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NITROGEN-REMOVING WASTEWATER TREATMENT SYSTEMS: MICROBIAL COMMUNITIES AND GREENHOUSE GAS EMISSIONS

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NITROGEN-REMOVING WASTEWATER TREATMENT SYSTEMS: MICROBIAL
COMMUNITIES AND GREENHOUSE GAS EMISSIONS

BY

SARA KATHERINE WIGGINTON

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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Abstract

Wastewater is a major source of anthropogenic nitrogen (N) pollution that causes groundwater contamination and eutrophication in coastal ecosystems. The negative effects of excess N from wastewater on human and environmental health have led the United States Environmental Protection Agency (USEPA) and many state and local agencies to set maximum N concentrations for treated wastewater before it can be discharged to ground and surface water bodies. Wastewater treatment systems that include biological nitrogen removal (BNR) can help meet these standards by promoting microbial N removal in centralized wastewater treatment plants (WTP) as well as decentralized, onsite wastewater treatment systems (OWTS; i.e., septic systems). Nitrogen removal in BNR wastewater treatment is accomplished by sequential nitrification in oxic zones and denitrification in hypoxic/anoxic zones. Wastewater treatment, including BNR, can produce the greenhouse gases (GHGs) CO₂, N₂O, and CH₄ as by-products, potentially threatening air quality. In Manuscripts 1 and 2 of this dissertation, I investigated the dynamics of GHGs and microbial communities in OWTS that have a lignocellulose-amended, N-removing layered soil treatment area (STA). These systems are designed to promote sequential nitrification and denitrification by stratifying the two processes into layers that promote microbial N removal. Layered, non-proprietary STAs are a cost-effective alternative to proprietary, advanced N-removal OWTS. In Manuscript 3, I compared the microbial community structure and composition of nine proprietary, advanced N-removal OWTS and a WTP with BNR. Although BNR in OWTS and WTPs is designed to promote the same microbial processes – and their

microbial communities assumed to be the same for OWTS management purposes – their nitrifying and denitrifying microbial communities have not been compared.

In Manuscript 1, I describe CO₂, N₂O, and CH₄ emissions from the septic tanks, Layered STAs, and Control STAs from three OWTS serving homes in southeastern MA, USA. Emissions did not differ significantly between Layered and Control STAs at any of the sites, and were controlled chiefly by temperature, soil moisture, and subsurface GHG concentrations. Per capita average emissions for these systems were higher than those reported by others, with mean values ranging from 0 to 1835 gCO₂e capita⁻¹ day⁻¹ and from 48 to 1400 gCO₂e capita⁻¹ day⁻¹ in septic tanks and STAs, respectively. These results suggest that Layered STAs are unlikely to have a negative impact on air quality compared to conventional STAs.

In Manuscript 2, I investigated the diversity, structure, and composition of ammonium oxidizing and nitrous oxide reducing bacterial communities in three Layered and Control STAs by targeting the functional genes *amoA* (ammonium monooxygenase) and *nosZ* (nitrous oxide reductase). The *amoA* community composition was similar throughout the profile of both STAs, but there was a somewhat different denitrifying communities between the sand and lignocellulose-amended layers within the Layered STA. The most common classified *amoA* taxon was *Nitrosospira* and the most common *nosZ* taxon was *Afipia*, with the most common taxa made up of unclassified bacterial strains for both *amoA* and *nosZ*. The septic tank pH and concentration of TN were correlated with differences in composition for both *amoA* and *nosZ* communities. A number of physical and chemical properties of the STA fill material were also correlated

with community differences, some of which may be adjusted to support and promote specific *amoA* and *nosZ* populations and increase N-removal efficiency in Layered STAs.

In Manuscript 3, I compared the bacterial communities responsible for N removal (ammonia oxidizers and denitrifiers) in a BNR WTP and nine advanced N-removal OWTS, all within the Greater Narragansett Bay watershed in RI, USA, targeting the bacterial genes *amoA* and *nosZ*. Diversity metrics were similar between oxic and anoxic zones within a type of treatment, suggesting the capacity for nitrification is present in hypoxic/anoxic zones, and the capacity for denitrification is present in oxic areas in both centralized and decentralized BNR wastewater treatment systems. The most widespread ammonia oxidizing genera at both the WTP and OWTS were *Nitrosomonas* and *Nitrosospira*, and the latter had higher relative abundance in OWTS than the WTP. The most widespread nitrous oxide reducers were in the genus *Pseudomonas* and *Aeromonas*. *Thauera*, *Alicyclophilus*, *Oligotropha*, *Sinorhizobium*, and *Rhodospseudomonas* were also common *nosZ* genera in both types of treatment. *Bradyrhizobium*, *Burkholderia*, *Massilia*, and *Paracoccus* were more frequently associated with OWTS, whereas *Azospirillum*, *Pseudogulbenkiania*, *Rhodoferrax*, *Shinella*, and *Thiobacillus* were more frequently associated with the WTP. These results point to major differences in the *amoA* and *nosZ* microbial communities between BNR treatment as a function of scale, even though they are designed to promote the same microbial processes. These differences need to be considered in the design and management of advanced N-removal OWTS to maximize N removal.

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Dedication

In memory of Jacob Anderson Wigginton

(1981-2017)

“Dying may not be so with me, Great Earth.
I dread to think it is—dying is change
I hope—back to a childhood or new birth;
Back to new life—to something fine and strange.
I’ve loved the Earth: I hate to lose the Earth.
The tree and bud, flower and leaf there-on;
The mist, the day, the dark and dusk and dawn.
I hate to lose the friendships I have made
With forms of flesh and graves of Earth destroyed;
I hate to lose this life when I have played
With joy of life more than the average boy.
I am in love with life—I love to live
One of the light and time, the dirt and sky,
And one of wind and rain—I hate to give
My life—I hate to think at death we die.

I am no better than those gone before.
Let me think death is process and a change
And I shall live again—be something strange.
Let me think anything; for no one knows.
And all we choose to think perhaps is vain.
But I do know I’ve come—come not to stay,
My body is a living house of clay,
It must go back as men’s have gone before
To Earth’s jail-house behind heavy doors.
The sentence there is long just for one crime
For birth and life—and we are crucified
When we are born and when our flesh has died.
We’re brought into immeasurable space and time;
Earth is our jail-house and who holds the key
Unlocking space and time and eternity!”

Jesse Stuart

“Man with a Bull-Tongue Plow”

Sonnets 613 and 614

Preface

Onsite wastewater treatment systems (OWTS), commonly known as septic systems, are used to treat domestic wastewater by about a quarter of U.S. households for whom centralized treatment at a wastewater treatment plant (WTP) is not available (U.S. Census Bureau, 2011). Conventional OWTS, consisting of a septic tank and soil treatment area (STA), can be significant contributors of nitrogen (N) pollution to groundwater and coastal ecosystems. Coastal southern New England is densely populated with households that rely on OWTS to treat wastewater before it is recharged through sandy soils to shallow ground water tables. Conventional OWTS remove between 0 and 30% of N in wastewater, and their high density in this region has resulted in harmful levels of N in groundwater that supplies drinking water and in coastal waters.

Areas that are most sensitive to N pollution in southern New England have been designated as critical resource areas, as they are referred to in Rhode Island, or areas of critical environmental concern, as these are called in Massachusetts. Many homes in these areas are not connected to a WTP and are required to upgrade from conventional OWTS to advanced N-removal OWTS upon failure of the conventional system or if the property is sold. Nitrogen removal at a WTP and in advanced N-removal OWTS generally rely on wastewater recirculation between aerated and anoxic/hypoxic components or zones to support sequential nitrification ($\text{NH}_4^+ \rightarrow \text{NO}_3^-$) and denitrification ($\text{NO}_3^- \rightarrow \text{N}_2$), a process known as biological N removal (BNR). Both types of systems are capable of removing high amounts of N, but connecting residents to a WTP or installing advanced OWTS are expensive options in terms of infrastructure, operation and maintenance, and energy requirements — with all of these costs usually

borne by the homeowner (USEPA, 2002). An alternative for enhancing N removal from residential wastewater are Lignocellulose-amended layered STAs. These systems include a conventional septic tank that supplies effluent to an STA that has two layers: a top layer consisting of sand to support aerobic nitrifying bacteria, and a lower, lignocellulose-amended layer consisting of sand and sawdust to support heterotrophic denitrification. Because these systems are non-proprietary and passive, their cost is greatly reduced compared to installation of an advanced N-removal OWTS or connection to a WTP.

I investigated three approaches that can be used to reduce N pollution from wastewater in southern New England: (1) a non-proprietary, passive, lignocellulose N-removal STA, (2) proprietary, advanced N-removal OWTS, and (3) a BNR WTP. In Manuscript 1, I assessed greenhouse gas (GHG) emissions from Lignocellulose layered N-removing STAs to determine how they impact air quality compared to control STAs. In Manuscript 2, I investigated the nitrifying and denitrifying microbial communities in Lignocellulose layered N-removing STAs, compared them to control STAs, and examined the relationship of microbial community structure and composition to environmental variables. In Manuscript 3, I compared the microbial communities in advanced, proprietary N-removing OWTS with that of a BNR WTP to elucidate similarities and differences in N dynamics between these two types of treatment.

This dissertation is prepared in manuscript format containing three papers. Manuscripts one and two are currently in preparation. Manuscript three has been submitted to the journal *Water*.

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WTP = systems 01 and 02; OWTS = systems 03 to 13. 99

Manuscript 1:
**Greenhouse Gas Emissions from Lignocellulose-Amended Soil
Treatment Areas for Removal of Nitrogen from Wastewater**

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In preparation for submission to Science of the Total Environment

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Abstract

Lignocellulose-amended, layered soil treatment areas (STAs) remove nitrogen (N) passively from wastewater by sequential nitrification and denitrification. As wastewater percolates through the STA, the top sand layer promotes nitrification, and the lower, lignocellulos-amended sand layer promotes heterotrophic denitrification. Layered STAs can remove large amounts of N from wastewater, which may increase their emissions of CO₂, N₂O, and CH₄ to the atmosphere. We measured greenhouse gas (GHG) flux from sawdust-amended (Experimental) and sand-only (Control) STAs installed in three homes in southeastern Massachusetts, USA. The Experimental STAs did not emit significantly more GHGs to the atmosphere than Control STAs receiving the same wastewater inputs, and both Control and Experimental STAs emitted more CO₂ and N₂O – but not CH₄ – than soil not treating wastewater. Median (range) flux ($\mu\text{mol m}^{-2} \text{s}^{-1}$) for all homes for the Control STAs was 7.6 (0.8-23.0), 0.0001 (-0.0004-0.004), and 0.0008 (0-0.02) for CO₂, CH₄ and N₂O, respectively, whereas values for the Experimental STAs were 6.6 (0.3-24.3), 0 (-0.0005-0.005), and 0.0004 (0-0.02) for CO₂, CH₄ and N₂O, respectively. Despite the absence of differences in flux between Control and Experimental STAs, the Experimental STA had significantly higher subsurface GHG levels than the Control STA, suggesting microbial consumption of excess gas levels near the ground surface in the Experimental STA. The flux of GHGs from Experimental and Control STAs was controlled chiefly by temperature, soil moisture, and subsurface GHG concentrations. Total emissions ($\text{gCO}_2\text{e capita}^{-1} \text{day}^{-1}$) were higher than those reported by others for conventional STAs, with mean values ranging from 0 to 1835 for septic tanks, and from 48 to 1400 for STAs. Our results suggest that, despite a higher capacity to remove N from

wastewater, layered STAs may have limited impact on air quality compared to conventional STAs.

Introduction

Coastal communities threatened by nutrient pollution from septic systems often require advanced onsite wastewater treatment systems (OWTS) that reduce C and N loads to sensitive ground and coastal waters to a greater extent than is possible with conventional OWTS (Bellone et al., 2017; FDOH, 2018; MassDEP, 2013; Murray et al., 2018; RIDEM, 2020). Like conventional systems, advanced OWTS rely on microbial processes that produce CO₂ and CH₄ to remove organic C from wastewater as part of primary treatment. In addition, most advanced septic systems provide conditions that support enhanced microbial transformations of wastewater C and N to gaseous forms that are released to the atmosphere, including the greenhouse gases CO₂, CH₄, and N₂O.

Although septic systems treat wastewater for over 20% of US households (Census Bureau, 2006) and produce GHG through microbial pollutant removal, quantification of their greenhouse gas emissions has received little attention (Truhlar et al., 2016). A number of recent studies have begun to address this knowledge gap and indicate that septic systems can be important contributors of GHGs (Diaz-Valbuena et al., 2011; Dubber and Gill, 2014; Truhlar et al., 2016, 2019; Somlai-Haase et al., 2017; Somlai et al., 2019). As such, onsite wastewater treatment should be better integrated into global GHG emission estimates. For example, Truhlar et al. (2016) estimated the carbon footprint for a septic system (including emissions from the septic tank, roof venting system, and STA) to be 0.27 tCO₂e capita⁻¹ year⁻¹, equivalent to 1.5% of the U.S. total annual per capita carbon footprint.

A more complete understanding of the contribution of septic systems to global GHG emissions requires that we quantify emissions from all treatment train components. This includes the STA, where emissions to the atmosphere can result from physical processes, such as outgassing of gases dissolved in effluent, and from *in situ* microbial production and consumption of gases (Somlai et al., 2019). Values of GHG flux from soils treating wastewater vary widely. Diaz-Valbuena et al. (2011) reported consistently similar concentrations of N₂O, CO₂, and CH₄ between flux chambers above a conventional STAs and ambient atmospheric samples. In contrast, Truhlar et al. (2016) found STAs can be a substantial source of N₂O – but not CO₂ or CH₄ – relative to soils not receiving wastewater, with flux values ranging from -0.012 to 0.17 g N₂O capita⁻¹ day⁻¹. Somlai et al. (2019) observed higher median CO₂ flux from STAs than from native soil, with STA values ranging from 0.66 to 22.72 μmol m⁻² s⁻¹. Beyond developing a sense of the magnitude of GHG emissions from STAs, a better understanding of the factors governing the direction and magnitude of GHG fluxes in STAs would help to design systems that minimize emissions without compromising treatment.

The STA can be engineered to enhance N removal from septic tank effluent. One design, often referred to as a Layered STA, involves vertically stratifying treatment processes, with a top layer of sand to promote oxic conditions that support nitrification placed above a layer of sand mixed with lignocellulosic material (e.g., sawdust or mulch), which promotes anoxic conditions and provides a source of C and energy for heterotrophic denitrification.

The Layered design contrasts markedly with that for a conventional STA, which consists of a pipe-on-stone trench underlain with either native soil or sand that promotes

oxic conditions and lacks a C source for denitrification, and is not designed to remove N. In contrast, Layered STAs can lower the total N concentration in septic tank effluent by as much as 85% (Heufelder, 2017). They are also non-proprietary, passive technologies, that provide a much less costly alternative to manufactured trade named advanced N-removal OWTS. As such, they represent a cost-effective solution to lowering N pollution in coastal areas. Assessing their GHG emissions will help evaluate their impact on the atmosphere relative to existing onsite N-removal technologies, which are net sources of GHG (Brannon et al., 2017; Ross et al., 2020).

