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ASSESSING TREATMENT PERFORMANCE OF ADVANCED NITROGEN-REMOVAL ONSITE WASTEWATER TREATMENT SYSTEMS

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ASSESSING TREATMENT PERFORMANCE OF ADVANCED NITROGEN-REMOVAL
ON-SITE WASTEWATER TREATMENT SYSTEMS

BY

BIANCA NOELLE ROSS

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN

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2020

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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2020

Abstract

Wastewater is a major source of nitrogen (N) to groundwater and coastal waterbodies, threatening both environmental and public health. Advanced N-removal onsite wastewater treatment systems (OWTS) are used to reduce effluent N concentration through biological N removal (BNR). Despite the important role that these systems play in treating nutrient- and pathogen-rich wastewater, few studies have targeted the mechanisms involved in N removal, their capacity to produce effluent to meet regulatory standards, or their impact on the atmosphere.

I evaluated effluent total N (TN) concentration, the structure and composition of N-removing microbial communities, and greenhouse gas fluxes of advanced N-removal OWTS in the town of Charlestown, Rhode Island, USA. To assess N outputs from advanced OWTS and compliance with the 19 mg N/L state regulatory standard for advanced-treated effluent, in Manuscript 1 I quantified TN concentration of effluent from 50 advanced N-removal OWTS between March 2017 and December 2019, and evaluated differences as a function of N-removal technology, home occupancy pattern (systems used seasonally *vs.* those used year-round), and various wastewater properties. Four N-removal OWTS technologies were included in this study: (i) Orenco Advantex® AX20 ($n = 33$), (ii) Orenco Advantex® RX30 ($n = 9$), (iii) BioMicrobics MicroFAST® ($n = 3$), and Norweco Singulair® (models TNT, 960, and DN; $n = 5$). RX30 systems produced the lowest median TN concentration (mg N/L) (13.2), followed by FAST (13.4), AX20 (14.9) and Norweco (33.8). Compliance with the state standard varied among technologies, with compliance rates of 78%, 73%, 67%, and 0% for RX30, AX20, FAST, and Norweco systems, respectively. Effluent TN concentration did not vary as a function

of occupancy pattern. Ammonium and nitrate were identified as predictors for effluent TN in all technologies; temperature and pH were also part of best-fit models for FAST and Norweco systems, respectively. Systems used year-round produced a significantly higher median daily (5.3 g N/d) and annual (2.3 kg N/yr) N load than did seasonally-used systems (3.7 g N/d and 0.41 kg N/yr), likely due to differences in home usage patterns, demographics, and associated differences in system flow.

Assessment of treatment capabilities of advanced N-removal OWTS requires fast, accurate methods of monitoring performance. Regular monitoring of OWTS using *in situ* rapid tests can provide an inexpensive option for assessing treatment performance. In Manuscript 2 I assessed the ability of a portable photometer to accurately measure ammonium and nitrate concentrations in final effluent from 46 advanced N-removal OWTS in 2017. By comparing measurements made using the photometer with values determined by standard laboratory methods, I determined that photometer-based analysis reliably estimates inorganic N (ammonium and nitrate) concentration in field and laboratory settings. Photometer-based analysis of the sum of inorganic N species also consistently approximated the total N concentration in the final effluent from the systems. A cost-benefit analysis indicated that the photometer is a more cost-effective option than having samples analyzed by commercial environmental testing laboratories after analysis of 8 to 33 samples. These results suggest that a portable photometer can be used to provide reliable, cost-effective measurements of ammonium and nitrate concentrations, and estimates of total N levels in advanced N-removal OWTS effluent in a way that helps to identify underperforming systems.

Advanced N-removal OWTS remove N from effluent via microbial nitrification and denitrification. Despite the important role that microorganisms play in N removal, few studies have investigated the nitrifying and denitrifying microbial communities in advanced N-removal OWTS. In Manuscript 3, I used high-throughput sequencing to evaluate the structure and composition of nitrifying and denitrifying bacterial communities in 44 advanced N-removal OWTS. I sampled effluent from these systems in June and September 2017, targeting the genes encoding ammonia monooxygenase (*amoA*) and nitrous oxide reductase (*nosZ*), and assessed differences in the diversity and taxonomy of nitrifying and denitrifying communities as a function of technology, occupancy pattern, and season. Alpha diversity (species diversity at a local scale) and beta diversity (differences in species diversity among communities/sites) for *amoA* varied strongly as a function of season. Differences in beta diversity for *nosZ* were also influenced by season, although to a lesser extent than for *amoA*. Alpha diversity for *nosZ* varied among technologies. *Nitrosospira* and *Nitrosomonas* were the main genera of nitrifying bacteria in advanced N-removal OWTS. The predominant genera of denitrifying bacteria included *Zoogloea*, *Thauera*, and *Acidovorax*. Differences in taxonomy for each gene generally mirrored patterns observed in alpha and beta diversity, highlighting the important role that season and technology play in shaping communities of *amoA* and *nosZ*, respectively.

Microbial activity is required for the removal of N, pathogens, and organic C in advanced N-removal OWTS. However, these processes produce greenhouse gases (GHGs) including carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O). In Manuscript 4 I measured GHG emissions from 27 advanced N-removal OWTS in the

towns of Jamestown (2016) and Charlestown (2018), Rhode Island, USA, and assessed differences in flux based on technology, occupancy pattern, and zone within the system (oxic vs. anoxic zone), as well as various wastewater properties. Flux values for CO₂, CH₄, and N₂O fluxes ranged from -0.44 to 61.8, -0.0029 to 25.3, and -0.02 to 0.23 μmol m⁻² s⁻¹, respectively. CO₂ and CH₄, but not N₂O, fluxes were significantly higher in the anoxic/hypoxic zone than the oxic zone. CO₂ and CH₄ fluxes from the anoxic/hypoxic zone were positively correlated with 5-day biochemical oxygen demand, and negatively correlated with dissolved oxygen and nitrate, suggesting that anaerobic respiration contributes significantly to CO₂ and CH₄ flux. CO₂ and CH₄ fluxes peaked at ~22 to 23°C, as expected for microbial processes. CH₄ flux was positively correlated with ammonium concentration in the anoxic/hypoxic zone, likely due to inhibition of methane oxidation by ammonium. N₂O flux was not significantly correlated to any wastewater parameter. I estimated that advanced N-removal OWTS contribute approximately 347 g CO₂ equivalents capita⁻¹ day⁻¹, comparable to emissions from conventional OWTS.

My findings show that advanced N-removal OWTS in Rhode Island vary in their compliance with the state's standard for final effluent TN, in the structure and composition of their N-cycling microbial communities, and in their contribution of GHGs to the atmosphere. Continued monitoring of effluent TN from these systems could be easily achieved using rapid tests such as a field photometer, which we found to be a reliable and cost-effective predictor of effluent TN concentrations.

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Loomis at the University of Rhode Island. I thank my regulatory partners at the Rhode Island Department of Environmental Management and the Town of Charlestown Office of Wastewater Management for assisting with technical aspects of the project and procuring study sites. I also thank the Charlestown homeowners who allowed us to study their advanced N-removal OWTS.

Although the information in this paper has been funded wholly or in part by the U. S. Environmental Protection Agency, it has not undergone the Agency's publications review process and therefore may not necessarily reflect the views of the Agency; no official endorsement should be inferred. The viewpoints expressed here do not necessarily represent those of the town of Charlestown, the Rhode Island Department of Environmental Management, or the U. S. Environmental Protection Agency, nor does mention of trade names, commercial products, or causes constitute endorsement or recommendation for use.

Preface

Wastewater from onsite wastewater treatment systems (OWTS) is a source of nitrogen (N) to coastal watersheds (Valiela et al., 2010). Left untreated, wastewater can contaminate groundwater with pathogens as well as nitrate (Ward et al., 2005; Lancellotti et al., 2017). Because N is a limiting nutrient in coastal ecosystems, increased inputs of N to coastal systems also promote eutrophication, which results in anoxic conditions that kills fish and shellfish. Eutrophication also threatens public health by boosting the growth of potentially toxic algal species (Carpenter et al., 1998; Gan, 2004; Sohail & Adeloju, 2016). The harmful effects of eutrophication are also detrimental to the local economy, as a result of financial losses in the fish and shellfish industry and mandated beach closures. Evidence of eutrophication throughout Rhode Island's Greater Narragansett Bay watershed, where OWTS serve approximately 38% of the population, has become increasingly apparent (Amador et al., 2018; Bergondo et al., 2005).

Advanced onsite wastewater treatment systems (OWTS) are used to lower the N concentration in wastewater through biological N removal (BNR). Advanced N-removal OWTS are designed to facilitate successive nitrification (the oxidation of ammonium to nitrate) and denitrification (the reduction of nitrate to inert dinitrogen gas and nitrous oxide), ultimately removing N from effluent and releasing it into the atmosphere (Oakley et al., 2010). According to the Rhode Island Department of Environmental Management (RIDEM), final effluent leaving these systems must contain ≤ 19 mg total N/L (RIDEM, 2016), a standard which is also used in other areas on the east coast, such as Barnstable County, MA (BCDHE, 2012) and Suffolk County, NY (Tomarken & Dawydiak, 2017). However, the few studies that have assessed treatment performance of advanced N-

removal OWTS have found that these systems do not always meet regulatory standards (Oakley et al., 2010; Lancellotti et al., 2017).

My dissertation is divided into four manuscripts that address different aspects of advanced N-removal OWTS. In Manuscript 1, I assessed the effluent total N (TN) concentration of 50 advanced N-removal OWTS in Charlestown, Rhode Island, USA, a coastal town in which homes and businesses rely exclusively on OWTS for wastewater treatment. I included four N-removal technologies in this study: Orenco Advantex® AX20 ($n = 33$), (ii) Orenco Advantex® RX30 ($n = 9$), (iii) BioMicrobics MicroFAST® ($n = 3$), and Norweco Singulair® (models TNT, 960, and DN; $n = 5$). Like many other coastal towns in New England, Charlestown is home to residents who occupy their homes year-round, as well as residents that only use their homes during the summer months (RIDEM, 2016). Whether seasonally-used systems can consistently reduce effluent TN as well as systems used year-round is a common question among wastewater industry regulators. Thus, I investigated system compliance with the state effluent standard for TN as a function of technology and home occupancy pattern. I also assessed whether wastewater properties (effluent pH, alkalinity, temperature, dissolved oxygen, 5-day biochemical oxygen demand, ammonium, and nitrate) can be used to predict effluent TN concentration. After ~1.5 years of sampling, technology manufacturers and service providers were asked to identify and adjust underperforming OWTS, and I evaluated how these actions affected effluent TN concentrations. In addition to assessing difference in effluent TN concentration, I also estimated the mass load of N contributed by seasonal and year-round systems to better understand their impact on the surrounding

environment. This manuscript will be submitted for publication in *Water, Air, & Soil Pollution*.

Wastewater management and regulatory agencies rely on operation and maintenance (O&M) performed by service providers. These visits include quantifying tank substrate levels (sludge and scum), inspecting electrical components, and evaluating system physical integrity (Bounds et al. 2004). However, O&M visits in Rhode Island do not require evaluation of effluent TN concentration (RIDEM, 2016). Frequently monitoring of effluent TN from advanced OWTS, in conjunction with communicating this information via outreach platforms to homeowners and service providers, has greatly improved OWTS performance in Barnstable County, MA (Lancellotti et al., 2017). Previous research efforts by Lancellotti et al. (2017) assessed the efficacy of using rapid tests for field monitoring of advanced OWTS. These can allow for *in situ* analysis of system treatment performance, and can provide some of the same information as a commercial laboratory would, but more quickly and at a lower cost to the service provider. Service providers need efficient, inexpensive methods for evaluating the performance of advanced OWTS in order to ensure that OWTS are not releasing effluent that exceeds the TN regulatory standard. In Manuscript 2, I assessed the accuracy and cost-effectiveness of using a portable photometer to measure inorganic N concentrations in wastewater. Total N is comprised of the sum of inorganic (ammonium and nitrate) and organic N, and I investigated whether the sum of ammonium and nitrate concentrations measured using the photometer can be used to approximate effluent TN concentration. I also performed a cost-benefit analysis to compare the cost of using the photometer to measure effluent N concentrations with the cost of having effluent analyzed by a

commercial laboratory. This manuscript was published in *Water, Air, & Soil Pollution* (Ross et al., 2018).

The successive nitrification and denitrification processes that advanced OWTS rely on for N-removal are facilitated by specific groups of microorganisms. The microbial communities in BNR wastewater treatment plants (WTPs) have been studied extensively to assess the effectiveness of system design in promoting nitrification and denitrification (Geets et al., 2007; Siripong & Rittmann, 2007; Jaranowska et al., 2013; Brannon et al., 2017). However, despite their shared goal of N-removal, few studies have characterized the microbial communities in advanced N-removal OWTS. In Manuscript 3, I used high-throughput sequencing to evaluate the structure and composition of nitrifying and denitrifying bacterial communities in advanced N-removal OWTS. I targeted two genes that play critical roles in the N cycle: *amoA*, which encodes ammonia monooxygenase and is responsible for the first step in nitrification, and *nosZ*, which codes for nitrous oxide reductase and reduces nitrous oxide to dinitrogen gas. I characterized differences in diversity and taxonomy as a function of technology, occupancy pattern, and season for both genes to investigate the influence that these variables have on the nitrifying and denitrifying communities in advanced N-removal OWTS. This manuscript will be submitted for publication in *Water*.

Advanced N-removal OWTS are designed to remove N from effluent and release it into the atmosphere through denitrification. When denitrification proceeds to completion, harmless dinitrogen gas (N_2) is produced, but incomplete denitrification produces nitrous oxide (N_2O), a potent greenhouse gas (GHG) (Brannon et al., 2017). The microbial degradation of organic matter that takes place in wastewater treatment can

also produce carbon dioxide (CO₂) and methane (CH₄), two other potent greenhouse gases (Diaz-Valbuena et al., 2011). Few studies have investigated whether advanced treatment of effluent shifts the pollution stream from receiving waters to the atmosphere. In Manuscript 4, I quantified the flux of CO₂, CH₄, and N₂O from five types of advanced N-removal OWTS in the towns of Charlestown and Jamestown, Rhode Island, USA. I assessed differences in emissions as a function of technology, occupancy pattern, and zone within the treatment train (oxic vs. anoxic zone). Because GHG emissions result from microbial processes that respond to environmental conditions and availability of nutrients, electron acceptors, and organic C, I also assessed the relationship between gas emissions and effluent temperature, pH, and the concentration of dissolved oxygen (DO), 5-day biochemical oxygen demand (BOD₅; a proxy for organic C), ammonium, nitrate, and TN. This manuscript will be submitted for publication in *Science of the Total Environment*.

This dissertation is prepared in manuscript format containing four papers. Manuscript 2 has been published, and all other manuscripts are currently in preparation.

