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Influence of Season, Occupancy Pattern, and Technology on Structure and Composition of Nitrifying and Denitrifying Bacterial Communities in Advanced Nitrogen-Removal Onsite Wastewater Treatment Systems

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Abstract: Advanced onsite wastewater treatment systems (OWTS) use biological nitrogen removal (BNR) to mitigate the threat that N-rich wastewater poses to coastal waterbodies and groundwater. These systems lower the N concentration of effluent via sequential microbial nitrification and denitrification. We used high-throughput sequencing to evaluate the structure and composition of nitrifying and denitrifying bacterial communities in advanced N-removal OWTS, targeting the genes encoding ammonia monooxygenase (*amoA*) and nitrous oxide reductase (*nosZ*) present in effluent from 44 advanced systems. We used QIIME2 and the phyloseq package in R to examine differences in taxonomy and alpha and beta diversity as a function of advanced OWTS technology, occupancy pattern (seasonal vs. year-round use), and season (June vs. September). Richness and Shannon's diversity index for *amoA* were significantly influenced by season, whereas technology influenced *nosZ* diversity significantly. Season also had a strong influence on differences in beta diversity among *amoA* communities, and had less influence on *nosZ* communities, whereas technology had a stronger influence on *nosZ* communities. *Nitrosospira* and *Nitrosomonas* were the main genera of nitrifiers in advanced N-removal OWTS, and the predominant genera of denitrifiers included *Zoogloea*, *Thauera*, and *Acidovorax*. Differences in taxonomy for each gene generally mirrored those observed in diversity patterns, highlighting the possible importance of season and technology in shaping communities of *amoA* and *nosZ*, respectively. Knowledge gained from this study may be useful in understanding the connections between microbial communities and OWTS performance and may help manage systems in a way that maximizes N removal.

Keywords: onsite wastewater treatment systems; *amoA*; *nosZ*; nitrification; denitrification; biological nitrogen removal

1. Introduction

Nitrogen pollution from wastewater poses a serious threat to surface and groundwater in coastal watersheds. Advanced onsite wastewater treatment systems (OWTS)—designed specifically to remove N from wastewater—are often required in areas vulnerable to excess N inputs [1]. These technologies vary in their design, but they all have an oxic zone to facilitate nitrification and an anoxic/hypoxic zone

for denitrification (Figure A1). Autotrophic microorganisms are primarily responsible for nitrification, in which ammonia is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB), and nitrite is then oxidized to nitrate by nitrite-oxidizing bacteria. Several other pathways (e.g., anaerobic ammonia oxidation (anammox)), and groups of organisms (e.g., ammonia-oxidizing archaea (AOA)) may also be involved in nitrification [2,3]. However, AOB are believed to be primarily responsible for ammonia oxidation in wastewater treatment settings [4]. Facultative anaerobic heterotrophic bacteria are thought to carry out denitrification [5]. The effluent is discharged to a soil treatment area (STA), which provides a final opportunity for treatment before it enters the surrounding environment.

Advanced N-removal OWTS are generally expected to meet certain regulatory standards. For example, in Rhode Island, the Department of Environmental Management (RIDEM) requires that the total N (TN) concentration in final effluent must not exceed 19 mg TN/L [6]. A similar standard is also used in other areas, including Barnstable County, MA, USA and Suffolk County, NY, USA [7,8].

The capacity of these systems to meet effluent total N standards differs among advanced N-removal technologies. Oakley et al. [5] found that 19 out of 20 advanced N-removal OWTS technologies were less than 50% likely to produce effluent with <10 mg TN/L. More recently, Lancellotti et al. [9] found that only 64 to 75% of advanced N-removal OWTS in the Greater Narragansett Bay watershed complied with the RIDEM regulatory standard of 19 mg TN/L, with compliance rates varying among technologies. Advanced technologies also differ in greenhouse gas (GHG) emissions, which represent the end products of microbial activity associated with carbon and N removal [10,11]. These differences among technologies may be associated with differences in the composition and structure of the microbial communities that drive wastewater treatment.

The microbial communities of wastewater treatment plants (WTPs) with biological N removal (BNR), which rely on the same processes as advanced N-removal OWTS to remove N from wastewater, have been studied extensively in an attempt to assess the relationship between a number of variables, including system design, geography, temperature, and other effluent properties, and N-cycling microbial communities [4,12–16]. Similar to WTPs, microorganisms play a critical role in the N-cycling processes facilitated by advanced N-removal OWTS. However, despite their reliance on nitrifiers and denitrifiers, few studies have investigated these microbial communities in advanced N-removal OWTS. In studies conducted within the Greater Narragansett Bay watershed, Brannon et al. [10] assessed the size of nitrifying and denitrifying microbial communities in BNR WTPs and advanced N-removal OWTS, and Wigginton et al. [17] analyzed the abundance, structure, and composition of these communities in advanced N-removal OWTS. Both studies targeted the functional genes *amoA* (encodes for ammonia monooxygenase, which carries out the first step in ammonia oxidation) and *nosZ* (encodes for nitrous oxide reductase, which reduces nitrous oxide to dinitrogen gas). These studies found that OWTS technology, component type (oxic vs. anoxic), and time of year can affect gene abundance. Wigginton et al. [17] observed no differences in richness and Shannon's diversity among technologies or between zones for nitrifying or denitrifying communities. However, they did find significant geographical and temporal differences in the structure and composition of *nosZ* communities. Further investigations specifically targeting the microbial communities in advanced N-removal OWTS may help elucidate the currently understudied mechanisms and driving factors of N removal in these systems.