We quantified emissions of CO₂, CH₄, and N₂O from Layered (also referred to as Experimental) and Control STAs, as well as the septic tank, at three single-family homes in Barnstable County, Massachusetts, USA. One home was occupied year-round, whereas the other two were occupied only in summer. We also measured subsurface concentrations of CO₂, CH₄, and N₂O to further characterize GHG dynamics patterns in the STAs. Finally, we examined the role of system and environmental variables in controlling both emissions and subsurface GHG dynamics. We expected that CO₂, CH₄, and N₂O emissions would be higher from the Layered STA than the Control STA, since the former is designed to increase heterotrophic denitrification, which should increase CO₂ and N₂O production. We also expected CH₄ would be produced at higher rates in the saturated lower layer of the STA, where anoxic conditions would promote methanogenesis. Finally, we expected that GHG flux from both the Control and Experimental STAs would be controlled by soil physical properties such as temperature, oxygen concentration, and belowground GHG concentration, since these factors modulate emissions in other soils (Davidson et al., 1998; Risk et al., 2002).

Methods

Gas Flux Measurements

We measured the flux of GHG at the soil surface and from septic tanks using a Picarro model G2508 cavity ring-down spectroscopy analyzer that simultaneously measures CO₂, CH₄, and N₂O flux in real time (Brannon et al., 2016). We measured GHG fluxes at the Acushnet site in July and December 2018 and April and July 2019, in May and July 2019 at the Sippewissett site, and in April and July 2019 at the Chappaquoit site. On every site visit we made at least seven gas flux measurements: three from the Experimental side, three from the Control side, and one from either the septic tank or pump chamber (Fig. 1.1). At Acushnet, we made three flux measurements from the Reserve STA in July 2019.

To measure gas flux at the soil surface, we used an opaque, closed PVC soil gas chamber (i.d. = 28 cm, height = 18 cm, fitted with a stainless steel pressure equilibration device) that was placed on a PVC collar (i.d. = 26.5 cm, height = 11.5 cm) and connected to the gas analyzer via nylon tubing. The chamber was fitted with a rubber silicon gasket to form an air-tight seal when it was placed on the collar. The collars were inserted ~10 cm into the soil on the day we measured fluxes. We measure GHG flux at the water surface of the septic tank or pump chamber as described in Brannon et al. (2017). A Hobo® data logger (Onset, Bourne, MA) was installed inside the flux chamber to measure the air temperature every 15 seconds.

The flow rate of gas from the chamber to the analyzer was ~223 standard cm³ min⁻¹ and the change in concentration over time was measured every second. Gas concentration data were collected for 5 to 15 minutes. The gas flux was calculated as outlined in Martin and Moseman-Valtierra (2015). We assigned a flux value of 0 to

measurements with no statistically significant change in gas concentration over time.

Calculations and assumptions for daily per capita conversions can be found in the

Supplementary Materials.

Subsurface Gas Concentration, Temperature, and Moisture Content

We measured the concentration of soil gases (CO_2 , CH_4 , N_2O , and O_2) as well as temperature and moisture content at the Acushnet site. Gas diffusion chambers were installed during STA construction at 15, 30, 60, and 75 cm below the infiltrative surface in six arrays: three in the Experimental STA and three in the Control STA (Fig. 1.1). The chambers were made of well screen PVC (i.d. = 2.5 cm; length = 17.1 cm; Atlantic-Screen Inc., Milton, DE), which allowed for the movement of soil gases while preventing water accumulation. The chambers were connected to the ground surface using nylon tubing, with a three-way stopcock valve placed on the end of the tubing that was closed to the atmosphere between sampling events.

We attached EC-5 soil moisture sensors (METER Group, Pullman, WA) to four of the gas diffusion chambers in one array each in the Experimental and Control STAs. We also attached Hobo® temperature probes (Onset, Bourne, MA) to the same four chambers as the moisture probes in the Experimental STA (Fig. 1.1). Moisture and temperature measurements were recorded every 30 minutes. The gas diffusion chambers, and associated moisture and temperature probes were installed during system construction using a wooden frame that stabilized the chambers and held them at the correct depth while media was added to build the STA.

Before sampling soil gases, we purged the system by removing 300 mL of gas using an air-tight, 60 mL syringe. The pore space gases were then mixed by drawing and

expelling a 60 mL gas volume five times. After mixing we transferred a 20 mL sample of the gases into pre-evacuated glass vials fitted with rubber septa and an aluminum collar, which were stored immersed in water until analysis. An additional 60 mL gas sample was collected for analysis of O₂ concentration.

Soil greenhouse gas concentrations were measured on a Shimadzu Gas Chromatograph-2014 Greenhouse Gas Analyzer (Shimadzu Scientific Instruments, Kyoto, Japan). A flame ionization detector (FID) was used to analyze CO₂ and CH₄ and an electron capture detector (ECD) was used to analyze samples for N₂O. The flow rate of the ultrapure N₂ used as carrier gas was 25 mL min⁻¹. Instrument temperatures were 250 °C for the FID, 325 °C for the ECD, 80 °C for the column, and 100 °C for the injection port.

To measure the concentration of oxygen, we injected a 60 mL of sample into a flow-through cell connected to an O₂ probe at a rate of ~1 mL s⁻¹ to obtain percent O₂ (Cooper et al., 2015). Calculations to converted oxygen concentration in the gas phase to dissolved oxygen (DO) are found in **Supplementary Materials**.

Statistical Analyses

We used RStudio (Version 1.2.1335; R Core Team, 2012) to perform all data analysis. We used a two-way ANOVA to determine differences in GHG flux among seasons and STA type at Acushnet and to examine the differences in GHG flux among the subsurface sampling depths and STA type. We used Student's t-tests to determine differences in flux between Layered and Control STAs at the seasonal sites and to assess differences in GHG flux between periods of use and nonuse at the seasonal sites. We

examined the relationship between subsurface gas concentrations, soil temperature, dissolved oxygen (DO) and GHG flux by constructing multiple linear models in RStudio. For all statistical analyses, we used a confidence interval of 95%.

Results & Discussion

GHG Emissions from the Septic Tank

We measured CO₂, CH₄, and N₂O from the primary treatment tank to compare emissions from different parts of the treatment system. Carbon dioxide flux from the septic tank at Acushnet, was consistently low with values ranging from 0 to 2.9 $\mu\text{mol m}^{-2} \text{s}^{-1}$. We did not detect a significant N₂O flux except during winter 2018, when the flux value was 0.0002 $\mu\text{mol m}^{-2} \text{s}^{-1}$. There was no significant CH₄ flux from the septic tank during summer 2018, three months post-installation. We subsequently observed a consistently high CH₄ flux from the tank, ranging from 0.24 to 0.82 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 1.2). At the seasonally-occupied sites GHGs tank emissions were between one and three orders of magnitude higher than at Acushnet during both periods of use (summer) and non-use (spring) (Fig. 1.2).

Daily per capita emissions for all gases from the septic tank at Acushnet were lower than others have reported from conventional septic tanks, but those from the seasonal sites were much higher (Diaz-Valbuena et al., 2011; Somlai-Haase, 2019), and were more comparable to values for roof vents reported by Truhlar et al. (2016). Differences in CO₂ flux among sites and studies may be due to differences in the curing time of concrete septic tanks, since longer curing times result in less formation of calcium carbonate from the reaction of CO₂ with calcium hydroxide present in cement (Balayssac et al., 1995; Dias, 2000; Han et al., 2011). The tank at Acushnet had a shorter curing time

and the wastewater had higher pH than at the other sites (data not shown), suggesting that increased calcium carbonate formation caused the low CO₂ flux. Differences in CH₄ flux are likely due to variations in development of a scum layer – a C-rich layer of oils and fats that accumulates on the wastewater surface between maintenance visits – and the stochastic nature of CH₄ off-gassing (Grinham et al., 2011; Somlai-Haase, 2019; Windsor et al., 1992).

GHG Emissions from the Soil Treatment Area

Carbon dioxide. Carbon dioxide flux from the Layered and Control STAs at the Acushnet site were not significantly different regardless of sampling period (Fig. 1.3). When flux values from the Control and Layered STA were included, the median CO₂ flux for the whole study period was 11 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and ranged from 1.56 to 24.3 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The CO₂ flux in summer 2019 was nearly twice that in summer 2018, likely because the system was installed 75 days before summer 2018 sampling and the microbial community and soil hydraulic properties were not yet stable. In addition, CO₂ flux was higher during both summers than during winter or spring. We did not observe significantly different CO₂ emissions between the Control and Experimental STAs at either of the seasonally-occupied sites, and these were also higher in the summer than in the spring (Fig 1.3). Truhlar et al. (2019) did not observe significant seasonal differences in CO₂ emissions from conventional STAs, but they did observe a general decrease in flux in late fall and early winter. Seasonal differences in our systems may be due to the use of a low-profile dispersal system, which places the infiltrative surface closer to the

ground surface and thus less buffered from variations in temperature than a conventional STA with an infiltrative surface placed deeper in the soil profile.

The median CO₂ flux (11.24 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in the Reserve STA at Acushnet – measured in summer 2019 – was significantly lower than for the Control (22.9 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or the Layered (22.7 $\mu\text{mol m}^{-2} \text{s}^{-1}$) STAs (Fig. 1.3). The Reserve STA is filled with carbon-poor, biochemically-inactive sand and did not receive wastewater, resulting in lower microbial activity that produces less CO₂, compared to both native soil and STAs receiving wastewater. In contrast, Diaz-Valbuena et al. (2011) observed that CO₂ concentrations above a conventional STAs were the same as ambient atmospheric concentrations, and Truhlar et al. (2016, 2019) did not observe significantly different CO₂ emissions from STAs and native soils. Somlai-Haase et al. (2017) and Somlai et al. (2019) also observed similar CO₂ flux between native and STA soils, but they did observe higher CO₂ flux from STAs on some sampling events.

The daily per capita CO₂ emissions from the STA at the three homes ranged from 0.28 to 24.3 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and were slightly above the range reported by Somlai et al. (2019) and Somlai-Haase (2019) for conventional systems in Ireland.

Nitrous oxide. Nitrous oxide flux at Acushnet ranged from 0 to 0.018 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with no significant differences observed between the Control and Layered STA for any of the sampling dates (Fig. 1.3). We also did not observe significant differences between the Control and Experimental STAs at the seasonally-occupied sites (Fig. 1.4). The N₂O flux was low in winter 2018 and spring 2019 for both the Control and Layered STA, with values ranging from 0.0008 to 0.001 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Flux values were slightly higher in summer 2018 ranging from 0 to 0.008 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 1.3) and they were an order of

magnitude higher than previous dates in the summer of 2019, with values ranging from 0.01 to 0.02 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The same temporal pattern was observed for CO_2 flux and is likely driven by higher temperatures once the microbial community becomes established in the STA.

The median flux of N_2O in the Reserve STA at Acushnet (0.006 $\mu\text{mol m}^{-2} \text{s}^{-1}$) – measured in summer 2019 – was significantly lower than in the Control and Experimental STAs, where median flux values were both 0.02 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 1.3). In contrast, Diaz-Valbuena et al. (2011) did not observe N_2O concentrations differing between ambient atmospheric samples and from air above conventional STAs. Differences depth to the infiltrative surface between our systems and conventional STAs may be responsible for differences in N_2O flux. Truhlar et al. (2016) observed significantly higher N_2O emissions above conventional STAs than above control soils, as we did. Higher N_2O flux from the STAs observed by us and others suggests that soil-based wastewater treatment may be an important source of N_2O .

Across all sites, times, and STAs, N_2O flux ranged from 0 to 0.02 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (equivalent to 0 to 0.47 g $\text{N}_2\text{O capita}^{-1} \text{day}^{-1}$). Although some of our lowest values are comparable to those reported in previous studies (Diaz-Valbuena et al., 2011; Somlai-Haase, 2019; Truhlar et al., 2016), most are higher than previously reported, especially during summer 2019. The low-profile design of the dispersal systems may explain this. The proximity of the oxic layer, where N_2O can be produced by ammonia oxidation, and of the infiltrative surface to the soil surface, may result in higher N_2O flux than for systems where the infiltration surface is at a lower depth, with a higher volume of soil where N_2O may be consumed before it is emitted to the atmosphere. The location of the

oxic layer relative to the ground surface may also explain the consistently low values of N₂O flux we observed at Sippewissett (Fig. 1.4), where fill material over the infiltrative surface is nearly four times deeper than at the other sites (Table 1.1). Cooper et al. (2016) also observed higher N₂O concentrations near the surface of mesocosms with low-profile dispersal systems compared to mesocosms with conventional STAs.

Methane. Methane flux values at Acushnet ranged from -0.0005 to 0.002 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with no significant differences between the Layered and Control STAs on any sampling date (Fig. 1.3). Similarly, there were no significant differences in the CH₄ emissions between the Control and Experimental STAs in the seasonally-occupied sites (Fig. 1.4). Methane emissions had seasonal patterns that were opposite to those for CO₂ and N₂O, with higher values in the spring and winter (0.0005 to 0.002 $\mu\text{mol m}^{-2} \text{s}^{-1}$) than in the summer (-0.0005 to 0.001 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The latter may be due to differences in the temperature sensitivity of microbial consumption and production of CH₄ in surface soil. Methane oxidation decreases significantly at temperatures under 10°C (Le Mer and Roger, 2001), while methanogenesis can continue in the septic tank – increasing the level of dissolved CH₄ in effluent – where the water temperature was 12°C in winter g, 6°C higher than the soil (data not shown). As was the case for CO₂, Truhlar et al. (2019) did not observe seasonal differences in CH₄ flux, though they did observe that the STA acted as sink in the spring and fall, and as a source during the summer.

Unlike CO₂ and N₂O, the median CH₄ flux ($\mu\text{mol m}^{-2} \text{s}^{-1}$) for the Reserve STA (-0.00017) at Acushnet was not significantly different from the values for the Experimental (-0.00029) or Control STA (-0.00025) in summer 2019, when all three STAs acted as

sinks (Fig. 1.3). Similarly, Truhlar et al. (2019) saw no differences in CH₄ flux between conventional STAs and native soil and that STAs frequently acted as CH₄ sinks.

Other studies have observed no net CH₄ flux from soils treating wastewater (Diaz-Valbuena et al., 2011), whereas we observed a wide range of flux values, depending on season and site. The range of CH₄ flux across all sites, dates, and STA types was -0.0005 to 0.005 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (-0.024 to 0.21 g CH₄ capita⁻¹ day⁻¹). Many of these values were within the range reported by others from conventional STAs (Somlai et al., 2019; Truhlar et al., 2019, 2016). Nevertheless, we observed some much higher flux values, especially during the winter and spring at Acushnet and in the summer at Chappaquoit. These high values could be explained by the low-profile dispersal systems in our STAs. It is likely that the soil above the low-profile dispersal system is not deep enough to allow for consumption of the CH₄ produced in the anoxic layer and that diffusing from the septic tank effluent dispersed to the STA. This is exacerbated in cold temperatures when CH₄ consumption decreases. The consistently low CH₄ flux at Sippewissett (Fig. 1.4) suggests that there is greater microbial consumption of the gas in the deeper soil above the dispersal system relative to the shallower soil in the other two systems (Table 1.1).

Subsurface Concentrations of Dissolved Oxygen and GHG at Acushnet

At Acushnet, we measured subsurface concentrations of CO₂, N₂O, CH₄, and DO at four depths within the layered and control STAs to better describe patterns of gas production and consumption and to develop a better understanding of what controls GHG emissions of Layered systems.

Dissolved oxygen. In the Layered STA at Acushnet, median DO concentrations ranged from 3.7 mg L⁻¹ at 75 cm in July 2019 to 11.9 mg L⁻¹ in March 2019 at 30 cm. Median DO concentrations in the Control STA ranged from 6.9 mg L⁻¹ at 30 cm in July 2019 to 13.6 mg L⁻¹ in March 2019 at 15 cm (Supplementary Fig. S1). Dissolved oxygen concentration decreased with increasing depth in the Layered STA, whereas DO levels in the Control STA varied little with depth. When compared across all dates, median DO values were significantly higher in the Control STA than in the Layered STA at the two lowest sampling points, where the Layered STA has the lignocellulose amendment, but no difference in DO was observed between STAs at the top two sampling depths, where both sides were filled with sand. These data suggest that the Layered STA acted as designed, lowering DO levels relative to the Control STA to promote denitrification.

