



# Comparative nitrogen removal via microbial ecology between soil and green sorption media in a rapid infiltration basin for co-disposal of stormwater and wastewater

Dan Wen, Andrea Valencia, Diana Ordonez, Ni-Bin Chang\*, Martin Wanielista

Department of Civil, Environmental, and Construction Engineering, University of Central Florida, Orlando, FL, USA

## ARTICLE INFO

### Keywords:

Biological nitrogen removal  
Rapid infiltration basins  
Microbial ecology  
Green sorption media  
Groundwater conservation

## ABSTRACT

In this study, a rapid infiltration basin (RIB) designed as green infrastructure for co-disposal of wastewater effluent and stormwater runoff was retrofitted for sustainable groundwater recharge after nitrogen removal. For comparison of nitrogen removal efficiency via different filtration media, the RIB was divided into two sub-basins for different filtration processes. One sub-basin was filled with a native sandy soil with about 2–4% clay (Control RIB), and the other sub-basin was modified with Biosorption Activated Media (BAM) (BAM RIB), for the enhancement of microbial nitrogen removal. The two sub-basins accept an equal amount of excess reclaimed wastewater in non-storm periods, and stormwater during periodic storm events. The infiltrate in both the BAM RIB and the Control RIB eventually reaches the Upper Floridan Aquifer. The seven microbial species involved in this microbial ecology study are nitrite oxidizing bacteria (NOB), ammonia oxidizing bacteria (AOB), anaerobic oxidation of ammonium (anammox) bacteria, complete ammonia oxidizer (Comammox) bacteria, denitrifiers, dissimilatory nitrate reduction to ammonium (DNRA) and ammonia-oxidizing archaea (AOA). The population dynamics study was conducted with the aid of the quantitative polymerase chain reaction (qPCR) for the quantification of the microbial gene population in support of microbial ecology discovery. The qPCR results demonstrated the competition effect between AOA, AOB, and Comammox, the inhibition effect between NOB and DNRA with the presence of anammox, and the complementary effect due to an abundance of NOB and AOB in the microbial ecology. Although, competition between denitrifiers and DNRA was expected to impact population dynamics, both microbial species were found to be the most predominant in both control and BAM RIBs. Research findings indicate that the use of BAM RIB achieves significantly efficient nitrogen removal driven by complementary effects in the microbial ecology.

## 1. Introduction

Nitrogen pollution from wastewater effluent, agricultural discharge, and stormwater runoff has seriously impacted aquatic environments, altered the natural nitrogen (N) cycle, and affected ecosystem integrity. The National Academy of Engineering in the United States has consistently indicated that understanding and managing the nitrogen cycle is one of 14 grand challenges for engineering in the 21st century (National Academy of Engineering, 2018). In addition to nitrogen, phosphorus is of concern because the commonly exist Karst regions in Florida presents a challenge for controlling groundwater contamination from phosphorus (Liao et al., 2019), nitrate (Denizman, 2018), and seawater (Xu et al., 2019) intrusion due to high permeability and porosity (Upchurch et al., 2019).

The primary sources of nitrogen and phosphorus pollution in

surface and groundwaters are fertilizers and wastewater effluents (Isobe and Ohte, 2014; Mueller and Helsel, 2017). Recent research trends regarding wastewater treatment have focused on improving the aerobic/anaerobic treatment process toward substantial biological nutrient removal (BNR) in the effluent (Isobe and Ohte, 2014). In BNR for nitrogen, the application of nitrification-denitrification through a few intertwined unit operations involving microbial organisms has been employed in many tertiary wastewater treatment plants (e.g., the four-stage Bardenpho process, Modified Ludzack–Ettinger Process, Anoxic Step-Feed Process, etc.) (Jeyanayagam, 2005; Qiu et al., 2010). These improved wastewater treatment processes have been applied worldwide to reduce nutrients in receiving water bodies at the expense of high energy consumption (Goldstein and Smith, 2002). Oftentimes, when there are acceptable disposal alternatives for wastewater effluent, a rapid infiltration basin (RIB) may be used in a wastewater treatment

\* Corresponding author.

E-mail address: [nchang@ucf.edu](mailto:nchang@ucf.edu) (N.-B. Chang).

<https://doi.org/10.1016/j.envres.2020.109338>

Received 5 August 2019; Received in revised form 16 January 2020; Accepted 2 March 2020

Available online 03 March 2020

0013-9351/ © 2020 Elsevier Inc. All rights reserved.

plant for final disposal and groundwater recharge. Some RIBs are commonly used for both wastewater effluent and stormwater runoff alternately, through which higher nitrogen contents can be partially removed by soil layers in RIBs before reaching groundwater aquifers.

The removal of nitrate in wastewater treatment processes and natural water bodies is associated with microbial interactions (Arriagada et al., 2017). Contamination of groundwater aquifers by nitrogen-laden wastewater effluent and stormwater runoff can trigger additional growth of ammonium oxidizing bacteria, nitrate oxidizing bacteria, nitrate reduction bacteria, and archaeal species. However, the microbial community in the soil layers of RIBs can play an important role in nitrogen removal as an integral part of the N-cycle in a microbial ecosystem (Isobe and Ohte, 2014). The microbial community includes, but is not limited to, anaerobic ammonium oxidation (anammox) bacteria, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), complete ammonia oxidation (Comammox) bacteria, dissimilatory nitrate reduction to ammonia (DNRA) and denitrifiers. The ammonia oxidation performed by AOA and AOB in the initial step of nitrification under aerobic conditions (presence of oxygen) provides nitrite for NOB to convert into nitrate (Isobe and Ohte, 2014; Yao et al., 2011). To avoid drastic competition, AOA and AOB prefer differing environments in terms of soil pH, oxygen availability, and carbon source (Qin et al., 2017; Yao and Peng, 2017; Yin et al., 2018). Although, the role of AOB in nitrification has been well-studied; the role of AOA in nitrification has only recently been realized (Tao et al., 2017).

New analyses have changed the conception of AOB as the only microorganism capable of ammonia oxidation, as AOA has also been found to take part in ammonia oxidation in wastewater treatment (Yin et al., 2018). Further, the recent discovery of Comammox bacteria (Daims et al., 2015; Van Kessel et al., 2015; Dang and Chen, 2017; Annavajhala et al., 2018) has transformed the understanding of nitrification, as previously it was believed that nitrification could only be performed by the two aforementioned microorganisms (Costa et al., 2006; Koch et al., 2019). Comammox bacteria have been found in wastewater and can oxidize ammonia while concurrently converting nitrite to nitrate (Annavajhala et al., 2018). The interaction between distinct microbial species in nitrification can negatively or positively impact NOB nitrite oxidation by forming competitive or cooperative relations among microorganisms while denitrifying bacteria contribute to the balance of input-output nitrogen by transforming nitrate to nitrogen gas released to the atmosphere (van den Berg et al., 2017). Lastly, the analysis of DNRA further contributes to link ammonium production to its use by nitrifying bacteria and the retention of nitrogen in the system (Friedl et al., 2018). Therefore, the evaluation of the role of AOA, Comammox, and DNRA in the microbial community could be critical for enhancing the understanding of the N-cycle, especially through comparison in two different types of sub-basins, one of which contains natural soil, while the other contains engineered green sorption media.

In this study, an existing RIB was divided into two sub-basins for infiltration of wastewater effluent and stormwater runoff alternately to recharge the groundwater aquifers. One sub-basin was called the Control RIB, which contained natural soil, and the other was called the Biosorption Activated Media (BAM) RIB (i.e., the BAM RIB), which contained 60 cm (2 feet) of BAM covering the surface of the sub-basin for enhanced nutrient removal. BAM are known as green sorption media due to their use of recycled tire crumb in conjunction with clay and sand. Chemophysical nitrogen and phosphorus removals for this site were investigated by Cormier and Duranceau (2019). However, no microbiological interactions responsible for nitrogen removal were considered.

The quantification of the population dynamics of the relevant microbial species was conducted by using the quantitative polymerase chain reaction (qPCR) to determine the relationship between the bacteria gene population and nitrogen removal in a recirculation filtration

tank, a passive septic tank underground drainfield, and a constructed wetlands with the inclusion of BAM media (Chang et al., 2010; Hossain et al., 2010; Xuan et al., 2010; Chang, 2011). However, none of the previous studies focused on microbial ecology to explore the possible complementary and inhibitory effects among microbial species at the community scale in both natural soil and BAM layers. In this context, microbial ecology is the ecology of microorganisms and their relationship with one another and with their environment, in which three major domains of life (Eukaryota, Archaea, and Bacteria) are of primary concern. It is thus essential to deepen the understanding of the microbial ecology in RIBs for future field applications.

We hypothesized that water at each sub-basin of a RIB serves as a source of groundwater recharge, where better nitrogen removal can be made possible through the use of BAM in the BAM RIB. The implementation of a RIB allows the slow infiltration of reclaimed wastewater and stormwater into the aquifers, reducing the impact of groundwater extracted for tap water consumption. We also hypothesized that NOB will inhibit the growth of anammox, as both depend on the same food source (nitrite) via microbial ecology. Both NOB and anammox compete for the acquisition of nitrite first under two oxygen availability environments. The research questions to be answered include: 1) Are there any complementary and/or inhibitory effects between any of the seven targeted microbial species? 2) How do the different microbial species responsible for aerobic ammonia oxidation interact and compete? 3) Do such complementary and inhibitory effects differ across the control RIB and BAM RIB? 4) Does denitrifying bacteria dominate over DNRA in the N-cycle? 5) How does the presence of AOA and comammox address NOB survival in the nitrification process? The objective of this study is thus to examine the microbial ecology comparatively among targeted microbial species that could significantly affect the nitrogen removal in the two sub-basins of the RIB with or without the presence of BAM in the soil layers.