To better understand the structure and composition of nitrifying and denitrifying communities and the factors that affect these, we investigated the bacterial communities of 44 advanced N-removal OWTS in the town of Charlestown, Rhode Island, USA. This is one of the first studies to investigate the microbial communities of advanced N-removal OWTS. We used primers targeting the genes *amoA* and *nosZ* and high-throughput sequencing to characterize alpha (species richness and Shannon's diversity index) and beta diversity and taxonomy as a function of technology, occupancy pattern, and season. Although the presence of these genes in effluent does not guarantee gene expression, we targeted functional genes to assess the potential for bacterial nitrification and denitrification in advanced N-removal OWTS. We sampled final effluent from four different advanced N-removal

technologies: Orenco Advantex® AX20 (Orenco Systems, Sutherlin, OR, USA), Orenco Advantex® RX30 (Orenco Systems, Sutherlin, OR, USA), BioMicrobics MicroFAST® (BioMicrobics, Inc., Lenexa, KS, USA), and Norweco Singulair® models DN, TNT, and 960 (Norweco, Norwalk, OH, USA).

We also investigated occupancy pattern as a potential source of variation in microbial communities. Like many coastal towns in New England, Charlestown, RI has year-round residents, as well as residents who occupy their homes only during the summer months, whose OWTS receive no wastewater inputs for approximately eight months. This dichotomy prompted us to examine whether occupancy pattern influences the structure and composition of OWTS microbial communities. Because they receive no effluent inputs between September and May, the microbial communities in seasonally-used systems may take some time to get re-established at the beginning of the summer, which may alter community structure and composition between June and September. Finally, we expected differences in effluent communities between June and September in year-round systems, possibly due to changes in household demographics associated with the summer season in this area.

By narrowing the geographic range of the study systems, we expected to improve our ability to detect differences as a function of technology, use pattern, and season that may be overshadowed by geography, which Wigginton et al. [17] found to partially drive community differences. The sites studied by Wigginton et al. [17] were located in six towns within the Greater Narragansett Bay watershed, covering an area of 855 km^2, nearly six-fold larger than Charlestown, which has an area of 154 km^2.

2. Materials and Methods

2.1. Study Systems

We sampled final effluent (prior to dispersal to the soil treatment area (STA)) in June and September 2017 from four different N-removal OWTS technologies in Charlestown, RI, USA: (i) Orenco Advantex® AX20, (ii) Orenco Advantex® RX30, (iii) BioMicrobics MicroFAST®, and Norweco Singulair® (models TNT, 960, and DN). Detailed descriptions of each technology can be found in Appendix A. Sampling in June (early summer) and September (early fall) allowed us to identify seasonal shifts in OWTS microbial communities. We sampled a total of 44 systems: 23 systems served homes occupied year round, while 21 systems served seasonally-occupied homes. The specific criteria used for determining home occupancy pattern can be found in Ross et al. [18]. All sites were single-family homes that rely on well water as their potable water source and had an advanced N-removal OWTS that uses a pressurized pump to discharge effluent to the STA. Sites were selected based on adherence to these criteria and the homeowners' willingness to participate in this study. Table A1 has a summary of effluent properties for June and September 2017.

2.2. Sample Collection

We sampled final effluent from the Advantex systems at the recirculating splitter valve assembly, while the FAST and Norweco systems were sampled from the STA pump basin. A grab sample was collected into an autoclaved, 250 mL plastic bottle and stored at 4 °C until transported to the laboratory (within 8 h of sampling). Within 24 h of sampling, ~100 mL of sample was vacuum filtered to pass a sterile nitrocellulose membrane filter with a pore size of 0.45 µm (MilliporeSigma, Burlington, MA, USA), and the filter stored in individual sterile Whirl-Pak bags (Uline, Pleasant Prairie, WI, USA) at −80 °C until analysis.

2.3. Sample Processing

Genomic DNA was extracted from each membrane filter using the PowerWater DNA Isolation Kit (MoBio Laboratories, Waltham, MA, USA). Gene fragments for *nosZ* and *amoA* were amplified using polymerase chain reaction (PCR) in a single, 50 µL reaction using the primer pairs *nosZ* 1F and *nosZ* 1662R, and *amoA* 1F and *amoA* 862R [12,17]. Reactions for *nosZ* amplification contained

1.25 µL of extracted DNA template, 25 µL of BIO-X-ACT™ Short Mix (Bioline, Taunton, MA, USA), 21.75 µL of water, and 1 µL of *nosZ* forward and reverse primers. Thermocycler conditions for *nosZ* amplification were as follows: an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Reactions for *amoA* amplification contained 1.25 µL of extracted DNA template, 25 µL of MyFi™ Mix (Thomas Scientific, Swedesboro, NJ, USA), 21.25 µL of water, and 1.25 µL of *amoA* forward and reverse primers. Thermocycler conditions for *amoA* amplification were as follows: 4 min at 94 °C, 35 amplification cycles (each 60 s at 94 °C, 60 s annealing at 58 °C, and 60 s 72 °C), and a final extension at 72 °C for 5 min. After quality checking the PCR products on an ethidium bromide agarose gel, products were sequenced using an Illumina MiSeq Next Generation Sequencer at the University of Rhode Island Genetic Sequencing Center (Kingston, RI, USA) [17]. Additional details of DNA extraction, PCR methods, and sample metadata can be found in Appendix A.

2.4. Downstream Analysis and Statistics

We used QIIME 2 (version 2019.10) for downstream sequence analysis, as outlined in Cox et al. [17]. After demultiplexing and quality filtering the raw forward and reverse reads using the q2-demux plugin, we identified and removed the primer sequences for *nosZ* and *amoA*, so that the individual gene amplicons could be analyzed. We then joined, filtered, and denoised the reads for each amplicon with the q2-dada2 plugin. Finally, we removed chimeras and generated unique amplicon sequence variants (ASVs). We used the number of unique ASVs as a proxy for species richness.

To assign sequence taxonomy, we followed the methods outlined in Cox et al. [19]. We created reference databases for *nosZ* and *amoA* by downloading all FASTA sequences and associated accession numbers from the NCBI nucleotide database for each gene. Sequence files were converted to 'DNAFASTAFormat' and imported into QIIME 2. We used Entrez Direct (E-utilities on Command Line) to obtain taxonomy strings for each accession number and formatted the strings to be imported into QIIME 2. We used the q2-feature-classifier to extract reference reads matching our PCR primer sequences, and then used a Bayesian probability approach to train the feature classifier to identify the appropriate taxonomy. Finally, we used the classify-sklearn command from the q2-feature-classifier plugin to assign taxonomy to each sequenced read. In the few situations where the classifier identified a genus that is known to not contain the gene analyzed, we removed those ASVs from our analysis.