Differences in DO concentration between the Layered and Control STAs were affected by season. There were no significant differences in DO levels between the control and layered STAs when the soil temperature fell below 7 °C, and we observed the lowest DO values during warm sampling dates, especially in the layered STA (Supplementary Fig. S1). This is consistent with other seasonal DO observations in shallow groundwater and denitrifying reactive barriers (Datry et al., 2004; Warneke et al., 2011). Warm temperatures lower DO solubility, and DO levels decrease further due to increased microbial activity, which consumes DO and produces CO₂.

Carbon dioxide. Median subsurface levels of CO₂ in the control STA ranged from 2,965 ppmv in March at 75 cm to 98,981 ppmv in June 2018 at 60 cm. In the Layered STA, CO₂ levels ranged from 420 ppmv in April at 75 cm to 171,168 ppmv at 75 cm in June 2018 (Supplementary Fig. S2). When samples from all dates were

considered, CO₂ levels were significantly higher in the Layered STA than in the Control STA at all depths except 15 cm. Carbon dioxide concentrations in the Control STA did not vary as a function of depth. In contrast, we found significantly lower CO₂ concentration at 15 cm than at 60cm or 75cm below the infiltrative surface – the denitrification zone – of the layered STA. High CO₂ concentrations in the denitrification zone and lower concentrations higher in the profile of the layered STA indicate CO₂ consumption in the nitrification zone. A small fraction of this could be attributed to chemoautotrophic organisms, such as nitrifiers, which use CO₂ as a carbon source (Robertson and Groffman, 2015). It is more likely that CO₂ is consumed as it reacts with silicate and carbonate compounds in the sand (Liu et al., 2011).

Carbon dioxide levels in the subsurface at Acushnet generally increased with increasing temperature, especially in the Layered STA (Supplementary Fig. S2) and were notably high in June 2018, approximately six weeks after installation, when levels of the gas in the Layered STA were significantly higher than on any other date. While CO₂ concentrations in the Control STA were also highest in June 2018, they were significantly lower than in the Layered STA at 60 and 75 cm. This suggests that sometime during the first few months of installation a large amount of CO₂ was produced in the Layered STA, possibly by aerobic decomposition of the lignocellulose amendment before hypoxic/anoxic conditions were established.

Nitrous oxide. Subsurface N₂O levels were lowest in March 2019 at 15 cm, when median values were 11 and 19 ppmv in the Control and Layered STA, respectively. The highest N₂O levels were observed in July 2019, when the median N₂O value in the control STA was 160 ppmv at 15 cm and 197 ppmv at 75 cm in the Layered STA

(Supplementary Fig. S3). When all dates were considered, there were no significant differences in N_2O concentration with depth in either STA. We did, however, observe significantly higher concentrations of N_2O in the Layered STA compared to the Control STA, especially in the lower, carbon-amended layer (Supplementary Fig. S3) where hypoxic/anoxic conditions support N_2O production by heterotrophic denitrification. Differences in N_2O levels between the Layered and Control STA are likely exacerbated in summer by warmer temperatures and low DO levels (Supplementary Fig. S5), both contributing to higher denitrification rates (Korom, 1992; Sutton et al., 1975). During the coldest months, when temperatures at the bottom of the STA were consistently below 5 °C and DO levels were consistently above 11 ppmv, we did not observe significant differences in N_2O levels between the STAs.

Methane. Subsurface CH_4 levels were lowest in May 2019, when the median value in the Control STA was 3.8 ppmv at 75 cm and 4.4 ppmv at 60 cm in the Layered STA. The highest CH_4 concentrations were observed in November 2018 with median values 520 and 1,832 ppmv at 75 cm in the Control and Layered STA, respectively (Supplementary Fig. S4). Across all months and dates, subsurface CH_4 levels were significantly higher in the Layered than in the Control STA (Supplementary Fig. S4) and differences in CH_4 concentrations between the two STAs were largest in August 2018, October 2018, and June 2019. There was no clear relationship between CH_4 concentration and depth in either STA. Subsurface CH_4 values were highest when temperatures were between 10 and 13 °C (Supplementary Fig. S5). Methane concentration in both STAs was slightly elevated in the first two months after installation, otherwise it

was very low except in November, when they were an order of magnitude higher than on all other sampling dates.

Predictors of GHG Flux at Acushnet

The flux of GHG from soil is partially controlled by soil physical properties (Ludwig et al., 2001; Oertel et al., 2016). Thus, we examined the relationship between surface GHG emissions and soil temperature and DO – a proxy for soil moisture – at a depth of 15 cm. We also investigated the relationship between surface flux and GHG level at 15 cm below the infiltrative surface to determine if GHG concentrations within the STA could help predict surface emissions.

Using multiple linear regressions, we found that soil temperature, DO, and GHG gas concentrations at 15 cm below the infiltrative surface were the best predictors of emissions of all three GHGs, with R^2 values of 0.98 for CO₂ and N₂O flux, and 0.68 for CH₄ flux. Soil moisture and temperature are important drivers of GHG emissions from soils (Davidson et al., 1998; Oertel et al., 2016). Greenhouse gas concentrations at 15 cm below the infiltrative surface were always positively correlated with surface flux (Fig. 1.5). This, paired with our observation of significantly higher subsurface GHG concentrations in the Layered STA, makes the lack of difference in flux between the Control and Experimental STAs even more surprising.

Somlai et al (2019) found that variation in CO₂ flux above STAs was mainly driven by daily fluctuations in soil temperature, in agreement with our results. Because CO₂ is a product of aerobic respiration, we expected to see a positive relationship between DO and CO₂; surprisingly, we observed high CO₂ flux associated with low DO

(Fig. 1.6). The interaction of temperature and DO may explain these results: at higher temperatures, oxygen solubility decreases but microbial respiration increases, and thus CO₂ production increases (Oertel et al., 2016). The increased microbial activity further lowers DO concentration in soil pores (Oertel et al., 2016). At lower temperatures, DO is more available, but microbial activity is slower so we see a lower CO₂ at lower temperatures (Fig. 1.7).

The association of high CH₄ flux with low temperatures (Fig. 1.7) and high DO was also expected (Fig. 1.6) since these conditions are opposite of those that promote methanogenesis in soils (Le Mer and Roger, 2001). There are, however, reports of negative relationships between CH₄ flux and soil temperature in grasslands (Van Den Pol-van Dasselaar et al., 1998). The low CH₄ flux in the summer may be because of increased methane oxidation consumption in the surface soil when the temperature is warmer, while higher flux in cold months could be because CH₄ oxidizing bacteria slow their consumption, whereas methanogenesis is less affected by temperature changes (Le Mer and Roger, 2001).

High N₂O flux was associated with low DO levels (Fig. 1.6) and high temperatures (Fig. 1.7). Cold temperatures adversely affect denitrification and N₂O production in other STA types and in denitrification barriers designed to remove N based on the same mechanisms as Layered STAs (Robertson et al., 2008; Truhlar et al., 2019; Warneke et al., 2011). Additionally, higher N₂O production is expected with low DO levels, since denitrification is limited by increasing oxygen levels (Korom, 1992).

Whole System Emissions

Total GHG emissions from septic tanks at three homes ranged from 0 to 1835.4 gCO₂e capita⁻¹ day⁻¹, and from 48.2 to 1400 gCO₂e capita⁻¹ day⁻¹ from the STAs (Table 1.2). While all the STAs were CH₄ sinks at some point, CH₄ consumption was never high enough to offset the GHG emissions produced by treatment. Many of the emission values we report here are much higher than those previously reported for conventional septic tanks or STAs (Table 1.2). This difference is likely because of our use of low-profile dispersal systems in the Control and Experimental STAs. These systems are designed to increase nutrient removal from wastewater but may result in high GHG emissions relative to conventional systems. The latter are installed deeper in the soil profile and have more soil separating the infiltrative surface and STA from the atmosphere. The reduced soil depth above the low-profile dispersal system used in the Layered STA, lowers the opportunity for microbial consumption of GHGs relative to conventional STAs.

Conclusions

We did not observe a significant difference in GHG emissions between the Experimental Layered STAs and Control STAs at any of the sites during any sampling date. We did find evidence that onsite wastewater treatment does impact air quality, as both Control and Experimental STAs had significantly higher CO₂ and N₂O flux than a Reserve STA engineered identically, but not receiving wastewater inputs. We found that the Layered STA at Acushnet had higher GHG levels in the subsurface than the Control STA, but this did not translate to a difference in flux between the STAs, likely because of GHG consumption near the ground surface. These findings suggest that using lignocellulose-amended STAs to remove N from wastewater is unlikely to have adverse

effects on air quality compared to other STAs with low-profile dispersal systems. Nevertheless, they may have higher GHG emissions than conventional STAs due to reduced soil depth above the infiltrative surface, which may lower the opportunity for microbial gas consumption. The GHG flux from the STA could be predicted based on subsurface temperature, DO level, and GHG concentration 15 cm below the infiltrative surface. This relationship may be used to estimate GHG emissions absent access to a continuous, real-time gas analyzer.

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Figures and Tables

Figure 1.1. (A) Schematic diagram soil treatment area (STA) at the Acushnet site. The STA was divided into thirds and gas flux measurements were made at three points in each STA: Experimental (E1-E3), Control (C1-C3), and Reserve (R1-R3). (B) Cross-section of the Experimental and Control STAs at the Acushnet site showing the placement of arrays of subsurface gas diffusion chambers, moisture probes, and temperature probes. Probes were installed during system construction at: 15 cm, 30 cm, 60 cm, and 75 cm below the infiltrative surface (GeomatTM - a low profile dispersal system). Double dashed lines (=) indicate moisture probes, single dashed lines (-) indicate temperature probes. Figures not to scale.

Figure 1.2. CH₄, CO₂, and N₂O flux at the water surface of the septic tank at Acushnet, Chappaquoit, and Sippewissett sites. Each symbol represents a single flux measurement.

Figure 1.3. Flux of (A) CO₂, (B) N₂O, and (C) CH₄ from Control and Experimental soil treatment areas (STAs) at the Acushnet site. An additional set of measurements was made in a Reserve STA in summer 2019.

Figure 1.4. Flux of (A) CO₂, (B) N₂O, and (C) CH₄ at the seasonally-occupied homes in spring and summer 2019 in Control and Experimental soil treatment areas (STAs).

Figure 1.5. Relationship between gas flux and gas concentration at 15 cm below the infiltrative surface for (A) CO₂, (B) N₂O, and (C) CH₄. Circles = Control STA; Triangles = Experimental STA. Data are for the Acushnet site. Grey shading represents 95% confidence interval.

Figure 1.6. Relationship between gas flux and dissolved oxygen concentration at 15 cm below the infiltrative surface for (A) CO₂, (B) N₂O, and (C) CH₄. Circles = Control STA; Triangles = Experimental STA. Data are for the Acushnet site. Grey shading represents 95% confidence interval.

Figure 1.7. Relationship between gas flux and soil temperature at 15 cm below the infiltrative surface for (A) CO₂, (B) N₂O, and (C) CH₄. Circles = Control STA; Triangles = Experimental STA. Data are for the Acushnet site. Grey shading represents 95% confidence interval.

Table 1.1. System and site characteristics for the Acushnet, Chappaquoit, and Sippewissett sites.

Characteristic	Site		
	Acushnet	Chappaquoit	Sippewissett
Occupancy	Year-round	Seasonal (May-Sept.)	Seasonal (June-Sept.)
Installation date	April 20, 2018	April 10, 2017	November 16, 2017
# of occupants	2 adults, 1 infant	2 adults	1 adult
Average flow while occupied (L day ⁻¹)	872	375	450
Depth of soil above Geomat™ (cm)	25	25	90
Type (width) of Geomat	1200 (0.3 m)	3900 (1 m)	3900 (1 m)
Total number of Geomat strips	9	4	4
Space between Geomats (m)	0.3	0	0
Soil treatment area dimensions (m)*			
Length	7.62	13	10.7
Width	6.64	4.3	3.9
Depth	0.9	0.9	0.9
Area (m ²)	14	54.6	41.73
Vegetative cover and management	Lawn grasses (<i>Festuca sp.</i> , <i>Lolium sp.</i> , <i>Poa sp.</i>) mown short (~5 cm height)	Lawn grasses (<i>Festuca sp.</i> , <i>Lolium sp.</i> , <i>Poa sp.</i>) mown short (~5 cm height)	Little cover in spring; vegetation grew without maintenance before summer sampling (~1 m height)

*length excludes soil on perimeter of STA; area excludes space between Geomat™ laterals (although this space is included when regulatory agencies determine the size of the treatment area). Depth excludes fill above the Geomat™

Table 1.2. Total greenhouse gas emission in our study compared to published values. Values for our study are geometric mean and standard deviation of all samples collected on a sampling date and include values for Control and Experimental STAs.

Source	Site	Date	Treatment train component	Total emissions gCO ₂ e cap ⁻¹ d ⁻¹
This study	Acushnet	Summer 2018	Septic tank	0
			STA	249 ±70.5
		Winter 2018	Septic tank	12.9
			STA	48.2 ±10
		Spring 2019	Septic tank	18.9
			STA	91.5 ±32
		Summer 2019	Septic tank	67.8
			STA	579.9 ±60
	Sippewissett	Spring 2019	Septic tank	1605.7
			STA	810.7 ±145
		Summer 2019	Septic tank	1835.4
			STA	1400 ±357.4
	Chappaquoit	Spring 2019	Septic tank	300.8
			STA	87.6 ±40
		Summer 2019	Septic tank	1175.9
			STA	1028 ±273.7
Diaz-Valbuena et al. (2011)			Septic tank	310
			STA	Not detectable above ambient
Truhlar et al. (2016)			Roof vent	470
			STA	122
Brannon et. al. (2017)			N-removal septic tank	17.9 (N ₂ O)
Somlai-Haase et al. (2017)			STA	78.2 (CO ₂)
Somali et al. (2019)			STA	83 (CO ₂ & CH ₄)
Somlai-Haase (2019)			Septic tank	20
			STA	37

Converting $\mu\text{mole}/\text{m}^2/\text{s}$ to $\text{g}/\text{capita}/\text{day}$

- We first converted the μmoles to moles by multiplying by 1000
- We then converted moles of each greenhouse gas to grams by multiplying the flux by the molar mass of the gas (44.01- CO_2 , 16.04- CH_4 , and 44.01- N_2O)
- We assumed constant flux across the whole STA or septic tank and we extrapolated flux for the whole STA by multiplying by the area of the STA or tank
- We divided this by the known number of residents
- We assumed constant flux throughout the day and extrapolated for the whole day by multiplying flux h^{-1} by 24 and flux s^{-1} 86400

Calculating total CO_2eq

- We converted N_2O and CH_4 $\text{g capita}^{-1} \text{ day}^{-1}$ to $\text{g CO}_2\text{eq g capita}^{-1} \text{ day}^{-1}$ by multiplying them each by their global warming potential (25- CH_4 and 298- N_2O).
- To calculate whole $\text{CO}_2 \text{eq}$ for each sample, we summed CO_2 emissions and CH_4 and N_2O emissions converted to CO_2eq

Conversion to Dissolved Oxygen Concentration

We converted gas-phase oxygen concentration to dissolved oxygen using Henry's

Law formula:

$$C = HP_{\text{gas}}$$

where C is the O_2 concentration in solution, P_{gas} is the oxygen partial pressure, and H is Henry's constant for oxygen. A pressure of 1 atm was assumed, and the Henry's constant value was corrected for temperature using the equation:

$$H(T) = H^\circ e^{\left[\frac{-\Delta_{\text{sol}}H}{R}\left(\frac{1}{T} - \frac{1}{T^\circ}\right)\right]}$$

where H° is Henry's constant at room temperature (298.15 K), $\frac{\Delta_{\text{sol}}H}{R}$ is the enthalpy of dissolution (1700 K constant for O_2), T is the soil temperature (K), and T° is 298.15 K.