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Manuscript 1:
**Effectiveness of Advanced Nitrogen-Removal Onsite Wastewater Treatment
Systems in a New England Coastal Community**

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In preparation for submission to Water, Air, & Soil Pollution

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nitrogen removal

Abstract

Wastewater is a major source of nitrogen to groundwater and coastal waterbodies, threatening both environmental and public health. Advanced N-removal onsite wastewater treatment systems (OWTS) are used to reduce effluent N concentration. Despite the important role that these systems play in treating N-rich wastewater, few studies have assessed their effectiveness. We evaluated the total N (TN) concentration of effluent from 50 advanced N-removal OWTS in the town of Charlestown, a coastal community in Rhode Island, USA for three years. We quantified differences in effectiveness as a function of N-removal technology, home occupancy pattern (seasonal *vs.* year-round use), and examined the relationship between effluent TN concentration and wastewater properties. Orenco RX30 systems produced the lowest median TN concentration (mg N/L) (13.2), followed by FAST (13.4), Orenco AX20 (14.9) and Norweco (33.8). Compliance with the state's regulatory standard for effluent TN concentration (19 mg N/L) was highest for RX30 systems (78%), followed by AX20 (73%), FAST (67%), and Norweco (0%). Effluent TN concentration was not affected by occupancy pattern. The best predictors of effluent TN concentration were ammonium and nitrate for AX20 and RX30 systems; ammonium, nitrate, and temperature for FAST systems; and ammonium, nitrate, and pH for Norweco systems. Median daily N load (g N/d) was significantly higher for year-round (5.3) than for seasonal (3.7) systems, as was the annual N load (kg N/yr), with values of 2.3 and 0.41 for year-round and seasonal systems, likely due to differences in system flow. Our results suggest that advanced N-removal OWTS vary in capacity to produce effluent that meets the state regulatory standard for TN, and can withstand long periods of non-use without compromising

effectiveness. Nevertheless, systems used year-round do produce a higher daily and yearly N load than those used seasonally.

Introduction

Effluent from onsite wastewater treatment systems (OWTS) is an important source of nitrogen to coastal watersheds (Valiela et al., 2010). Because N is a limiting nutrient in coastal ecosystems, increased inputs of N to groundwater and poorly flushed coastal systems promote eutrophication, which results in anoxia that kills fish and shellfish, and can boost the growth of toxic algal species, which threatens public health (Carpenter et al., 1998; Gan, 2002; Sohail & Adeloju, 2016). The harmful effects of eutrophication have become increasingly evident in Rhode Island's Greater Narragansett Bay Watershed, where OWTS serve approximately 38% of the population (Amador et al., 2018; Bergondo et al., 2005). Compromising the health of coastal waterbodies threatens environmental and public health as well as the economy, through mandated beach closures and financial losses in the fish and shellfish trade (Gan, 2002). Without adequate treatment, residential wastewater can also contaminate groundwater with nitrate and pathogens (Ward et al., 2005; Lancellotti et al., 2017).

Commercially-available advanced N-removal OWTS are designed specifically to lower the high concentration of N in wastewater through biological N removal (BNR), which relies on sequential nitrification and denitrification. Ammonia-oxidizing and nitrifying bacteria oxidize ammonium to nitrate, which is then reduced to inert dinitrogen gas and nitrous oxide (a greenhouse gas) by denitrifying bacteria, at which point N is emitted into the atmosphere. Although the configuration and design of advanced OWTS treatment trains (the sequential arrangement of OWTS components) differ among

technologies, they all have an oxic zone (for nitrification) and an anoxic zone (for denitrification) to promote N removal (Oakley et al., 2010). Treated effluent from the advanced OWTS is dispersed to a soil treatment area (STA), which provides a final opportunity for treatment before effluent reaches the groundwater table.

The use of advanced N-removal OWTS is particularly important in areas that rely on groundwater wells as their source of drinking water, and in coastal areas where groundwater is hydrologically connected to coastal water bodies (Bowen et al., 2007; Lancellotti et al., 2017). The state of Rhode Island requires the use of advanced N-removal OWTS in critical resource areas, which are particularly vulnerable to excess nutrients and/or pathogens present in effluent. State regulations require that the effluent dispersed to the STA (“final effluent”) contain no more than 19 mg total N/L (TN; RIDEM, 2016). A similar standard is also used in other areas, including Barnstable County, MA (BCDHE, 2012) and Suffolk County, NY (Tomarken & Dawydiak, 2017).

Advanced N-removal OWTS do not always produce effluent that meets regulatory standards (Oakley et al., 2010). For example, analysis of data from Barnstable County, MA shows advanced N-removal OWTS fail to meet the 19 mg/L standard approximately 30% of the time (BCDHE, 2012). A recent study by Lancellotti et al. (2017) found between 25 and 36% of the 42 advanced N-removal systems they evaluated in 6 towns in Rhode Island did not meet the 19 mg N/L standard, with effectiveness depending on technology. They recommended monitoring of effluent TN concentration to improve treatment effectiveness (Lancellotti et al., 2017), based on the positive impact of monitoring in areas such as Barnstable County, MA. The study by Lancellotti et al. is the only one to quantify the extent to which advanced N-removal OWTS meet the Rhode

Island state regulatory standard, and to consider how system performance can be improved.

In this study, we assessed the effluent TN concentration of advanced N-removal OWTS in the town of Charlestown, Rhode Island, USA. Charlestown is a town on the southern coast of the state, where homes and businesses rely exclusively on OWTS for wastewater dispersal and treatment, with a population of ~7,800 people (U.S. Census Bureau, 2018). While many of the homes are occupied year-round, a portion are only used during the summer months (RIDEM, 2016). The town has a large number of advanced N-removal OWTS that employ different technologies. We investigated system compliance with the state effluent standard for TN as a function of technology and home occupancy pattern. We also determined whether wastewater properties (effluent pH, alkalinity, temperature, dissolved oxygen, 5-day biochemical oxygen demand, ammonium, and nitrate) can be used to predict effluent TN concentration. In addition, we assessed how adjustment of underperforming systems affected effluent TN. Finally, we estimated daily and annual mass N loads from systems as a function of occupancy pattern. Our study extends on the work of Lancellotti et al. (2017) by narrowing the geographic range to a single town, expanding the number of N-removal technologies assessed, evaluating OWTS used both seasonally and year-round, and quantifying the mass load of TN from each system as a function of occupancy.

Methods

Study Systems

We sampled final effluent from 50 advanced N-removal OWTS serving single-family, residential homes in Charlestown, Rhode Island, USA. Twenty-two systems

serve homes used year-round and 28 systems serve seasonally-used homes. We assessed occupancy pattern based on homeowner self-identification, as well as daily flow data from each system. We worked with the Rhode Island Department of Environmental Management (RIDEM) and the Town of Charlestown Onsite Wastewater Management department to identify sites that rely on well water for potable water and use a pressurized pump to discharge effluent to the STA. Sites adhering to these criteria were selected after the homeowners agreed to participate in the study. Four N-removal OWTS technologies were included in this study: (i) Orenco Advantex® AX20 (n = 33), (ii) Orenco Advantex® RX30 (n = 9), (iii) BioMicrobics MicroFAST® (n = 3), and Norweco Singulair® (models TNT, 960, and DN; n = 5). Detailed descriptions of individual technology designs can be found in the Appendix.

Sample Collection

We sampled final effluent (effluent to be dispersed to the STA) from each system four times a year from March 2017 to December 2019. Systems used year-round were sampled in March, June, September, and December, and seasonally-used systems were sampled in June, July, August, and September. The Advantex systems were sampled at the recirculating splitter valve assembly, while the FAST and Norweco technologies were sampled from the STA pump basin. One grab sample was collected into a clean 1-L plastic bottle. Part of the sample was used for field analysis, and the remainder of the sample was stored at 4°C until transported to the laboratory (within 8 hours of sampling) for further analysis. The pH, dissolved oxygen (DO), and temperature of final effluent samples were measured in the field using a Hanna Instruments HI9828 Multiparameter

Meter (Woonsocket, RI). A second grab sample was collected into a clean 250-mL plastic bottle and was also stored at 4°C until transported to the laboratory for analysis of alkalinity.

Effluent Analyses

Detailed descriptions of methods and quality control criteria for laboratory analyses can be found in Ross et al. (2018). Upon arrival at the laboratory, 25 mL of sample was passed through a 0.45- μ m-pore-size membrane filter and the filtrate was frozen until analyzed for ammonium and nitrate concentration, and 25 mL of unfiltered sample was frozen for analysis of TN concentration. Ammonium (Weatherbern, 1967) and nitrate (Doane and Horwath, 2003) concentrations were measured colorimetrically using a BioTek Synergy HTX multi-mode microplate reader (Winooski, Vermont). Total N concentration was determined using the persulfate oxidation method (APHA, 1998), and the resulting nitrate was measured colorimetrically using the microplate reader.

BOD₅ was analyzed with an OxiTop BOD₅ measurement system (Weilheim, Germany) immediately upon return from the field. Within two weeks of sample collection, we measured alkalinity content with an Automatic Titration System (Woonsocket, RI), using an HCl solution to titrate a diluted wastewater sample.

Summary statistics for the wastewater parameters measured from each technology can be found in Table 1.

Nitrogen Load

Flow data was obtained from the OWTS control panels (elapsed time meter and cycle counter readings for pumps in the treatment train) at each sampling event. Average forward flow and recirculation ratio were calculated as described in Lancellotti et al. (2017). We calculated average daily and annual N load for our systems based on average forward flow data from three years of sampling. We multiplied the average daily forward flow by the TN concentration measured at each sampling event to determine average daily N load. Due to insufficient replication of occupancy patterns across technologies, we grouped systems within occupancy pattern category without regard for technology when investigating flow parameters for seasonal and year-round sites. Annual mass N load was calculated by first multiplying the average daily load by the number of days in each particular sampling month for each system to calculate the total monthly load. We then averaged the total monthly load across all sampling months, and multiplied that average by the number of months during which the systems are used – 4 months for seasonal sites and 12 months for year-round sites – to quantify the total annual N load.

Statistical Analysis

To assess differences in effluent TN concentration as a function of occupancy pattern, we used a Mann-Whitney U test. We assessed increases in median effluent TN concentration to evaluate differences in effluent TN concentration in underperforming systems before (March 2017 – August 2018) and after (June 2019 – December 2019) system adjustments by manufacturers/service providers.

We performed multiple linear regressions to select the best wastewater properties (ammonium, nitrate, pH, alkalinity, BOD₅, DO, and temperature) as predictors of effluent TN. The criteria for wastewater property selection was based on Lancellotti et al. (2017): to include a new parameter in the regression, it had to improve the R² value by at least 10% and/or increase the *p* value to greater than 0.05, otherwise it was not included in the model. This allowed us to choose the best model with the fewest variables, highlighting the most time- and cost-effective options from a management perspective. We created a correlation matrix for each technology to identify variables that covary with each other, and those that covaried ($r \geq 0.70$) were not considered together in the model.

We assessed differences in average daily forward flow, as well as differences in daily and annual mass N load, as a function of occupancy pattern using a Mann-Whitney U test.

We used a *p* value of less than or equal to 0.05 as a measure of statistical significance for all statistical analyses.

Results & Discussion

Final Effluent Total Nitrogen Concentration

We assessed system final effluent TN concentration as a function of technology and occupancy pattern. Technologies varied in effluent TN concentration and in compliance with the 19 mg N/L regulatory standard (Fig. 1.1). Norweco systems produced the highest median final effluent TN concentration (mg TN/L) (median 33.8; range 5.5 – 60.6; *n* = 47), followed by AX20 (median 14.9; range 0.6 – 87.4; *n* = 319), FAST (median 13.4; range 2.6 – 62.3; *n* = 32), and RX30 (median 13.2; range 3.0 – 60.7; *n* = 84). Values for AX20 and FAST systems are comparable to those reported by

Lancellotti et al. (2017), who evaluated the effectiveness of 42 advanced N-removal OWTS in six Rhode Island towns within the Greater Narragansett Bay watershed between March 2015 and August 2016.

When values for all sampling events and both occupancy patterns are considered, 78% of RX30 systems produced effluent with a median TN concentration of ≤ 19 mg N/L, followed by AX20 (73%), FAST (67%), and Norweco (0%) (Fig. 1.2). Although the proportion of systems in compliance varied among technologies, almost all of the individual systems were capable of producing final effluent at or below the standard at some point during the study. The proportion of systems in compliance with the 19 mg N/L standard was higher in our study than for those studied by Lancellotti et al. (2017), who found compliance values of 71% and 64% for AX20 and FAST systems, respectively. The difference may be partly attributed to the broader geographic range studied by Lancellotti et al (2017), which assessed advanced N-removal OWTS in six towns within the Greater Narragansett Bay Watershed, while we sampled systems in a single town. Different towns employ different practices when it comes to wastewater management, and Charlestown have a more effective approach to managing and regulating N-removal OWTS. Furthermore, differences in demographics and quality of potable water sources among towns may have contributed to variations in effluent TN concentration.

Our results show that effluent TN concentration varies as a function of treatment train design. Norweco systems, which produced the highest median TN concentrations, rely on temporal separation of nitrification and denitrification. Both processes take place in the same compartment: intermittent periods of aeration of effluent allow for a switch

between anoxic and oxic conditions. In contrast, AX20, RX30, and FAST systems have these processes separated in space, rather than time. AX20s and RX30s rely on a textile media filter as an oxic environment to facilitate nitrification, and an anoxic/hypoxic processing tank for denitrification. Effluent is recirculated between the oxic and anoxic/hypoxic components multiple times before dispersal to the STA to maximize N removal. FAST systems use submerged, fixed-film activated sludge to treat wastewater. As air is blown into the oxic zone of FAST systems and effluent is nitrified, it is then transferred into an adjacent anoxic zone where denitrification takes place. Spatial separation of oxic and anoxic components may favor the establishment of microbial communities that are more effective at N removal. In a study of nitrifying and denitrifying microbial communities of these technologies, Ross et al. (2020) suggested that treatment train design may drive differences in the diversity of microbial communities of *nosZ*, the gene that encodes for the enzyme responsible for the final step in denitrification. Technologies using textile filters (AX20s and RX30s) had communities with higher diversity indices than other designs.

Norweco systems also produced effluent with higher median BOD₅ than the other three technologies (Table 1). A high concentration of BOD₅ is unusual in advanced N-removal OWTS final effluent, since oxygen that is introduced to promote nitrification also promotes microbial oxidation of organic C (Bounds et al., 2004). A high concentration of BOD₅ in final effluent suggests that organic matter is not being oxidized, possibly due to insufficient aeration. This is supported by a lower median DO concentration in final effluent from Norweco systems than from that of AX20, RX30, and FAST systems (Table 1). Effluent from Norweco systems also had a higher ammonium

concentration than the other technologies (Table 1), suggesting that nitrification was limited.

Home occupancy pattern did not significantly influence final effluent TN concentration (Fig. 1.3). Across all technologies and sampling events, the median TN concentration in effluent from seasonally-used systems and for systems used year-round were nearly identical (16.1 and 15.3 mg N/L, respectively). This contradicts the commonly-held belief among industry manufacturers and regulators that a lag time prior to re-establishment of the microbial community at the beginning of the summer compromises the capacity of seasonally-used systems to remove TN (Postma et al., 1992; Baumgaertel, 2016). An assessment of three seasonally-used advanced N-removal OWTS in Barnstable County, MA found similar results: seasonally-used systems produced effluent TN concentrations comparable to those produced by OWTS used year-round (Baumgaertel, 2016). Ross et al. (2020) found no difference in diversity or taxonomy between OWTS used seasonally and those used year-round, suggesting that OWTS maintain similar microbial communities regardless of occupancy pattern. It appears that the accumulation of resources necessary for microbial growth and activity, such as organic carbon (C) and N, when the systems are in use during the summer is sufficient to sustain the necessary microbial communities during periods of non-use. These results speak to the robustness of seasonally-used systems which, even after months of not receiving wastewater, maintain a microbial community that is capable of treating wastewater to the same level as systems receiving wastewater inputs throughout the year.