## 2. Materials and methods

In the southeastern United States, the system of aquifers in Florida provides potable water to approximately 10 million people (Bouchard et al., 1992; Berndt et al., 2014). Excess nitrate concentrations in groundwater and surface waters that are used as sources of drinking water can seriously impact the health of the human population if consumed or exposed. One salient example is a toxic response to nitrite concentrations in drinking water, called methemoglobinemia, which involves the inhibition of oxygen transport in the blood, particularly for infants (Bouchard et al., 1992). In most cases, surface water systems are affected by eutrophication caused by excess nitrogen and/or phosphorus pollution (Wang et al., 2015). By applying discharge to basins containing porous soil that enables infiltration, RIBs have been utilized for the final disposal of wastewater effluent or stormwater runoff at locations where direct discharge to surface waters is limited (EPA, 2003). The primary uses of RIBs include groundwater recharge and storage of treated water for later reuse via aquifer storage and recovery facilities; RIBs can therefore be considered green infrastructure for sustainable groundwater recharge (Crites, 1981; O'Reilly et al., 2012a; O'Reilly et al., 2012b).

### 2.1. Study location and RIB operation

This study was conducted between January 2017 and February 2018, and the study site has two sub-basins modified from an existing RIB previously utilized for storage of excess stormwater runoff collected from the city of DeLand, Florida. The Water Reclamation Facility (DeLand WRF hereafter) was originally designed to treat 22,712 Cubic Meters a Day (CMD) (or 6 MGD (million gallons per day)). The DeLand WRF treatment process train includes bar screen, grit chamber, pumping station, aeration carousel, media filter, chlorine contact basin for disinfection, and storage tank, all of which is in parallel with the

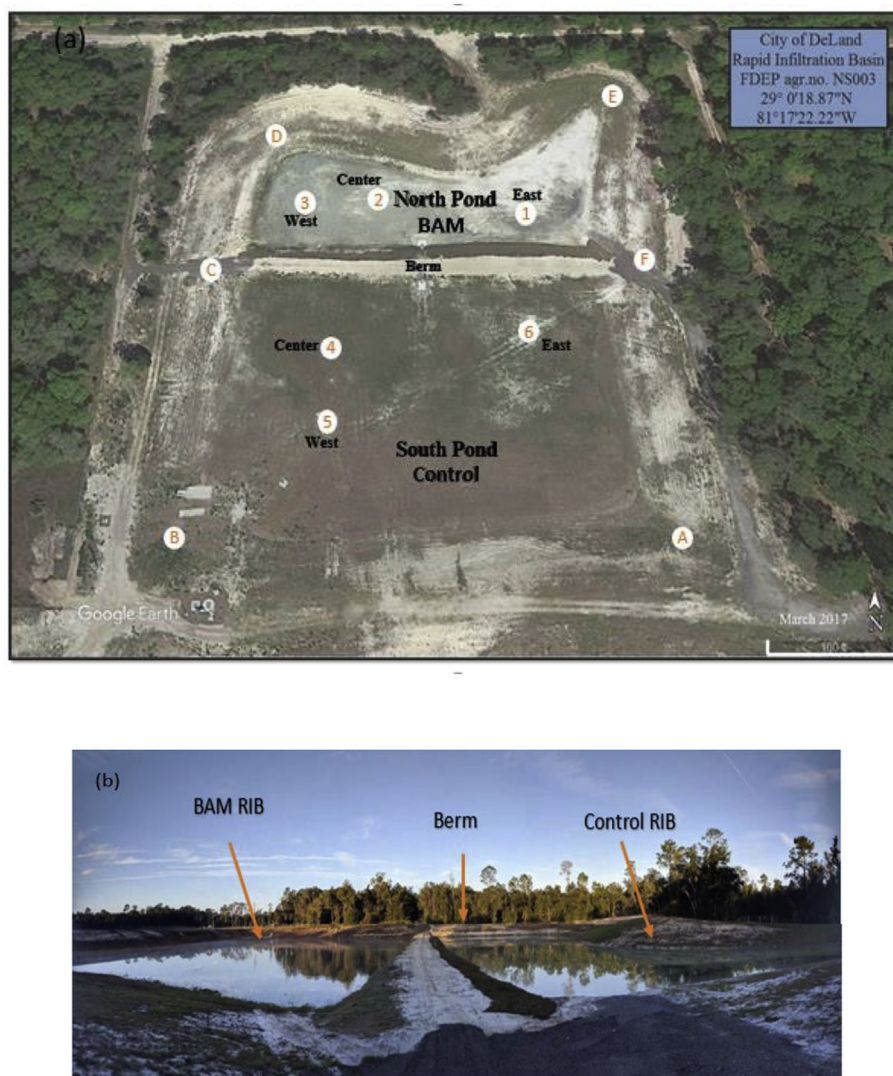


Fig. 1. (a) Sampling Locations of BAM and Control RIBS (source: EFIRD Surveying Group, November 11, 2016, provided by the City of DeLand), (b) 3D photo of RIB (Source: Google Maps (reference time March 2017)).

sludge treatment train including sludge storage and belt press operation. It is operated to produce reclaimed water for irrigation or final disposal to groundwater aquifers. In the process design, the DeLand WRF reduces nitrogen using a combination of aerobic and anoxic treatment zones. Currently it operates at about half its treatment capacity, thus providing an opportunity for additional nutrient removal. When there is not sufficient reclaimed water to meet irrigation demands, additional water is delivered from the St. Johns River. When there is excess reclaimed water in the storage tank, it is discharged to the RIBs. During flood conditions, the storage water can consist of a blend of reclaimed, river, and stormwater. Thus, the RIBs, while usually only disposing of excess reclaimed water, can also dispose of a blend of water, including excess stormwater. An above ground storage tank was added to reduce effluent discharge to the St. Johns River and return more water to the aquifer. The water in the storage tank of the facility is discharged to the RIBs.

The difference in nitrogen removal between the two sub-basins was analyzed through a holistic comparison (Fig. 1a). The Control RIB maintained the original sandy soil that contained approximately 2–4% clay, whereas the BAM RIB contained 60 cm (2 ft) of green sorption media (BAM). The soil characteristics of each RIB, such as media uniformity, are expected to impact nitrogen removal. The BAM RIB, constructed on the north side of the existing RIB, has an area of about

0.405 ha (1 acre or 43,600 square feet (SF)), while the Control RIB has an area of about 0.680 ha (1.68 acres or 73,200 SF). The construction cost was \$400,000 per acre, with a unit removal cost of \$352/Kg (\$160/pound) in a 20-year operational time frame, given that there are minimal operating and maintenance costs for the BAM-modified RIB.

When RIBs were periodically loaded, a measure of the water in a monitoring well was conducted to compare the groundwater and surface water table depths. The well is located in the separation berm between the BAM RIB and the Control RIB (Fig. 1b). The RIBs were used to store excess stormwater during and immediately after Hurricane Irma (Landfall date: Sept. 10, 2017). The depth of storage for the excess Hurricane Irma stormwater was greater than the top of the well used to measure groundwater and was in a flooded condition until the end of December 2017. Thus, no data on well depths are available for that time period. In total, 12 events in a 13-month time frame were investigated, including 10 reclaimed wastewater disposal and 2 stormwater events courtesy of Hurricane Irma (Fig. 2). However, in the months of January and February 2018 the water table rose above the bottom of the RIBs by 30–60 cm (1–2 feet). The rate of infiltration during this period decreased to about 0.32–0.64 cm/h (0.125–0.25 inch/hour). During other times in 2017, the infiltration rates varied between 1.12 and 5.08 cm/h (0.5 and 2.0 inches/hour).



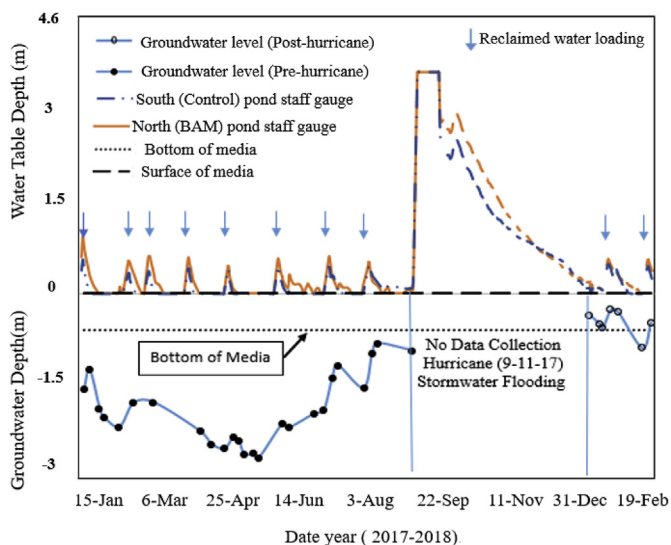


Fig. 2. Depth of water in the BAM RIB and the Control RIB with the bottom of the media as the reference point; reclaimed water loading is marked by downward blue arrows (Chang, 2018). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. Material characteristics

The BAM in this study are a mixture of clay, tire crumb, and sand (also called “CTS” specification) with a ratio of 5%, 10%, and 85% by volume, respectively. The measured bulk density was 1,009 kg/cubic meter (63 pounds/cubic foot) with a porosity of 32% at a dry condition without compaction. The compacted CTS at the BAM RIB was about 1,441 kg/cubic meter (90 pounds/cubic foot). The depth was 60 cm (2 feet). The BAM, which have been proven cost-effective and sustainable, were manufactured with natural and recycled materials. At least 2%, but less than 6%, of the final product size passed the #200 sieve. The BAM mix was composed of 85% poorly graded sand and 15% sorption materials by volume. The sorption materials were composed of recycled tire crumb with no metal content and mined clay that had no less than 99% clay content. Percentages were determined by in place volume. The mix was non-flammable, as tested up to 250 °C (482 °F). Water passing through the media did not exhibit acute or chronic toxicity and did not change the pH of the filtered water by more than 1.0 unit. The BAM mix has a water holding capacity (amount of water that the media can hold for crop use) of at least 10%, and a total porosity of 35%. The permeability, as measured in the laboratory, was greater than 2.54 cm per hour (1.0 inch per hour) at maximum compaction.