Figure S1. Subsurface concentration of dissolved oxygen, CO_2 , N_2O , and CH_4 at four depths within the control and experimental soil treatment areas. Values are means ($n=3$) and bars and whiskers signify one standard deviation.

Figure S2. Subsurface concentration of CO_2 at four depths within the control and experimental soil treatment areas. Values are means ($n=3$) and bars and whiskers signify one standard deviation.

Figure S3. Subsurface concentration of N_2O at four depths within the control and experimental soil treatment areas. Values are means ($n=3$) and bars and whiskers signify one standard deviation.

Figure S4. Subsurface concentration of CH_4 at four depths within the control and experimental soil treatment areas. Values are means ($n=3$) and bars and whiskers signify one standard deviation. Data has been log transformed to improve visual comparison among sampling events.

Figure S5. Relationship between flux and concentration of (A) dissolved oxygen, (B) CO₂, (C) N₂O, and (D) CH₄ 15 cm below the infiltrative surface in Acushnet STA. Circles represent measurements from the control STA and triangles represent measurements from the experimental STA. Grey shading represent 0.95 confidence interval.

Manuscript 2:
**Structure and Composition of Nitrifying and Denitrifying Bacterial
Communities of Lignocellulose-Amended Soil Treatment Areas
Receiving Wastewater**

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Abstract

Soil-based wastewater treatment is a low-cost, passive treatment option for the 25% of US households that rely on onsite wastewater treatment systems (OWTS; i.e., septic systems). Conventional soil-based treatment, however, does not remove enough N to protect ground and coastal water bodies, endangering human and environmental health. Lignocellulose-amended layered soil treatment areas (STAs) provide enhanced N removal by stratifying nitrification and denitrification into a top layer that is filled with sand and promotes nitrification, and a bottom layer that contains sand and sawdust, which provides a C source and electron donor for heterotrophic denitrification. We collected soil cores from three Experimental (layered) STAs and associated Control STAs – containing only sand – and sequenced bacterial ammonia monooxygenase (*amoA*) and nitrous oxide reductase (*nosZ*) genes using high-throughput MiSeq Illumina sequencing. We observed that *amoA* and *nosZ* diversity and richness were similar between Experimental and Control STAs for all sampling periods and sites, except at one site during a summer sampling event where *nosZ* diversity and richness was higher in the Control than the Experimental STA. The most common classified *amoA* taxon was *Nitrosospora* and the most common *nosZ* taxon was *Afipia*; unclassified bacterial strains were the most common taxa for both *amoA* and *nosZ*. The *amoA* community composition was similar throughout profile of both STAs, but there was a somewhat different denitrifying community between the sand and lignocellulose-amended layers within the Experimental STA. In both Experimental and Control STAs, the composition of *amoA* and *nosZ* communities was correlated with site, sampling event, septic tank N concentrations and pH level, dissolved oxygen and soil temperatures. Using this

information, we can begin to alter the design of lignocellulose-amended layered STAs to promote specific *amoA* and *nosZ* populations and increase N-removal efficiency.

Introduction

In the United States, wastewater from one in five homes is treated using an onsite wastewater treatment system (OWTS; i.e., septic systems) (U. S. Census Bureau, 2006), consisting of a septic tank and a soil treatment area (STA). Water leaving the STA can be a major source of nitrogen (N) pollution to groundwater and coastal ecosystems, threatening human and environmental health (Oakley et al., 2010; Valiela et al., 1997). In Barnstable County, MA, USA, over 85% of homes and businesses rely on OWTS to treat wastewater before it enters the area's only aquifer, which supplies drinking water for its population (Swartz et al., 2006). The high density of OWTS has resulted in harmful levels of N in groundwater, threatening the region's drinking water supply and coastal ecosystems (Costa et al., 2017). Many other coastal communities in the eastern United States face similar problems, including Rhode Island (Lancellotti et al., 2017), Suffolk County, New York (NYSCCWT, 2016), Chesapeake Bay (Maryland and Virginia) (Reay, 2004), and coastal Florida (FDOH, 2018).

The use of proprietary advanced biological N-removal in OWTS can significantly reduce N loads from domestic wastewater (Amador et al., 2018; Oakley et al., 2010), but these systems have a high cost that is borne by homeowners. Lignocellulose-amended layered STAs provide a less expensive, non-proprietary alternative to these systems. They are designed to remove N by stratifying nitrification and denitrification in two layers to maximize coupled nitrification-denitrification (Tobias et al., 2007). Septic tank effluent is first delivered to a top layer of medium coarse sand that promotes aerobic

nitrification. The water then moves through a layer of sand amended with sawdust, which serve as a carbon source and electron donor to support heterotrophic denitrification.

In addition to sequential nitrification-denitrification in the two layers, there may be tightly coupled nitrification-denitrification occurring in microsites in both layers (Sexstone et al., 1985). Other N transformation and removal pathways may also be important in layered STAs, including, but not limited to: (1) anaerobic ammonia oxidation (anammox), which simultaneously removes NO_2^- and NH_4^+ under anoxic conditions (Robertson and Groffman, 2015); (2) nitrifier denitrification, which can be a major source of N_2O in hypoxic conditions (Zhu et al., 2013); (3) complete ammonia oxidation (comammox) by some *Nitrospira* (Daims et al., 2015); (4) ammonia oxidation by methanotrophs when CH_4 concentrations are high (Mandernack et al., 2009); (5) and autotrophic processes in the sand layer including Fe-coupled nitrification, and S^{2-} - or CH_4 -coupled denitrification (Cooper et al., 2016).

The broad range of possible microbial N transformations warrants the investigation of N removal pathways in layered STAs so that we develop a better understanding of the specific N removal mechanisms at play and the factors that control them, allowing us to refine system design to maximize N removal efficiency. An important initial step is characterization of the structure and composition of nitrifying and denitrifying microbial communities, and the relationship between community metrics and physical and chemical properties of the STA, such as nutrient concentration, pH, soil temperature, and dissolved oxygen (DO).

We analyzed the bacterial ammonia monooxygenase (*amoA*) and nitrous oxide reductase (*nosZ*) community structure and composition in STAs at three homes in

southeastern Massachusetts, USA, between July 2018 and July 2019 using high-throughput MiSeq Illumina sequencing. Each STA was split into an Experimental (lignocellulose-amended layered) and Control STAs; the Control STA was designed identically to the Experimental STA and received the same wastewater but did not contain a sawdust amendment. We also collected data on the physical and chemical properties of STA soil and septic tank effluent to examine relationships between community composition and their environment.

Methods

Study systems

We took soil cores from three homes with lignocellulose-amended layered STAs in coastal southeastern Massachusetts, USA. One of the homes (Acushnet) was occupied year-round and we sampled it four times between July 2018 and July 2019. The other two homes (Sippewissett and Chappaquoit) were occupied seasonally, from late spring through the fall, and were sampled once during a period of non-use in spring 2019, and again in summer 2019, when the homes were occupied.

Wastewater leaving the homes flowed by gravity to the septic tank and then the pump chamber, where it was time-dosed to the STA via a GeomatTM dispersal system. The dispersal system is composed of a geotextile fabric surrounding perforated PVC pipes designed to increase the surface area of the infiltrative surface, maximizing the treatment area. This design creates an environment with frequent wetting events that oscillates continuously between wet and dry when the system is in use. All three systems were dosed with wastewater even when they were not in use. Dosing occurred less frequently during periods of non-use at the seasonally-occupied sites, while dosing

frequency at Acushnet was consistent year-round (Table 2.1). Additional details on site design and characteristics can be found in Table 2.1.

There were two STAs at each site, a Control STA and an Experimental (i.e., lignocellulose-amended layered) STA, which abutted each other and received effluent from the same septic tank (Fig. 2.1). The Experimental STA had two layers: a 45-cm layer of sand on top of a 45-cm layer of sand amended with sawdust (1:1 by volume). The sand was medium coarse (0.30 mm effective particle size; D_{10}) and the sawdust was mainly *Quercus spp.* The Control STA had a single, 90-cm deep layer of sand. A plastic liner separated the amended layer of the Experimental STA and the bottom portion of the Control STA (Fig. 2.1).

At the Acushnet site we installed gas sampling ports and EC-5 soil moisture sensors (METER Group, Pullman, WA) at four depths in the Experimental and Control STAs. We also installed Hobo® temperature probes (Onset, Bourne, MA) at the same four depths as the moisture probes in the Experimental STA. DO samples were collected during each sampling events following Wigginton et al. (2020a). Moisture and temperature measurements were recorded every 30 minutes. A summary of the physical and chemical properties of the soil cores and water samples can be found in the Supplementary material (Supplementary Table S1).

Water and Soil Samples

We collected dip water samples for microbial community analyses in autoclaved, 0.5-L plastic bottles from the primary treatment tank during every collection event. At Acushnet we sampled the septic tank, and at Sippewissett and Chappaquoit we sampled

the pump chamber because the septic tank manhole was not accessible. Water samples were stored at 4°C and filtered within 24 hours.

Samples for determination of septic tank effluent properties were collected monthly by personnel from the Massachusetts Alternative Septic System Testing Center, Sandwich, MA (MASSTC), within a two-week window of collection of soil and water samples for microbial community analyses. Total Kjeldahl Nitrogen (TKN) was determined following EPA Method 351.2 (O'Dell, 1996). Nitrate (NO_3^-) and nitrite (NO_2^-) levels were determined following EPA Method 300.0 (Pfaff, 1993). Total nitrogen (TN) concentration was calculated using the following formula:

$$TN = (\text{NO}_3^- - \text{NO}_2^-) + \text{TKN}.$$

We took four soil cores during each sampling event, two from the Control STA and two from the Experimental STA. To collect the cores, we removed surface soil, located the edge of the dispersal system and drove a 3.2 x 102-cm gouge auger (AMS, Inc, American Falls, ID) adjacent to the edge at a 73° angle with the vertical (Fig. 2.1). We used two, 61-cm extenders and a bracing mechanism, which produced cores between 50 and 90-cm long. An additional soil sample was collected from the surface soil above the dispersal system.

Each core was divided into eight to ten subsamples: six for analysis of microbial and physical properties, and two to four for bulk density analysis. We collected samples for microbial and physical properties analysis at 0, 15, 30, 45, 60, and 75 cm below the infiltrative surface; some cores were long enough to obtain a sample at 90 cm. For microbial sampling, we used flame-sterilized tools and sampled the center of the soil core to avoid contamination from soil transported by the coring device. Bulk density samples

were collected in 10.2-cm long sections at 5, 20, 50, and 65 cm. All samples were stored in sterile Whirl-Pak bags (Uline, Pleasant Prairie, WI) at 4 °C during transportation to the laboratory, and at -10 °C or below until extraction.

Soil Properties

We extracted NO_3^- and NH_4^+ from soil samples using a 2 M KCl solution (4:1 ratio of extractant to soil) (Keeney and Nelson, 1982) and determined NH_4^+ (Weatherburn, 1967) and NO_3^- (Doane and Horwáth, 2003) concentration in the extract colorimetrically. The organic matter content of the STA fill material was determined by loss-on-ignition at 505 °C for 5 hours (Dean, 1974). Soil moisture was determined from the difference between wet soil and soil dried overnight at 105 °C. We calculated bulk density from the dry soil weight and the core volume (80.44 cm³) (Black and Blake, 1965).

Subsurface Dissolved Oxygen and Soil Temperature

We measured the concentration of soil O_2 as well as temperature and moisture content at the Acushnet site. Gas diffusion chambers were installed during STA construction at 15, 30, 60, and 75 cm below the infiltrative surface in arrays near the soil core locations. We attached Hobo® temperature probes (Onset, Bourne, MA) to four chambers in the Experimental STA. Soil temperature measurements were recorded every 30 minutes from April 2018 through July 2019. Oxygen samples were collected monthly from November 2018 to July 2019.

To measure the concentration of oxygen, we injected a 60 mL of sample into a flow-through cell connected to an O₂ probe at a rate of ~1 mL s⁻¹ to obtain percent O₂ (Cooper et al., 2015). We converted the oxygen concentrations in the gas phase to dissolved oxygen (DO) using Henry's Law formula:

$$C=HP_{gas}$$

where C is the O₂ concentration in solution, P_{gas} is the oxygen partial pressure, and H is Henry's constant for oxygen. A pressure of 1 atm was assumed, and the Henry's constant value was corrected for temperature using the equation:

$$H(T) = H^{\circ} e^{\left[\frac{-\Delta_{sol}H}{R}\left(\frac{1}{T}-\frac{1}{T^{\circ}}\right)\right]}$$

where H° is Henry's constant at room temperature (298.15 K), $\frac{\Delta_{sol}H}{R}$ is the enthalpy of dissolution (1700 K constant for O₂), T is the soil temperature (K), and T° is 298.15 K.

DNA Extraction, PCR, and Sequencing

For analysis of aqueous samples, we vacuum-filtered ~100 mL of water using a sterile, 0.22-μm-pore-size nitrocellulose filter (Millipore Corporation, Burlington, MA) and extracted DNA from the filter using a PowerWater DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). We extracted DNA from soil using PowerSoil DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA) and DNeasy PowerSoil Kits (Qiagen, Hilden, Germany). We used a modified protocol to increase DNA yield from the STA fill material which was low in genetic material compared to other samples (Supplemental material).

For soil samples, we extracted DNA from 0.25 g of surface soil, but extracted DNA from 1 g of STA fill material (i.e., sand and sand amended with sawdust) because

these had a lower DNA yield. We assessed the quality and concentration of extracted DNA using a Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA) and stored samples at -80 °C until PCR analysis.

We amplified fragments of *amoA* and *nosZ* in single, 50- μ L reactions containing: 2.5 μ L DNA template, 25 μ L MyFiTM Mix (Thomas Scientific, Swedesboro, NJ), 21.25 μ L water, and 1.25 μ L (10 μ M) of forward and reverse *amoA* primers or 1 μ L (10 μ M) of *nosZ* forward and reverse primers. We used primer pairs *amoA* 1F and *amoA* 862R (Junier et al., 2009) and *nosZ* 1F and *nosZ* 1662R (Geets et al., 2007) as described in Wigginton et al. (2018).

We used the following thermocycler settings for *amoA*: 4 min at 94°C, 35 amplification cycles (each 60 s at 94°C, 60 s annealing at 58°C, and 60 s 72°C), and a final extension at 72°C for 5 min. Thermocycler settings for *nosZ* were: 4 min at 94°C, 35 amplification cycles (each 60 s at 94°C, 60 s annealing at 61°C, and 60 s at 72°C), and a final extension at 72°C for 10 min. We checked the quality of the resulting amplicons on a 1% ethidium bromide agarose gel. All samples yielding a single band of the correct size were sequenced at the University of Rhode Island Genomic Sequencing Center using an Illumina Miseq Next Generation Sequencer. PCR products were purified and quantified, then *amoA* and *nosZ* fragments were pooled equimolarly by sample before sequencing.