Differences in compliance with the 19 mg N/L effluent standard among technologies were apparent after ~1.5 years of sampling, at which point the Town of Charlestown's Office of Wastewater Management asked service providers and technology representatives to adjust underperforming systems. After adjustment and a period of equilibration, we assessed effluent TN concentration in the underperforming systems. Measurements made between March 2017 and August 2018 were used to initially identify underperforming systems. We compared those measurements to TN concentrations measured between June and December, 2019 to assess the effectiveness of the adjustments made. Out of the 13 underperforming systems, eight had a lower median effluent TN concentration after adjustments, while the remaining five had a higher concentration (Fig. 1.4). There are several possible reasons for why effluent TN concentrations paradoxically increased for some systems after adjustment. It is possible that the request for adjustment was ignored, and no adjustments were made to the systems. It is also possible that the types of adjustments made did not have a substantial impact on the capacity of OWTS to lower TN concentration in effluent. Lancellotti et al. (2017) also observed no consistent improvement in effluent TN concentration pre- and post-adjustments. They suggested that these results may have been due to an inability of service providers to identify the reason(s) for systems' reduced capacity for N removal, and/or due to system physical conditions (e.g., liquid levels, tank settling, etc.) limiting the microbial activity responsible for N removal.

Predictors of Total Nitrogen Concentration

We evaluated the best-fit multiple linear regression model for each technology to identify the combination of properties that best predicted effluent TN (Table 2).

Ammonium and nitrate, not surprisingly, were significant predictors for all technologies, in agreement with Ross et al. (2018), who found that the sum of ammonium and nitrate in effluent are strong predictors of TN. Effluent TN concentration for both AX20 and RX30 systems had a significant positive correlation with ammonium and nitrate concentration (Table 2). Ammonium and nitrate were also part of the best-fit model observed for AX20s by Lancellotti et al. (2017).

For FAST systems, TN concentration was positively correlated with ammonium and nitrate, and negatively correlated with temperature (Table 2). FAST was the only technology to have temperature as a significant predictor. Lancellotti et al. (2017) found that, although effluent temperature did not significantly predict TN, TN concentrations were lower in FAST systems in winter when ambient temperatures were the lowest, than in spring, summer, and fall. Because the activity of nitrifying and denitrifying microorganisms increases with increasing temperature (Shammas, 1986; Seitzinger, 1988), we would expect to find a negative relationship between temperature and TN in all technologies. Although effluent median temperatures were similar across all technologies (Table 1), FAST systems may provide conditions in which nitrifying and denitrifying microorganisms are more sensitive to changes in temperature than other technologies. For example, the FAST treatment train design relies on fixed activated sludge, and sensitivity to low temperatures has been observed in some N cycling microbial communities in activated sludge BNR WTPs (Randall & Buth, 1984).

Effluent TN in Norweco systems was positively correlated with ammonium and nitrate concentrations, and negatively correlated with pH (Table 2). While pH has varying impacts on communities of nitrifying and denitrifying communities in OWTS, these typically perform best within a certain range: 6.5 – 8.0 for nitrifiers and 7.0 – 8.5 for denitrifiers (Haandel & Lubbe, 2007). In our study, effluent pH values typically fell between ~6 and 8 (data not shown). The microbial communities in Norweco systems may be more sensitive to pH than communities in other technologies, but the reasons for this are unclear. The negative relationship between TN and pH suggests that microbial N removal in Norweco systems is more effective as effluent shifts from acidic to alkaline conditions.

Mass N Load

The current concentration-based regulatory standard employed by regulatory agencies helps to mitigate N pollution from advanced OWTS; however, because systems vary in the volume of effluent they produce, the total amount of N emitted by OWTS is a better indicator of their impact on the environment (Amador et al., 2018). We calculated daily and annual mass N load of TN from systems (Fig. 1.5) using the concentration of TN in effluent from advanced OWTS and the volume effluent dispersed by OWTS to the STA. Year-round sites produced a significantly higher average daily mass load (g N/system/day) (median 5.3; range 0.01 – 50.6; $n=176$) than seasonal sites (median 3.7; range 0.03 – 133; $n = 172$). The values for year-round sites are lower than those reported by Amador et al. (2018), who assessed daily N loads from 42 advanced N-removal OWTS technologies used year-round within the Greater Narragansett Bay watershed and

reported median daily loads ranging from 9.6 to 10.8 g N/system/day. Differences in demographics, wastewater management practices, system effectiveness, and quality of potable water sources likely contribute to this discrepancy.

For both seasonal and year-round systems in our study, average daily forward flow was significantly positively correlated with daily mass N load, highlighting the importance of flow in determining TN outputs from advanced OWTs. Owners of summer homes in Charlestown do not necessarily live in these homes for the entire duration of the summer: many people may only use them on weekends or for a few weeks during the summer. This results in a significantly lower average daily forward flow from seasonal systems during periods of use. The difference in mass N load as a function of occupancy pattern, despite the lack of difference in effluent TN concentration between seasonal and year-round systems, indicates that flow controls mass N load. The median daily flow value for year-round systems in our study (318 L/system/d) was slightly lower than the ranges reported by Amador et al., 2018 (374 – 476 L/system/d), possibly due to differences in wastewater management strategies employed by Charlestown compared to those employed throughout the entirety of the Greater Narragansett Bay Watershed.

Year-round systems produced a significantly higher estimated annual mass N load (kg N/system/yr) (median = 2.3; range 0.20 – 12.2; $n = 58$) than seasonal systems (median = 0.41; range 0.020 – 4.8; $n = 59$) – nearly five times less than year-round systems (Fig. 1.5). The higher annual mass N load from year-round systems is a function of the higher daily N load and the longer usage. Our results point to system usage and associated flow – rather than differences in effluent TN concentration – as controlling the

differences in mass of N discharged from advanced N-removal OWTS used seasonally and year-round, and their environmental impact.

Conclusions

We found that median effluent TN concentration varied among the different N-removal OWTS technologies we studied in the town of Charlestown. RX30 systems produced effluent with the lowest median TN concentration (mg N/L) (13.2), followed by FAST (13.4), AX20 (14.9), and Norweco systems (33.8). Variations may be attributed to differences in treatment train design, such as whether nitrification and denitrification are separated in space or time. Although technologies varied in the proportion of systems in compliance with the regulatory standard of 19 mg TN/L, almost all of the systems were capable of meeting the standard at some point in the study. Approximately two thirds of the underperforming systems exhibited a reduction in effluent TN concentration after adjustments, while the remaining systems increased in effluent TN concentration. Wastewater properties that best predicted effluent TN concentration included ammonium and nitrate for all four technologies, with temperature and pH as additional significant predictors for FAST and Norweco systems, respectively.

Despite the prevalence of similar home occupancy patterns in many coastal towns that experience a significant influx of summer tourists throughout the United States, our study is one of the first to examine the relationship between home usage and performance of advanced OWTS. Our results suggest that occupancy pattern does not significantly influence the concentration of TN in effluent from advanced N-removal OWTS, and that these systems are capable of withstanding long periods of non-use without compromising performance. Nevertheless, year-round systems produced significantly higher daily and

annual N loads than systems used seasonally, likely due to the higher flow discharged from year-round systems. These results point to the need to quantify mass N loading from advanced OWTS to assess their impact on receiving waters. Monitoring of advanced OWTS effluent TN concentration in combination with recursive adjustments to meet the regulatory standard, and quantifying mass N load, will help to mitigate the threat that N-rich wastewater poses to public and environmental health.

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Figures and Tables

Figure 1.1. Final effluent total N concentration for each technology across all sampling events (March 2017 to December 2019) and occupancy patterns. The red dashed line represents the 19 mg N/L standard required for final effluent. The solid line in the middle of each box represents the median and the edges of each box represents the first and third quartiles. Box whiskers extend $1.5 \times$ the inter-quartile range beyond the edges of the box and the dots represent outliers beyond $1.5 \times$ the inter-quartile range. AX20 = Orenco Advantex® AX20 (33 systems; $n = 319$); FAST = BioMicrobics MicroFAST® (3 systems; $n = 32$); NOR = Norweco Singulair® (5 systems; $n = 46$); RX30 = Orenco Advantex® RX30 (9 systems; $n = 84$)

Figure 1.2. Final effluent total N concentration for individual AX20 ($n = 3 - 12$), FAST ($n = 9 - 12$), Norweco ($n = 1 - 12$), and RX30 ($n = 3 - 12$) systems, in order of increasing median value, across all occupancy patterns and sampling events (March 2017 – December 2019). The red dashed line represents the 19 mg N/L standard required for final effluent. Description of box and whisker plots can be found in Fig. 1.1.

Figure 1.3. Final effluent total N concentration for seasonal (28 systems; $n = 273$) and year-round (22 systems; $n = 208$) across all technologies and sampling events. The red dashed line represents the 19 mg N/L standard required for final effluent. Description of box and whisker plots can be found in Fig. 1.1.

Figure 1.4. Final effluent total N concentration of underperforming systems pre- and post-system adjustments. Data for both occupancy patterns are included. Pre-adjustment data are for samples taken between March 2017 and August 2018, representing the data presented to service providers to help them target underperforming systems. Post-adjustment analyses are for samples taken between June and December 2019, after adjustments were made to these underperforming systems. The red dashed line represents the 19 mg N/L standard. Description of box and whisker plots can be found in Fig. 1.1.

Figure 1.5. Daily (top) and annual (bottom) nitrogen load for seasonal ($n = 59 - 172$) and year-round ($n = 58 - 176$) systems across all sampling events and technologies. Descriptions of box and whisker plots can be found in Fig. 1.1.

Table 1.1. Summary statistics of effluent properties across all sampling events and occupancy patterns for each technology. Units are mg/L except for pH and temperature ($^{\circ}$ C).

Property	AX20				RX30				FAST				Norweco			
	<i>n</i>	Min	Med	Max	<i>n</i>	Min	Med	Max	<i>n</i>	Min	Med	Max	<i>n</i>	Min	Med	Max
pH	267	3.8	6.8	8.5	76	5.7	7.0	9.5	32	6.2	7.2	7.8	47	4.5	7.1	8.6
Alkalinity	218	0	75.3	391	64	7.0	87.5	260	20	11.6	54.5	276	34	70.7	179	368
Temperature	275	5.0	20.9	27.1	69	5.3	21.2	25.0	30	14.9	21.2	24.6	46	10.4	20.1	23.1
BOD ₅	209	0	0	100	54	0	0	92	19	0	0	86	35	0	34	94
DO	257	0.8	6.9	12.1	64	0.8	6.3	10.4	29	0	3.8	7.1	43	0	1.7	5.5
Ammonium	289	0	0.5	28.6	81	0	0.4	51.6	28	0	0.4	47.1	45	0.10	19.0	53.6
Nitrate	331	0	10.0	46.9	86	0	8.7	33.9	30	0	9.9	20.4	44	0	4.9	38.0
TN	319	0.6	14.9	87.4	84	3.0	13.2	60.7	32	2.6	13.4	62.3	47	5.5	33.8	60.6

Table 1.2. Results of multiple regression best-fit models selected for each technology. Units for wastewater properties can be found in Table 1.1.

Technology	R^2	Property	Coefficient	p value
AX20	0.64	Ammonium	1.30	< 0.001
		Nitrate	0.89	< 0.001
RX30	0.72	Ammonium	1.01	< 0.001
		Nitrate	1.02	< 0.001
FAST	0.92	Ammonium	1.21	< 0.001
		Nitrate	0.79	< 0.001
		Temperature	-0.86	0.033
Norweco	0.76	Ammonium	0.74	< 0.001
		Nitrate	0.80	< 0.001
		pH	-0.44	0.86

Appendix

Advanced OWTS technology descriptions

Technology descriptions for the Orenco Advantex® AX20 and RX30, BioMicrobics FAST®, and Norweco Singular® systems were taken from Ross et al. (2018). 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 timed-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 tank media insert and creates an oxic environment. Nitrified effluent moves upward through a honeycomb-like plastic insert in the tank and splashes onto a trough, allowing the effluent to be transferred into an adjacent area where denitrification takes place.

Finally, 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 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.

Manuscript 2:
**User-Based Photometer Analysis of Effluent from Advanced Nitrogen-Removal
Onsite Wastewater Treatment Systems**

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Abstract

Advanced nitrogen-removal onsite wastewater treatment systems (OWTS) are used to reduce total nitrogen (N) levels in domestic wastewater. Maintaining system performance requires regular monitoring and in situ rapid tests can provide an inexpensive option for assessing treatment performance. We used a portable photometer to measure ammonium and nitrate concentrations in final effluent from 46 advanced N-removal OWTS, sampling each site at least three times in 2017. To assess photometer accuracy, we compared measurements made using the photometer with those determined by standard laboratory methods using linear regression analysis and a two-tailed t-test to compare regression parameters to those for a perfect linear relationship (slope = 1, intercept = 0). Our results show that photometer-based analysis reliably estimates inorganic N (ammonium and nitrate) concentration in field and laboratory settings. Photometer-based analysis of the sum of inorganic N species also consistently approximated the total N concentration in the final effluent from the systems. A cost-benefit analysis indicated that the photometer is a more cost-effective option than having samples analyzed by commercial environmental testing laboratories after analysis of 8 to 33 samples. A portable photometer can be used to provide reliable, cost-effective measurements of ammonium and nitrate concentrations, and estimates of total N levels in advanced N-removal OWTS effluent. This method can be a viable tool for triaging system performance in the field, helping to identify systems that are not functioning properly and may need to be adjusted or repaired by an operation and maintenance service provider in order to meet treatment standards.

Introduction

Advanced nitrogen-removal onsite wastewater treatment systems (OWTS) are used to mitigate the impact of residential wastewater on ecosystems. Because nitrogen (N) is a limiting nutrient in coastal watersheds, increased N inputs from wastewater promote eutrophication, which can result in fish and shellfish kills (Carpenter et al. 1998; Sohail and Adeloju 2016). Advanced N-removal OWTS aim to reduce N levels in wastewater by cycling it through an advanced treatment train that promotes successive nitrification (the conversion of ammonium to nitrate) and denitrification (the conversion of nitrate to nitrous oxide and/or dinitrogen gas), ultimately resulting in N being removed from the wastewater and emitted into the atmosphere (Fig. 2.1; Oakley et al. 2010).

Wastewater management and regulatory agencies rely on service providers – trained professionals responsible for carrying out operation and maintenance of OWTS, which can include quantifying tank substrate levels (sludge and scum), inspecting system electrical components, and evaluating system physical integrity (Bounds et al. 2004). Although measuring total N (TN) is not usually required during system maintenance, frequent monitoring of advanced OWTS TN concentrations in Cape Cod, MA has significantly improved system performance (BCDHE 2012). Previous efforts have assessed the efficacy of using rapid tests for field monitoring of advanced OWTS. Rapid tests allow for in situ analysis of system treatment performance, and can provide much of the same information as an external laboratory would, but faster and at less cost to the service provider (Bounds et al. 2004). Rapid tests for in situ measurement of N levels are particularly important for advanced OWTS because of the harmful threat that N-rich residential wastewater poses to coastal watersheds (Valiela et al. 1992). Service

providers need efficient, inexpensive methods for evaluating N-removal performance of advanced OWTS.

TN is the sum of ammonium, nitrate, and organic N. The few field tests capable of quantifying TN are time- and labor-intensive (Hach 2017; Hanna 2018), such that measuring ammonium and nitrate levels in the field may be a feasible alternative to quantifying TN. Several types of rapid field tests exist to measure the inorganic fraction of TN. Brannon et al. (2017) measured ammonium, nitrate, TN, and 5-day biochemical oxygen demand (BOD₅; a proxy for organic N) in effluent from advanced OWTS. Based on these measurements, it appears that BOD₅ does not significantly influence TN concentration in effluent with less than 19 mg/L TN, while BOD₅ does have a significant contributing influence on TN concentration in effluent with a TN concentration was higher than 19 mg/L. Because organic N is unlikely to make up a significant fraction of the TN present in treated effluent of advanced systems, quantifying ammonium and nitrate levels could potentially be used as a proxy for TN levels in effluent. In addition, evaluating ammonium and nitrate levels may provide insight into how the OWTS is treating the wastewater. Extremely high or low concentrations of either inorganic N species may indicate the extent to which specific N transformations are occurring in the system. For example, a system producing effluent from the nitrification component of the treatment train that is high in ammonium and low in nitrate is likely not facilitating nitrification, which will ultimately impede system N-removal.