We used the phyloseq package in R (version 3.6.2) [20] to rarefy data to the lowest sequencing depth for each gene (914 sequences for *nosZ* and 163 sequences for *amoA*) and to calculate species richness and Shannon's diversity. We used a one-way ANOVA on ranks to assess differences in species richness (based on unique ASVs) and Shannon's diversity index among OWTS technologies for *amoA*, and a one-way ANOVA to assess these differences for *nosZ* [20]. Because we did not have sufficient replication of occupancy patterns for two of the technologies in the study, we grouped systems within a technology without regard for occupancy pattern by system when assessing differences among technologies (and subsequently grouped technologies when assessing differences between occupancy patterns). We used a Mann–Whitney U test to assess differences in richness and Shannon's diversity as a function of occupancy pattern and season for *amoA*, for which data could not be normalized, and a paired *t*-test to assess these differences for *nosZ*, for which data were normally distributed. Differences in beta diversity as a function of technology, occupancy pattern, and season were determined based on Bray–Curtis dissimilarity values and cluster analysis using Ward's cluster method with the vegan package in R [20]. Significant clustering, as a function of technology, occupancy pattern, and/or season, was identified using nonparametric permutational multivariate ANOVAs (PERMANOVAs) on Bray–Curtis distance matrices using the Adonis function (with 999 permutations for each correlation) [17,19,20].

3. Results and Discussion

3.1. amoA: Species Richness and Diversity

Species richness (the number of ASVs) for *amoA* across technologies, occupancy patterns, and seasons (n = 55) ranged from 3 to 33 ASVs, with a median of 25 (Figure 1). Richness was significantly different between seasons, with higher species richness in September than in June (Figure 1). In contrast, there were no significant differences in richness among technologies or between occupancy patterns. The median value of Shannon's diversity index across all samples was 2.81 (range of 0.27—3.25). As was the case for richness, diversity indices in September were significantly higher than in June (Figure 1). There were no significant differences in Shannon's diversity index as a function of technology or occupancy pattern.

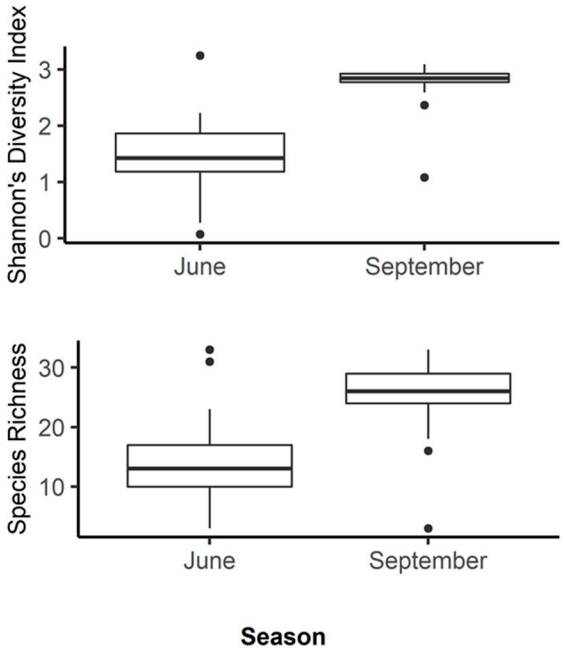

Figure 1. Median values of Shannon's diversity index and species richness for *amoA* genes in effluent from advanced N-removal OWTS in June (n = 13) and September (n = 42) across all technologies and occupancy patterns. Box whiskers extend 1.5 × the interquartile range beyond the edges of the box and the dots represent outliers beyond 1.5 × the interquartile range.

When we assessed differences in *amoA* beta diversity, e.g., differences in diversity across communities, samples clustered significantly only by season (33% variation explained) (Figure 2), indicating that nitrifying microbial communities in June are more similar to one another than to those in September. This seasonal shift was apparent in OWTS nitrifying communities regardless of occupancy pattern or technology.

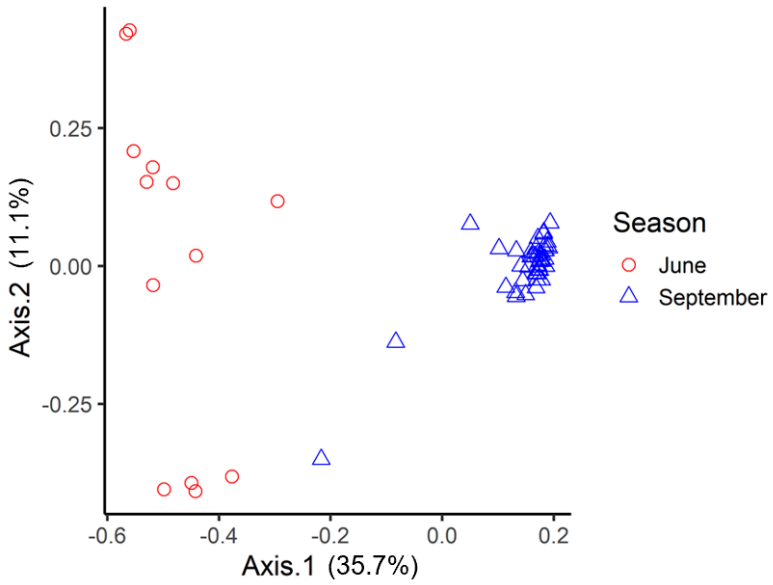

Figure 2. Principal coordinate analysis based on Bray–Curtis dissimilarity distances for *amoA* communities across all advanced N-removal OWTS technologies and occupancy patterns (n = 55).