The particle size distribution for BAM and natural soil, as well as the physical properties of natural soil and BAM, are presented in Fig. 3 and

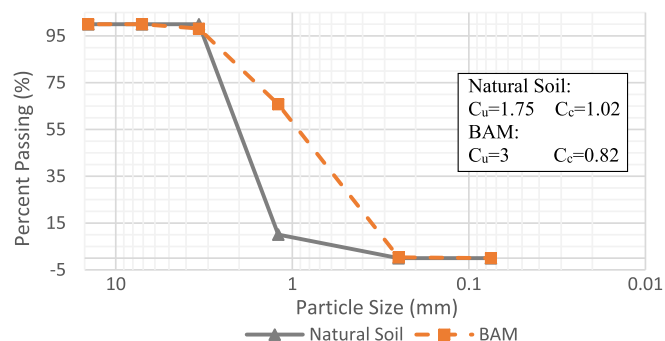


Fig. 3. Particle size distribution for BAM and natural soil.

Table 1 Physical properties of BAM and natural soil.

	BAM	Natural Soil
Density (g/cm <sup>3</sup> )	1.39	1.56
BET Surface Area (m <sup>2</sup> /g)	0.7059	0.3150
Porosity (%)	40.10 ± 2.28	34.22 ± 0.49
Hydraulic Conductivity (cm/s)	0.026 ± 0.48	0.020 ± 0.0007

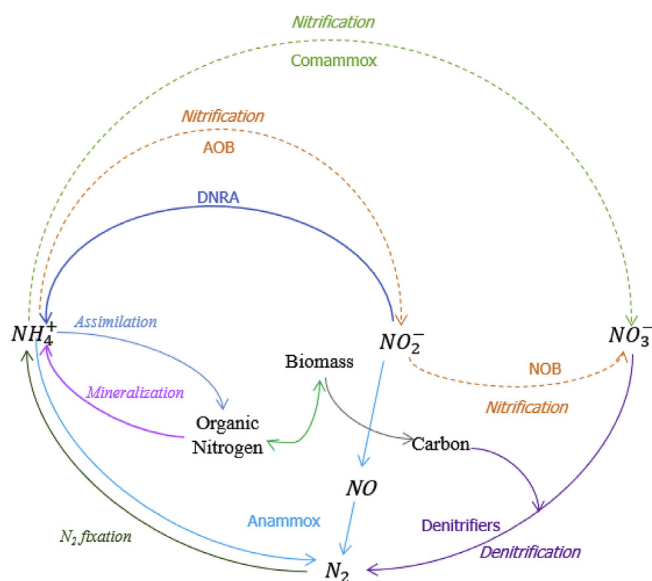
Table 1, respectively. The uniformity coefficient ( $C_u$ ) and coefficient of curvature ( $C_c$ ) were derived from the gradation curves (Fig. 3). The  $C_u$  for both BAM and natural soil laid below 4, indicating that they are uniformly graded. Furthermore, BAM has a higher  $C_u$  value in comparison to natural soil, implying a higher range of particle size. The  $C_c$  values laid below 1 or approximately equal to 1, confirming that natural soil and BAM are uniformly graded. The BET surface area of natural soil was approximately half of that of BAM as a consequence of clay particles contributing to more having difference in particle sizes in BAM. The larger BET surface area and porosity of BAM can be associated with a greater capacity for adsorption based on the availability of pores and exterior particle surface. Lastly, the hydraulic conductivity provides insight as to the ease that water travels through the porous spaces. BAM and natural soil possess similar hydraulic conductivity values, although the hydraulic conductivity of natural soil is slightly slower.

2.3. Microbial pathways for nitrogen removal

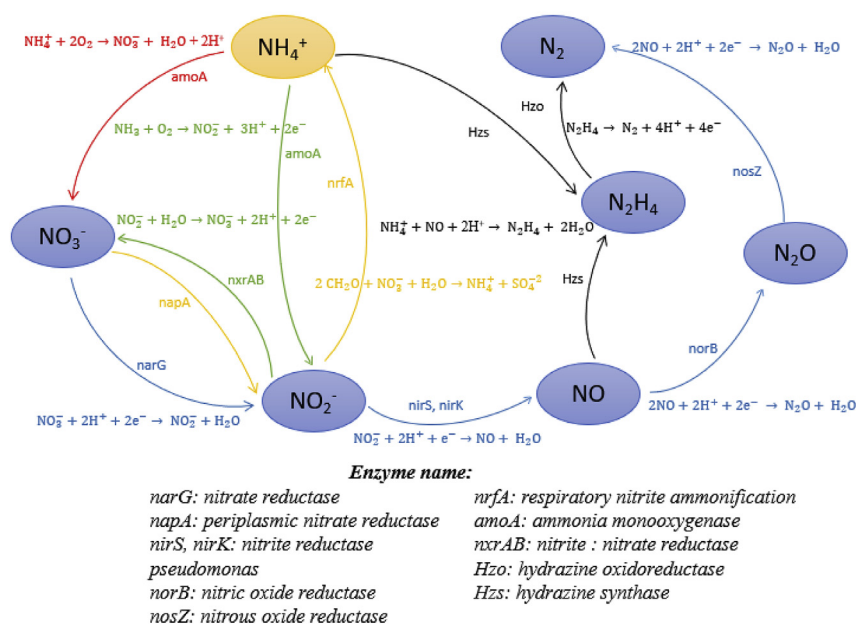
To explore the microbial interactions for nitrogen removal, two nitrification and denitrification pathways were investigated in congruence to dissimilatory nitrate reduction to ammonia or nitrate/nitrite ammonification. The first nitrification pathway consists of the familiar nitrification species of AOB, AOA, and NOB. The second nitrification pathway corresponds to Comammox bacteria, which is completely responsible for the two nitrification steps ( $NH_3/NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$ ). The nitrogen removal pathways described in Fig. 4a were explored given the current understanding of the nitrogen cycle with respect to known microorganisms in nitrification, denitrification, and DNRA. Whereas the two nitrification pathways are driven by Comammox and AOB, the two denitrification pathways involve anammox and denitrifying bacteria when nitrate/nitrite ammonification is completed by DNRA. The chemical equations and enzymes analyzed for nitrifying and denitrifying bacteria via qPCR are also delineated in Fig. 4b. Note that the overall anammox pathway is divided into two reactions corresponding to the conversion of  $NH_4^+$  and  $NO$  to  $N_2H_4$  (enzyme *Hzs*) and  $N_2H_4$  to  $N_2$  (enzyme *Hzo*) (Kartal et al., 2011; Zhou et al., 2018).

2.4. Sample collection

To determine inhibitory and/or complementary effects among the microbial species across both sub-basins, soil and water samples were collected. Once excess water was discharged into the sub-basins of the RIB and the water infiltrated the soil gradually, water samples were collected from the lysimeters (20 L bucket with a filter lid, buried 60 cm underground for collecting infiltrated water) at multiple vertical locations in the Control and BAM RIBs, partitioned by a berm in the middle (Fig. 1). Additional inlet samples were collected after loading. Soil samples from three vertical locations adjacent to the lysimeters were extracted from the BAM and Control RIBs at the surface of the basin and depths of 30 cm (1 foot) and 60 cm (2 feet). Further, soil samples around the edge of the Control and BAM RIBs were collected to analyze the microbial communities in the natural soil. The soil samples collected at the BAM and Control RIBs are labeled locations 1–6, while the soil samples extracted around the RIBs are referred to as locations A-F.



(a) Interactive metabolic pathways of microbial species for nitrogen removal



(b) Microbial enzymes relevant to qPCR analysis in this paper

Fig. 4. Microbial ecology analysis (a) Interactive metabolic pathways of microbial species for nitrogen removal in the nitrogen cycle (b) major microbial enzymes relevant to qPCR analysis with respect to AOA, AOB, Comammox, NOB, denitrifiers, DNRA, and anammox. Arrows in both graphs represent the reactions from ammonia, nitrite, nitrate oxidation, and denitrification.

Table 2  
Standard methods for wastewater and lysimeter analysis.

Parameter	City of DeLand Wiley Nash WRF	Environmental Research and Design, Inc (ERD)
TKN	EPA method 351.2	-
NOx	EPA method 353.2	SM-21 Sec. 4500-NO3, F
TP	EPA method 365.4	SM-22, Sec. 4500 P F
TN	-	SM-21, Sec 4500 N C

These samples were collected at the same depths as the RIB samples for comparison.

### 2.5. Sample analysis

Influent and lysimeter samples were collected and delivered to the City of DeLand Wiley Nash WRF and a certified laboratory (Environmental Research and Design, Inc.) for chemical analyses, respectively. Water quality constituents include TKN (Total Kjeldahl Nitrogen), TN (total nitrogen = TKN + Nitrite + Nitrate), TP (total

**Table 3**  
Primer used in this study for targeting the key species in nitrification and denitrification.

Bacteria	Primer	Sequence	Thermocycling	Reference
AOA	Arch-amoA-for, Arch-amoA-rev	5'-CTGAYTGGGCGYTGACATC -3', 5'- TTCTCTTTGTTGCCAGTA-3'	50 °C for 2 min, 95 °C for 5 min holding stage, 40 cycles at 95 °C for 30 s, 56 °C for 45 s and 72 °C for 45s	Wuchter et al. (2006) Marshall et al. (2018)
AOB	amoA-1F, amoA-2R	5'-GGGGTTTCTACTGGTGGT-3', 5'-CCC CTC KGS AAA GCC TTC TTC-3'	50 °C for 2 min, 95 °C for 2 min holding stage, 40 cycles at 95 °C for 15 s, and 60 °C for 60 s	Rotthauwe et al. (1997); Shimomura et al. (2012)
NOB	NSR1113f, NSR1242r	5'- CCTGCITT CAGTTGCTACCG- 3', 5'-GTTTGACGCGCTTTGTACCG-3'	50 °C for 2 min, 95 °C for 2 min holding stage, 40 cycles at 95 °C for 15 s, and 63.8 °C for 60 s	Dionisi et al. (2001); López-Goldstein and Smith (2002)
Comammox	A387f, C616r	5'-TGGTGGTGGTGGTCNAAYTAT-3', 5'- ATCATCCGRATGTACTCHGG-3'	50 °C for 2 min, 95 °C for 5 min holding stage, 45 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s	Xia et al. (2018)
Denitrifier	narG-1960m2f, narG-2050m2r	5'-TACTGTGCGGGCAGGAAGAACTG-3', 5'-CGTAGAAGCTGGTGCTGT-3'	50 °C for 2 min, 95 °C for 2 min holding stage, 40 cycles at 95 °C for 15 s, and 60 °C for 60 s	Bru et al. (2007);
Anammox	amx809 F, amx1066 R	5'- GCCGTAAACGATGGGCACT-3', 5'- CAGCTCGTGTGCTGAGACGTT-3'	50 °C for 2 min, 94 °C for 5 min holding stage, 40 cycles at 94 °C for 15 s, and 62 °C for 60 s	Tsushima et al. (2007)
DNRA	nrfA2F, nrfA2R	5'-CACGACAGCAAGACTGCCG-3', 5'-CCGGCACTTTCGAGCCC-3'	50 °C for 2 min, 95 °C for 10 min holding stage, 40 cycles at 95 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s	Yin et al. (2017)