Test strips are commonly used to measure ammonium and nitrate concentrations in freshwater samples (Isbell et al. 2006; Murphy et al. 2014); however, few studies have investigated the efficacy of these tests with wastewater effluent. Lancellotti et al. (2016)

measured ammonium and nitrate concentrations in effluent from advanced N-removal systems in an outdoor (field) and indoor (laboratory) setting using both test strips and wet chemistry test kits and reported that ammonium and nitrate test strips only provide accurate results in the laboratory. A variety of factors – including contamination, temperature, precipitation, humidity, wind, and differences in ambient sunlight – can interfere with measurements that are not made in a controlled environment, one of the drawbacks of using field-based rapid tests (Taylor and Kerr 1941). Human bias may also lead to inaccurate rapid test results, especially for tests that require interpretation of color (Fellers et al. 2015).

One rapid field method for analysis of ammonium and nitrate in effluent that has not yet been evaluated is spectrophotometry. A portable photometer can be used to quantify ammonium and nitrate concentrations colorimetrically, based on the principle that light absorbance at a particular wavelength is directly proportional to the concentration of the compound analyzed (Harris 1991). Using a portable photometer to measure the concentration of N species removes the human bias associated with tests that require color interpretation and reduces interference of environmental factors. User manuals containing step-by-step instructions, as well as troubleshooting options and data management suggestions, help make photometer usage possible for OWTS operation and maintenance staff. Portable photometers and kits containing the reagents necessary for making these measurements are available commercially (Fig. 2.2).

To determine the feasibility of using a portable photometer to measure inorganic N levels in wastewater, we analyzed effluent from 46 advanced N-removal OWTS in Charlestown, Rhode Island. Twenty-four of the systems serve homes that are occupied

year-round, while 22 systems serve seasonally-occupied homes. We analyzed effluent in field and laboratory settings using a portable photometer and analyzed the same samples using standard laboratory methods. We first compared measurements generated by standard methods with those obtained using the photometer in the field vs. in the laboratory setting to ascertain whether the test setting (outdoor vs. indoor) influences photometer results. To assess the accuracy of ammonium and nitrate measurements generated by the photometer, we then compared results obtained by the photometer to results obtained via standard methods. We also evaluated whether the sum of ammonium and nitrate measured with the photometer could predict TN measured using standard methods. Finally, we performed a cost-benefit analysis evaluating the cost-effectiveness of measuring ammonium and nitrate on the photometer vs. having samples analyzed for the same N species by commercial environmental testing laboratories.

Methods

Study Systems

We sampled final effluent from four different N-removal OWTS technologies: (i) Orenco Advantex® AX20, (ii) Orenco Advantex® RX30, (iii) BioMicrobics MicroFAST®, and Norweco Singulair® (models TNT, 960, and DN). We sampled from a total of 46 sites in Charlestown, Rhode Island. Twenty-four of the systems served homes occupied year-round, while 22 systems served seasonally-occupied homes. Detailed descriptions of the systems can be found in the Supplementary Materials.

Sample Collection

Final effluent samples (effluent to be dispersed to the drainfield) were collected in June, September, and December 2017 for systems serving homes occupied year-round,

and in June, July, August, and September for systems serving seasonally-occupied homes. The Advantex systems were sampled at the recirculating splitter valve assembly, while the FAST and Singulair technologies were sampled from the drainfield pump basin. A grab sample was collected into a clean 1-L plastic bottle. Part of the sample was used for field analysis, and the remainder of the sample was stored at 4° C until transported to the laboratory (within 8 hours of sampling). Upon arrival at the laboratory, 25 mL of sample was passed through a 0.45- μ m-pore-size membrane filter and the filtrate was frozen until analyzed for ammonium and nitrate using standard methods (described below).

Analyses

Standard Methods

The concentration of ammonium (range of 0-5 mg NH_4^+ -N/L; Weatherbern 1967) and nitrate (range of 0-2 mg NO_3^- -N/L; Doane and Horwath 2003) was determined colorimetrically using a BioTek Synergy HTX multi-mode microplate reader (Winooski, Vermont). Total N concentration was determined using the persulfate oxidation method (APHA 1998), and the resulting nitrate measured colorimetrically using the microplate reader. Effluent samples were diluted with deionized distilled water as necessary so that they would fall within the detection range.

Data collected using standard methods were required to meet certain performance criteria in order to ensure that results complied with established standards of accuracy and precision. The calibration curve for each plate was required to have an R^2 of at least 0.99. All samples were analyzed in triplicate and were reanalyzed if the coefficient of variation among the triplicates was greater than 20%. The dilution factor of samples that

measured out of range of the calibration curve was adjusted and samples were reanalyzed. Method blanks analyzed for ammonium could not exceed 200 $\mu\text{g NH}_4^+\text{-N/L}$, blanks analyzed for nitrate could not exceed 100 $\mu\text{g NO}_3^-\text{-N/L}$, and blanks analyzed for TN could not exceed 50 $\mu\text{g N/L}$. A laboratory control standard was also analyzed with the effluent samples and its ammonium, nitrate, and TN concentrations could not deviate more than 20% from the established values. One field duplicate per 10 sites was analyzed for ammonium, nitrate, and TN, and the relative percent difference between the field duplicate and original sample could not exceed 100%.

Photometer Method

Ammonium and nitrate concentrations were measured with a photometer in field and laboratory settings using a model 83214 Hanna Instruments Multiparameter Bench Photometer (Woonsocket, Rhode Island). For both the ammonium and nitrate analyses, a 5 mL syringe was used to add 1 mL of unfiltered effluent to the test vials. Once effluent had been added to a vial, it was inverted several times, inserted into the photometer, and read as a blank. The test-specific reagent was then added to the vial and the vial was inverted several times and inserted into the photometer. The photometer has a built-in timer programmed for each test, and determined the concentration after the required amount of time had passed. Ammonium was determined using the Nessler method, in which 4 drops of Nessler's reagent (dipotassium tetraiodomercurate (II) in dilute NaOH) were added to each test vial, which reacts with ammonium to produce a yellow-brownish color (Jeong et al. 2013; Hanna Instruments 2016). Concentrations of up to 100 mg $\text{NH}_4^+\text{-N/L}$ can be detected using this method. Nitrate concentration was measured using the chromotropic acid method, in which a powdered reagent (chromotropic acid disodium

salt and sodium metabisulfite) is added to a test vial and reacts with sulfuric acid and nitrate in the effluent to produce a yellowish color (Sims and Jackson 1971; Hanna Instruments 2016). A nitrate concentration of up to 30 mg NO_3^- -N/L can be measured using this method. Photometer measurements were made in the field during the months of June, July, and August 2017. Samples obtained in September and December were kept in the dark at 4° C and analyzed with the photometer in the laboratory within 24 hours of collection. One field duplicate per 10 sites was analyzed for ammonium and nitrate, and the relative percent difference between the field duplicate and original sample could not exceed 100%.

Statistical Analyses

Linear regression analysis was used to evaluate the accuracy of measurements made with the photometer. Because the upper limits for detection using the photometer were 100 mg NH_4^+ -N/L and 30 mg NO_3^- -N/L, samples with values above this threshold (as determined using standard methods) were excluded from the data set. We used the values obtained using the photometer method as the dependent variable and values generated by standard laboratory methods as the independent variable. First, we assessed the influence of test setting (field vs. laboratory) on photometer-based measurements by comparing regression lines for each setting. A two-tailed t test ($\alpha = 0.01$) was then used to identify which regression parameters (slope and intercept) differed between settings. To assess accuracy of measurements using the photometer, we compared ammonium and nitrate regression lines to a line representing the ideal relationship between concentrations measured with the photometer and with standard methods, with a y-intercept of 0, a slope of 1, and an R^2 value of 1. A two-tailed t test ($\alpha = 0.05$) was used

to identify regression parameters that deviated significantly from the ideal values for slope and intercept. For our analysis, a two-tailed t test with $\alpha = 0.05$ is a more stringent test than one with $\alpha = 0.01$, because utilizing an α of 0.05 generates a smaller confidence interval for each regression parameter, narrowing the window of comparison to the ideal regression parameters. We also investigated the validity of estimating TN concentrations from the sum of ammonium and nitrate concentrations measured by the photometer compared to the same sum generated by standard methods by performing a linear regression using the sum of ammonium and nitrate values as the independent variable, and the corresponding TN value obtained via standard methods as the dependent variable.

Cost-benefit Analysis

A cost-benefit analysis was performed to compare the cost of analyzing effluent samples for ammonium and nitrate using a photometer with the cost for the same analyses performed by two commercial environmental testing laboratories in Rhode Island (Lab A and Lab B). We considered the fixed and variable costs associated with both methods of analysis. For the photometer analysis, the fixed costs include the photometer and miscellaneous operational supplies (scissors and syringes). Because sending samples to a commercial laboratory does not require purchasing any instruments or supplies, this method has no fixed costs associated with it. Variable photometer costs consist of the cost of the reagents required to run ammonium and nitrate analyses, as well as the cost of reagent disposal, since hazardous waste disposal is required for the ammonium tests due to the use of Nessler's reagent, which contains mercury (N. Paterson, personal communication, April 24, 2018). These costs are variable because

they depend on how many samples require analysis. The variable costs for commercial analysis are determined by each laboratory, and are incorporated in the rate charged per sample analyzed for a particular analyte.

Results & Discussion

Analysis of accuracy in a field vs. laboratory setting

To assess the photometer's ability to accurately measure effluent ammonium and nitrate concentrations, we performed a linear regression that compared the concentrations obtained via standard methods (independent variable) to those obtained by the photometer method (dependent variable) and compared the regression to that of an ideal 1:1 relationship between the two variables. Our first consideration was whether the external environment influenced photometer accuracy. To assess this, we performed separate regressions for ammonium and nitrate based on photometer measurements determined in the field, and then performed the same regressions for ammonium and nitrate values generated by the photometer in the laboratory in order to investigate the potential benefits of performing the analysis in a climate-controlled environment. We then conducted a two-tailed t test ($\alpha = 0.01$) to determine whether the regression parameters differed between values obtained in field and laboratory settings for ammonium and nitrate.

When plotted against each other, the regression lines were very similar for ammonium concentrations measured using the photometer in field and in laboratory settings (Fig. 2.3). The nearly identical regression parameters observed suggest that the setting of this test (indoor vs. outdoor environments) does not significantly impact the

photometer measurements (Table 2.1). The slope of the regression of the values determined in the laboratory did not differ significantly from that of the values determined in the field. The regression intercept for the field-based photometer measurements (1.51 mg NH_4^+ -N/L) was slightly higher than that generated by lab-based measurements (0.67 mg NH_4^+ -N/L). The 99% confidence interval for field intercept (0.57 – 2.46) and laboratory intercept (0.02 – 1.31) do overlap, suggesting minimal differences in the data.

The regression parameters for our comparison of nitrate concentrations determined using the photometer in the field and laboratory were nearly identical (Fig. 2.3; Table 2.1). Neither the slope nor the intercept of the laboratory-obtained values differed significantly from that of the field-obtained values, indicating that the photometer can be used to measure nitrate in both the field and laboratory settings. Based on the minimal effects of environment setting on ammonium measurements and the lack of effects of setting on nitrate measurements, we grouped photometer measurements made in the field and laboratory settings for subsequent analyses.

Comparison of photometer method accuracy for ammonium vs. nitrate

When ammonium values obtained using the standard method were plotted against those obtained using the photometer method, the resulting regression line was nearly identical to the 1:1 line representing the ideal relationship between the two methods (Fig. 2.4). This highlights the photometer's capability for accurately measuring ammonium. The regression line resulting from a comparison of nitrate values obtained using standard methods to those obtained using the photometer method deviated slightly from the 1:1 line (Fig. 2.4).

Analysis of the nitrate model's regression residuals showed that the values are not normally distributed; rather, it skews positively. This suggests that the photometer tends to overestimate effluent nitrate concentration. The difference may be due to differences in how effluent samples are processed prior to analysis. Analysis by the standard method requires that samples be filtered prior to analysis, whereas samples are not filtered prior to analysis by the photometer method. The measurement of ammonium and nitrate concentrations using the photometer is based on Beer's Law ($A = \epsilon bc$), which states that absorbance is directly proportional to the concentration of the compound for which the sample is being analyzed (where A = absorbance, ϵ = molar absorptivity, b = path length, and c = concentration). Effluent contains organic and inorganic particles which block the light's path through the effluent, and is measured as the light being absorbed. This reduced transmittance can result in the photometer reporting a higher concentration than the sample actually has (Harris 1991).

We conducted a two-tailed t test ($\alpha = 0.05$) to determine which of the regression parameters generated by our comparison of photometer method and standard method measurements could be corresponded with ideal regression parameters (Table 2.2). Although the slope of the ammonium test's regression line was the only value deemed statistically similar to the regression parameters of a 1:1 line, the slope generated by the nitrate test regression was also close to the acceptable range of values. Intercepts for both the ammonium and nitrate regressions were greater than 0, indicating that the photometer test may overestimate effluent ammonium and nitrate concentration of the analytes, likely due to the presence of particles and their effect on photometer absorbance calculations. Mathematically calculating the offset between photometer and standard method

measurements and incorporating this calculated offset value into the photometer measurements may improve their accuracy.

Sum of ammonium and nitrate as a predictor of total N

We also examined whether the sum of ammonium and nitrate could predict effluent TN levels accurately. We compared the ability of the photometer and standard methods to predict effluent TN by summing the ammonium and nitrate values obtained by each method, plotting the sum against the TN content, and performing a regression analysis for each dataset (Fig. 2.5).

The sum of ammonium and nitrate generated by both the photometer and standard methods was strongly correlated with TN concentration (Table 2.3). Nevertheless, because organic N is also a component of the total amount of N in wastewater, quantifying only the inorganic forms of N cannot, in theory, predict TN levels accurately. Approximately 58% of sampling sites reported an average BOD₅ value greater than 0 mg/L, indicating that organic N is at least a minor contributor to TN levels for some sites. The regression line generated by the photometer analysis has a steeper slope than that of the standard methods regression line (Table 2.3). The difference may be due to the fact that sample filtration is required for standard method analysis of ammonium and nitrate, while filtration is not required prior to analyzing samples with the photometer. As stated earlier, both organic and inorganic N species are present in wastewater in particulate form. Organic N can take the form of both macroscopic and microscopic particles. Because the photometer measures light absorbance, particles blocking the light's path through the sample could be misconstrued as sample absorbance. Thus, the presence of organic N could cause the photometer to slightly overestimate TN concentrations for

effluent containing an appreciable amount of organic N in particulate form. The photometer method's tendency to overestimate effluent nitrate concentrations could also contribute to an overestimating TN measurements. The photometer method's overestimation of TN contrasts with our expectation that the method would underestimate TN, since it only quantifies inorganic N, effectively excluding the organic component of TN. These opposing factors, combined with the overall strength of the model ($p < 0.001$ and $R^2 = 0.68$), suggest that the photometer is capable of reliably approximating TN concentrations. In a practical application, the sum of inorganic N measured with the photometer can be used to identify systems that are clearly not meeting performance standards, as well as those that should be targeted for more detailed analysis – those with an inorganic N concentration that is approaching or exceeding regulatory threshold values.