The relationship between season and *amoA* diversity in wastewater treatment appears to differ at different system scales. For example, Wigginton et al. [17] analyzed effluent from 38 advanced N-removal OWTS in the Greater Narragansett Bay Watershed and found no differences in species richness or Shannon's diversity indices for *amoA* among June, August, and September samples. In contrast, Siripong and Rittman [13] used *amoA* to assess shifts in nitrifying communities in seven WTPs as a function of season, solids retention time, and type of influent wastewater (residential versus industrial). Season was the only variable found to influence the nitrifying communities significantly. They observed a shift in the dominance of nitrifying species (*Nitrosospira* spp. and *Nitrosomonas* spp.) found in the summer and winter months and suggested that season has a significant effect on selecting for certain nitrifiers. Our results also suggest that season partly drives differences in the richness and diversity of *amoA* communities in advanced N-removal OWTS. Changes in household demographics and/or activity between the beginning and end of the summer may consistently alter the structure of *amoA* communities in advanced OWTS. Charlestown is an active summer tourist destination, and the influx of individuals who do not normally reside in Charlestown, including visitors and residents returning home from college, may contribute novel species to OWTS microbial communities, increasing species richness and diversity [17].

The lack of significant differences in alpha and beta diversity among technologies suggests that nitrifying communities are consistent despite differences in treatment train design and specific configuration for N removal (i.e., textile filters vs. aerobic treatment units). Furthermore, despite the period of non-use that seasonally-used systems experience prior to June, similarities in community structure observed in both seasonal and year-round systems, as well as the temporal clustering observed regardless of occupancy pattern, suggest that the *amoA* communities of advanced N-removal OWTS do not experience a lag when system operation resumes in early summer. Ross et al. [18] assessed the performance of 50 advanced N-removal OWTS in Charlestown, RI, and found no significant differences in performance between seasonal and year-round systems. The consistent metrics of alpha and beta diversity exhibited by the microbial communities of both OWTS occupancy patterns may translate into robust capacity for wastewater treatment.

3.2. *nosZ: Species Richness and Diversity*

Species richness across technologies, occupancy patterns, and seasons ranged from 4 to 85 ASVs, with a median of 35 (n = 65) (Figure 3). Richness differed significantly among technologies and was

significantly higher in RX30 systems than all other technologies, and was higher in AX20 than FAST systems. Species richness did not differ based on season or occupancy pattern. Shannon's diversity index values across all samples (n = 65) ranged from 0.03 to 3.27, with a median of 1.98 (Figure 3).

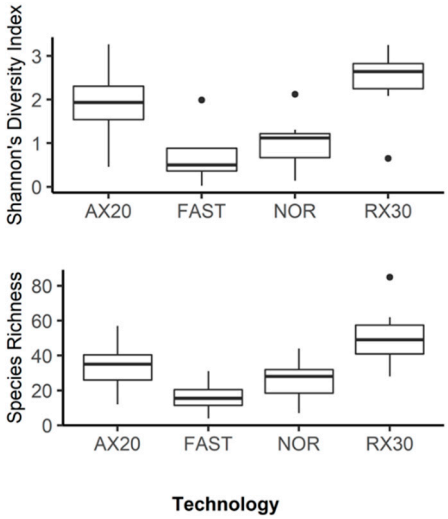

Figure 3. Shannon's diversity index and species richness for *nosZ* genes for different advanced N-removal OWTS technologies across all occupancy patterns and seasons. AX20 = Orenco Advantex® AX20 (n = 43); FAST = BioMicrobics MicroFAST® (n = 4); NOR = Norweco Singulair® (n = 7); RX30 = Orenco Advantex® RX30 (n = 11). Description of box-and-whisker plots can be found in Figure 1.

We observed significant differences in diversity among technologies, with values for AX20 systems significantly higher than those for FAST and Norweco systems, and values for RX30 systems significantly higher than those for the other three technologies. In contrast, diversity did not differ significantly based on occupancy pattern or season. Finally, when we assessed differences in beta diversity of *nosZ* communities based on technology, occupancy pattern, and season, samples clustered significantly by technology (8.2% variation explained) and season (3.0% variation explained) (Figure 4), indicating that *nosZ* communities are influenced by these two factors.

Unlike *amoA* communities, which were only influenced by season, *nosZ* communities appear to respond to differences in both technology and season. Differences in alpha and beta diversity among technologies suggest that some aspect of treatment train design significantly influences *nosZ* communities in advanced N-removal OWTS. In contrast, Wigginton et al. [17] did not observe differences in *nosZ* richness or diversity among technologies. However, they did observe differences in *nosZ* community structure among geographical locations, which may have occluded any differences among technologies.

Jaranowska et al. [14] observed trends in species richness similar to ours in the *nosZ* communities of nine Polish N-removal BNR WTPs that represented a broad range of design characteristics. They found that the community structure of *nosZ* was significantly influenced by facility design; namely, *nosZ* species richness was highest for WTPs with separate denitrification tanks compared to all other design schemes. Although BNR WTPs and advanced N-removal OWTS differ in design, size, flow, and the number of people served, the BNR processes they promote are the same, suggesting that the comparison may be instructive. In our study, AX20 and RX30 systems—which had significantly higher Shannon's diversity than FAST and Norweco systems—rely on a textile filter and recirculation for nitrification and subsequent denitrification of effluent, with these processes taking place in different tanks. In contrast, FAST and Norweco systems rely on submerged fixed activated sludge and intermittent cycles of suspended growth aeration, respectively, for N removal. The compartmentalization of nitrification and denitrification, in conjunction with highly porous media, and recirculation of wastewater in AX20

and RX30 systems, may increase microbial habitat available as well as the likelihood of novel species being introduced throughout the system, thus promoting more diverse *nosZ* communities.