phosphorus), and NO<sub>x</sub> (Nitrite + Nitrate). The standard methods for the water quality analyses performed by the City of DeLand Wiley Nash WRF and the certified laboratory are outlined in Table 2. The RIB soil samples collected on February 2, 2017 (i.e., initial sampling event), and February 16, 2018 (i.e., final sampling event), and the natural soil around the RIBs collected on October 9, 2018 (i.e., background soil investigation), were utilized for qPCR analysis. In each event, three samples were collected per lysimeter location, totaling 18 soil samples per event (Fig. 1).

## 2.6. DNA extraction and qPCR analysis

Quantitative Polymerase Chain Reaction analysis (qPCR) was used to quantify the population dynamics of AOB, NOB, AOA, Comammox, denitrifiers, DNRA, and anammox (Dionisi et al., 2001; Henry et al., 2004; López-Gutiérrez et al., 2004; Rotthauwe et al., 1997) in the BAM and Control RIBs in addition to the natural soil around the two sub-basins. The primer sequences and thermocycling conditions associated with the AOA, AOB, NOB, Comammox, denitrifiers, DNRA, and anammox are listed in Table 3. The 16 S RNA gene sequencing was used for the classification of all the microbial species.

The DNeasy PowerSoil kit (Qiagen, Germany) was used for DNA extraction according to the manufacturer's instructions for all the soil/media samples. The SYBR Green master mix (Applied Biosystems®) was used for NOB and denitrifier gene quantification, while PowerUp SYBR Green master mix (Applied Biosystems®) was used for the analysis of AOB, AOA, anammox, DNRA, and Comammox. The qPCR mix for the NOB and denitrifier analysis for each well consisted of 10 µL of SYBR Green master mix, 0.8 µL of DNA specific forward primer, 0.8 µL of DNA specific reverse primer, 5 µL sample DNA, and 3.4 µL of qPCR water. The same quantities were utilized for the AOB and AOA analyses, apart from the use of the PowerUp SYBR Green master mix. The qPCR mix for the anammox analysis of each well consisted of 10 µL of SYBR Green master mix, 0.8 µL of DNA specific forward primer, 0.8 µL of DNA specific reverse primer, 4 µL sample DNA, and 4.4 µL of qPCR water. The final primer concentration in each reaction well was 0.4 µM for each analysis. The samples were analyzed in a 48 well plate; each well plate was composed of 5 duplicated standards, 12 triplicate samples from each location and depth, and 1 negative control.

## 3. Results

### 3.1. Media characteristics in rapid infiltration sub-basins

The different physical characteristics of the media in the Control and BAM RIBs impacted the cultivation capacity and the ultimate nutrient removal performance. In natural soil the smaller porosity due to a lower range in particle size provided smaller void spaces between the

soil particles, impacting and decreasing the hydraulic conductivity. This contributed to the decreased influent infiltration of the natural soil of the Control RIB, and in return, decreased the hydraulic retention time (HRT) of the influent and diminished the oxygen and nutrient source availability, thereby decreasing the treatment capability of the soil. On the other hand, the BAM RIB contains small clay content and homogeneous media composition with a unique texture that permits equal distribution of the influent to maintain the required HRT, providing sufficient contact time for biological reactions given the passage of oxygen and availability of nutrients (Fig. 5). Moreover, a larger porosity from BAM mixture indicates larger void spaces allowing for faster infiltration. In addition, the infiltration of stormwater and reclaimed wastewater delivers carbon sources as electron donors, providing an adequate source for denitrifier growth (Chang, 2011). Table 4 summarizes the average nitrate percent removal for both BAM RIB and Control RIB.

Nevertheless, influent treatment, and consequently nitrogen removal, in both sub-basins is dependent on microbial communities that are present in media composition. The microbial population dynamics are impacted by media composition through the formation of biofilms. The cultivation or deterioration of biofilm impacts the interactions among microbial species by transporting nutrients and oxygen. The stratification of microbial species such as AOB, NOB, Comammox, denitrifiers, anammox, DNRA, and AOA within the developed biofilms can occur in the BAM and Control RIBs when constrained by the availability of oxygen and nutrients (Fig. 5).

### 3.2. Microbial populations of natural soil in the surrounding area of RIB

qPCR analysis revealed a small quantity of microbial populations of AOB and AOA, with larger quantities of NOB, Comammox, denitrifiers, and DNRA in the undisturbed natural soil, confirming the difference in physical characteristics between the natural soil and the green sorption media utilized in the BAM RIB. Anammox was found to be under the detection limit, thus it was not included in the results. As observed at two locations around the sub-basins, the AOB microbial population was scarce, while AOA was present at all localities, albeit in small quantities (Fig. 6a), highlighting the lean resource availability in the soil. However, the NOB, Comammox, denitrifier and DNRA populations were more predominant than AOB populations (Fig. 6b). Overall, DNRA was most prominent at the soil surface followed by NOB and Comammox. Albeit, Comammox was not detected at all the sampling locations in comparison to denitrifiers, NOB and AOA. The observed population dynamics substantiate the stratification and the role each micro-organism plays in the environmental conditions.

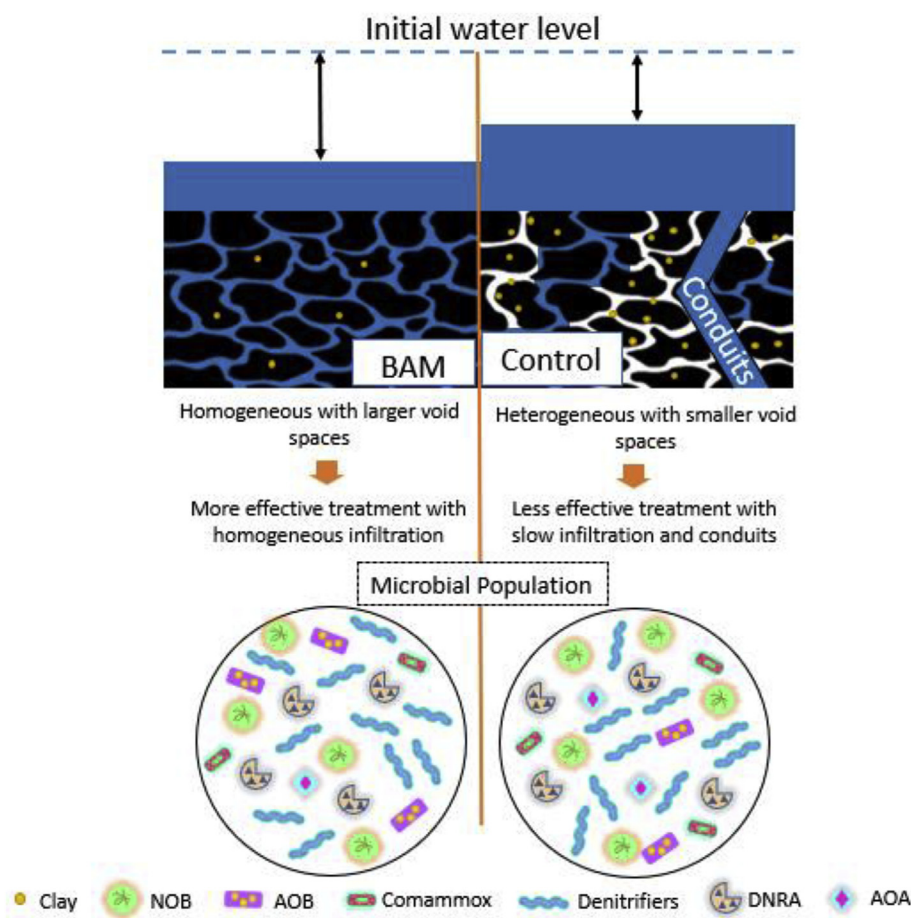


Fig. 5. Media environment and texture characteristics leading to different microbial population dynamics in biofilm formation at the soil or BAM surface in the Control and BAM RIBs.

**Table 4**  
Average nitrate percent removal (Chang, 2018).

Type of RIB	Percent Removal (%)	
	Reclaimed Wastewater	Stormwater
BAM	83	95
Control	40	90

### 3.3. Population dynamics in the two sub-basins of the RIB

The population dynamics of the BAM and Control RIBs differed greatly from the initial to the final sampling events. The AOB population was below the qPCR detection limit during the initial stage but was significantly improved after one-year in operation, especially for the BAM RIB. The initial sampling events observed AOB populations at only 3 out of 6 locations, with two of the locations corresponding to the Control RIB. However, in the second sampling event, an AOB population was observed in all 6 locations (Fig. 7a). Nevertheless, there was a decrease in population density in the Control RIB between the initial and final sampling for the majority of the vertical sample depths. The population of AOA was observed for all sampling locations for the final sampling event rather than the initial sampling; however, these quantities were minor when compared to AOB (Fig. 7b).