Sequence and Data Analysis

We processed sequences using QIIME 2 (v. 2019.10; Bolyen et al., 2019) following Cox et al. (2020). We used the q2-demux plugin to demultiplex and quality filter raw reads and then separate reads. To separate *amoA* and *nosZ* sequences that were

pooled during sequencing, we used the q2-cutadapt plugin allowing us to search and filter sequences based on primers and then trim primers from sequences. After the gene sequences were separated, we used the q2-dada2 plugin (Callahan et al., 2016) to join and denoise reads, remove chimeras, and group sequences into unique Amplicon Sequence Variants (ASVs). We used the q2-diversity plugin to rarefy data to the lowest sequencing depth, 17 and 1447 sequences per sample for *amoA* and *nosZ*, respectively, and calculate alpha diversity indices. We assigned taxonomy to ASVs by creating custom *nosZ* and *amoA* reference databases from the National Center for Biotechnology Information Database (NCBI) (Cox et al., 2020) to use in the q2-feature-classifier QIIME2 plugin (Bokulich et al., 2018). To assign taxonomy to representative sequences, we used the classify-sklearn function in the q2-feature-classifier plugin using the classifier we constructed from the NCBI database.

We used RStudio (v. 3.6.1, R Core Team, 2012) and the packages vegan, phyloseq (Bioconductor), and ggplot2 in to calculate and rarefy Bray-Curtis dissimilarity indices, ordinate canonical coordinate analysis (CCA) plots, and construct taxonomic bar graphs. To determine if physical or chemical soil and water properties were correlated with beta diversity distances, we used a non-parametric permutational analysis of variance (PERMANOVA) with 999 permutations for each correlation. We used a confidence interval of 0.95 to determine significance of PERMANOVAs.

Results & Discussion

amoA

Diversity and richness. Richness (the number of unique ASVs after rarefaction) and Shannon's diversity index (a measure of ASV richness and relative abundance) were

both similar between Control and Experimental STAs within a site and sampling event (Fig. 2.2). In spring and summer 2019, when all sites were sampled, alpha diversity metrics (i.e. diversity measures within a sample, e.g. species richness and diversity) were similar in both STA types among all sites. In all sites *amoA* alpha diversity metrics decreased between samples collected 5-cm and 20-cm below the infiltrative surface. Interestingly, we also observed high alpha diversity metrics 50 cm below the infiltrative surface. This pattern of high diversity near the infiltrative surface and closer to the bottom of the STAs may reflect selective pressure with distance from septic tank inputs. The higher diversity of the community directly under the infiltrative surface is likely because it receives inputs of bacteria from the surface soil and septic tank effluent, both of which have a high level of *amoA* richness and diversity (data not shown). The decrease in diversity between 5 and 20-cm below the infiltrative surface could reflect a shift away from the composition of the STE and surface soil communities and towards a community that only oxidize NH_4^+ , as NH_4^+ is likely still high at the 20-cm depth. Higher *amoA* diversity at 50 cm may be due to increased niche space availability for nitrifiers that can oxidize NO_2^- in addition to NH_4^+ , those that can co-oxidize NO_2^- and NH_4^+ , and those capable of nitrifier-denitrification. In the present study, we could not determine if this is occurring using taxonomic data because the majority of *amoA* bacteria observed are unclassified and we do not know which of these strains are capable of the above processes.

At Acushnet, where we had four sampling campaigns, there was evidence of seasonal shifts, with the highest alpha diversity values observed during summer 2019 and the lowest in spring 2019 and winter 2018. Because ammonium oxidizers are sensitive to

low temperatures (Shammas, 1986), it is not surprising that the diversity would decrease, presumably because species that are more sensitive to lower temperatures are not present during colder seasons. In the Experimental STA in winter, however, diversity values in the lignocellulose-amendment remained comparable to other sampling dates, while diversity in the Control STA decreased with depth during this date. It may be that the sawdust amendment helps to insulate this layer, keeping it warmer than in the Control STA.

Community composition. Of the 1,951 unique ASV we observed, none was present in all samples. Twelve *amoA* ASVs occurred in 70% or more of the samples (Table 2.2). Additionally, 350 ASVs were significantly correlated with beta diversity (i.e. diversity measures among samples, e.g. Bray Curtis dissimilarity) ($r^2 = 0.07$ to 0.34), suggesting these strains contribute to the differences among *amoA* communities. Ten of the ASVs driving community differences were also among the most widespread and abundant organisms in the STAs (Table 2.2). This group included *Nitrosospira lacus* APG3, *Nitrosospira* sp., unclassified Nitrosomonadaceae, and uncultured bacteria. *Nitrosospira lacus* strain APG3 is capable of growth at 4°C and a pH of 5 (Garcia et al., 2013). Adaptations to grow at low temperatures and pH likely gives this strain an advantage over other ammonium oxidizers and may explain its consistent presence in the STAs. Some of the *Nitrosospira* sp. had a higher relative abundance over time at Acushnet (Table 2.2), suggesting some strains become more dominant over time in STAs (Fig. 2.3).

At the genus level, the communities at all sites were dominated by *Nitrosospira* and uncultured bacteria (Fig. 2.3). We also observed transient populations of

Nitrosomonas at all sites, which may be at a competitive disadvantage compared to *Nitrosospira* in the soil environment where the latter are the dominant taxa (Aigle et al., 2019). In contrast, analysis of nitrifying microbial communities from layered systems similar to ours by Langlois et al. (2020) found that *Nitrosomonas* was a more important ammonium oxidizing bacterial taxon than *Nitrosospira*. The difference in our results could be due to differences in the wastewater applied to the Experimental STA in the Langlois et al. (2020) study, which was collected from a wastewater treatment plant. *Nitrosomonas* is a faster-growing taxon and can outcompete other nitrifiers in high NH_4^+ environments (Chen et al., 2017), like WTPs. Continued inputs of wastewater from the plant may allow *Nitrosomonas* to build populations that are more competitive in those STAs. Wigginton et al. (2020) observed that *Nitrosomonas* and *Nitrosospira* were both important ammonia oxidizers in advanced N-removal, OWTS but *Nitrosomonas* dominated *Nitrosospira* in a WTP with BNR. Together, these results suggest that both genera can be important in onsite wastewater treatment, but *Nitrosomonas* are likely to be more competitive in aquatic environments. The prevalence of *Nitrosospira* in the septic tank of our systems (Fig. 2.3) may be associated with inputs of soil from the surrounding area, which unavoidably enters the tanks during installation, inspection, and sampling. The long hydraulic retention time (8-10 days) in the tank allows this slow-growing genus to maintain dominance, even in an aquatic tank environment.

We examined differences in *amoA* beta diversity among the three sites during the spring and summer 2019, when all sites were sampled, and found no significant patterns based on STA type (Control vs. Experimental) or fill material (sand vs. sand amended with sawdust). The strongest clustering pattern in *amoA* beta diversity was based on

sampling event, which was significantly correlated with community composition ($r^2 = 0.10$; Fig. 2.5). The pH of effluent was correlated with community composition in spring and summer 2019 at Sippewissett and Acushnet ($r^2 = 0.10$; Supplementary Fig. S1) (pH data were not available for the Chappaquoit site). The total N concentration in the input to the STA and NH_4^+ concentration in the sand fill material in the STA were both correlated with community composition ($r^2 = 0.03$ and 0.05 , respectively; Fig. 2.5). Input characteristics like pH and NH_4^+ affect the microbial community structure in municipal wastewater systems (Chen et al., 2017; Gao et al., 2014). Thus, it is not surprising these properties influence the community composition in the STAs.

Community composition differences were also correlated with site ($r^2 = 0.06$; Fig. 2.5). Communities at the three sites were more similar in summer 2019 than in spring 2019. This could be because all the systems were in use and receiving frequent inputs of wastewater during the summer, resulting in communities adapted to an environment receiving wastewater inputs. In the spring, however, only Acushnet was in use and the other two systems were dormant, receiving infrequent doses from septic tanks that were not receiving inputs from the homes. We expected these differences in community structure among sites since they vary in location, proximity to the coast, and home occupancy patterns.

Other properties including DO and temperature have been found to significantly impact N transforming bacteria community structure in municipal wastewater treatment systems (Chen et al., 2017). To investigate differences in community composition based on these variables we installed additional instrumentation at Acushnet to collect soil temperature, moisture, and DO at depths corresponding to the depths at which microbial

samples were collected. We found that *amoA* community structure at this site was correlated with DO soil concentrations ($r^2 = 0.12$), soil temperature ($r^2 = 0.1$), and soil moisture ($r^2 = 0.09$; Supplementary Fig. S1) which is not surprising as these are important variables that are known to contribute to the structure of soil microbial communities (Fierer, 2017).

nosZ

Diversity and richness. Diversity and richness of nitrous oxide reducing bacteria was similar between Control and Experimental STAs at each site within a sampling date, except for summer 2019, when the alpha diversity metrics at Acushnet were higher in the Control STA than in the Experimental STA (Fig. 2.2). Lower alpha diversity values in the Experimental STA in summer may reflect the establishment of a stable community where a few ASVs maintain robust populations that outcompete other denitrifiers. Alternatively, denitrifiers that do not contain *nosZ* may outcompete those that do when a steady C source is available, and temperatures are above 20 °C. Warneke et al. (2011a) observed an increase in nitrite reductase gene abundances relative to nitrous oxide reductase gene abundances in warm, relative to cold, mesocosms with a design similar to ours. They suggested that populations of bacteria with incomplete denitrifying pathways (i.e., those that end with nitrite reductase) increase when temperatures are high and outcompete denitrifiers with full pathways, which ends with *nosZ*.

In spring 2019, we observed lower diversity and richness at Acushnet than at the seasonally-occupied sites (Fig. 2.2). This is interesting because Acushnet was the only home that was occupied during this sampling event. It may be that a system that receives

wastewater inputs consistently maintains a less diverse community of nitrous oxide reducers than systems that go long periods without receiving frequent wastewater inputs. We also observed a general increase in alpha diversity metrics between the spring and summer 2019 sampling events in all the STAs, indicating that warmer temperatures promote increased richness and diversity of *nosZ*, which has been reported by Wu et al. (2018).

Community composition. Of the 17,443 unique *nosZ* ASVs that we observed, 11 occurred in 50% or more of the samples, and 425 were significantly correlated with beta diversity dissimilarity indices ($r^2 = 0.02$ to 0.97). Many of the ASVs contributing to the differences in *nosZ* community composition were among the most commonly occurring strains (Table 2.3). Most of the *nosZ* ASVs at all sites were uncultured bacteria, highlighting the diversity in undescribed soil organisms (Delgado-Baquerizo et al., 2018). The most common classified genus was *Afipia*, which decreased in relative abundance over time at Acushnet, especially in the Control STA. *Mesorhizobium* was also important among classified strains (Table 2.3) and was most abundant at Acushnet in winter 2018 and at Chappaquoit near the bottom of the STA (Fig 2.4).

Other important genera included *Bradyrhizobium*, present at all sites on all sampling events, and *Azospirillum*, which was widespread but transient. The relative abundance of unclassified *nosZ* bacteria communities increased over time and represented most of the community in summer 2019 at all the sites. The uncultured bacteria at seasonally-occupied sites also increased in relative abundance between the spring and summer of 2019. *Acidovorax* was present in low relative abundances in both seasonally-occupied sites, but was not observed at Acushnet. We observed multiple

genera at Sippewissett that were absent from the other sites, including *Paracoccus*, *Thauera*, and *Zooglea*, all at low relative abundances (Fig. 2.4). The denitrifying communities at our sites differed from those for similar STA systems described by Langlois et al. (2020), who found *Arcobacter*, *Rhodoferrax*, *Sulfuritalea*, and *Dechloromona* as the main denitrifiers. The lack of overlap between our study and that by Langlois et al. (2020) highlights the diversity of denitrifying communities in Experimental STAs.

When we compared beta diversity from all sites in spring and summer 2019, we found that the community composition was correlated with site ($r^2 = 0.05$) and sampling date ($r^2 = 0.05$) (Fig. 2.6). As with *amoA*, site differences were expected because of site specific inputs, differences in environmental variables, and in occupancy patterns. Similarly, Wigginton et al. (2018) found that differences in the *nosZ* bacteria communities in 38 advanced N-removing OWTS in six towns within the Greater Narragansett Bay watershed in Rhode Island were weakly driven by geographical location. When only spring and summer 2019 dates were included, the property with the strongest correlation with *nosZ* community composition was septic tank effluent pH ($r^2 = 0.07$; Supplemental Fig. S1). Fill material (i.e., sawdust vs sand), NH_4^+ concentrations, and soil moisture were all significantly correlated with beta diversity ($r^2 = 0.03$). Additionally, NO_3^- concentration in the STA fill material, bulk density, soil organic matter, effluent TN concentration, and depth below the dispersal system were all weakly, but significantly, correlated with *nosZ* beta diversity ($r^2 < 0.02$). When we included samples from winter 2018, spring 2019, and summer 2019 at Acushnet in the analysis, there was a significant correlation between *nosZ* community composition and soil

temperature ($r^2 = 0.07$), soil moisture ($r^2 = 0.08$), and DO ($r^2 = 0.04$) at the corresponding depth (Fig. 2.6). The large number of variables that significantly affect the community composition of nitrous oxide reducers indicates that these communities are plastic and can vary significantly with small changes in environmental conditions.

Conclusions

We did not observe differences in *amoA* or *nosZ* richness or diversity patterns between the Experimental and Control STAs or among the three sites. As others have observed (Langlois et al., 2020) we saw a large overlap in the nitrifying and denitrifying microbial communities among systems and sampling periods. Most of the *amoA* and *nosZ* ASVs in STAs were unclassified bacteria. The most common classified *amoA* taxon was *Nitrosospira* and the most common classified *nosZ* taxon was *Afipia*; this was true across site and dates for both genes. While dissimilarities in community composition were low, fill material (i.e., sand or sand amended with sawdust) contributed to differences in the *nosZ* community, with the sawdust-amended fill material forming a slightly different *nosZ* community than the Control STA or the nitrification layer in the Experimental STA. The composition of both communities was correlated with the concentration of total N and pH in septic tank effluent, and with DO in soil. Soil characteristics including $\text{NH}_4^+/\text{NO}_3^-$ concentration, temperature, bulk density, soil moisture, organic matter, and the depth below the infiltrative surface were also correlated with differences in the composition of one or both communities, although these explained comparably less of the community variability than effluent N, pH, or soil DO.

Some of the variables that we found to be significantly correlated with community are adjustable. For example, soil moisture conditions can be altered by changing the frequency and volume of dosing, and bulk density and organic matter content can be altered by adjusting the level of compaction during installation and/or by changing the ratio of sand to sawdust. An important next step in improving the design and promoting a productive N removing microbial community will be assessing the extent to which differences in community composition and correlated environmental variables are related to N-removal.

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Figures and Tables

Figure 2.1. (A) Aerial view of soil treatment area showing the location of system components and Control and Experimental STAs. Soil cores were collected at two points in each STA: Experimental (E1 & E2) and Control (C1 & C2). (B) Cross-section of the Experimental and Control STAs showing the location and angle of soil cores.

Figure 2.2. Alpha diversity metrics (richness and Shannon's diversity index) for *amoA* and *nosZ* in the Control and Experimental STAs at the Acushnet, Chappaquout, and Sippewissett sites. Dashed lines represent the top of the lingnocellulose-amended layer in the Experimental STA.

Figure 2.3. *amoA* taxonomy bar plots showing the relative abundance of taxa in Control STAs, and in Experimental STAs, surface soil above the STAs, and in septic tank effluent (STE) applied to STAs, at the Acushnet (top), Chappaquoit (middle), and Sippewissett (bottom) sites. Labels indicate were samples were collected (STE, Control STA, or Experimental STA), the depth below the infiltrative surface, and the replicate (A or B). All y-axis values within a box are relative abundance (0-100%).