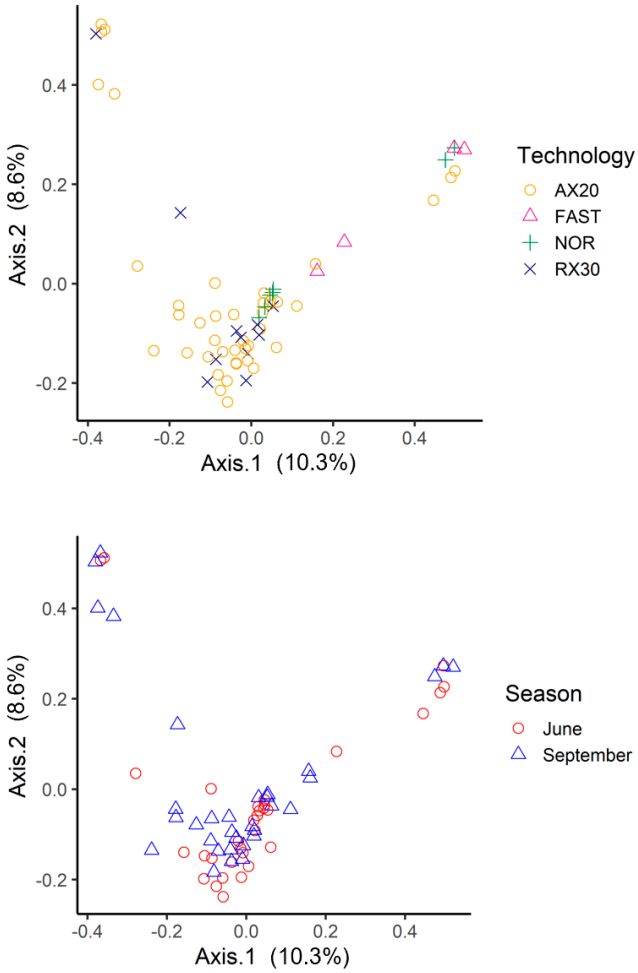

Figure 4. Principal coordinate analysis based on Bray–Curtis dissimilarity distances for *nosZ* communities in effluent from different advanced N-removal OWTS technologies across occupancy patterns (n = 65). AX20 = Orenco Advantex® AX20; FAST = BioMicrobics MicroFAST®; NOR = Norweco Singulair®; RX30 = Orenco Advantex® RX30.

The higher diversity and species richness exhibited by RX30 systems compared to all other technologies also suggests that specific design differences may help shape *nosZ* communities. Although RX30 and AX20 both rely on textile filters for nitrification, the orientation of those filters differs: RX30s utilize horizontal racks of lightweight, absorbent textile pieces, while AX20s use vertical hanging textile sheets. Because the textile filters in RX30s can become saturated due to their horizontal orientation, the manufacturer developed the AX20 systems, in which water trickles freely down the vertically hanging sheets by gravity, preventing saturation. Although textile filters are designed to establish oxic conditions and promote nitrification, Wigginton et al. [17] found that denitrifiers were ubiquitously distributed in both nitrifying and denitrifying components of advanced OWTS treatment trains, likely due to their nature as facultative anaerobes. Thus, differences in the nitrification component of AX20s and RX30s may promote differences in *nosZ* community richness. Visual inspections conducted during the course of our research indicates that liquid and organic materials often accumulate to a greater extent in RX30 textile filters than in AX20 textile filters. A higher concentration and diversity of organic substrates may augment the opportunity for different denitrifying species to establish themselves, as more niches become available within the substrates.

System age may also drive the higher Shannon diversity and species richness observed in RX30s relative to AX20s. Although both technologies had higher *nosZ* diversity than the other two technologies, the median age of AX20s (6.8 years) was lower than that of the RX30s (11.3 years). Changes in microbial diversity over time have been observed in many natural environments, as communities increase in diversity until they reach a point of stability [21]. Increased time of OWTS operation may provide a greater opportunity for new and/or different niches with unique *nosZ* communities to develop—possibly through the increased accumulation of liquid and organic substrate in RX30 textile filters—ultimately increasing species richness in these systems.

The *nosZ* samples clustered significantly by season, mirroring the results in Wigginton et al. [17]. Although less of the temporal variation was explained for *nosZ* communities (3.0%) than for *amoA* communities (33%), the significant clustering suggests that time of year has some influence on *nosZ* communities. Similar to *amoA*, differences in demographics and increased tourism activity may introduce different and novel species to OWTS, causing the shift observed in OWTS denitrifiers between June and September. The consistent clustering of samples by season regardless of occupancy pattern suggests that, similar to *amoA* communities, communities of *nosZ* in seasonal OWTS require little time for re-establishment once system use resumes at the beginning of the summer.

3.3. amoA: Taxonomy

We observed a total of 241 unique ASVs for *amoA* across all samples (n = 55) (Figure 5), 88 (37%) of which could be matched with a known genus. Of these, the most common genera (based on the number of ASVs) were *Nitrosospira* (78) and *Nitrosomonas* (8). The dominance of unidentified bacteria containing *amoA* suggests that more research is required in order to describe a large portion of the nitrifying community in OWTS. Three ASVs were not matched with any sequences deposited in the NCBI database, suggesting the presence of three previously undescribed bacterial strains capable of ammonium oxidation.