The BAM RIB appears to favor the cultivation of AOA, as noted by the increase of the gene population between the initial and final sample events (Fig. 7b). Yet, even with the decrease of AOA population density in the Control RIB after one-year, this density was higher than that in the BAM RIB. At locations 1–3, there were few to no gene copies

recorded for the initial event, followed by an observable increase in the final sample event in the BAM RIB. At the final sampling, the AOA population in the Control RIB increased in most locations and depths. Although AOB and AOA populations increased between the initial and final sample events, AOB had a more prominent growth in the BAM RIB, becoming the principal aerobic ammonia oxidizing organism.

The NOB population density observed for the BAM RIB in the final sample event was very minimal compared to the Control RIB (Fig. 8a). Only 3 out of the 6 locations displayed abundant NOB populations in the initial sample event, and the locations with abundant NOB corresponded to the Control RIB, while 5 locations had NOB gene quantification for the second sample event (Fig. 8b). In general, there was an increase of NOB in the BAM RIB, but a large decrease of NOB in the Control RIB after one-year in operation. Furthermore, the Comammox population was observed only at location 2 of the BAM RIB during the final sampling event, with population densities under detection limits in the initial sampling event (Fig. 9a). Overall, Comammox was more abundant in the Control RIB, being quantified at 2 locations (Fig. 9b).

The presence of denitrifier microorganisms confirms their contribution to nitrogen removal via the denitrification pathway. All 6 locations corresponding to the BAM and Control RIBs had denitrifier populations from the initial to final sample events. However, only 4 locations experienced an increase in denitrifier population at the surface layer after one-year of operation. Deviating from this trend, the vertical location of 30 cm (1 foot) below the surface had the highest denitrifier population for the BAM media samples at locations 1 and 2 for the initial sample date, as opposed to the final sampling event (Fig. 10a). While the population dynamics of the final sample event showed the largest denitrifier gene population at the surface of location



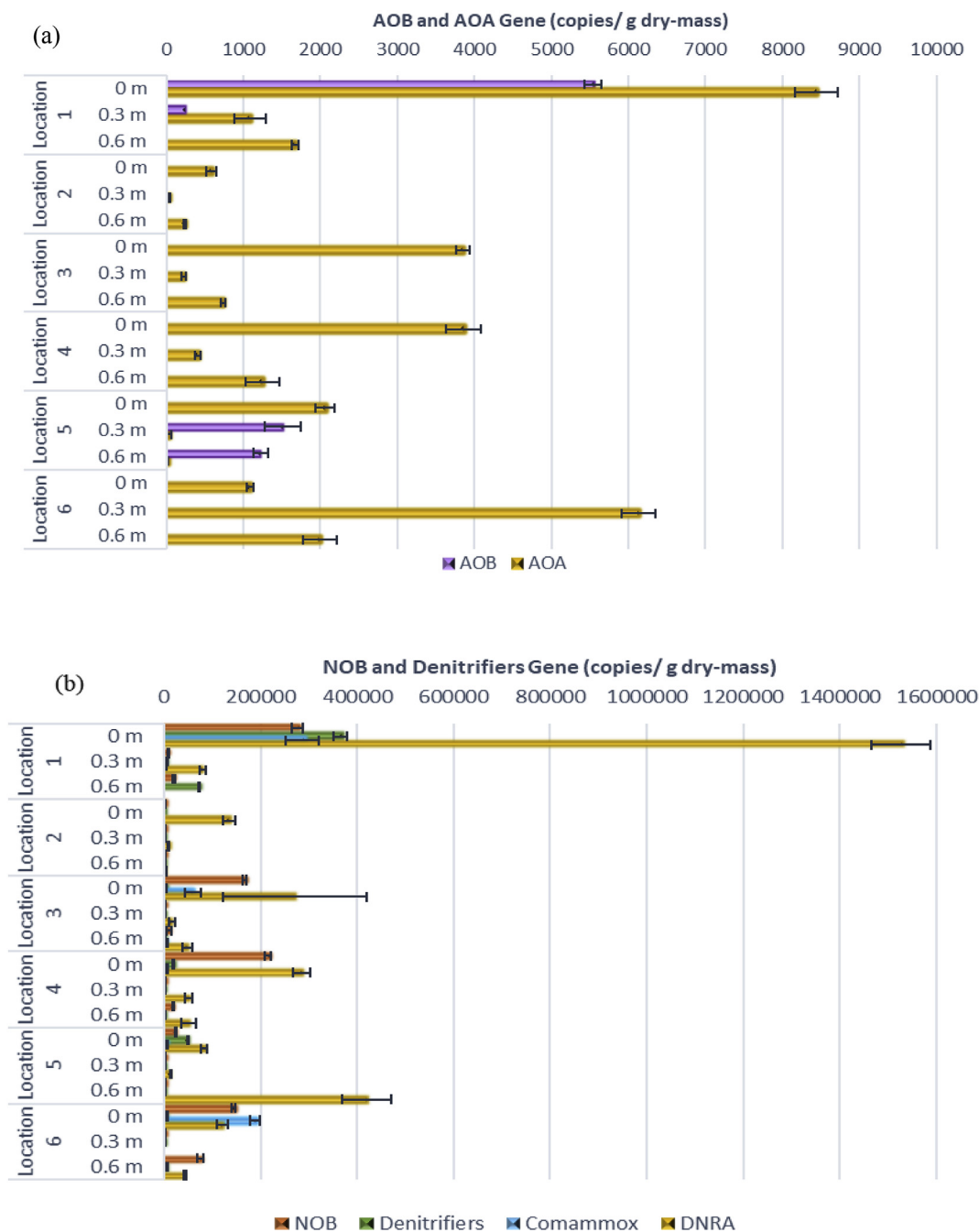


Fig. 6. Background microbial population at the surrounding RIB basin for (a)AOB, AOA, and (b) NOB, denitrifiers, Comammox and DNRA.

2 in the BAM RIB, followed by location 6 in the Control RIB. Further, the quantification of denitrifier genes determined that the greatest population was present at the top layer for all the soil samples for both the BAM and Control RIBs.

The DNRA population substantially increased between the initial and final sampling events, with higher population density occurring at the surface of both BAM and Control RIBs (Fig. 11a) and their locations (Fig. 11b). Further, according to the total microbial population the most abundant microbial species at the Control and BAM RIBs for the initial sampling events are NOB and denitrifiers, respectively (Fig. 12), which comprise 55% and 98% of the total microbial population. After one-year operation, the most abundant microbial species from the overall population at all locations and vertical depths were denitrifiers accounting for 62–86% of the total microbial population of the Control and BAM RIBs, respectively.

#### 4. Discussion

##### 4.1. Microbial community, population dynamics, and microbial ecology analysis

Current studies have shifted focus from traditional nitrifying bacteria and archaea (AOA, AOB) responsible for ammonia oxidation to the recently discovered Comammox. The activity of Comammox in nitrification in natural soil was investigated by Wang et al. (2019), while its presence (Spasov et al., 2019) and effect in nitrogen removal in wastewater systems (Cotto et al., 2020) was also explored. It has also been found that the competition between anammox and denitrifiers for nitrite and their dependence on NOB for nitrite can be avoided in wastewater treatment by utilizing partial denitrification (Cao et al., 2019). Additionally, the exploration of DNRA and its interaction further