Figure 2.4. *nosZ* taxonomy bar plots showing the relative abundance of taxa in Control STAs, and in Experimental STAs, surface soil above the STAs, and in septic tank effluent (STE) applied to STAs, at the Acushnet (top), Chappaquoit (middle), and Sippewissett (bottom) sites. Labels indicate were samples were collected (STE, Control STA, or Experimental STA), the depth below the infiltrative surface, and the replicate (A or B). All y-axis values within a box are relative abundance (0-100%).

Figure 2.5. Canonical correspondence analysis (CCA) biplots relating *amoA* community composition to environment variables for all three sites (top) and for Acushnet (bottom), where additional environmental data was collected. Bray-Curtis distance indices for each sample are represented by a point. The distance between points corresponds to the similarity of the community composition in the sample: shorter distance indicates higher community similarity. Grey lines and arrows represent the environmental data; line length indicates the strength of the correlation between community composition and the environmental variable with longer arrows indicating stronger correlations. The perpendicular distance between microbes and environmental variable axes in the plot reflects their correlations, and the angles between the variable axes corresponds to the correlation between variables. The smaller the distance, the stronger the correlation.

Figure 2.6. Canonical correspondence analysis (CCA) biplots relating *nosZ* community composition to environment variables for all three sites (top) and for Acushnet (bottom), where additional environmental data was collected. Bray-Curtis distance indices for each sample are represented by a point. The distance between points corresponds to the similarity of the community composition in the sample: shorter distance indicates higher community similarity. Grey lines and arrows represent the environmental data; line length indicates the strength of the correlation between community composition and the environmental variable with longer arrows indicating stronger correlations. The perpendicular distance between microbes and environmental variable axes in the plot reflects their correlations, and the angles between the variable axes corresponds to the correlation between variables. The smaller the distance, the stronger the correlation.

Table 2.1. System and site characteristics for sites in the towns of Acushnet and Falmouth (Chappaquoit and Sippewissett) in Massachusetts, USA

Characteristic	Site		
	Acushnet	Chappaquoit	Sippewissett
Occupancy	Year-round	Seasonal (May-Sept.)	Seasonal (June-Sept.)
Installation date	April 20, 2018	April 10, 2017	November 16, 2017
# of occupants	2 adults, 1 infant	2 adults	1 adult
Average daily flow during occupied months (L day ⁻¹)	872	375	450
Average daily flow during unoccupied months (L day ⁻¹)	NA	64	28
Type (width) of Geomat TM	1200 (0.3 m)	3900 (1 m)	3900 (1 m)
Total number of Geomat strips	9	4	4
Vegetation cover and management	Lawn grasses (<i>Festuca sp.</i> , <i>Lolium sp.</i> , <i>Poa sp.</i>) mown short (~5 cm height)	Lawn grasses (<i>Festuca sp.</i> , <i>Lolium sp.</i> , <i>Poa sp.</i>) mown short (~5 cm height)	Little cover in spring; vegetation grew without maintenance before summer sampling (~1 m height)

NA- Not applicable

Table 2.2. Species present in at least 70% of *amoA*. Genus, species and strain of closest cultured specimen is shown (n=172). Samples were collected at Acushnet in summer 2018, winter 2019, spring 2019, and summer 2019, and in spring and summer 2018 at Sippewissett and Chappaquoit.

Genus, species and strain of closest cultured specimen (% identity match)	Sampling Event	Acushnet				Chappaquoit				Sippewissett			
		STE	Surface	Sand	Sawdust	STE	Topsoil	Sand	Sawdust	STE	Topsoil	Sand	Sawdust
<i>Nitrosospira</i> sp. (96%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Nitrosospira</i> sp. (87%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Nitrosospira</i> sp. (96%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Nitrosospira lacus</i> APG3 (98.1%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Nitrosospira</i> sp. (87%)	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Nitrosospira lacus</i> strain APG3 (99.7%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Nitrosospira lacus</i> strain APG3 (99.4%)	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Nitrosospira lacus</i> APG3 (100%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Nitrosospira lacus</i> APG3 (99.4%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Unclassified Nitrosomonadaceae (72%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												

Legend	
# of sequences	Shade
0	
1-250	
251-500	
501-750	
751-1000	
1001+	

*significantly correlated with dissimilarities among samples

Table 2.3 Species present in at least 50% of *nosZ* samples. Genus, species and strain of closest cultured specimen is shown (n=183). Samples were collected at Acushnet in summer 2018, winter 2019, spring 2019, and summer 2019, and in spring and summer 2018 at Sippewissett and Chappaquoit.

Genus, species and strain of closest cultured specimen (% identity match)	Sampling Event	Acushnet				Chappaquoit				Sippewissett			
		STE	Surface	Sand	Sawdust	STE	Topsoil	Sand	Sawdust	STE	Topsoil	Sand	Sawdust
Uncultured bacterium*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Mesorhizobium sp.</i> (99.9%)	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Afipia sp.</i> (99.9%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
<i>Afipia sp.</i> (99.9%)*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												
Uncultured bacterium*	Summer 18												
	Winter 18												
	Spring 19												
	Summer 19												

Legend	
# of sequences	Shade
0	
1-250	
251-500	
501-750	
751-1000	
1001+	

Legend	
# of sequences	Shade
0	
1-250	
251-500	
501-750	
751-1000	
1001+	

*significantly correlated with dissimilarities among samples

Supplemental Material Manuscript 2:

Increasing DNA yield from PowerSoil kit

Info from rep Dr. Tetreault Carlson and from Tips and FAQ portion of MoBio website

Alternative PowerSoil Protocol for RNA and DNA from Low Biomass Soil

1. If starting with the dry glass bead tube from the PowerLyzer PowerSoil Kit, add 0.25 grams soil followed by 500 ul of Bead Solution and 200 ul of phenol:chloroform:isoamyl alcohol pH 7-8 (We buy Amresco brand, catalog# 0883- it comes with the buffer for the phenol). If using the original PowerSoil Kit, remove 200 ul of bead solution from the tube and add in 200 ul of PCI.
2. Add the 60 ul of Solution C1.
3. Vortex 10 minutes. Centrifuge to pellet (1 minute full speed).
4. Remove the supernatant or upper aqueous layer if you have one, to the new tube. Add Solution C2. For low humic soils, reduce it to 100 ul. Add 100 ul of Solution C3 next and mix, and incubate at 4C for 5 minutes or on ice.
5. Centrifuge to pellet (1 minute full speed) and remove the supernatant to a new tube.
6. Ideally you will have 650 ul of lysate and can add 650 ul of Solution C4 and 650 ul of 100% ethanol. If you have 700 ul, add 700 ul of Solution C4 and 600 ul of 100% ethanol.
7. Load the lysate 650 ul at a time and bind in three steps, alternating with centrifugation or using a vacuum manifold.
8. If the membrane is not stained brown, wash with 650 ul of 100% ethanol and then 500 ul of solution C5. If the membrane is stained, per sample, prepare a mix of 300 ul of Solution C4 and 370 ul of 100% ethanol. Wash the column with this mixture first. Follow this wash with the 100% ethanol and the Solution C5.
9. Dry the spin column for 2 minutes full speed. Transfer to a clean tube.
10. Elute in 60 ul of buffer C6. Let the buffer sit on the membrane 5 minutes before elution.

Table S1: Mean (SD) of chemical septic tank effluent (STE) properties. Data at Acushnet collected in between summer 2018, winter 2018, spring 2019 and summer 2019. Data at Chappaquoit and Sippewissett were collected in spring 2019 and summer 2019. n= 2-4.

	Acushnet	Chappaquoit	Sippewissett
STE TN	52 (7)	76 (15)	51 (6)
STE pH	7.6 (0.6)	*	6.8 (0.2)

*Not determined

Table S2: Mean (SD) of physical and chemical properties of STA sand and sawdust-amended fill. Data at Acushnet collected in between summer 2018, winter 2018, spring 2019 and summer 2019. Data at Chappaquoit and Sippewissett were collected in spring 2019 and summer 2019. n= 4-14.

	Acushnet		Chappaquoit		Sippewissett	
	<i>Sand</i>	<i>Sawdust</i>	<i>Sand</i>	<i>Sawdust</i> <i>t</i>	<i>Sand</i>	<i>Sawdust</i>
Moisture (%)	1.8 (2)	4.3 (2.2)	1.0 (0.4)	3.0 (2.6)	1.2 (<0.1)	2.5 (0.01)
Bulk density (g/cm³)	1.2 (0.1)	1 (0.2)	1.2 (0.1)	0.9 (0.1)	1.2 (0.1)	1.1 (0.1)
Dissolved Oxygen (mg/L)**	8.9 (2.4)	6.9 (3.2)	*	*	*	*
NH₄⁺ (μg g⁻)	0.71 (0.7)	0.53 (0.3)	0.09 (0.1)	0.49 (0.4)	0.11 (0.1)	0.14 (0.1)

NO₃⁻ (µg g⁻¹)	0.0018 (0.002)	0.0034 (0.002)	0.001 (0.001)	0.001 (0.001)	0.53 (0.7)	0.66 (0.9)
Organic matter (%)	0.6 (1.9)	4.9 (2.9)	0.8 (1.5)	3.0 (2.6)	0.37 (0.5)	3.0 (0.7)
Soil temperature (°C)**	16.2 (8.1)	15.8 (7.2)	*	*	*	*
Soil Moisture (Volumetric soil content, 240 cm³ cm⁻³)**	0.09 (0.04)	0.15 (0.02)	*	*	*	*

*Not determined

**collected at four depths within each STA. Microbial samples were matched with soil moisture values from the probe nearest to the sampling location.

Supplemental Figure S1 *amoA* (top) and *nosZ* (bottom) CCA including only Acushnet and Sippewissett because STE pH was not collected at Chappaquoit. Only variables significantly correlated to community composition were included in the ordination.

Manuscript 3: Microbial Communities in Centralized and Decentralized Biological Nitrogen Removing Wastewater Treatment Systems

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Abstract

Biological nitrogen removal (BNR) in centralized and decentralized wastewater treatment systems are assumed to be driven by the same microbial processes and to have communities with a similar composition and structure. There is, however, little information to support these assumptions, which may impact the effectiveness of decentralized systems. We used high-throughput sequencing to compare the structure and composition of the nitrifying and denitrifying bacterial communities of nine onsite wastewater treatment systems (OWTS) and one wastewater treatment plant (WTP) by targeting the genes coding for ammonia monooxygenase (*amoA*) and nitrous oxide reductase (*nosZ*). *amoA* diversity was similar between the WTP and OWTS, but *nosZ* diversity was generally higher for the WTP. Beta diversity analyses showed the WTP and OWTS promoted distinct *amoA* and *nosZ* communities, although there is a core group of N-transforming bacteria common across scales of BNR treatment. Differences were driven by soil taxa that were more common in OWTS, and by taxa adapted to low C environments in the WTP. Our results suggest that advanced N-removal OWTS have microbial communities that are sufficiently distinct from those of WTP with BNR, which may warrant different management approaches.

Introduction

Wastewater treatment plants (WTP) and onsite wastewater treatment systems (OWTS) with biological nitrogen removal (BNR) can lower the concentration of N in effluent before it is discharged to receiving waters [1], lowering the public health and environmental risks associated with N pollution of ground and surface waters [2–4]. In both cases the BNR

process employs some type of microbial growth surface and achieves removal by engineering conditions that promote sequential nitrification ($\text{NH}_4^+ \rightarrow \text{NO}_3^-$) in an oxic zone and denitrification ($\text{NO}_3^- \rightarrow \text{N}_2\text{O}, \text{N}_2$) in a hypoxic/anoxic zone. Nitrogen removal is maximized by recirculation of wastewater between the oxic and hypoxic/anoxic zones.

Because they are designed to promote the same microbial processes and conditions, OWTS with BNR – commonly referred to as advanced N-removal systems – are considered to be a scaled-down version of a WTP with BNR [1], with designs explicitly based on engineering principles underlying for a WTP [5,6]. This is based on the expectation that environmental selection – in this case oxic and hypoxic/anoxic conditions – drives microbial community structure [7]. The validity of this assumption can have consequences for effective management of OWTS with BNR.

There are considerable differences between these two types of treatment. Centralized WTPs serve populations ranging from 10^3 to greater than 10^6 and receive inputs from a broad range of uses (e.g., homes, businesses, restaurants, manufacturing facilities, stormwater runoff), resulting in wastewater flows that range from 10^6 to 10^9 L per day [8,9]. In contrast, most OWTS serve single homes with fewer than 10 people, resulting in wastewater flows of $\sim 10^3$ L per day [1], between 1,000 and 1,000,000 $\times$ lower than for a WTP. In addition, the hydraulic retention time (HRT) is shorter in a WTP (1 h or less) relative to an OWTS with BNR (8 - 12 days) [10]. Operation and maintenance conditions at WTPs are closely controlled and monitored continuously in terms of inputs (e.g., flow, C and N levels, pH), process conditions (e.g., aeration rate, dissolved O_2 , availability of organic C, temperature), and concentration of N in final effluent [11]. In contrast, advanced N-removing OWTS are maintained once or twice a year, with maintenance limited to the

physical and mechanical aspects of the systems, and are generally not monitored for N levels in treated effluent [1]. Differences in the magnitude and temporal variability of flow, in sources of microorganisms, type and concentration of electron donors and acceptors, and in the control and monitoring of system operations can result in divergent microbial communities involved in N removal in OWTS *vs.* WTP. Erroneous assumptions about the similarities between these systems with different scales of treatment could result in management and maintenance practices that interfere with, rather than promote, the capacity of advanced N-removing OWTS to lower effluent N levels.

The microbiome of WTPs has been well described using culture-independent, high-throughput sequencing techniques [12] that can identify low abundance and transient taxa in WTP communities more accurately than culture-dependent techniques [13,14]. These advances in molecular microbial probing have allowed for analyses of the metagenome of WTPs, which have shown that communities vary as a function of geography [15–17], time [18–20], influent type [21], and zone within a treatment facility [22]. A number of the N-transforming communities of WTP – including ammonia-oxidizers[23], anammox [24], comammox [25], and denitrification [26] – have been described. Physical and chemical water properties, including levels of dissolved oxygen [27,28] and of NO_3^- and NH_4^+ [29,30], pH [20,29], organic C concentration [31], and temperature [27,32], have been identified as important factors shaping the microbial communities responsible for N removal.

The microbiome of OWTS has been the topic of comparatively few studies, mostly using culture-dependent [33,34] or low-throughput sequencing methods [33,35–37]. Studies describing the metagenome of OWTS using high-throughput sequencing have

focused on the microbial community of the soil treatment area [38–40] or of mesocosms representing conventional septic tanks[41,42].

To our knowledge, only two studies have described the microbiome of BNR OWTS at the whole system scale. Brannon et al.[10] compared the abundance of N ammonia-oxidation and nitrous oxide reduction genes among nine advanced N-removal OWTS and a BNR WTP using quantitative PCR. They found that the abundance of nitrification and denitrification genes normalized by nucleic acid concentration differed between the WTP and OWTS, with higher abundance of nitrifiers at the WTP and higher abundance of denitrifiers in the OWTS. Wigginton et al. [43] reported on the structure and composition of nitrifying and denitrifying communities in 38 advanced N-removal OWTS within the Greater Narragansett Bay watershed, and found that the most prevalent taxa for both genes were also associated with municipal wastewater treatment plants. They also found that the composition of denitrifying, but not nitrifying, communities was weakly driven by geographical location.