Cost-benefit Analysis

After considering the fixed and variable costs associated with analyzing samples on the photometer and sending samples to be analyzed by commercial environmental testing laboratories, we set up a linear function for each method and calculated the break-even point: the number of samples for which using the photometer would begin saving money (Table 2.4). The break-even point for ammonium analysis ranged from 27 to 33 samples. Because costs for nitrate analysis in a commercial laboratory are even higher, the photometer proved to be the more cost-effective option after having 8 samples analyzed by Lab A, and 20 samples analyzed by Lab B. Because Lab A does not actually offer individual nitrate testing (rather, nitrate is measured as a component of their TN analysis), the cost per sample is significantly higher than that offered by Lab B. Because

we found that TN levels can be estimated from the sum of ammonium and nitrate determined using the photometer, we also calculated the break-even point for TN, which is 8 samples for Lab A and 11 samples for Lab B.

Conclusions

Our results show that that analysis using a portable photometer can be a viable method for measuring inorganic N levels in effluent, and users can operate the photometer in both indoor and outdoor settings without compromising accuracy. The photometer method can predict ammonium concentrations with great accuracy, and although it sometimes overestimates nitrate concentrations, it can also approximate nitrate concentrations. Reliable photometer ammonium and nitrate measurements can also provide valuable information about the systems' performance and capacity for facilitating N cycle processes. We also found that the sum of ammonium and nitrate measurements made using the photometer can be used as a proxy for TN concentration in effluent. This value can be used to help identify systems that are not performing optimally.

The photometer-based analysis of ammonium, nitrate, and TN is also a more financially-viable option than sending samples to be analyzed at a commercial laboratory. Not only does it become the most cost-effective option after a small number of samples, the photometer method provides users, service providers, and regulatory decision makers with immediate information about system performance, vs. having to wait for a commercial laboratory to return analysis results (typically a turnaround time of 2-3 weeks). Our results have the potential to benefit researchers, service providers, and

operation and maintenance staff in the advanced OWTS community. Utilizing the photometer will not only save them money, but it will also allow service providers to quickly monitor system performance, diagnose system problems, and facilitate real time adjustments to the system to help it meet treatment standard goals. This would provide regulatory agencies with added assurances that compliance is being met to help meet public and environmental health objectives.

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mention of trade names, commercial products, or causes constitute endorsement or recommendation for use.

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Figures and Tables

Figure 2.1. Schematic diagram of advanced N-removal onsite wastewater treatment systems, illustrating components, treatment type, and treatment processes. Components are shown separately for clarity, but can also exist within one multi-compartment tank (BOD refers to biochemical oxygen demand; TSS refers to total suspended solids)

Figure 2.2. Multiparameter bench photometer and supplies: photometer (A), instruction manual (B), scissors (C), Nessler's reagent (D), powdered reagent (E), syringe (F), ammonium test vial (G), and nitrate test vial (H)

Figure 2.3. Regression analyses of the accuracy of photometer ammonium and nitrate measurements made in the field vs. in the laboratory setting (illustrated by the solid lines). The dashed line represents the ideal 1:1 relationship between measurements obtained via the photometer and standard methods (with a slope of 1 and an intercept of 0)

Figure 2.4. Regression analyses of ammonium and nitrate concentration measurements made using photometer and standard methods (illustrated by the solid lines). The dashed line represents the ideal 1:1 relationship between measurements obtained via the photometer and standard methods (with a slope of 1 and an intercept of 0)

Figure 2.5. Regression analysis of the relationship between TN and the sum of ammonium and nitrate values generated by either the photometer or standard methods

Table 2.1. Parameters of regressions comparing photometer accuracy in measuring ammonium and nitrate in field and laboratory settings ($n = 54-64$). A two-tailed t test ($\alpha = 0.01$) was used to determine if test setting significantly influenced measurements reported. Values in bold differ significantly between settings. R^2 values were not included in this deviation analysis.

Property	Test setting	Regression parameters		
		Intercept	Slope	R^2
Ammonium	Field	1.51	0.99	0.93
	Laboratory	0.67	1.01	0.98
Nitrate	Field	3.49	0.83	0.55
	Laboratory	5.13	0.79	0.51

Table 2.2. Parameters of regressions comparing photometer accuracy in measuring ammonium ($n = 118$) and nitrate ($n = 115$) concentrations to standard methods. Samples measured by the photometer both in the field and laboratory settings were considered in this analysis. A two-tailed t test ($\alpha = 0.05$) was used to determine which values differed significantly from ideal regression parameters (0 for intercept, 1 for slope). Values in bold are significantly different from ideal parameters.

Property	Regression parameters		
	Intercept	Slope	R^2
Ammonium	1.12	1.00	0.95
Nitrate	4.32	0.81	0.52

Table 2.3. Parameters of regressions comparing photometer accuracy in measuring ammonium ($n = 118$) and nitrate ($n = 115$) concentrations to standard methods. Samples measured by the photometer both in the field and laboratory settings were considered in this analysis. A two-tailed t test ($\alpha = 0.05$) was used to determine which values differed significantly from ideal regression parameters (0 for intercept, 1 for slope). Values in bold are significantly different from ideal parameters.

Method of analysis	Regression parameter			
	Intercept	Slope	R^2	p-value
Photometer	0.62	1.01	0.68	<0.001
Standard	5.86	0.86	0.64	<0.001

Table 2.4. Comparison of the cost-effectiveness of analyzing effluent samples with a portable photometer vs. sending samples to commercial environmental testing labs to be analyzed.

Method of analysis	Parameter	Fixed cost	Variable cost per sample
Photometer	Ammonium	\$560	\$2.87
	Nitrate	\$560	\$1.14
	Total nitrogen	\$560	\$4.01
Lab A	Ammonium	-	\$24.00
	Nitrate	-	\$76.00
	Total nitrogen	-	\$76.00
Lab B	Ammonium	-	\$20.00
	Nitrate	-	\$30.00
	Total nitrogen	-	\$60.00

Appendix

Advanced OWTS technology descriptions

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 FAST® 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 tank and creates an oxic environment. Nitrified effluent moves upward through a corrugated plastic insert in the tank and splashes onto a trough, allowing the effluent to be transferred into an adjacent area where denitrification takes place. Finally, 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 anoxic environment, thus promoting both nitrification and denitrification. The third compartment of the

Norweco systems is a clarification chamber, in which promotes solids removal from the effluent.

**Manuscript 3: 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 N 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 systems. We examined differences 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* significantly. Season also had a strong influence on differences in species diversity among *amoA* communities, and has less influence on *nosZ* communities, whereas technology has a stronger influence. *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 patterns observed in diversity patterns, highlighting the possible importance of season and technology in shaping communities of *amoA* and *nosZ*, respectively. Our results suggest that season and technology are important factors in shaping the microbial communities of advanced OWTS. This knowledge 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.

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 (Amador et al., 2018). These technologies vary in their design, but they all have an oxic zone to facilitate nitrification and an anoxic/hypoxic zone for denitrification. Aerobic autotrophic bacteria are thought to be primarily responsible for nitrification, with facultative anaerobic heterotrophic bacteria carrying out denitrification (Oakley et al., 2010). The effluent is discharged to a soil treatment area (STA), which provides a final opportunity for treatment before effluent 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 (RIDEM, 2016). A similar standard is also used in other areas, including Barnstable County, MA, and Suffolk County, NY (BCDHE, 2012; Tomarken & Dawydiak, 2017).

The capacity of these systems to meet effluent total N standards differs among advanced N-removal technologies. Oakley et al. (2010) 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. (2017) found 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 (C) and N removal (Brannon et al., 2017; Ross et al., 2020a). 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 WTPs with 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 effectiveness of system design in promoting nitrification and denitrification (Geets et al., 2007; Siripong & Rittmann, 2007; Jaranowska et al., 2013; Brannon et al., 2017). In contrast, few studies have investigated the microbial communities of advanced N-removal OWTS. In studies conducted within the Greater Narragansett Bay watershed, Brannon et al. (2017) assessed the size of nitrifying and denitrifying microbial communities in BNR WTPs and advanced N-removal OWTS, and Wigginton et al. (2018) 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. (2018) observed no differences in richness and Shannon's diversity among technologies or between component types for nitrifying or denitrifying communities. However, they did find significant geographical and temporal differences in the structure and composition of *nosZ* communities.

To better understand the factors that affect the structure and composition of nitrifying and denitrifying communities, we investigated the microbial communities of 44 advanced N-removal OWTS in the town of Charlestown, Rhode Island, USA. We used primers targeting the genes *amoA* and *nosZ* and high-throughput sequencing to characterize alpha 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 nitrification and denitrification exhibited by OWTS microbial communities. We sampled final effluent from four different advanced N-removal technologies: Orenco® Advantex AX20, Orenco® Advantex RX30, BioMicrobics MicroFAST®, and Norweco Singlair® models DN, TNT, and 960.

We also investigated occupancy pattern as a potential source of variation in microbial communities. Like many coastal towns in New England, Charlestown 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, 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. (2018) found to partially drive community differences. The sites studied by Wigginton et al. (2018) were located in 6 towns within the Greater Narragansett Bay watershed, covering an area of 855 km², nearly six times larger than Charlestown, which has an area of 154 km².

Methods

Study Systems

We sampled final effluent (prior to dispersion to the 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 Singlair® (models TNT, 960, and DN). Detailed descriptions of each technology can be found in the Appendix. 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. (2020b). 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 in the Appendix has a summary of effluent properties for June and September 2017.

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 hours of sampling). Within 24 hours of sampling, ~100 mL of sample was vacuum-filtered to pass a sterile 0.45-μm-pore-size nitrocellulose membrane filter (MilliporeSigma, Burlington, MA), and the filter stored in individual sterile Whirl-Pak bags (Uline, Pleasant Prairie, WI) at -80° C until analysis.

Sample Processing

Genomic DNA was extracted from each membrane filter using the PowerWater DNA Isolation Kit (MoBio Laboratories, Waltham, MA). Gene fragments for *nosZ* and *amoA* were amplified using a single, 50-μL reaction using the primer pairs *nosZ* 1F and *nosZ* 1662R, and *amoA* 1F and *amoA* 862R (Geets et al., 2007; Wigginton et al., 2018). 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). Details of DNA extraction, PCR methods, and sample metadata can be found in the Appendix.

Downstream Analysis and Statistics

We used QIIME 2 (version 2019.10) for downstream sequencing analysis, as outlined in Cox et al. (2020). 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. (2020). 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 (R Core Team, 2012) 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* (R Core Team, 2012). 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* (data could not be normalized), and a paired *t*-test to assess these differences for *nosZ* (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. Significant clustering as a function of technology, occupancy pattern, and/or season was identified using nonparametric permutational multivariate ANOVAs (PERMANOVAs) Bray-Curtis distance matrices using the *Adonis* function (with 999 permutations for each correlation) (R Core Team, 2012; Wigginton et al., 2018; Cox et al., 2020).

Results & Discussion

Species Richness and Diversity

amoA. Species richness (number of ASVs) for *amoA* across technologies, occupancy patterns, and seasons ($n = 55$) ranged from 3 to 33 ASVs, with a median of 25 (Fig. 3.1). Richness was significantly different between seasons, with higher species richness in September than in June (Fig. 3.1). 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 (Fig. 3.1). There were no significant differences in Shannon's diversity index as a function of technology or occupancy pattern.

When we assessed differences in *amoA* beta diversity, or differences in diversity across communities, samples clustered significantly only by season (33% variation explained) (Fig. 3.2), indicating that nitrifying microbial communities in June are more similar to one another than to those from September. This seasonal shift was apparent in OWTS nitrifying communities regardless of occupancy pattern or technology.

The relationship between season and *amoA* in wastewater treatment appears to differ at different system scales. For example, Wigginton et al. (2018) 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 (2006) 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 (*Nitrospira 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 don't 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 (Wigginton et al., 2018).

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. fixed activated sludge). Furthermore, despite the period of inactivity 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. (2020b) 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.

nosZ. Species richness across technologies, occupancy patterns, and seasons was generally higher for *nosZ* than for *amoA*, ranging from 4 to 85 ASVs, with a median of 35 ($n = 65$) (Fig. 3.3). 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 (Fig. 3.3). 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) (Fig. 3.4), 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. (2018) 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 prevented identification of differences among technologies.

Jaranowska et al. (2013) 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 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 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.

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 vertical hanging sheets by gravity, preventing saturation. Although textile filters are designed to establish oxic conditions and promote nitrification, Wigginton et al. (2018) 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. Upon visual inspection, we have found 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 in increased/potentially novel organic 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 has been observed in many natural environments, as communities increase in diversity until they reach a point of stability (Fierer et al., 2010). Increased time of OWTS operation may provide a greater opportunity for new and/or different niches where unique *nosZ* communities can 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. (2018). 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.

Taxonomy

amoA. We observed a total of 241 unique ASVs for *amoA* across all samples ($n = 55$) (Fig. 3.5), 88 (37%) of which could be matched with a known genus. Of these, the most common genera (based on number of ASVs) were *Nitrosospira* (78) and *Nitrosomonas* (8). The dominance of unidentified bacteria containing *amoA* suggest 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 species capable of nitrification.

We identified the 20 most abundant *amoA* ASVs, based on abundance across all technologies, occupancy patterns, and seasons (Table 3.1). Although many of the ASVs were present in effluent samples from all technologies, occupancy patterns, and seasons, the most obvious differences were apparent 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.

Although Wigginton et al. (2018) observed a higher number of ASVs (711) in samples from all 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 (Mertoglu, 2008; Dionisi et al., 2011; Gao et al., 2014; Wigginton et al., 2020). A study by Wigginton et al. (2020) assessed *amoA* communities in a BNR WTP and nine advanced N-removal OWTS in the Greater Narragansett Bay Watershed and 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 (Aigle et al., 2019; Wigginton et al., 2020). The overlap in prevalent nitrifiers observed in OWTS between systems in the Wigginton et al. (2018 & 2020) study 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 suggest systems designed for N-removal support taxonomically consistent nitrifying communities, regardless of system design, scale, or geographical location.

nosZ. We observed a total of 1,307 unique ASVs for *nosZ* across all samples ($n = 65$) (Fig. 3.6), 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 number of ASVs) were *Zoogloea* (57), *Thauera* (35), and *Acidovorax* (34).

We identified the 20 most abundant *nosZ* ASVs, based on abundance across all technologies, occupancy patterns, and seasons (Table 3.2). Several of the top ASVs were found in at least one sample from all technologies, occupancy patterns, and seasons. All of 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 in 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 (Thomsen et al., 2007; Wang et al., 2018), was the only genus with a high relative abundance in both our study and in Wigginton et al. (2018). Three other genera (*Aeromonas*, *Azospirillum*, and *Sinorhizobium*) observed in Wigginton et al. (2018) were also present in at least one of our samples.

Two of the most common genera present in our samples, *Zoogloea* and *Acidovorax*, have not been identified previously in N-removal OWTS (Brannon et al., 2017; Wigginton et al., 2018 and 2020). *Zoogloea spp.* assist with the formation of flocculated materials in activated sludge in WTPs (Wagner et al., 1995), 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 (Kraigher et al., 2008; Schulzel et al., 2020). Although *Acidovorax spp.* and *Zoogloea spp.* are present in WTPs, their capacity for denitrification was not identified until recently (Bellini et al. (2012). These two species may play an important role in denitrification in advanced N-removal OWTS.

Conclusions

The influence of technology and season differed between the nitrifying and denitrifying communities of advanced N-removal OWTS. Species richness and diversity were largely driven by season for *amoA* communities, with higher richness and diversity observed in September samples than in June samples. Species richness and diversity did not vary among technologies for *amoA*. Conversely, species richness and diversity varied significantly among technologies, which suggests that denitrifiers are influenced by differences in treatment train design. Differences in beta diversity of denitrifying communities were also weakly driven by season. The shifts observed between June and September may be due to changes in household demographics. Occupancy pattern did not influence the structure or composition of *amoA* or *nosZ* communities, suggesting that N-cycling microbial communities in OWTS remain stable despite varying temporal patterns of wastewater inputs.