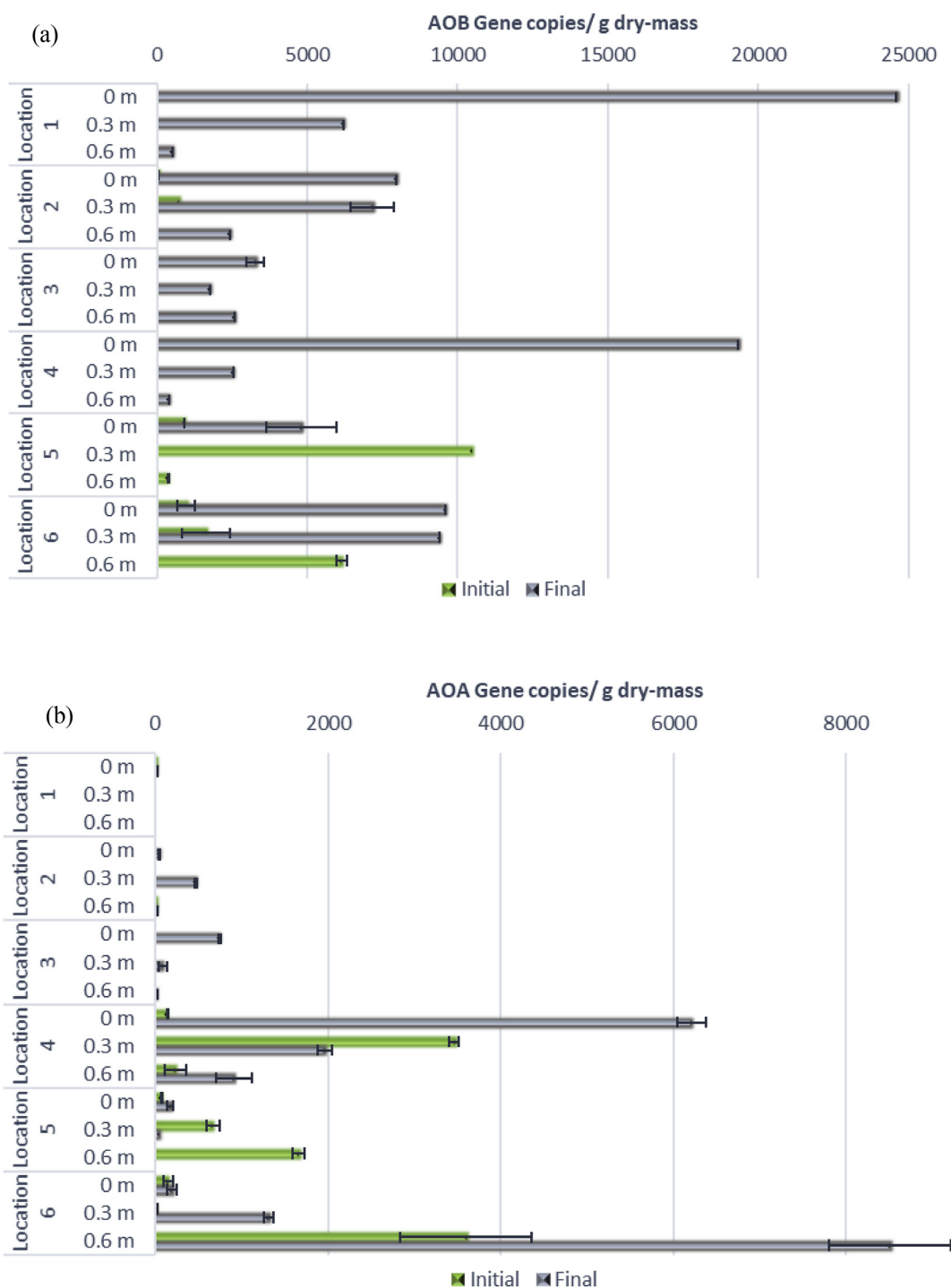
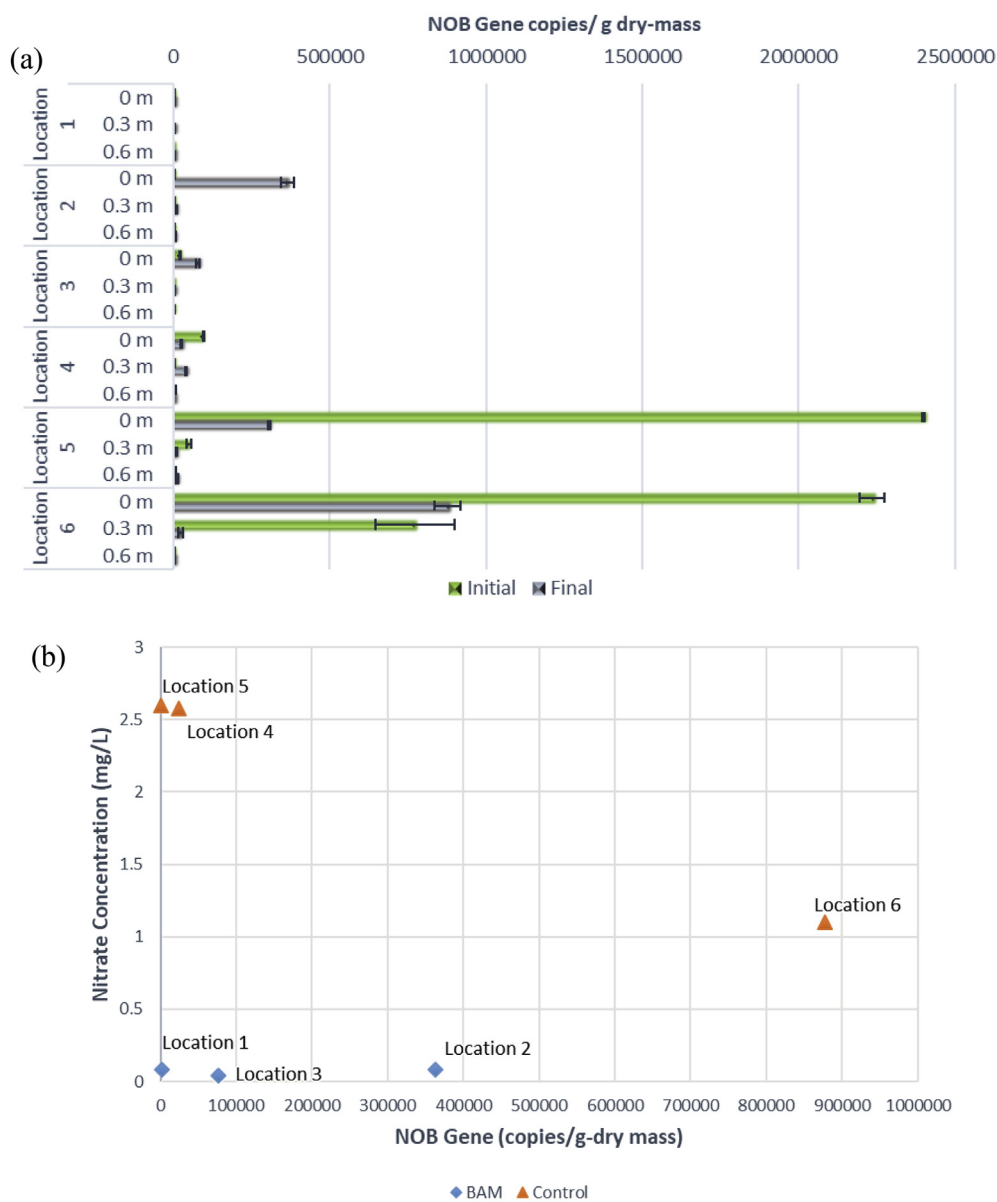


Fig. 7. (a) AOB Gene and (b) AOA (copies/g-dry mass) for BAM (Locations 1, 2, and 3) and Control (Locations 4, 5, and 6) in the initial (February 2, 2017) and final (2/16/2018) sampling. Error bars represent the standard deviation.

contributes to the interconnection of microbial species in the microbial community as the competition between DNRA and denitrifiers for oxidized nitrogen compounds such as nitrite and nitrate can be investigated by this study (Putz et al., 2018; van den Berg et al., 2017). Hence the interrelationship between the reactants and products required for nitrification and denitrification can be elucidated via a holistic approach in microbial ecology.

In this study, seven microbial species present in the biofilm were linked to the required sources for the nitrification, denitrification and nitrate/nitrite ammonification processes conducted in nitrogen removal under aerobic and anaerobic conditions (Fig. 13). In the aerobic

process, the microbial species require the presence of the preceding microbe for food source availability, resultant of prior chemical reactions. On the other hand, the conversion of ammonium to nitrogen gas is completed by anammox alone in oxygen deprived conditions. Hence, the interaction and interdependence between distinct bacteria in the microbial community motivates the exploration of research question 1. The need for nitrite by anammox as an electron acceptor in the absence of oxygen requires the dependence of AOB, AOA, and Comammox to produce nitrite, while fostering a competitive relationship with NOB and DNRA for the accessibility of nitrite. However, the qPCR results revealed the absence of anammox in the microbial community,



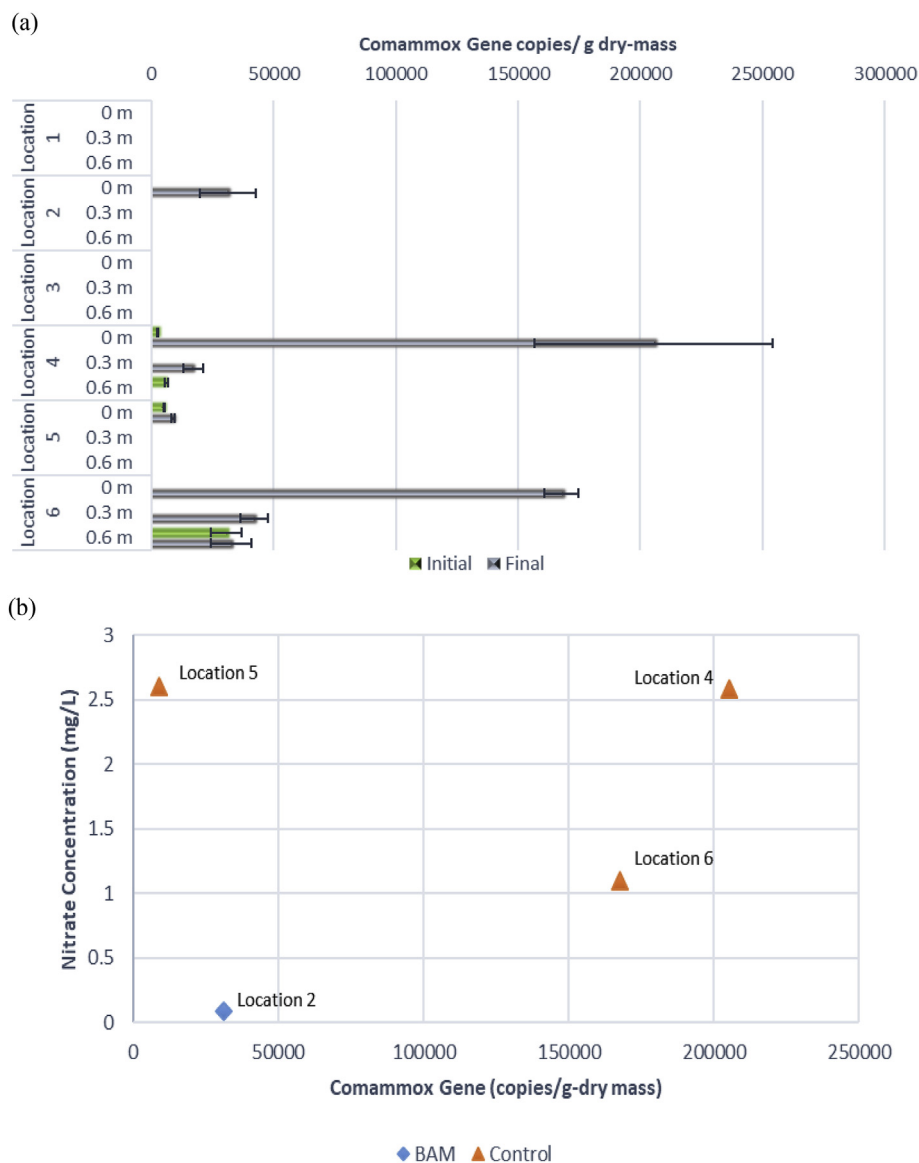
**Fig. 8.** (a) NOB Gene (copies/g-dry media) for BAM (Locations 1, 2, and 3) and Control (Locations 4, 5, and 6 in Fig. 2(a)) in the initial (February 2, 2017) and final (2/16/2018) sampling, (b) Comparison of nitrate concentration and NOB genes at the surface layer for BAM and Control RIB for locations 1–6 for the second sample event for nitrate removal. Error bars represent the standard deviation (Chang, 2018).

suggesting inhibitory effects from the environment and the presence of the other microbial species. As nitrite could have been primarily utilized by NOB and DNRA reducing the available quantity remaining for anammox uptake. Furthermore, the oxygen requirement greatly influences which microbial species utilizes nitrite first, thus enabling population survival and growth. In addition, anammox are slow growing organisms which prevent the microbial population from being cultivated at a fast rate compared with the other microbial species. This is because AOB, NOB, and anammox are autotrophic, whereas denitrifiers are heterotrophic. Moreover, denitrifying bacteria utilize nitrate, fostering a dependence on NOB and Comammox for its nitrate source while competing with DNRA for nitrate or nitrite. Thus, the complementary and inhibitory effects of the seven microbial species were determined and interpreted from the population dynamics obtained from the qPCR analysis.