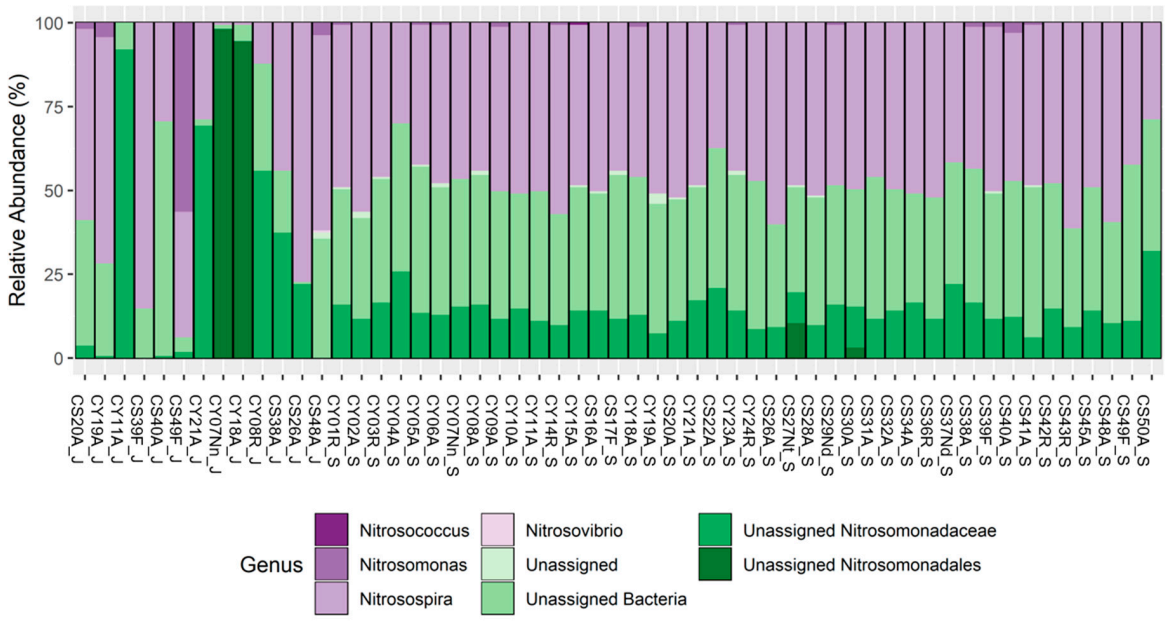

Figure 5. Relative abundance of *amoA* genera based on rarefied abundance. Sample labels indicate the study location (C = Charlestown), occupancy pattern (Y = year round; S = seasonal), site number, technology (A = AX20, R = RX30, F = FAST, Nn/Nt/Nd = Norweco), and sampling month (_J = June; _S = September).

We identified the 20 most abundant *amoA* ASVs, based on relative abundance across all technologies, occupancy patterns, and seasons (Table 1). Although many of the ASVs were present in effluent

samples from all technologies, occupancy patterns, and seasons, the most obvious differences were between seasons, with five of the most abundant ASVs present only in September samples. This pattern mirrors the increases in alpha and beta diversity observed in September samples, and is likely also due to shifts in household demographics between the beginning and end of the summer.

Table 1. Top 20 most abundant *amoA* ASVs identified across all advanced N-removal OWTS technologies, occupancy patterns, and season. AX20 = Orenco Advantex® AX20 (n = 37); RX30 = Orenco Advantex® RX30 (n = 8); FAST = BioMicrobics MicroFAST® (n = 5); NOR = Norweco Singulair® (n = 5). U.A. = unassigned; x = ASV present in at least one sample within a category.

Genus	Technology				Occupancy Pattern		Season	
	AX20	RX30	FAST	NOR	Year Round	Seasonal	June	September
U.A. *Nitrosomonadaceae*	x	x	x	x	x	x	x	x
U.A. Bacteria	x	x	x	x	x	x	x	x
Nitrosospira	x	x	x	x	x	x		x
U.A. Bacteria	x	x	x	x	x	x	x	x
Nitrosospira	x	x	x	x	x	x	x	x
Nitrosospira	x	x	x	x	x	x	x	x
Nitrosospira	x	x	x	x	x	x		x
Nitrosospira	x	x	x	x	x	x		x
U.A. *Nitrosomonadales*	x			x	x	x	x	x
Nitrosospira	x	x	x	x	x	x	x	x
U. A. Bacteria	x	x	x	x	x	x	x	x
Nitrosospira	x	x	x	x	x	x	x	x
Nitrosospira	x	x	x	x	x	x	x	x
Nitrosospira	x		x		x	x	x	x
Nitrosospira	x	x	x		x	x	x	x
Nitrosospira	x	x	x	x	x	x	x	x
U.A. Bacteria	x	x	x	x	x	x		x
U.A. Bacteria	x	x	x	x	x	x	x	x
U.A. Bacteria	x	x	x	x	x	x		x
U.A. Bacteria	x					x	x	x

Other groups of organisms may play a role in nitrification in wastewater treatment. For example, Wigginton et al. [22] assessed *amoA* communities in a BNR WTP and nine advanced N-removal OWTS in the Greater Narragansett Bay Watershed and observed relatively low abundances of *Nitrospira* in the advanced N-removal OWTS. Several other studies have also found *Nitrospira* in aquatic environments with low concentrations of ammonium [23,24]. Limpiyakorn et al. [25] assessed the abundance of AOA and AOB in seven WTPs in Thailand, and found significantly greater abundances of AOA in effluent with lower concentrations of ammonium (0.2–3.0 mg N/L), while AOB dominated effluent high in ammonium (5–29 mg N/L). Because the ammonium concentration in our systems in this study tends to be higher than that in WTP (Table A1), AOB are likely to play a more important role in nitrification than other groups of ammonia-oxidizers.

Although Wigginton et al. [17] observed a higher number of ASVs (711) in samples from 38 advanced N-removal OWTS, they also identified *Nitrosospira* and *Nitrosomonas* as two of the most common genera present in effluent. Both genera have also been observed in BNR WTPs throughout the world [4,22,26,27]. Wigginton et al. [22] also found *Nitrosomonas* and *Nitrosospira* to be the most prevalent genera across both settings. *Nitrosospira* is most commonly found in soil environments, which suggests that these bacteria are likely introduced to OWTS through soil that enters treatment train components during the initial system installation as well as during periodic maintenance inspections [22,28]. The overlap in prevalent nitrifiers observed in OWTS between systems in the Wigginton et al. [17,22] studies and those in our systems suggests that certain genera dominate the nitrification process in N-removal OWTS. The presence of these genera in different advanced OWTS technologies and in BNR WTPs at broad geographic scales suggests that systems designed for N

removal support taxonomically consistent nitrifying communities, regardless of system design, scale, or geographical location.

3.4. *nosZ*: Taxonomy

We observed a total of 1307 unique ASVs for *nosZ* across all samples (n = 65) (Figure 6), of which 222 (17%) could be matched with a known genus. As was the case for *amoA*, most *nosZ* ASVs were classified as unknown bacterial species. Of those that could be matched to a genus, the most common genera (based on the number of ASVs) were *Zoogloea* (57), *Thauera* (35), and *Acidovorax* (34).

