as a function of DO concentration in anoxic zones of the WWTP. In addition, the production mechanism of N_2O emissions may be more complicated than simple production by autotrophic nitrification or heterotrophic denitrification. For instance, nitrifier denitrification, the reduction of NO_2^- to N_2O and N_2 by nitrifiers, is another potential source of N_2O (Wrage et al., 2001). In this study, single relationships were examined between wastewater properties and *amoA* and *nosZ* abundance. However, a study in soil pointed out the difficulty of predicting N_2O emissions based on static soil properties in a dynamic system (Breuillin-Sessoms et al., 2017). Future studies in both wastewater and soil should include models that consider multiple dynamic properties at once.

The lack of relationship between *nosZ* and N_2O fluxes may be related to variations that exist in the *nosZ* gene and/or denitrifying gene pathways. For example, denitrifiers that lack *nosZ* genes (Philippot et al., 2011) or one or more genes in the entire denitrification pathway (Roco et al., 2016) have been reported in soils, in which case N_2O fluxes may be a consequence of the functional diversity of the community rather than a single gene. In addition, the primer set used in this study may not have captured atypical *nosZ* genes that have previously been reported (Sanford et al., 2012). Although examining additional genes was outside the scope of this study, future studies that do so can help identify the contribution of alternative pathways to N_2O fluxes.

Conclusion

This preliminary evaluation of N_2O emissions from three advanced OWTS technologies indicates that they are generally lower (on a mole per area basis) relative to an IFAS BNR system at a centralized WWTP. However, when the N_2O emissions were normalized per population served and area of treatment tanks, they were similar between the WWTP and OWTS. Among the three technologies of advanced OWTS that were evaluated, the one with the highest N_2O emissions was the Advantex system. Additional studies are needed to determine the mechanisms that drive the differences in N_2O emissions between the systems. Overall, the BNR systems examined in this study do not produce large N_2O emissions relative to the amount of N removed, mostly <1%. The WWTP had higher *amoA* abundance and lower *nosZ* abundance compared with the OWTS. However, N_2O emissions were not directly related to *amoA* nor *nosZ* abundance or to the wastewater properties we evaluated.

Further evaluation of N_2O emissions from emerging BNR technologies and their microbial sources should be conducted, particularly as they become increasingly numerous as wastewater treatment demands increase.

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