Further, the specific interactions between aerobic ammonia oxidizing microbial species address research question 2. Biofilm cultivation and competition for food sources between these microbial species

could contribute to their absence in specific sample regions. This is a possible consequence of competition between the Comammox bacteria, AOA, AOB, and anammox species in the sampling location, as they compete for the procurement of ammonia substance in the biofilms. The competition between AOA and AOB is severe, and AOB seems to be the winner. However, the competition between AOB, AOA, and Comammox did not hinder AOB or Comammox cultivation, thus primarily impacting the population dynamics of AOA. The AOA genes detected were relatively low in quantity compared to those of AOB, which supports the competition assumption between aerobic ammonia oxidizing species. However, the population densities of Comammox are observably larger than both AOA and AOB, although the presence of Comammox is not consistent throughout all the locations. This suggests that 1) the competition for ammonia uptake by the three species did not completely impact an individual microbial specie, and 2) these microbial species possibly utilized different sources of ammonia (organic, inorganic) which forced the continual oxidation of ammonia to nitrite.

Since AOB and Comammox are the dominant ammonia oxidizing



**Fig. 9.** Comammox Gene (copies/g-dry-media) for BAM (Locations 1, 2, and 3) and Control (Locations 4, 5, and 6 in Fig. 2(a)) in the initial (February 2, 2017) and final (2/16/2018) sampling, (b) Comparison of nitrate concentration and Comammox genes at the surface layer for BAM and Control RIB for locations 1–6 for the second sample event for nitrate removal. Error bars represent the standard deviation (Chang, 2018).

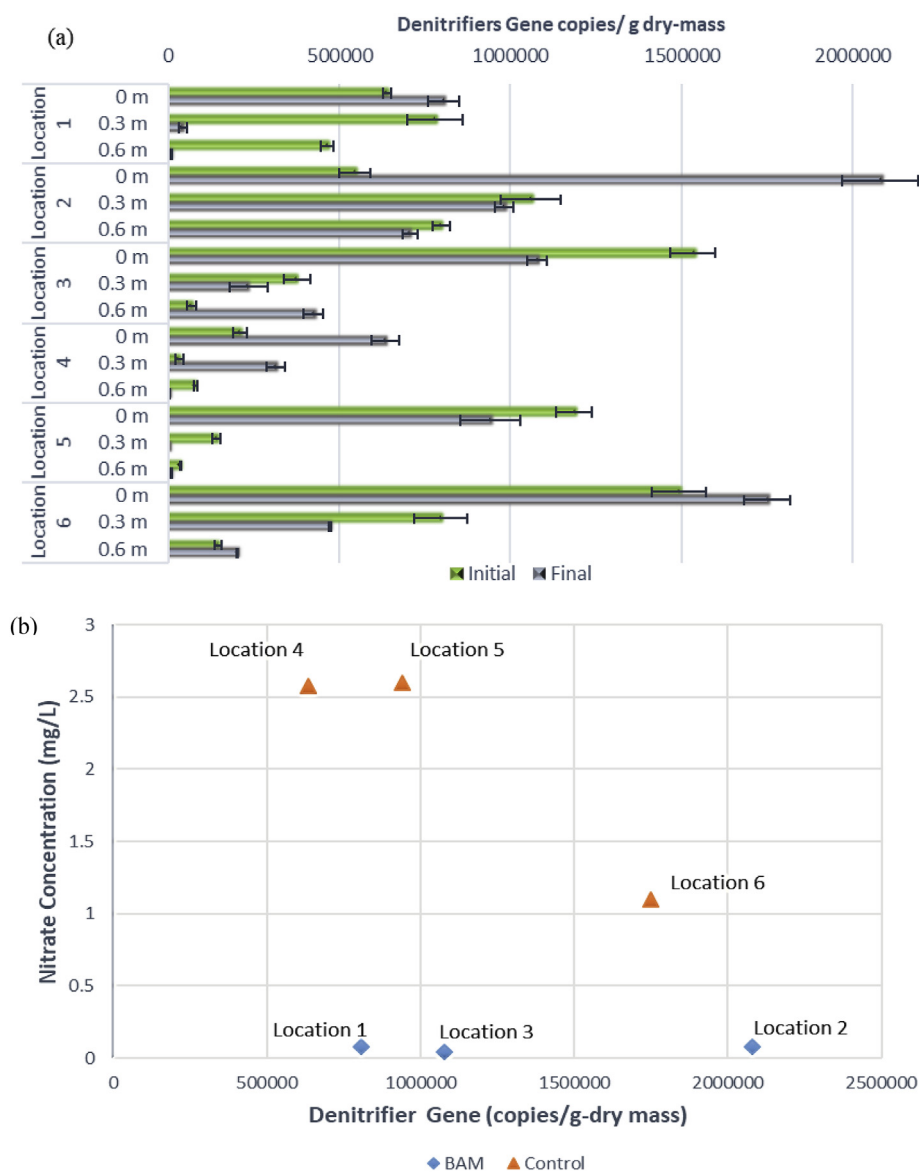
species, the inhibition effect of Comammox and AOB on anammox was also observed in the gene quantification. The gene population densities of anammox were determined to be under detection limits, thus reducing its influence in the microbial community. This confirms the hypothesis regarding the inhibition of anammox by nitrifying microorganisms. Anammox requires ammonium and nitrite for the production of nitrogen gas under anaerobic conditions (Tsushima et al., 2007), thereby being indirectly impacted by AOB, AOA, and Comammox, as the necessary constituent required for the microbial processes of each of these microbial species was primarily consumed by aerobic ammonia oxidizing bacteria, leaving anammox disengaged. Therefore, in addition to nitrite availability and competition between the species, an aerobic and anaerobic environment would impact the process.

Also, there were notable differences in the microbial population densities in the Control and BAM RIBs, leading to consideration of research question 3. The BAM RIB experienced a small decay in the denitrifier population from the initial to the final sample event for the majority of sample location depths in comparison to the BAM RIB. Where the observed increase in NOB population would aid in the

increase of denitrifying bacteria, as opposed to an increase in DNRA population which could decrease the growth of denitrifier population density. The increase in both NOB and DNRA is observed for the BAM RIB, with only an increase of DNRA achieved by the Control RIB after one-year operation.

The abundance of denitrifiers compared to AOB and AOA (Figs. 7 and 9a) may be attributed to the high nitrate concentrations of the influent and the nitrifying bacteria available in the soil layers of the BAM and Control RIBs under the aerobic conditions produced in the biofilm structure (Mustafa, 2009). Note that the denitrifying bacteria utilize nitrate as an electron acceptor in the absence of oxygen. Nitrate may have been more abundant in the influent than ammonia, promoting the denitrifier population instead of the growth of ammonia oxidizing species.

Moreover, the gene density of AOB and AOA were relatively low compared to that of NOB. The AOB and AOA gene populations were not observed for some of the layers in the Control and BAM RIBs, indicating the complementary effect NOB and AOB or AOA have on each other. This interaction can change with the inclusion of nitrification by Comammox, since Comammox bacteria can perform both nitrification



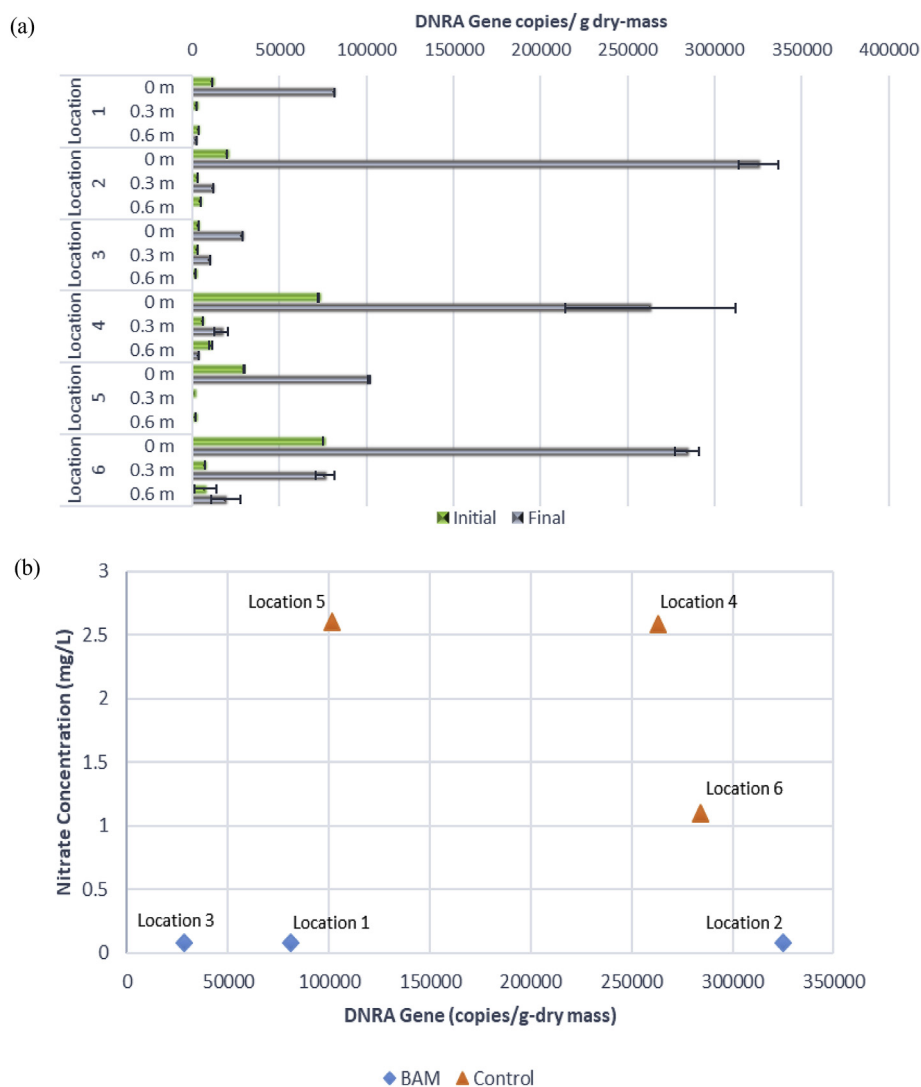
**Fig. 10.** (a) Denitrifier Gene (copies/g-dry-mass) for BAM (Locations 1, 2, and 3 in Fig. 2(a)) and Control (Locations 4, 5, and 6 in Fig. 2(a)) in the initial (February 2, 2017) and final (2/16/2018) sampling. (b) Comparison of nitrate concentration and denitrifier genes at the surface layer for BAM and Control RIB for Locations 1–6 in Fig. 2(a) for the second sampling event for nitrate removal. Error bars represent the standard deviation (Chang, 2018).