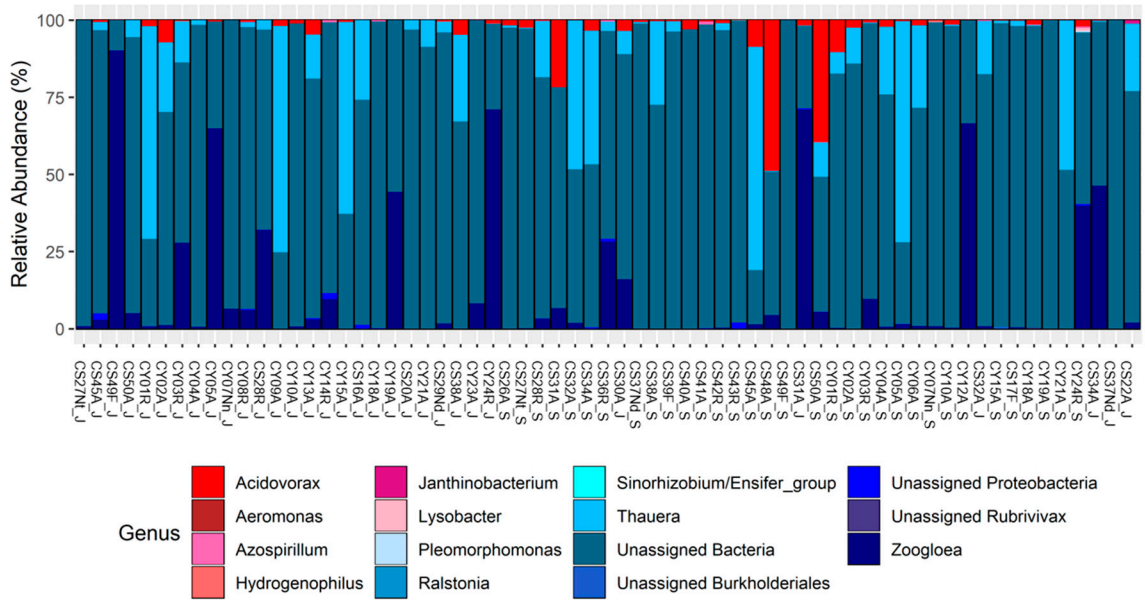

Figure 6. Relative abundance of nosZ genera based on rarefied abundance. Sample labels indicate the study location (C = Charlestown), occupancy pattern (Y = year round; S = seasonal), site number, technology (A = AX20, R = RX30, F = FAST, Nn/Nt/Nd = Norweco), and sampling month (_J = June; _S = September).

We identified the 20 most abundant *nosZ* ASVs, based on relative abundance across all technologies, occupancy patterns, and seasons (Table 2). Several of the top ASVs were found in at least one sample from all technologies, occupancy patterns, and seasons. All of the top ASVs were found in samples of both occupancy patterns and seasons; the only taxonomy differences we observed were among technologies. This pattern mirrors the variation in alpha and beta diversity observed among technologies and is likely driven by differences in treatment train design.

Thauera, which has been highlighted for its functional importance in denitrification in WTPs [29,30], was the only genus with a high relative abundance in both our study and in Wigginton et al. [17]. Three other genera (*Aeromonas*, *Azospirillum*, and *Sinorhizobium*) observed in Wigginton et al. [17] were also present in at least one of our samples.

Table 2. Top 20 most abundant *nosZ* ASVs identified across all advanced N-removal OWTS technologies, occupancy patterns, and season. AX20 = Orenco Advantex® AX20 (n = 42); RX30 = Orenco Advantex® RX30 (n = 13); FAST = BioMicrobics MicroFAST® (n = 4); NOR = Norweco Singulair® (n = 7). U.A. = unassigned; x = ASV present in at least one sample within a category.

Genus	Technology				Occupancy Pattern		Season	
	AX20	RX30	FAST	NOR	Year Round	Seasonal	June	September
U.A. Bacteria	x	x	x	x	x	x	x	x
U.A. Bacteria	x	x	x	x	x	x	x	x
U.A. Bacteria	x	x			x	x	x	x
Thauera	x	x			x	x	x	x
Zoogloea	x		x	x	x	x	x	x
Thauera	x	x	x	x	x	x	x	x
Thauera	x	x	x	x	x	x	x	x
U.A. Bacteria	x	x		x	x	x	x	x
U.A. Bacteria	x	x	x	x	x	x	x	x
U.A. Bacteria	x	x			x	x	x	x
U.A. Bacteria	x	x		x	x	x	x	x
U.A. Bacteria	x			x	x	x	x	x
U.A. Bacteria	x	x	x	x	x	x	x	x
Zoogloea	x	x	x	x	x	x	x	x
U.A. Bacteria	x	x		x	x	x	x	x
Zoogloea	x				x	x	x	x
U.A. Bacteria	x		x	x	x	x	x	x
U.A. Bacteria	x	x	x	x	x	x	x	x
U.A. Bacteria				x	x	x	x	x
Acidovorax	x	x	x	x	x	x	x	x

Two of the most common genera present in our samples, *Zoogloea* and *Acidovorax*, have not been identified previously in N-removal OWTS [10,17,22]. *Zoogloea* spp. assist with the formation of flocculated materials in activated sludge in WTPs [31], and thus may be involved in the formation of biofilms and flocs in advanced N-removal OWTS. A high abundance of *Acidovorax* has also been observed in the bacterial communities of activated sludge in WTPs with BNR [32,33]. Although *Acidovorax* spp. and *Zoogloea* spp. are present in WTPs, their capacity for denitrification was not identified until recently [34]. These two species may play an important role in denitrification in advanced N-removal OWTS.

4. Conclusions

Our study expands current understanding of the structure and composition of nitrifying and denitrifying microbial communities in advanced N-removal OWTS, suggesting that technology and season vary in how they affect these communities. While nitrifying communities were largely influenced by season, possibly due to changes in household demographics, the structure of denitrifying communities varied significantly among technologies, suggesting that denitrifiers are influenced by differences in treatment train design. The taxonomic trends observed for *amoA* and *nosZ* reflected those observed in species richness and diversity, with seasonal differences observed for *amoA*, and technology differences for *nosZ*. Conversely, home occupancy pattern does not appear to influence bacterial nitrifying/denitrifying communities in OWTS, suggesting a robust capacity for wastewater treatment in seasonally-used OWTS despite long periods of non-use.