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*. The key nitrifying genera in our study – *Nitrosospira* and *Nitrosomonas* – were similar to those reported by others for advanced N-removal OWTS (Wigginton et al., 2018; Wigginton et al., 2020) and WTPs with BNR (Mertoglu, 2008; Dionisi et al., 2011; Gao et al., 2014).

One of the most common *nosZ* genera identified in our study, *Thauera*, has been observed in advanced N-removal OWTS (Thomsen et al., 2007; Wang et al., 2018; Wigginton et al., 2018), while all three of the top genera identified (*Thauera*, *Zoogloea*, and *Acidovorax*) have been identified in WTPs (Wagner et al., 1995; Kraigher et al., 2008; Schulzel et al., 2020). 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.

Our study expands our understanding of the structure and composition of nitrifying and denitrifying microbial communities in advanced N-removal OWTS. Our results suggest that technology and season vary in how they affect these communities. The relationship between the structure and composition of nitrifying and denitrifying communities with 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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Figures and Tables

Figure 3.1. Median values of Shannon's diversity index and species richness for *amoA* genes in final effluent from advanced N-removal OWTS in June ($n = 13$) and September ($n = 42$) across all technologies and occupancy patterns. Box whiskers extend $1.5 \times$ the inter-quartile range beyond the edges of the box and the dots represent outliers beyond $1.5 \times$ the inter-quartile range.

Figure 3.2. Principal coordinate analysis based on Bray-Curtis dissimilarity distances for *amoA* communities across all advanced N-removal OWTS technologies and occupancy patterns ($n = 55$).

Figure 3.3. Shannon's diversity index and species richness for *nosZ* genes for each technology across all occupancy patterns and seasons. Description of box and whisker plots can be found in Fig. 3.1. AX20 = Orenco Advantex® AX20 ($n = 43$); FAST = BioMicrobics MicroFAST® ($n = 4$); NOR = Norweco Singulair® ($n = 7$); RX30 = Orenco Advantex® RX30 ($n = 11$).

Figure 3.4. Principal coordinate analysis based on Bray-Curtis dissimilarity distances for *nosZ* communities in effluent from advanced N-removal OWTS technologies across occupancy patterns ($n = 65$).

Figure 3.5. 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).

Figure 3.6. 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).

Table 3.1. Top 20 most abundant *amoA* ASVs identified across all advanced N-removal OWTs technologies, occupancy patterns, and sampling events. U.A. means “unassigned”. An “x” indicates the presence of the ASV in at least one effluent sample from the technology, occupancy pattern, or season listed. AX20 = Orenco Advantex® AX20 ($n = 37$); RX30 = Orenco Advantex® RX30 ($n = 8$); FAST = BioMicrobics MicroFAST® ($n = 5$); NOR = Norweco Singlair® ($n = 5$)

Genus	Technology				Occupancy Pattern		Season	
	AX20	RX30	FAST	NOR	Year-round	Seasonal	June	September
U. A. <i>Nitrosomonadaceae</i>	x	x	x	x	x	x	x	x
U. A. Bacteria	x	x	x	x	x	x	x	x
<i>Nitrospira</i>	x	x	x	x	x	x		x
U. A. Bacteria	x	x	x	x	x	x	x	x
<i>Nitrospira</i>	x	x	x	x	x	x	x	x
<i>Nitrospira</i>	x	x	x	x	x	x	x	x
<i>Nitrospira</i>	x	x	x	x	x	x		x
<i>Nitrospira</i>	x	x	x	x	x	x		x
U. A. <i>Nitrosomonadales</i>	x			x	x	x	x	x
<i>Nitrospira</i>	x	x	x	x	x	x	x	x
U. A. Bacteria	x	x	x	x	x	x	x	x
<i>Nitrospira</i>	x	x	x	x	x	x	x	x
<i>Nitrospira</i>	x	x	x	x	x	x	x	x
<i>Nitrospira</i>	x		x		x	x	x	x
<i>Nitrospira</i>	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

Table 3.2. Top 20 most abundant *nosZ* ASVs identified across all across all advanced N-removal OWTS technologies, occupancy patterns, and sampling events. U.A. means “unassigned”. An “x” indicates the presence of the ASV in at least one effluent sample from the technology, occupancy pattern, or season listed. AX20 = Orenco Advantex® AX20 ($n = 42$); RX30 = Orenco Advantex® RX30 ($n = 13$); FAST = BioMicrobics MicroFAST® ($n = 4$); NOR = Norweco Singulair® ($n = 7$)

Genus	Technology				Occupany 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
<i>Thauera</i>	x	x			x	x	x	x
<i>Zoogloea</i>	x		x	x	x	x	x	x
<i>Thauera</i>	x	x	x	x	x	x	x	x
<i>Thauera</i>	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
<i>Zoogloea</i>	x	x	x	x	x	x	x	x
U. A. Bacteria	x	x		x	x	x	x	x
<i>Zoogloea</i>	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
<i>Acidovorax</i>	x	x	x	x	x	x	x	x

Appendix

Advanced OWTS technology descriptions

Technology descriptions for the Orenco Advantex® AX20 and RX30, BioMicrobics FAST®, and Norweco Singular® systems were taken from Ross et al. (2018). 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 tank and creates an oxic environment. Nitrified effluent moves upward through a corrugated plastic insert in the tank and splashes onto a trough, allowing the effluent to be transferred into an adjacent area where denitrification takes place. Finally, 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 anoxic environment, thus promoting both nitrification and denitrification. The third compartment of the Norweco systems is a clarification chamber, in which promotes solids removal from the effluent.

DNA Extraction and PCR

We followed the protocol in Wigginton et al. (2018) for DNA extraction and PCR analysis methods for *nosZ*, and Wigginton et al. (2020) for *amoA* methods. After filtering effluent samples, we extracted DNA from filters using PowerWater DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA) 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 BIO-X-ACT™ Short Mix (Bioline, Taunton, MA, USA), 21.75 µL 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 MyFi™ Mix (Thomas Scientific, Swedesboro, NJ), 21.25 µL 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 sec. at 94°C, 60 sec. annealing at 58°C, and 60 sec. 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.

Sample Metadata

amoA

Sample	Site	Technology	Occupancy pattern	Sampling Month
SKW338	CS20A	AX20	Seasonal	June
SKW339	CY19A	AX20	Year-round	June
SKW340	CY11A	AX20	Year-round	June
SKW341	CS39F	FAST	Seasonal	June
SKW342	CS40A	AX20	Seasonal	June
SKW343	CS49F	FAST	Seasonal	June
SKW344	CY21A	AX20	Year-round	June
SKW345	CY07Nn	NOR	Year-round	June
SKW346	CY18A	AX20	Year-round	June
SKW347	CY08R	RX30	Year-round	June
SKW348	CS38A	AX20	Seasonal	June
SKW349	CS26A	AX20	Seasonal	June
SKW350	CS48A	AX20	Seasonal	June
SKW351	CY01R	RX30	Year-round	September
SKW352	CY02A	AX20	Year-round	September
SKW353	CY03R	RX30	Year-round	September
SKW354	CY04A	AX20	Year-round	September
SKW355	CY05A	AX20	Year-round	September
SKW356	CS06A	AX20	Seasonal	September
SKW357	CY07Nn	NOR	Year-round	September
SKW358	CY08A	AX20	Year-round	September
SKW359	CY09A	AX20	Year-round	September
SKW360	CY10A	AX20	Year-round	September
SKW361	CY11A	AX20	Year-round	September
SKW362	CY14R	RX30	Year-round	September
SKW363	CY15A	AX20	Year-round	September
SKW364	CS16A	AX20	Seasonal	September
SKW365	CS17F	FAST	Seasonal	September
SKW366	CY18A	AX20	Year-round	September
SKW367	CY19A	AX20	Year-round	September
SKW368	CS20A	AX20	Seasonal	September
SKW369	CY21A	AX20	Year-round	September
SKW370	CS22A	AX20	Seasonal	September
SKW371	CY23A	AX20	Year-round	September
SKW372	CY24R	RX30	Year-round	September
SKW373	CS26A	AX20	Seasonal	September
SKW374	CS27Nt	NOR	Seasonal	September
SKW375	CS28A	AX20	Seasonal	September
SKW376	CS29Nd	NOR	Seasonal	September

SKW377	CS30A	AX20	Seasonal	September
SKW378	CS31A	AX20	Seasonal	September
SKW379	CS32A	AX20	Seasonal	September
SKW380	CS34A	AX20	Seasonal	September
SKW381	CS36R	RX30	Seasonal	September
SKW382	CS37Nd	NOR	Seasonal	September
SKW383	CS38A	AX20	Seasonal	September
SKW384	CS39F	FAST	Seasonal	September
SKW385	CS40A	AX20	Seasonal	September
SKW386	CS41A	AX20	Seasonal	September
SKW387	CS42R	RX30	Seasonal	September
SKW388	CS43R	RX30	Seasonal	September
SKW389	CS45A	AX20	Seasonal	September
SKW390	CS48A	AX20	Seasonal	September
SKW391	CS49F	FAST	Seasonal	September
SKW392	CS50A	AX20	Seasonal	September

nosZ

Sample	Site	Technology	Occupancy Pattern	Sampling Month
BNR001	CS27Nt	NOR	Seasonal	June
BNR002	CS28A	AX20	Seasonal	June
BNR003	CS29Nd	NOR	Seasonal	June
BNR004	CS30A	AX20	Seasonal	June
BNR005	CS31A	AX20	Seasonal	June
BNR006	CS32A	AX20	Seasonal	June
BNR007	CS34A	AX20	Seasonal	June
BNR008	CS37Nd	NOR	Seasonal	June
BNR009	CS22A	AX20	Seasonal	June
BNR010	CS45A	AX20	Seasonal	June
BNR011	CS49F	FAST	Seasonal	June
BNR012	CS50A	AX20	Seasonal	June
BNR013	CY01R	RX30	Year-round	June
BNR014	CY02A	AX20	Year-round	June
BNR015	CY03R	RX30	Year-round	June
BNR016	CY04A	AX20	Year-round	June
BNR017	CY05A	AX20	Year-round	June
BNR018	CY07Nn	NOR	Year-round	June
BNR019	CY08R	RX30	Year-round	June
BNR020	CY09A	AX20	Year-round	June
BNR021	CY10A	AX20	Year-round	June
BNR022	CY13A	AX20	Year-round	June
BNR023	CY14R	RX30	Year-round	June

BNR024	CY15A	AX20	Year-round	June
BNR025	CS16A	AX20	Seasonal	June
BNR026	CY18A	AX20	Year-round	June
BNR027	CY19A	AX20	Year-round	June
BNR028	CS20A	AX20	Seasonal	June
BNR029	CY21A	AX20	Year-round	June
BNR030	CS38A	AX20	Seasonal	June
BNR031	CY23A	AX20	Year-round	June
BNR032	CY24R	RX30	Year-round	June
BNR033	CS26A	AX20	Seasonal	September
BNR034	CS27Nt	NOR	Seasonal	September
BNR035	CS28A	AX20	Seasonal	September
BNR036	CS31A	AX20	Seasonal	September
BNR037	CS32A	AX20	Seasonal	September
BNR038	CS34A	AX20	Seasonal	September
BNR039	CS36R	RX30	Seasonal	September
BNR040	CS37Nd	NOR	Seasonal	September
BNR041	CS38A	AX20	Seasonal	September
BNR042	CS39F	FAST	Seasonal	September
BNR043	CS40A	AX20	Seasonal	September
BNR044	CS41A	AX20	Seasonal	September
BNR045	CS42R	RX30	Seasonal	September
BNR046	CS43R	RX30	Seasonal	September
BNR047	CS45A	AX20	Seasonal	September
BNR048	CS48A	AX20	Seasonal	September
BNR049	CS49F	FAST	Seasonal	September
BNR050	CS50A	AX20	Seasonal	September
BNR051	CY01R	RX30	Year-round	September
BNR052	CY02A	AX20	Year-round	September
BNR053	CY03R	RX30	Year-round	September
BNR054	CY04A	AX20	Year-round	September
BNR055	CY05A	AX20	Year-round	September
BNR056	CS06A	AX20	Seasonal	September
BNR057	CY07Nn	NOR	Year-round	September
BNR058	CY10A	AX20	Year-round	September
BNR059	CY12A	AX20	Year-round	September
BNR060	CY15A	AX20	Year-round	September
BNR061	CS17F	FAST	Seasonal	September
BNR062	CY18A	AX20	Year-round	September
BNR063	CY19A	AX20	Year-round	September
BNR064	CY21A	AX20	Year-round	September
BNR066	CY24R	RX30	Year-round	September

Table A1 Summary statistics of wastewater parameters measured in each season, across all technologies (adapted from Ross et al. (B), 2020). Units are mg/L except for pH and temperature (°C).

Parameter	June				September			
	<i>n</i>	Min	Med	Max	<i>n</i>	Min	Med	Max
pH	45	3.75	7.04	8.14	45	4.74	6.87	7.83
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
DO	45	1.1	6.83	9.93	44	0	5.84	8.73
Ammonium	42	0	0	41.6	38	0	0.10	51.6
Nitrate	43	0	8.40	32.8	40	0.10	9.40	25.3
TN	43	3.60	18.7	60.9	41	0.60	11.9	52.0

**Manuscript 4: Greenhouse Gas Emissions from Advanced Nitrogen-Removal Onsite
Wastewater Treatment Systems**

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Abstract

Advanced onsite wastewater treatment systems (OWTS) designed to remove nitrogen from residential wastewater play an important role in protecting environmental and public health. Nevertheless, the microbial processes involved in treatment produce greenhouse gases (GHGs) that contribute to global climate change, including CO₂, CH₄, N₂O. We measured GHG emissions from 27 advanced N-removal OWTS in the towns of Jamestown and Charlestown, Rhode Island, USA, and assessed differences in flux based on OWTS technology, home occupancy (year-round *vs.* seasonal), and zone within the system (oxic *vs.* anoxic/hypoxic). We also investigated the relationship between flux and wastewater properties. Flux values for CO₂, CH₄, and N₂O ranged from -0.44 to 61.8, -0.0029 to 25.3, and -0.02 to 0.23 $\mu\text{mol GHG m}^{-2} \text{ s}^{-1}$, respectively. CO₂ and N₂O flux varied among technologies, whereas occupancy pattern did not significantly impact any GHG fluxes. CO₂ and CH₄ – but not N₂O – flux was significantly higher in the anoxic/hypoxic zone than in the oxic zone. Greenhouse gas fluxes in the oxic zone were not related to any wastewater properties. CO₂ and CH₄ flux from the anoxic/hypoxic zone peaked at ~22-23°C, and was negatively correlated with dissolved oxygen levels, the latter suggesting that CO₂ and CH₄ flux result primarily from anaerobic respiration. Ammonium concentration and CH₄ flux were positively correlated, likely due to inhibition of CH₄ oxidation by NH₄⁺. N₂O flux in the anoxic/hypoxic zone was not correlated to any wastewater property. We estimate that advanced N-removal OWTS contribute 347 g CO₂ equivalents capita⁻¹ day⁻¹, comparable to emissions from conventional OWTS. Our results suggest that technology influences CO₂ and N₂O flux and zone influences CO₂ and CH₄ flux, while occupancy pattern does not appear to

impact GHG flux. Manipulating wastewater properties, such as temperature and dissolved oxygen, may help mitigate GHG emissions from these systems.