Here, we used high-throughput sequencing to describe the ammonium-oxidizing and nitrous oxide-reducing bacterial communities in nine advanced OWTS with BNR in Jamestown, RI, USA and the Field's Point WTP BNR in Providence, RI, USA. Because Wigginton et al. [43] suggested that differences in denitrifying communities may be driven by geography, the OWTS included in the present study were all within a small island (90 km²; Jamestown, RI) about approximately 40 km of the WTP, as the crow flies. We sampled the oxic and hypoxic/anoxic zones in the centralized and decentralized systems in June and October of 2016, and compared the structure and composition of the bacterial functional genes ammonia monooxygenase (*amoA*) – which carries out the first step of

ammonia oxidation – and nitrous oxide reductase (*nosZ*), which carries out the last step of denitrification. This information will improve understanding of the differences and similarities in the structure and composition of nitrifying and denitrifying communities in decentralized and centralized BNR systems, and may lead to management approaches that improve the performance of BNR OWTS.

Methods

Study Systems

The advanced OWTS and the WTP we studied are in the Rhode Island, USA portion of the Greater Narragansett Bay watershed. The OWTS were located in the town of Jamestown, RI, USA, and served three-bedroom dwellings that used wells as their source of potable water, and represented three of the most commonly used types of advanced N-removing OWTS in the state: Orenco Advantex AX-20® (recirculating textile media filter, $n = 3$), BioMicrobics MicroFAST® (fixed activated sludge aerobic treatment unit, $n = 3$), and SeptiTech D Series® (recirculating trickling filter, $n = 3$). All three designs include a hypoxic/anoxic (denitrification) zone, and an oxic (nitrification) zone. The AX-20s are media filters that promote nitrification as water is dosed over hanging textile sheets during recirculation. Similarly, SeptiTech systems are trickling media filter systems that promote nitrification as the water is dosed onto the filter media. FAST systems have a submerged, fixed-film activated sludge design that promotes nitrification via a surface blower that introduces air into a submerged aerobic treatment insert with a ridged-block type media. Wastewater is recirculated between the nitrification component and the denitrification reactor component via time-dosed pumps in the AX-20 and SeptiTech systems; in the FAST systems, air from the blower forces nitrified effluent from the insert back to the

denitrification reactor component through a channel. Further description of the systems can be found in Appendix A and Lancellotti et al. [44].

The Field's Point WTP is in Providence, RI, USA and serves approximately 226,000 residents [10]. It provides treatment for combined domestic and industrial wastewater, and includes an Integrated Fixed Activated Sludge (IFAS) BNR system as part of secondary treatment. The WTP contains 10 identical open-air tanks each consisting of four zones: pre-anoxic, aerated IFAS, post-anoxic, and re-aeration. Further description of the system can be found in Appendix A and in Brannon et al. [10].

Sample collection and DNA extraction

Samples were collected from the WTP and OWTS in June and October of 2016. The oxic and anoxic zones of the nine advanced OWTS were sampled, and one of the IFAS tanks at Field's Point was sub-sampled by zone (Supplemental Fig. A1). Samples from the oxic zone of OWTS were obtained at the recirculating splitter valve, drainfield pump basin, and discharge pump basin within the processor for Advantex, FAST and SeptiTech technologies, respectively (Supplemental Fig. A2). Samples from the anoxic/hypoxic zone of OWTS were collected from the primary processing tank (Supplemental Fig. A2). Samples were collected in sterilized, 1-L plastic Nalgene bottles from just below the water surface and stored at 4°C for a maximum of 8 h before filtering a known volume (~100 mL) of sample onto a sterile, 0.22- μ m-pore-size nitrocellulose membrane filter (MilliporeSigma, Burlington, MA). DNA was extracted from the filters using a PowerWater DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA).

Samples from the WTP were collected from just below the water surface in one of the tanks in the IFAS from each of the four zones: (1) pre-anoxic, (2) aerated IFAS, (3) post-anoxic, and (4) re-aeration (Supplemental Fig. A1). June samples were collected in triplicate and October samples were collected in duplicate. A known volume (~50 mL) of sample was centrifuged at $3,000 \times g$ for 15 min, the supernatant liquid decanted, and DNA was extracted from the solid pellet using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). DNA samples were stored at -20 °C or below until analyzed.

Miseq Illumina Sequencing

Before sequencing, we optimized PCR reactions to amplify *nosZ* and *amoA* target amplicons using the primer pairs in Table A1 [45,46]. Each 50 μ L reaction contained: 2.5 μ L DNA template, 25 μ L BIO-X-ACTTM Short Mix (Bioline, Taunton, MA), 21.25 μ L H₂O, and 1.25 μ L (10 μ M) of each *amoA* primer or 1 μ L (10 μ M) of *nosZ* primers. Thermocycler settings for *nosZ* were: 4 min at 94°C, 35 amplification cycles (each 60 s at 94°C, 60 s annealing at 61°C, and 60 s at 72°C), and a final extension at 72°C for 10 min. Thermocycler settings for *amoA* were: 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. The resulting amplicons were visualized on a 1% ethidium bromide gel and sequenced at the University of Rhode Island Genomic Sequencing Center (Kingston, RI) on an Illumina Miseq Next Generation Sequencer.

Data Analyses

We used QIIME (version 1.9.1; [47]) to process all sequence data following Wigginton et al. [43]. Briefly, we quality-filtered sequences following Bokulich et al. [48], checked for chimeras using USEARCH in de novo mode [49], and clustered sequences into representative OTUs (operational taxonomic units, a proxy for species-level distinction) using swarm[50]. Clusters were based on a 90% identity similarity threshold for *nosZ* [51] and an 85% identity similarity threshold for *amoA*[52]. We rarefied data to the lowest sequencing depth – 2,028 and 1,430 sequences per sample for *nosZ* and *amoA*, respectively – and calculated alpha diversity metrics (species richness and Shannon’s diversity index) using QIIME (version 1.9.1). To identify representative OTUs for *nosZ*, we used the nucleotide-nucleotide Basic Local Search Alignment Tool (BLASTn) to determine the closest sequences in the National Center for Biotechnology Information database [53]. To determine the closest *amoA* identity matches, we cross referenced each representative sequence against the Ribosomal Database Project (RDP) *amoA* bacterial database [54]. Sequence data from our study have been deposited in the Sequence Read Archive (Accession no.: SRP149713; <https://www.ncbi.nlm.nih.gov/sra>).

We used the vegan package (version 2.5-6; [55]) in RStudio (version 3.6.1, [56]) to rarefy samples and calculate beta diversity. We used the packages phyloseq (version 1.30.0, [57]) and ggplot2 (version 3.2.1, [58]) to calculate and graph principal coordinate analyses (PCoA) and taxonomy bar plots.

Results and Discussion

Species Richness and Diversity

amoA

The median number of unique OTUs of bacteria containing *amoA* across all dates and zones was 16.5 in OWTS samples (Fig. 3.1), nearly identical to that for all dates and zones in the WTP (16.2). Richness for *amoA* in the WTP varied considerably among samples, and was highest in the anoxic/hypoxic zone in June (37.8 OTUs), with the lowest value (10.5 OTUs) in the anoxic/hypoxic zones in October. The lowest value of *amoA* richness in the OWTS was 6.7 OTUs in the hypoxic/anoxic zone in October, and the highest value (36.6 OTUs) occurred in the oxic zone in October.

The median value of Shannon's diversity index (a measure of species richness and relative abundance) for *amoA* was higher in OWTS (2.2) than for the WTP (1.6). We did not observe a clear pattern in diversity in OWTS based on either season or zones. In contrast, *amoA* diversity at the WTP was higher in June than October in both oxic and anoxic zones, suggesting a seasonal effect on diversity that was not observed in OWTS (Fig. 3.1). The tanks at the WTP are open to the air, and thus more likely affected by weather variables, such as temperature and precipitation, compared to OWTS tanks, which are installed underground. Others have observed lower *amoA* abundance and phylogenetic diversity with decreasing temperature in NH_4^+ -rich wastewater in WTPs [59]. The similarity in *amoA* richness and diversity between OWTS and WTP may result, at least in part, from the limited number of taxa that are capable of ammonia oxidation [60].

We observed a high diversity of *amoA* bacteria in zones engineered to promote anoxic/hypoxic conditions. The transfer of wastewater from aerated to hypoxic/anoxic components may promote diversification of *amoA* communities in response to low oxygen, by selecting for taxa that are capable of nitrifier denitrification, and/or by consuming O_2 that is produced during anaerobic ammonium oxidation [61,62].

nosZ

Across all dates and zones, the median *nosZ* richness was lower in the OWTS (87 OTUs) than at the WTP (141 OTUs) (Fig 3.1). At the WTP, the lowest (47 OTUs) and highest (225 OTUs) observed richness both occurred in the oxic zone in June. In the OWTS, the lowest observed richness (25 OTUs) was in the oxic zone and the maximum observed richness (188 OTUs) was in the anoxic/hypoxic zone, both during the June sampling event.

Like species richness, the Shannon's diversity index for *nosZ* was consistently higher for the WTP than the OWTS, which had median values of 3.0 and 4.7, respectively (Fig. 3.1). The lowest WTP diversity was 2.4 and the highest value was 5.5, both in the oxic zones during the June sampling event. The lowest diversity value in the OWTS (0.05) was in the anoxic/hypoxic zone in October, and the highest value was 4.4 in the anoxic/hypoxic zone in June.

The capacity, or size, of a WTP is positively related to microbial diversity [63,64]. Our results are consistent with these findings, with higher diversity of *nosZ* at the WTP. This is in line with the taxa-space relationship, a well-established ecological niche principle important in structuring microbial communities [65] which, in this case, is related to the amount of niche space available for denitrifiers in wastewater treatment. In addition to providing more niche space, the WTP has inputs of microorganisms from thousands more inhabitants and from industrial waste streams [10], making it more likely to have greater microbial diversity. Differences in diversity metrics among WTPs with different inputs have been reported, with higher diversity in WTP treating domestic wastewater compared to those treating industrial wastewater [20,21].

Taxa possessing nitrous oxide reductase were similarly diverse between oxic and hypoxic/anoxic zones within a type of treatment, suggesting that *nosZ* communities within a treatment type are not affected by the level of oxygen present. Indeed, strains of *Pseudomonas*, a genus often associated with wastewater treatment and present in our samples (discussed below), can grow and express *nosZ* rapidly in NO_3^- growth media, even in the presence of high oxygen levels [66–68]. *nosZ* diversity is likely not affected by the presence of oxygen because denitrifiers are facultative anaerobes, and some can reduce oxidized N compounds under oxic conditions [69]. Chen et al. [70] suggested that some denitrifiers can simultaneously perform aerobic and anaerobic respiration in a single metabolic pathway under dynamic oxygen conditions. Our observations highlight the plasticity and range of metabolic pathways possessed by facultative anaerobic bacteria.

Community Structure

Beta diversity patterns for *amoA* and *nosZ* showed clear clustering by treatment type, with OWTS samples clustering separately from WTP samples (Fig. 3.2). The chemical composition of influent – i.e., the type and concentration of substrates and inhibitors – drive community differences in WTPs [71], and likely contribute to differences between treatment types for both genes.

We observed tighter clustering patterns among WTP samples compared to samples collected from OWTS. This is not unexpected, since we subsampled a single WTP *vs.* nine OWTS, which were separate in space and affected by unique household inputs. It is surprising, however, that all the WTP samples, even those collected from different tanks on different months, cluster more closely together than many of the OWTS samples collected from the same system, on the same month, but in different components (e.g.,

systems 04 and 07 in Fig. 3.2). This is especially notable, considering the differences in the scale of treatment between WTP and OWTS: BNR treatment zones at the WTP ranged in capacity from 1.4×10^5 to 1.5×10^6 L, whereas the capacity of the largest OWTS component was less than 8.5×10^3 L. The high level of homogeneity in the WTP may be explained by the high flow and low HRT in this type of treatment compared to the OWTS, which likely causes more mixing of species between WTP zones than takes place between OWTS zones.

Four *amoA* samples from the anaerobic tanks were distinct from the main WTP cluster (Fig. 3.2). This, in conjunction with the findings of high *amoA* diversity in anoxic/hypoxic zones discussed above, reinforces the idea that *amoA* may diversify in response to a low oxygen environment.

Taxonomy

amoA

We recovered a total of 494 unique *amoA* sequences from 45 samples collected from the WTP and the nine OWTS. Of these, 195 strains could be matched (85% identity match) to a species in the Ribosomal Database Project (RDP) *amoA* database [72]. All the *amoA* strains we observed were present in at least one WTP and one OWTS sample pointing to the narrow phylogenetic distribution and broad geographic distribution of nitrification capacity. The most ubiquitous ammonia-oxidizing bacteria were in the genera *Nitrosomonas* and *Nitrospira*. Two ammonia-oxidizing species – *Nitrosomonas oligotropha* and an unidentified nitrifier (OTU 1096) – were present in both types of treatment, in both seasons, and in the oxic and anoxic zones of both treatment types. *Nitrosomonas oligotropha* is ubiquitous in WTPs [23,73]. Two other *Nitrosomonas* strains

were also ubiquitous across sampling dates and OWTS locations in both treatment types (Table 3.1). Fan et al. [31] found that *Nitrosomonas* was among the top four genera detected in the entire metagenome of an activated sludge WTP.

Nitrospira – the most common nitrifying bacterium found in soil [74] – is likely introduced into an OWTS from soil that enters the tank initially during installation, and possibly when the tank is periodically opened for inspection. *Nitrosomonas* has a faster growth rate than *Nitrospira* in WTPs [71], which may explain why *Nitrospira* makes up a higher proportion of the community in OWTS, which have a much longer HRT than the WTP, which selects for slow-growing organisms. *Nitrospira* strains had higher relative abundance in the OWTS than at the WTP, accounting for most of the population in most OWTS samples (Fig. 3.3). Similarly, *Nitrosovibrio* was better represented in OWTS than in the WTP. *Nitrospira*, which can co-oxidize ammonium and nitrite [25], was present in the anoxic zones of two OWTS in June, but was not present in the WTP (Fig. 3.3).

Many OTUs abundant in all WTP samples did not match any *amoA* strains in the RDP database. Most of these were present transiently or completely absent from the OWTS samples. Most sequences do not match strains from any database [75]; however, it is interesting that two wastewater treatment systems designed to promote the same microbial processes would have such different relative proportions of identified and unidentified species represented among the most abundant taxa. As suggested previously, these differences may be caused by the introduction of soil when OWTS are installed and inspected, suggesting that soil is an important source of ammonia-oxidizing bacteria for these systems. This would result in a larger number of identified strains in the OWTS

because a portion of their community comes from soil – an ecosystem that has been comparatively better studied than wastewater. For example, a search of ‘*amoA* soil bacteria’ on the NCBI nucleotide database returned 25,224 submissions, whereas searching ‘*amoA* wastewater bacteria’ returned only 6,320 submissions, suggesting that soil *amoA* communities have been better studied than communities in wastewater.

nosZ

In 50 samples from the WTP and OWTS, we identified 3,515 unique OTUs of *nosZ*, of which 1,681 could be matched to a sequence on the NCBI database using a 90% identity similarity threshold [72]. The WTP contained all 3,515 OTUs, while the OWTS contained 2,348 unique sequences. Although only one *nosZ* strain – *Pseudomonas* sp. CC6-YY-74 (Table 3.1) – was present in all OWTS, we found 26 OTUs present across all WTP samples, many of which did not match any *nosZ* species in the NCBI database and were found in few or none of the OWTS. These differences are likely due to the differences in wastewater sources discussed previously, as well as the widespread distribution of the capacity to reduce nitrous oxide [60]. Because the WTP receives inputs from more sources and with a greater microbial diversity, a widespread gene such as *nosZ* is likely to contribute to the higher diversity of the WTP.