The large number of organisms that could not be matched with a known genus for either gene highlights the need for more in-depth assessment of the composition of OWTS microbial communities. Furthermore, the relationship between the structure and composition of nitrifying and denitrifying communities and the concentration of N in effluent may help elucidate how season and technology affect N removal by advanced N-removal OWTS.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

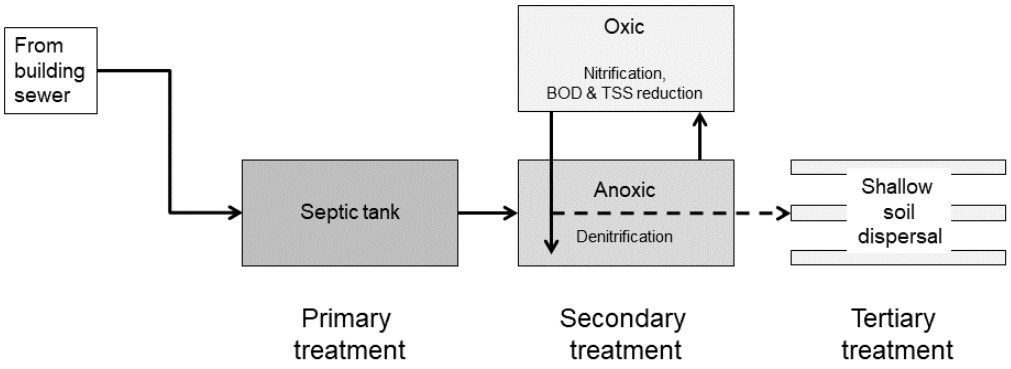

Figure A1. Diagram of components and treatment processes of advanced N-removal OWTS, as illustrated in Ross et al. [35]; BOD refers to biochemical oxygen demand; TSS refers to total suspended solids. Components are shown separately for clarity but can also exist within one multicompartment tank.

Appendix A.1. Technology Descriptions

Technology descriptions for the Orenco Advantex® AX20 and RX30, BioMicrobics FAST®, and Norweco Singular® systems are described in Ross et al. [18]. The Orenco Advantex® AX20 and RX30 systems both utilize a timed-dosed textile media filter. The textile filters serve as an oxic environment, which allows nitrification to occur. The AX20 system contains vertically hanging textile sheets, while the RX30 system's textile filter consists of horizontally packed textile "coupons". Denitrification occurs in the processing tank of both AX20 and RX30 systems, from which wastewater is dosed to the textile filters and recirculated throughout the systems multiple times to optimize N-removal potential. The processing tank also functions as a primary treatment area in which sedimentation processes promote separation of solids in the wastewater. The BioMicrobics MicroFAST® system is socially dosed rather than time dosed, and utilizes submerged fixed-film activated sludge in order to treat wastewater. The FAST system consists of two compartments; the first compartment facilitates primary treatment, while the second compartment facilitates both nitrification and denitrification. Nitrification occurs when air is forced into the ridged corrugated plastic block media insert within the

second compartment of the tank and creates an oxic environment. Nitrified effluent moves upward through a corrugated plastic block media insert and splashes onto a trough, allowing the effluent to be transferred into an adjacent area where denitrification takes place. The three Norweco Singular® models (TNT, 960, and DN) are also socially-dosed systems that utilize suspended growth technology to treat wastewater. The Norweco systems consist of a three-compartment tank, the first of which facilitates primary treatment. Nitrification and denitrification occur in the second compartment, which is intermittently aerated. Intermittent aeration causes the compartment to alternate between serving as an oxic and an anoxic environment, thus promoting both nitrification and denitrification. The third compartment of the Norweco systems is a clarification chamber, which promotes solids removal from the effluent.

Appendix A.2. DNA Extraction and PCR

We followed the protocol in Wigginton et al. [17] for DNA extraction and PCR analysis methods for *nosZ*, and Wigginton et al. [22] for *amoA* methods. After filtering effluent samples, we extracted DNA from filters using PowerWater DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA, USA) and stored samples at −80 °C until PCR analysis. We amplified *nosZ* fragments in single, 50 µL reactions containing 1.25 µL of extracted DNA template, 25 µL of BIO-X-ACT™ Short Mix (Bioline, Taunton, MA, USA), 21.75 µL of water, and 1 µL of *nosZ* forward and reverse primers. Thermocycler conditions for *nosZ* amplification were as follows: an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. We amplified *amoA* fragments in single, 50 µL reactions containing 1.25 µL of extracted DNA template, 25 µL of MyFi™ Mix (Thomas Scientific, Swedesboro, NJ, USA), 21.25 µL of water, and 1.25 µL of *amoA* forward and reverse primers. Thermocycler conditions for *amoA* amplification were as follows: 4 min at 94 °C, 35 amplification cycles (each 60 s at 94 °C, 60 s annealing at 58 °C, and 60 s 72 °C), and a final extension at 72 °C for 5 min. After PCR, we quality checked the amplicons on a 1% ethidium bromide agarose gel and sent samples that produced a single band of the correct size to the University of Rhode Island's Genetic Sequencing Center.

Table A1. Summary statistics of advanced treated wastewater parameters measured in each season, across all technologies and occupancy patterns. Units are mg/L except for pH and temperature (°C). Adapted from Ross et al. [18].

Parameter	June				September			
	n	Min	Median	Max	*n*	Min	Median	Max
pH	45	3.8	7.0	8.1	45	4.7	6.9	7.8
Temperature	45	12.6	16.1	20.9	44	18.9	21.2	25.4
BOD_5	20	0	0	54	20	0	0	76
Dissolved oxygen	45	1.1	6.8	9.9	44	0	5.8	8.7
Ammonium-N	42	0	0	41.6	38	0	0.1	51.6
Nitrate-N	43	0	8.4	32.8	40	0.1	9.4	25.3
Total N	43	3.6	18.7	60.9	41	0.6	11.9	52.0

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