Introduction

Wastewater can be a major source of nitrogen to groundwater and coastal waters. Decentralized, advanced N-removal onsite wastewater treatment systems (OWTS) are used to mitigate the impact of excess N from wastewater on receiving waters, which includes eutrophication, fish and shellfish kills, and threats to public health (Carpenter et al., 1998; Sohail & Adeloju, 2016; Ward et al., 2005). Advanced OWTS remove N by cycling wastewater through a treatment train that includes oxic and anoxic/hypoxic zones that promote successive nitrification (microbial oxidation of NH_4^+ to NO_3^-) and denitrification (microbial reduction of NO_3^- to N_2O and N_2). When denitrification proceeds to completion, NO_3^- is reduced to harmless N_2 ; however, incomplete denitrification produces N_2O , a potent greenhouse gas (GHG) with 265 times the global warming potential of CO_2 (Jones et al., 2013; Tomaszek and Czarnota, 2015; Wrage et al., 2001). Advanced N-removal OWTS also emit CO_2 , which is produced by microbial respiration – including processes that remove organic matter – and methane (CH_4), which is produced by Archaea under anoxic conditions (Kong et al., 2016).

Greenhouse gas emissions from centralized wastewater treatment plants (WTPs) have been quantified (Cakir & Stenstrom, 2005; Foley et al., 2010; Kong et al., 2016; Parravicini et al., 2016). Nationwide, the U.S. Environmental Protection Agency (USEPA) estimates that centralized WTPs treating domestic wastewater in the U.S. contribute 9.2 and 4.9 million metric tons of CO_2 equivalents of CH_4 and N_2O , respectively, to the atmosphere every year (USEPA, 2015). In 2010, domestic

wastewater treatment at WTPs accounted for 1.4% of U.S. N₂O emissions, and domestic and industrial wastewater treatment combined accounted for 2.4% of CH₄ emissions (USEPA, 2015). Assessment of GHG emissions from WTPs typically excludes CO₂ due to its biogenic origins (Doorn et al., 2006). However, recent studies have shown that non-biogenic CO₂ emissions from various C-containing household and personal care products can significantly contribute to GHG emissions from WTPs, and should be included in emissions analysis (Griffith, et al., 2009; Law et al., 2013; Tseng et al., 2016).

Unlike WTPs, few studies have quantified GHG emissions from OWTS, which serve nearly one quarter of households in the U.S. (U.S. Census Bureau, 2017). Diaz-Valbuena et al. (2011) investigated CO₂, CH₄, and N₂O emissions from conventional OWTS – consisting of a septic tank where gravity-separation of wastewater solids takes place, and the clarified effluent is treated below ground using soil-based treatment – and found net emission of all three GHGs at all study sites, with average values (g capita⁻¹ day⁻¹) of 33.3 for CO₂, 11 for CH₄, and 0.005 for N₂O. Truhlar et al. (2016) assessed CO₂, CH₄, and N₂O emissions from various components of a conventional OWTS treatment train, including the roof vent (which releases gases generated in the septic tank to the atmosphere) and the soil treatment area (STA). They also found net emission of GHGs from all components, with 0.17 and 0.050 tonnes CO₂ equivalents capita⁻¹ year⁻¹ from the roof vent and STA, respectively. Somlai-Haase et al. (2017) found that the STA for a conventional OWTS emitted 15.0 kg more CO₂ per year than a similarly sized control area that received no wastewater inputs, further highlighting the contribution of OWTS to global GHG emissions.

Emissions of N₂O from advanced N-removal OWTS were first quantified by Brannon et al. (2017). They compared emissions of N₂O from nine advanced N-removal OWTS – representing three different technologies – to emissions from a WTP with a biological N removal (BNR) component that also relies on successive nitrification and denitrification. They found that the flux of N₂O ranged from -4×10^{-3} to 3×10^{-1} $\mu\text{mol N}_2\text{O m}^{-2} \text{s}^{-1}$ and was typically higher for the WTP, followed by the three advanced N-removal treatment technologies. However, when emissions were normalized by treatment tank area and number of people served, the ranges of emissions for the WTP and the advanced N-removal technologies overlapped. Carbon dioxide equivalents – calculated based on N₂O emissions from the N-removal OWTS – ranged from 1.6×10^{-3} to 8×10^{-2} tonnes CO₂ equivalents capita⁻¹ yr⁻¹.

As the world's population increases, the number of OWTS will also increase (Amador & Loomis, 2018). The relatively low cost of installation and limited maintenance required make them viable wastewater treatment options, especially in areas with low population densities. Advanced N-removal OWTS will be particularly critical in addressing eutrophication of coastal ecosystems and groundwater contamination. Because wastewater treatment produces GHGs, we need to consider whether their use shifts the pollution stream from ground and surface waters to the atmosphere. As such, we need to develop a better understanding of the magnitude and composition of GHG emissions from OWTS – including advanced N-removal systems – and the factors that control them.

We quantified the flux of CO₂, CH₄, and N₂O from five different advanced N-removal OWTS technologies in the towns of Charlestown and Jamestown, Rhode Island,

USA: SeptiTech® Series D, Orenco Advantex® AX20, Orenco Advantex® RX30, BioMicrobics MicroFAST®, and Norweco Singulair®. We measured GHG flux in the summer and fall of 2016, and summer and winter of 2018. Because differences in home occupancy pattern may drive differences in microbial activity and the GHGs produced by this activity, we assessed the relationship between home occupancy patterns and GHG emissions by sampling systems used year-round and systems only used during the summer season. GHG emissions result from microbial processes that respond to environmental conditions and availability of nutrients, electron acceptors, and organic C, and thus we assessed the relationship between gas emissions and effluent temperature, pH, and dissolved oxygen (DO), as well as effluent 5-day biochemical oxygen demand (BOD₅; a proxy for organic C), ammonium, nitrate, and total N concentration. We also quantified emissions per capita in terms of CO₂ equivalents to allow for comparison with other types of wastewater treatment.

Methods

We measured CO₂, CH₄, and N₂O emissions, and determined wastewater properties from a total of 27 advanced N-removal OWTS serving single-family homes in the towns of Jamestown and Charlestown, Rhode Island, USA. Sites were chosen in collaboration with the Rhode Island Department of Environmental Management (RIDEM) and the Office of Wastewater Management in each town. All the sites included in this study rely on well water for potable water and discharge effluent to a low-pressure dosed soil treatment area using a pump. Sites were selected based on adherence to these criteria, as well as the homeowners' willingness to participate in the study. Data from

Jamestown sites were collected in June and October of 2016, and data from Charlestown sites were collected in July/August and December of 2018. We sampled 9 systems in Jamestown (3 SeptiTech® Series D, 3 Orenco Advantex® AX20, and 3 BioMicrobics MicroFAST®), and 18 sites in Charlestown (6 Orenco Advantex® AX20, 6 Orenco Advantex® RX30, 3 BioMicrobics MicroFAST®, and 3 Norweco Singulair® (models 960 and DN)). All 9 systems sampled in 2016 served homes occupied year-round. Of the systems sampled in 2018, 11 served homes occupied year-round and 7 served seasonally-occupied homes (June to September). Sampling from systems occupied seasonally and year-round allowed us to examine differences due to home occupancy. We assessed occupancy pattern based on daily flow data obtained from each system, as well as homeowner self-identification (Ross et al., 2020a).

Although specific design configurations vary by technology, all of the systems in this study have an oxic zone for nitrification (referred to as SP-2) and an anoxic/hypoxic zone for denitrification (referred to as SP-1) (see Appendix for individual technology designs/descriptions). On every sampling event we measured the flux of GHG and obtained a sample of wastewater from the oxic (SP-2) and anoxic/hypoxic (SP-1) zones of the treatment train. To avoid impacting GHG emissions from disturbance of the wastewater, GHG flux measurements were made prior to wastewater sampling.

Greenhouse Gas Flux Measurements

At each study site CO₂, CH₄, and N₂O flux measurements were made using a closed chamber connected to a Gas Concentration Analyzer (Model G2518; Picarro, Inc., Santa Clara, CA), which uses cavity ring-down spectroscopy to measure GHG

concentration every 2 seconds, providing real-time flux measurements in the field (Crosson, 2008). To measure gas flux, we placed an open-bottom cylindrical PVC chamber (0.13-m internal dia., 0.40-m length) on the water so that the bottom of the chamber was submerged 7.5 cm below the water surface (Brannon et al., 2017). Air temperature inside the chamber was measured every 10 seconds using a Hobo® data logger (Onset, Bourne, MA). To maintain a consistent submersion depth, the chamber was equipped with a stabilizing bar that rested across the top of the system's access riser. The chamber was deployed for 5 to 10 minutes at each site, providing ample time for gas emissions to travel between the system and the gas analyzer (Brannon et al., 2017). The concentration of each gas was plotted against time and the data fitted using a linear regression as part of the flux calculation. We used R statistical software to determine the statistical significance of each gas flux value (R Core Team, 2017), as described in Brannon et al. (2017). GHG flux values below the detection limits of the instrument, and those with a p value > 0.05 for the slope of the linear regression, were assigned a value of zero. Fluxes above the upper detection limits of the instrument were excluded from our analysis. We calculated per capita CO₂ equivalents (CO₂e) as described in Brannon et al. (2017) using global warming potential values published by the IPCC (2014).

Wastewater Analysis

Effluent collected from each zone was transferred to a clear, 1-L plastic bottle. Part of the sample was used for *in situ* determination of temperature, pH and dissolved oxygen concentration (DO), and the remainder was stored in the dark at 4°C and transported to the laboratory for analysis (within 8 h of sampling). A Hanna Instruments

HI9828 Multiparameter Meter (Woonsocket, RI) was used to determine effluent temperature, pH, and DO. Upon arrival at the laboratory, effluent was analyzed for BOD₅ with an OxiTop BOD₅ measurement system (WTW, Weilheim, Germany). Ammonium, nitrate, and total N were analyzed using standard laboratory colorimetric analysis (Ross et al., 2018). Values below the detection limit of a method were assigned a value of zero. A summary of wastewater properties for each zone can be found in the Appendix (Table S1).

Statistical Analyses

We used R (R Core Team, 2017) to perform a one-way ANOVA on ranks to examine statistical differences in GHG fluxes among technologies, and a Mann-Whitney U test to assess flux differences between zones (SP-1 vs. SP-2) and between home occupancy patterns (seasonal vs. year-round). Dunn's test was used for ANOVA post-hoc analysis. We used SigmaPlot v11.0 and Microsoft Excel to carry out linear and nonlinear regression analyses to assess the relationship between wastewater parameters and GHG fluxes. We added 1 as necessary to flux, DO, and nitrate measurements to eliminate negative and zero values, which preclude the use of some nonlinear regression models. We used a *p* value of less than or equal to 0.05 as a measure of statistical significance.

Results & Discussion

Carbon dioxide flux

Across all technologies, zones, occupancy patterns, and sampling events, CO₂ flux ranged from -0.44 to 61.8 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ($n = 78$) (Fig. 4.1). The Advantex AX20

systems produced the highest median CO₂ flux ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (range of -0.23 to 61.8; $n = 24$), followed by Norweco Singlair (range of -0.44 to 20.42; $n = 6$), Advantex RX30 (range of 0 to 33.64; $n = 19$), BioMicrobics MicroFAST (range of 0.1 to 10.62; $n = 18$), and SeptiTech (range of 0 to 5.32; $n = 11$). Negative CO₂ flux values were observed in two systems, likely due to the alkaline pH of effluent (8.51 and 7.38) in these systems, which promotes the dissolution of atmospheric CO₂ in water (Renforth, 2019).

The flux of CO₂ varied significantly by technology, with AX20 systems producing significantly higher CO₂ emissions than FAST systems, suggesting that system design – which differs considerably between AX20 and FAST systems (Appendix) – may influence CO₂ emissions. FAST systems rely on fixed activated sludge for advanced effluent treatment and are socially-dosed, while AX20 systems utilize a textile filter and are time-dosed. Variations in system dosing mechanism and treatment train design may drive differences in the microbial activity responsible for CO₂ emissions.

When values for all technologies were considered, CO₂ emissions were significantly higher in SP-1 (anoxic/hypoxic zone) than in SP-2 (oxic zone) (Fig. 4.2). SP-1 typically contains significantly higher BOD₅ concentrations than SP-2, which can fuel anaerobic respiration in SP-1, where anoxic/hypoxic conditions prevail. The lower amount of organic substrate available for aerobic respiration in SP-2 may result in lower CO₂ emissions.

When we considered values across all technologies, CO₂ flux did not differ based on home occupancy pattern (Fig. 4.3). Studies by Ross et al. (2020) found that home occupancy pattern did not impact treatment performance (Ross et al., 2020a) or microbial community structure or composition (Ross et al., 2020b). These results suggest that the

seasonally-used systems' microbial communities are robust enough to endure months of not receiving wastewater inputs without compromising their biological activity, as evidenced by similar fluxes of CO₂.

Methane Flux

Across all technologies, zones, home occupancy patterns, and sampling events, CH₄ flux ranged from -0.0029 to 25.3 $\mu\text{mol CH}_4 \text{ m}^{-2} \text{ s}^{-1}$ ($n = 79$) (Fig. 4.1). The Advantex RX30 systems produced the highest median CH₄ flux ($\mu\text{mol CH}_4 \text{ m}^{-2} \text{ s}^{-1}$) (0 to 25.3; $n = 19$), followed by Norweco Singulair (0 to 13.4; $n = 6$), Advantex AX20 (0 to 24.7; $n = 25$), SeptiTech (0 to 2.78; $n = 11$), and BioMicrobics MicroFAST (0 to 8.42; $n = 18$). Flux did not differ significantly among technologies or between occupancy patterns (Fig. 4.3), but SP-1 consistently produced significantly higher CH₄ fluxes than SP-2 (Fig. 4.2). Yan et al. (2014) investigated GHG emissions from WTPs employing various methods of wastewater treatment. They found that for all methods assessed, CH₄ emissions were significantly higher in the “grit tank” of the treatment trains, which serves a similar purpose as that of the anoxic/hypoxic zone of advanced N-removal OWTS (SP-1), than those from the oxic zones.

Nitrous Oxide Flux

Across all technologies, zones, home occupancy patterns, and sampling events, N₂O flux ranged from -0.02 to 0.23 $\mu\text{mol N}_2\text{O m}^{-2} \text{ s}^{-1}$ ($n = 78$; Fig. 4.1). Seventeen N₂O flux values were either statistically insignificant or below the instrument's detection limit, and were thus assigned a value of 0. The Norweco Singulair systems produced the

highest median N₂O flux ($\mu\text{mol N}_2\text{O m}^{-2} \text{ s}^{-1}$) (range of -0.01 to 0.1; $n = 6$), and exhibited far more variability than the other technologies, all of which had a median N₂O flux of 0: Advantex AX20 ($n = 24$) had a range of -0.01 to 0.07; BioMicrobics MicroFAST ($n = 18$) had a range of -0.02 to 0.23; Advantex RX30 ($n = 19$) had a range of 0 to 0.03; and the range for SeptiTech ($n = 11$) was 0 to 0.01. The variability observed in flux measurements from Norweco systems may be due to intermittent on/off cycles of aeration to facilitate successive nitrification and denitrification, and when we sampled within that cycle.

Nitrous oxide flux varied significantly by technology, with RX30 systems producing significantly higher N₂O emissions than SeptiTech systems, suggesting that system design, which differs considerably between these two technologies (Appendix), may influence N₂O flux. Although both technologies utilize textile filters for advanced effluent treatment, they differ in the type of media material used, as well as in wastewater dosing mechanism. As was the case for differences in dosing mechanism between AX20 and FAST systems (which differed significantly in their CO₂ emissions), RX30 systems are time-dosed while SeptiTech systems are socially-dosed. The porous nature of the textile material and the time dosing employed by RX30 systems may enhance N₂O production by denitrification and/or nitrification.