Because there were many *nosZ* taxa with low relative abundances, we only included the 50 most abundant taxa in bar plots to increase clarity and visibility (Fig. 3.4). *Aeromonas* and *Pseudomonas* were widespread N₂O reducing genera in both types of treatment. Some OWTS completely lacked one genus or the other, whereas *Pseudomonas* dominated other systems to the point of near exclusion of all other genera (Fig. 3.4). It is

unsurprising that *Aeromonas* was present throughout both types of treatment systems as it is ubiquitously found in water environments, has been detected in many types of food, is a facultative anaerobe, and includes many strains that are human pathogens [76]. *Pseudomonas* sp. CC6-YY-74, the ubiquitous N₂O reducer, has been cultured under aerobic conditions and its complete genome described, which shows that it is capable of full denitrification as well as five other energy-yielding N-transforming pathways [77]. This ability to use a variety of N compounds for energy and growth likely gives this strain a competitive advantage in high N environments like wastewater. Many of the most common N₂O-reducing strains that we observed have been cultivated aerobically (e.g., *Aeromonas*, *Pseudomonas*, *Shinella*), which agrees with our observation of similarities in the *nosZ* communities of oxic and anoxic/hypoxic zones in the centralized and decentralized treatment systems. Homogeneity in microbial community composition across zones and oxygen gradients was also observed by Zhu et al. [20] at four WTPs in China.

Thauera, *Alicyclophilus*, *Oligotropha*, and *Rhodospseudomonas* were also important *nosZ* genera in both types of treatment systems, although again *Pseudomonas* generally outnumbered these in OWTS systems, especially system 04 (Fig. 3.4 and Table 3.1). *Bradyrhizobium*, *Sinorhizobium*, *Burkholderia*, and *Paracoccus* were associated more often with OWTS, whereas *Pseudogulbenkiania*, *Rhodoferax*, *Shinella*, and *Thiobacillus* were more often associated with the WTP (Fig. 3.4). A number of genera found in higher relative abundances in OWTS are usually found in soil, including N-fixers like *Bradyrhizobium* and *Sinorhizobium*. This suggests that the soil entering OWTS during inspection is important to inoculate systems with denitrifying bacteria. Genera that were in

higher relative abundances in the WTP generally had the ability to survive in environments with lower C concentrations than those in the OWTS. For example, *Thiobacillus*, a genus that includes facultative and obligate chemolithotrophs and facultative anaerobes [78], may be more competitive in a WTP, where the organic C levels are lower (due to mixing with inputs with low C concentration) relative to OWTS [1]. In contrast, a higher concentration of organic C in OWTS [1] may favor heterotrophic over autotrophic denitrifiers. Other bacteria found preferentially in WTP include the genus *Rhodoferrax*, a purple non-sulfur bacterium that include strains capable of phototrophy [79] and oxidation of acetate [80], and *Shinella zoogloeoides* BC026, a strain commonly found in WTP that can use pyridine – a common pollutant in industrial wastewater [81]– aerobically as its sole C, N, and energy source (Bai et al., 2009).

As was the case for *amoA*, we observed a higher ratio of strains with a close match on NCBI's database in the OWTS than the WTP for the most common species of *nosZ* (Fig. 3.4), possibly because soil organisms are more likely to be present in higher proportion in the OWTS, and many more soil samples have been submitted to the NCBI database compared to WTP samples. A search of “*nosZ* soil” on the NCBI nucleotide database returned 15,796 submissions, whereas searching “*nosZ* wastewater” returned only 791 submissions, suggesting that soil *nosZ* communities have been better studied than communities in wastewater.

Conclusions

There were major differences in ammonia-oxidizing and nitrous oxide-reducing community composition and structure between centralized and decentralized BNR

wastewater treatment systems. *amoA* richness and diversity were similar at the two treatment scales, but *nosZ* diversity and richness were higher in the WTP than in the OWTS. Ordination analysis of beta diversity showed clear differences in the *amoA* and *nosZ* communities between the WTP and the OWTS. Relative abundances of *Nitrosomonas* and *Nitrospira* were different between the WTP and the OWTS. The higher diversity and closer clustering of beta diversity for *nosZ* in the WTP suggests that the larger scale of treatment supports a wider variety of denitrifiers in sufficiently large numbers to maintain more heterogeneous communities compared to OWTS. We also observed *nosZ* genera with more diverse metabolic strategies in the WTP. Together, these factors may make the WTP more resilient to environmental changes such as shifts in climate and influent properties. Like *nosZ*, *amoA* community composition was more similar within a scale of treatment, but the community of WTP and OWTS had similar alpha diversity metrics, likely because there is a limited number of nitrifying taxa.

The structure and composition of *nosZ* and *amoA* communities were similar between oxic and hypoxic/anoxic zones in both types of treatment, suggesting that differences in oxygen concentration within components are not the main drivers of microbial community composition. Although the WTP and OWTS communities were distinct, a small number of ammonium-oxidizing and nitrous oxide-reducing species were ubiquitous across all treatment types, sampling dates, and replicates. Our results also suggest that the introduction of soil bacteria in the OWTS may drive factor differences in *amoA* and *nosZ* communities between centralized and decentralized treatment systems. If soil is in fact an important inoculum for N-transforming bacteria in OWTS, soil inputs during installation

and the two annual operation and maintenance visits may be important not only to mechanical function, but also to the biological N-removal function of the systems.

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Figures and Tables

Figure 3.1. Species richness (left) and Shannon's diversity index (right) for *amoA* (top) and *nosZ* (bottom) genes in the oxic and anoxic compartments of a wastewater treatment plant (WTP) and of onsite wastewater treatment systems (OWTS) – both with biological N removal – in June (white box) and October 2016 (grey box). *amoA*: $n = 5-7$ for WTP and $5-8$ for OWTS; *nosZ*: $n = 5-7$ for WTP and $5-9$ for OWTS.

Figure 3.2. Principal coordinate analysis based on Bray-Curtis dissimilarity distances for (A) *amoA* and (B) *nosZ* communities in a wastewater treatment plant (WTP) and in onsite wastewater treatment systems (OWTS), both with biological nitrogen removal (BNR). Labels indicate whether sample was from oxic (AER) or hypoxic/anoxic (AN) zone and system or tank number. For WTP: AER1 = activated sludge; AER2 = re-aeration tank; AN1 = pre-anoxic tank; AN2 = post-anoxic tank. For OWTS: AER3 to AER13 = oxic zone; AN3 to AN 13 = hypoxic/anoxic zone.

Figure 3.3. Relative abundance of *amoA* in a biological nitrogen removal (BNR) wastewater treatment plant (WTP) and in advanced N-removal onsite wastewater treatment systems (OWTS). Labels indicate zone (AER = oxic; AN = hypoxic/anoxic), system number, month sampled, and replicate letter (A, B, C; WTP only). WTP = systems 01 and 02; OWTS = systems 03 to 13.

Figure 3.4. Relative abundance of the 50 most abundant *nosZ* taxa in a biological nitrogen removal (BNR) wastewater treatment plant (WTP) and in advanced N-removal onsite wastewater treatment systems (OWTS). Labels indicate component (AER = oxic; AN = anoxic), system number, month sampled, and replicate letter (A, B, C; WTP only). WTP = systems 01 and 02; OWTS = systems 03 to 13.

Table 3.1. Species present in at least 70% of samples. Genus, species and strain of closest culture specimen based on sequencing of *nosZ* and *amoA* in OWTS and a WTP. The total number of samples analyzed was 36 for OWTS and 18 for WTP. Data from June and October 2017 samples were combined for this analysis.

Gene	Genus, species and strain of closest cultured specimen	Id. match (%)	System type					
			Combined		OWTS		WTP	
			# samples with gene	Mean # seq./sample	# samples with gene	Mean # seq./sample	# samples with gene	Mean # seq./sample
<i>amoA</i>	<i>Nitrosomonas oligotropha</i> Nm75	97	45	10,899	25	13,061	20	8,197
	<i>Nitrosomonas</i> sp. Is79A3	94	44	2,710	24	1,987	20	3,577
	<i>Nitrosomonas</i> sp. JL21	86	43	52	24	77	19	20
	<i>Nitrosomonas</i> sp. JL21	97	32	252	12	157	15	20
	<i>Nitrosomonas</i> sp. Nm59	85	41	137	21	117	20	158
	<i>Nitrosomonas</i> sp. Nm59	92	36	649	16	129	20	1,064
	<i>Nitrosomonas</i> sp. Nm59	88	38	313	18	59	20	542
	<i>Nitrosomonas</i> sp. PY1	90	35	87	20	281	20	570
	<i>Nitrosomonas</i> sp. SN1	92	40	140	20	254	20	25
	<i>Nitrospira</i> sp. CT2F	86	36	55	20	80	16	24
	<i>Nitrospira</i> sp. L115	91	40	119	22	186	18	36
	<i>Nitrospira</i> sp. Wyke2	96	44	2,522	25	4,093	19	455
	<i>Nitrosovibrio</i> sp. RY3C	99	39	73	22	106	17	30
<i>nosZ</i>	Unclassified sp. (denovo 1096)	- *	45	18,114	25	5,075	20	34,412
	<i>Aeromonas media</i> WS	99	46	1,553	28	1,622	18	1,444
	<i>Alicyclophilus denitrificans</i> K601	99	40	111	24	176	16	13
	<i>Hyphomicrobium denitrificans</i> ATCC	99	41	66	25	102	16	11
	<i>Oligotropha carboxidovorans</i> strain C1S131/132.2	99	42	192	25	170	17	223
	<i>Pseudomonas</i> sp. CC6-YY-74	99	50	4,626	32	7,059	18	298
	<i>Sinorhizobium fredii</i> USDA 257	90	40	113	22	103	18	125
	<i>Sinorhizobium fredii</i> USDA 257	90	44	302	27	481	17	20
	<i>Thauera phenylacetica</i> strain TN9	90	45	845	27	1,374	18	53
	<i>Thauera phenylacetica</i> strain TN9	92	48	594	30	599	18	587
	Unclassified <i>Alphaproteobacteria</i>	77	45	375	27	459	18	249

<i>Unclassified</i>	74	37	223	19	96	18	357
<i>Alphaproteobacteria</i>							
<i>Unclassified</i>	76	40	125	22	38	18	230
<i>Betaproteobacteria</i>							
<i>Unclassified</i>	76	38	475	21	154	17	871
<i>Betaproteobacteria</i>							
<i>Unclassified</i>	72	38	203	20	59	18	363
<i>Betaproteobacteria</i>							
<i>Unclassified</i>	76	36	259	18	43	18	474
<i>Betaproteobacteria</i>							

* No match was found.

Supplemental Material Manuscript 3:

Supplemental Table A1. Primer sets used for qPCR analysis of nitrous oxide reductase (*nosZ*) and bacterial ammonia monooxygenase (*amoA*) genes.

Gene	Forward primer	Reverse primer	Reference
nosZ1F/1662R	CGY TGT TCM TCG ACA GCC AG	CGS ACC TTS TTG CCS TYG CG	Geets et al. (2007)
amoA1F/862R	GGG GTT TCT ACT GGT GGT	GAA SGC NGA GAA GAA SGC	Junier et al. (2009); Geets et al. (2007)

Figure A1. Aerial view of one of the ten Integrated Fixed Film Activated Sludge (IFAS) tanks at the Field's Point WTP. Black bars represent barriers. Water flows from left to right. Redrawn from Brannon et al. [10].

Supplemental Figure A2: Schematic diagram of Advantex, FAST, and SeptiTech technology treatment trains showing sampling locations. P = pump; SP1 = anoxic component; SP2 = oxic component. From Lancellotti et al. [44].

Epilogue

To address the issue of N pollution from wastewater treatment systems, I investigated lignocellulose-amended layered STA designed to remove N from onsite wastewater treatment systems (OWTS) in Manuscripts I and II. These papers focused on the GHG emissions of layered STAs and the N-transforming microbial community in these systems. I also compared the microbial community of advanced N-removal OWTS and a WTP designed to support BNR in Manuscript III to investigate the similarities and differences in these two types of treatment.

I investigated the greenhouse gas emissions of lignocellulose-amended (Experimental) and unamended (Control) STAs to compare their impact on air quality. The latter is the standard STA in Massachusetts. As the number of systems increase in southern New England and along the eastern U.S. coast – to lower N inputs from septic systems – it is important to ensure we are not compromising air quality as a result of improved N removal. There was no significant difference in GHG emissions between the Experimental STAs and Control STAs at any of the three sites during any sampling date, suggesting that lignocellulose-amended layered STAs will not negatively impact air quality relative to a standard STA. I did find evidence that onsite wastewater treatment in general does impact air quality, as both Control and Experimental STAs had significantly higher CO₂ and N₂O flux than a Reserve STA engineered identically, but not receiving wastewater inputs. Both Control and Experimental STAs had higher flux values than those reported by others, possibly due to the use of low-profile dispersal systems in both STAs. Low-profile systems may produce higher GHG emissions than STAs installed deeper in the profile simply because there is less soil between the wastewater dispersal area and the atmosphere for gases to be consumed.

I also investigated the N transforming microbial communities in lignocellulose-amended layered (Experimental) and Control STAs to elucidate similarities and differences in community structure and composition. I did not observe differences in *amoA* or *nosZ* richness or diversity patterns between the Experimental and Control STAs within a site, or among the three sites I studied. There was also considerable overlap in the nitrifying and denitrifying microbial communities among systems and sampling periods. While dissimilarities in community composition were low, fill material (i.e., sand or sand amended with sawdust) contributed to differences in the *nosZ* community, with the sawdust-amended material forming a slightly dissimilar *nosZ* community than the Control STA or the sand layer in the Experimental STA. Community differences were correlated with the concentration of N and pH in septic tank effluent and DO in soil. Some of the variables that were significantly correlated with community, such as bulk density and organic matter content, could be controlled during system installation to enhance desired N transformations.

An important next step in improving the design of lignocellulose-amended layered STAs that remove N effectively will be assessing the extent to which differences in community composition, GHG emissions, and the environmental variables controlling these, are related to N removal.

When I compared the ammonia-oxidizing and N₂O-reducing microbial communities of a BNR WTP and nine proprietary advanced N-removal OWTS, I observed major differences in community composition and structure between the two types of systems. Relative abundances of *Nitrosomonas* and *Nitrosospira* were different between the WTP and the OWTS. The higher diversity and closer clustering of beta

diversity for *nosZ* in the WTP suggests that the larger scale of treatment supports a wider variety of denitrifiers in sufficiently large populations to maintain more heterogeneous communities compared to OWTS. *nosZ* and *amoA* community composition and structure were similar between oxic and anoxic zones in both types of systems, suggesting that differences in oxygen within components may not be the sole driver of microbial community composition. Although the WTP and the OWTS communities were distinct, a small number of ammonium-oxidizing and nitrous oxide-reducing species were ubiquitous across all treatment types, sampling dates, and replicates. Because these differences in community are likely driven by capacity and influent characteristics that we cannot control, I suggest that OWTS professionals focus on designing systems that maximize treatment in systems with a small capacity and few input sources instead of attempting to design systems based on conventional knowledge from WTPs.

Addressing wastewater originated N pollution in southern New England and beyond will require a multifaceted approach and solutions will likely differ by community and by homeowner. In communities that have adequate land for infrastructure homes may connect to a centralized WTP if enough stakeholders buy into this solution. In communities that have very shallow water tables and small properties, homeowners will likely replace conventional OWTS with tank-based proprietary advanced N-removal OWTS. Communities that do not connect to a WTP and have deep enough water tables and large enough properties to accommodate a non-proprietary, passive lignocellulose-amended STA may choose to use these as replacements for conventional STAs. Together these solutions will significantly lower the N loading from OWTS to ground and coastal waters (Amador et al., 2018).