System zone did not significantly impact N₂O flux (Fig. 4.2) indicating that processes that produce N₂O, such as nitrification and incomplete denitrification, take place to the same extent in both zones. Wigginton et al. (2020) found that the structure and composition of communities of ammonia oxidizing and denitrifying bacteria in these two zones in nine advanced N-removal OWTS did not differ significantly, suggesting a

similar potential for N₂O production via these processes. As was the case for the other two gases, N₂O flux was not influenced by home occupancy pattern, mirroring the fact that the structure and composition of the microbial communities of seasonal and year-round systems were similar (Fig. 4.3).

Our values are comparable to those reported by Brannon et al. (2017) of -0.004 to 0.3 $\mu\text{mol N}_2\text{O m}^{-2} \text{s}^{-1}$, which included nine of the same advanced N-removal OWTS sampled in this study. Negative N₂O fluxes were reported from a BNR wastewater treatment plant by Brannon et al. (2017), and net N₂O consumption has been observed in soil and aquatic environments (Beaulieu et al., 2015; Chapuis-lardy et al., 2007; Soued et al., 2016). The final step of denitrification consumes N₂O as it is reduced to N₂, which takes place under hypoxic and anoxic conditions when nitrate is available as the terminal electron acceptor (Chapuis-Lardy et al., 2007). Because complete denitrification increases with decreased DO, the amount of N₂O produced from incomplete denitrification is largely dependent on DO. Six of the 7 negative N₂O values came from SP-1, the anoxic/hypoxic zone, suggesting that DO concentration played an important role in controlling N₂O emissions from OWTS.

Relationship between GHG flux and wastewater properties

Understanding the relationships between effluent properties and GHG flux may be useful in controlling GHG emissions from advanced N-removal OWTS while also maintaining effective treatment. To this end, we performed regression analyses between wastewater properties and GHG flux using data across technologies, occupancy patterns, and sampling events, allowing us to examine the relationship between wastewater

properties and flux at a broader scale. Because there were clear differences between zones in wastewater properties and GHG flux values, we performed separate analyses for SP-1 and SP-2.

No relationships were found between any wastewater properties and GHG flux in SP-2, the oxic zone. SP-2 contains little organic matter and produced very low CO₂ and CH₄ fluxes in comparison with SP-1. In addition, we found no significant relationships between N₂O flux and any wastewater property. N₂O flux values were typically very low, which likely made it difficult to identify a relationship if one exists.

The flux of CO₂ and CH₄ in the anoxic/hypoxic zone was correlated with a number of wastewater properties (Fig. 4.4). There was a positive linear relationship between the BOD₅ concentration and both CO₂ and CH₄ flux (Fig. 4.4), in line with the expectation that organic C in wastewater serves as substrate for heterotrophic microbial respiration. The relationship between flux of CO₂ and CH₄ and wastewater temperature was similar for both gases (Fig. 4.4). The flux increased with increasing temperature, peaking at 22-23°C, and declining at higher temperatures, following a typical response of microbial activity to temperature (Apple et al., 2006).

There was an inverse relationship – best described by a negative power function – between DO levels and the flux of CH₄ and CO₂ (Fig. 4.4). This indicates that, as expected, emissions of these two gases in the anoxic/hypoxic zone result from anaerobic respiration processes, including methanogenesis. Acetoclastic methanogenesis contributes significantly to anaerobic respiration in anoxic wastewater treatment environments (Qiao et al., 2015). The process produces equimolar amounts of CH₄ and CO₂. Linear regression analysis comparing CH₄ flux with CO₂ flux in SP-1 confirmed

this 1:1 relationship (slope = 1.2; intercept = 3.5; $R^2 = 0.32$), suggesting that acetoclastic methanogenesis is an important driver of CH₄ and CO₂ emissions. Anaerobic respiration processes are sensitive to the introduction of O₂, resulting in inhibition at DO levels greater than 2 mg/L (John, 1977; Hernandez & Rowe, 1988; Vaquer-Sunyer & Duarte, 2008). The presence of O₂ interferes with methanogenesis through at least two mechanisms: (i) by favoring aerobic metabolic processes, which have a more favorable energy yield than CO₂, and (ii) through toxic effects on methanogenic Archaea, which are obligate anaerobes (Mer et al., 2001).

We also observed an inverse relationship between CO₂ and CH₄ flux and nitrate concentration (Fig. 4.4). Others have reported negative effects of N on CO₂ and CH₄ flux. For example, the flux of CO₂ from agricultural soil decreases in response to amendments with inorganic N (Ramirez et al., 2010), and Anderson et al. (2019) found that nitrate addition to soil amended with anoxic septic tank effluent lowered the flux of both CO₂ and CH₄. Nitrate is potentially toxic to some methanogens (Bollag & Czlonkowski, 1973; Klüber & Conrad, 1998), and its favorable energy yield as a terminal electron acceptor relative to CO₂ can suppress methanogenesis. The inverse relationship between nitrate concentration and the flux of CO₂ and CH₄ in the anoxic/hypoxic zone may also reflect the intermittent introduction of aerated wastewater with a high concentration of DO from the oxic zone via recirculation.

Methane flux increased linearly with ammonium concentration, but there was no clear relationship between ammonium level and CO₂ flux (Fig. 4.4). The flux of CH₄ is the net result of gross methane production by methanogenic Archaea and gross consumption by methanotrophic bacteria. Ammonium is a competitive inhibitor of

methane monooxygenase, the enzyme used by methanotrophs to oxidize CH₄ (Norton et al., 2008; O'Neill & Wilktnson, 1977; Schnell & King, 1994). Thus, as the concentration of NH₄⁺ in wastewater increases, we would expect greater inhibition of CH₄ oxidation and an increase in CH₄ flux, assuming gross rates of methanogenesis remain constant.

The apparent response of CH₄ and CO₂ emissions to wastewater properties suggests that changes in some of these properties may be used to control the flux of these gases. For example, maintaining system temperature below 22-23°C – through the use of insulation around and over treatment compartments – may help limit CH₄ and CO₂ flux, provided the temperature is not so low that it interferes with performance. Increasing DO levels in wastewater – by changing recirculation ratios and/or mechanical aeration – could help also help mitigate CO₂ and CH₄ emissions from these systems. This would also increase nitrate levels, which may also curb emissions of these gases. However, the effluent would have to maintain oxygen levels low enough to allow for denitrification to take place to maintain effective N removal. Limited introduction of oxygen into the anoxic/hypoxic zone may also promote ammonia oxidation, reducing its inhibitory effect on methane oxidation. In contrast, although emissions increase with increasing concentration of BOD₅, the latter is a function of homeowner lifestyle and cannot be controlled by changes in system operation. Manipulation of BOD₅ levels is thus not a practical management strategy for limiting CH₄ or CO₂ emissions.

CO₂ Equivalents and Comparisons to Other Systems

Greenhouse gases differ in global warming potential (GWP) due to differences in their residence time and reactivity in the atmosphere. According to the

Intergovernmental Panel on Climate Change's Fifth Assessment (2014), CH₄ and N₂O have 28 and 265 times the GWP of CO₂, respectively. Converting GHG flux values into CO₂ equivalents (CO₂e) accounts for these differences in GWP and allows us to assess the relative impact of individual gas fluxes on the atmosphere, and to compare emissions from advanced N-removal OWTS those from other wastewater treatment technologies. We calculated per capita emissions (CO₂e; Brannon et al., 2017) for each gas and for the sum of the three gases based on: (i) the average flux from SP-1 and SP-2 at each system across all sampling events, (ii) the cross-sectional area of the tank, and (iii) the assumption that each system serves a household of 3 people (Amador et al., 2018). Advanced N-removal OWTS contributed an average of 60, 266, and 21 g CO₂e capita⁻¹ day⁻¹ for CO₂, CH₄, and N₂O, respectively (Fig. 4.5), with methane emissions accounting for the bulk of the impact of these systems on the atmosphere. The sum of mean emissions from advanced N-removal systems based on our data is 347 g CO₂e capita⁻¹ day⁻¹ (Fig. 4.5). This is within the range of values reported by Diaz-Valbuena et al. (2011) of 277 g CO₂e capita⁻¹ day⁻¹ for a conventional OWTS, and by Truhlar et al. (2016), who found that the roof vent of a conventional OWTS, which releases gases produced in the septic tank, produced an average of 469 g CO₂e capita⁻¹ day⁻¹. Our systems produced more CH₄ (266 CO₂e capita⁻¹ day⁻¹) and less N₂O (21 CO₂e capita⁻¹ day⁻¹) when compared with values for centralized WTPs in the U.S of 99 and 52 CO₂e capita⁻¹ day⁻¹, for CH₄ and N₂O, respectively (USEPA, 2015). Higher emissions of CH₄ from OWTS are not surprising, since organic C, which is a major driver behind CH₄ emissions, is present in higher concentrations in OWTS than in centralized WTPs, where

organic C inputs are diluted by large volumes of water with low organic C content (Amador & Loomis, 2018).

Our results indicate that, despite the enhanced microbial activity facilitated by increased aeration and system design, advanced N-removal OWTS do not have higher total GHG emissions than conventional OWTS. Advanced N-removal OWTS may have an even lower impact than conventional systems on the atmosphere if we consider emissions from the soil treatment area (STA). Final effluent from advanced OWTS has a much lower organic C concentration than septic tank effluent from a conventional system (Amador and Loomis, 2018). This translates into lower amounts of substrate and lower CO₂ emissions from heterotrophic microbial processes when effluent is dispersed to the STA. A lower concentration of organic compounds such as acetate – the substrate for acetoclastic methanogenesis – in effluent from advanced N-removal OWTS may also result in lower CH₄ emissions from the STA.

Conclusions

Advanced N-removal OWTS rely on microbial processes for wastewater treatment, and thus inevitably emit GHGs. Our study provides the first comprehensive assessment of GHG emissions from advanced N-removal OWTS. We observed differences in CO₂ and N₂O, but not CH₄, flux based on technology, suggesting that system design and/or dosing mechanism may influence emissions of these gases. Home occupancy did not significantly impact the flux of any of the three gases measured, suggesting that daily GHG emissions are not affected by seasonal and year-round usage patterns and associated differences in the volume of wastewater inputs. The flux of CO₂ and CH₄ – but not N₂O – was significantly higher in the anoxic/hypoxic zone than in the

oxic zone. The flux of both gases – but not N₂O – in the anoxic/hypoxic zone was related to wastewater properties, including BOD₅ concentration, temperature, DO and inorganic N levels, providing an opportunity to mitigate GHG emissions through manipulation of some of these properties. N₂O fluxes from advanced N-removal OWTS appear to be minimal, accounting for a small fraction of total CO_{2e} from these systems.

Daily per capita CO_{2e} for advanced N-removal OWTS were comparable to those for conventional OWTS. It appears that, despite the enhanced biological N removal that takes place in advanced OWTS, their contribution to atmospheric pollution is not different from that for systems that remove considerably less N from wastewater.

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Figures and Tables

Figure 4.1. Carbon dioxide, methane, and nitrous oxide flux from different advanced N-removal OWTS technologies across all home occupancy patterns, sampling dates, and system zones (SP-1 and SP-2). The solid line in the middle of each box represents the median and the edges of each box represents the first and third quartiles. Box whiskers extend $1.5 \times$ the inter-quartile range beyond the edges of the box and the dots represent outliers beyond $1.5 \times$ the inter-quartile range. AX20 = Orenco Advantex® AX20 (9 systems, $n = 30$); FAST = BioMicrobics MicroFAST® (6 systems, $n = 19$); NOR = Norweco Singulair® (3 systems, $n = 6$); RX30 = Orenco Advantex® RX30 (6 systems, $n = 20$); SEPT = SeptiTech® Series D (3 systems, $n = 12$).

Figure 4.2. Methane, carbon dioxide, and nitrous oxide fluxes from each system zone ($n = 25-36$ for SP-1; $n = 42-43$ for SP-2) across all technology types, home occupancy patterns, and sampling dates. Description of box and whisker plots can be found in Fig. 4.1.

Figure 4.3. Methane, carbon dioxide, and nitrous oxide fluxes from systems used seasonally ($n = 28-29$) and year-round ($n = 49-50$) across all technologies, system zones, and sampling events. Description of box and whisker plots can be found in Fig. 4.1.

Figure 4.4. Relationship between wastewater properties and CH₄ and CO₂ flux in the anoxic/hypoxic zone (SP-1) of advanced N-removal OWTs. Best-fit line is shown in blue.

Figure 4.5. Relationship between wastewater properties and CH₄ and CO₂ flux in the anoxic/hypoxic zone (SP-1) of advanced N-removal OWTS. Best-fit line is shown in blue.

Appendix

Advanced OWTS technology descriptions

Technology descriptions for the Orenco Advantex® AX20 and RX30, BioMicrobics FAST®, and Norweco Singular® systems were taken from Ross et al. (2018). 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 tank and creates an oxic environment. Nitrified effluent moves upward through a corrugated plastic insert in the tank 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 anoxic environment, thus promoting both nitrification and denitrification. The third compartment of the Norweco systems is a clarification chamber, in which promotes solids removal from the effluent. The SeptiTech ® Series D systems are time-dosed systems that rely on a trickling filter for effluent treatment. The SeptiTech systems consist of two tanks, the first of which is responsible for primary treatment and denitrification. The second tank contains a non-submerged trickling filter that facilitates nitrification, as well as two pumps; one pump recirculates effluent to the trickling filter, while the second pump recirculates effluent and sludge back to the first tank, where the nitrified effluent undergoes denitrification (Brannon et al., 2017).

Table A1 Summary statistics of wastewater parameters measured in each system zone, across all technologies and sampling events. BOD₅, DO, ammonium, nitrate, and TN are measured in mg/L and temperature is measured in degrees Celsius.

Parameter	SP-1				SP-2			
	<i>n</i>	Min	Med	Max	<i>n</i>	Min	Med	Max
pH	41	5.95	6.80	7.91	46	3.74	6.75	8.51
Temperature	40	9.30	20.8	26.0	46	8.50	19.8	26.7
BOD ₅	32	0	0	400	37	0	0	100
DO	35	0	0	9.8	41	0	4.32	9.5
Ammonium	41	0.7	9.1	107.5	46	0	1.2	34.4
Nitrate	41	0	1	56.7	46	0	6.3	29.8
TN	39	2.1	17.6	83.1	45	1.9	15.8	61.1

Conclusions

My results show that, although median effluent TN concentration varies among technologies, advanced N-removal OWTS studied in Charlestown, RI are capable of producing effluent in compliance with the state regulatory standard. There were no differences in median effluent TN concentration between systems used seasonally and those used year-round; however, year-round systems did produce significantly higher daily and annual mass N loads than seasonal systems. These results highlight the impact of system flow on mass N load outputs from advanced OWTS, and suggest that, although TN concentrations are important to monitor, the mass inputs of N from these systems should also be considered when evaluating their impact. Increased monitoring of advanced N-removal OWTS has been found to improve effluent TN concentrations, and my results also show that it can be achieved in a fast, cost-effective, and accurate way using a portable photometer.

My assessment of microbial N-transformation processes in advanced OWTS and their GHG emissions to the atmosphere provide insight into an area of onsite wastewater treatment that has received little attention. Understanding the influence of season and technology on the nitrifying and denitrifying microbial communities and identifying key microorganisms present in these systems are important steps towards better understanding of the factors that drive removal. This information can be incorporated into design and management decisions to improve system performance.

Quantifying the GHG emissions from advanced OWTS and examining their relationship with wastewater properties also provides insight into the mechanisms involved in effluent treatment. My results suggest that managing temperature and

oxygen levels in advanced OWTS may help mitigate CH₄ and CO₂ emissions. Despite the enhanced BNR that takes place in these systems were comparable to those of conventional OWTS. My findings suggest that the contribution of advanced N-removal OWTS to atmospheric pollution may not outweigh the benefits they provide by lowering N inputs to receiving waters.