steps. Additional causes for microbial community differences are the physical characteristics of the natural soil and BAM media at each sub-basin. BAM has a much faster infiltration rate than the control side, which provided the biofilm more nutrients in a certain period of time and in turn caused the whole microbial community to flourish. However, since AOA, AOB, and Comammox mainly live within the upper layers in biofilm, they might be affected most via possible biofilm detachment. However, even with these differences and complementary and inhibitory relationship, denitrifiers, DNRA, and NOB were found to be the three most prominent microbial species in both sub-basins according to the total population density. This result moves to address research question 4, suggesting denitrifying bacteria to be more predominant than DNRA in the BAM and natural soil biofilms. Proposing denitrifiers to be more dominant than DNRA in the N-cycle of both sub-basins. Even when considering the presence of both microbial species at each location of the RIBS, denitrifiers population are visibly more consistently present. Additionally, the large presence of DNRA allowed for additional ammonia production and contribution for nitrifying bacteria utilization providing a continual and closed cycling of

nitrogen.

Further, the NOB population was affected by the nitrite concentration in the soil media in the Control RIB. This phenomenon results from the amount of nitrite generated by AOB, AOA, Comammox, and denitrifiers. However, most denitrifiers live at the deeper layer of biofilm, and the nitrite they produce may not cause any significant impact on NOB at the upper layer of biofilm. This prompts the consideration of research question 5. If insufficient AOB or Comammox populations are present in the soil, a deficit of nitrite is salient due to the absence of nitrite formed from ammonia oxidation. However, the presence of Comammox can impact NOB by independently transforming ammonia to nitrate without the inclusion of NOB for nitrite oxidation. Consequently, the absence of nitrite either from its absence in the influent or from microbial competition inhibits the cultivation of NOB. In the traditional two-step nitrification process corresponding to three bacteria types, AOB or AOA and NOB (Mustafa, 2009; Xia et al., 2008), the reactants and products of each reaction affect the proceeding step. According to Yao and Peng (2017), having more growth of AOB compared to NOB yields a higher ammonium oxidizing rate than the nitrite





**Fig. 11.** DNRA Gene (copies/g-dry-mass) for BAM (Locations 1, 2, and 3 in Fig. 2(a)) and Control (Locations 4, 5, and 6 in Fig. 2(a)) in the initial (February 2, 2017) and final (2/16/2018) sampling, (b) Comparison of nitrate concentration and DNRA genes at the surface layer for BAM and Control RIB for locations 1–6 for the second sample event for nitrate removal. Error bars represent the standard deviation (Chang, 2018).

oxidizing rate. However, this growth is related to the quantity of ammonium present in the environment available for utilization. Also, the thriving NOB population may also indicate the existence of abundant nitrite in the liquid phase, which could be toxic to many other species in the community when its concentration is high enough (Bollag and Henninger, 1978). Thus, the coexistence of aerobic ammonia oxidizing species can be a reason for abundant nitrite availability for NOB.

#### 4.2. Comparison of nitrogen removal

The nitrogen removal performance of the BAM and Control RIBs was analyzed to determine which soil media provided enhanced nitrogen removal in addition to promoting microbial activity. The media of the BAM RIB assisted in the improved reduction of nitrogen in comparison to the Control RIB. The BAM RIB consisted of the green sorption media containing a mixture of tire crumb, sand, and clay, which provided a homogeneous environment with carbon sources for microbial activity, compared to the heterogeneous natural soil composition of the Control RIB, which consisted of more compact sand and clay soil with less void spaces, thus retaining more moisture than the BAM RIB. As a result, the BAM RIB has a homogeneous infiltration rate that constantly provided the appropriate conditions for biofilm

development, enabling better nitrogen removal due to having more denitrifier population density in a moist environment, as opposed to the Control RIB. As larger denitrifier population provides greater conversion of nitrate to nitrogen gas. Additionally, BAM has been found to have large surface area per volume derived from its fine-grained texture (O'Reilly et al., 2012b). Natural soil is usually very non-homogeneous in soil structure and soil moisture distribution, causing preferential flows and oxygen addition that disturbed the denitrification process. Hence, some removal of nitrate was observed at the Control RIB due to natural clay providing both physiochemical and microbiological effects.

The BAM RIB surpassed the Control RIB in reclaimed wastewater and stormwater nitrogen removal with 83% (± 4%) and 40% (± 10%) for reclaimed wastewater as well as with 95% (± 1%) and 90% (± 4%) for stormwater, respectively. The overall nitrate removal of 89% for BAM RIB corroborates the efficiency of BAM for removing nitrogen. This level of treatment is not unique to reclaimed wastewater, as research has been conducted on BAM for stormwater and groundwater treatment. Other field applications of BAM media have obtained suitable nitrogen removals and explored the microbial biofilm communities, as in the case for linear ditch located beside a road and farm land (Wen, 2018, 2019a; Chang et al., 2019), and blanket filters placed in a retention basin (Wen et al., 2019b).

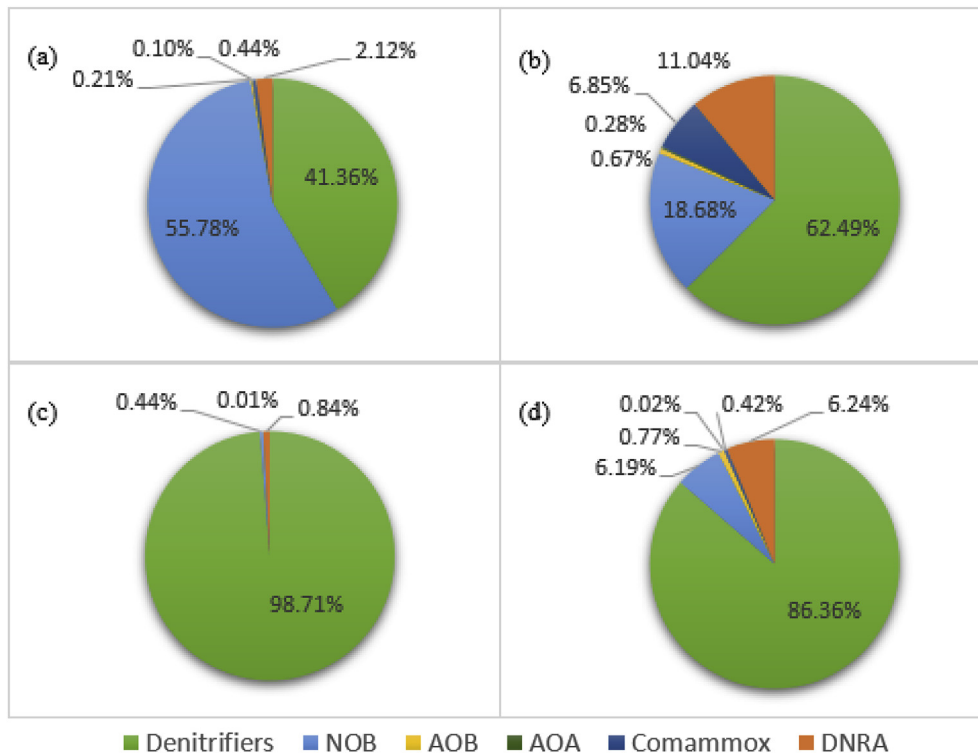


Fig. 12. Percent denitrifiers, NOB, AOB, AOA, Comammox, and DNRA as total microbial population at (a) Control RIB for initial sampling (February 2, 2017), (b) Control RIB for final sampling (2/16/18), (c) BAM RIB for initial sampling (February 2, 2017), and (d) BAM RIB for final sampling (2/16/2018).

5. Conclusion

The nitrogen removal in the two sub-basins of the RIB were compared based on lysimeter samples with BAM RIB surpassing the Control RIB in reclaimed wastewater and stormwater nitrogen removal. The quantification of NOB, AOA, AOB, Comammox, denitrifiers, DNRA, and anammox bacteria through qPCR enabled the analysis of microbial ecology when these microbial species coexisted in the same environment. The population dynamics of microbial communities contributed to the understanding of the complementary and inhibitory effects of nitrifying and denitrifying microbial species in the BAM and natural soil. NOB, denitrifier and DNRA population dynamics contributed to better nitrogen removal efficiency in the BAM RIB due to their complementary effects. The interaction between the three aerobic ammonia oxidations was found to support NOB population, which indicates that

microbial ecology in BAM RIB does play a critical role in polishing the effluent for nutrient removal, leading to sustainable groundwater recharge.

Acknowledgements

The authors are grateful for the help from City of Deland (Jodi Harrison, Alex Konoval, Larry Nordman, Keith Riger, and Shawn Wahrenberger), UCF students, and the staff of Environmental Conservation Solutions Inc. The authors are thankful for the funding support from the Florida Department of Environmental Protection (under the agreement No. NS 003), the St. Johns River Water Management District, and the City of DeLand.

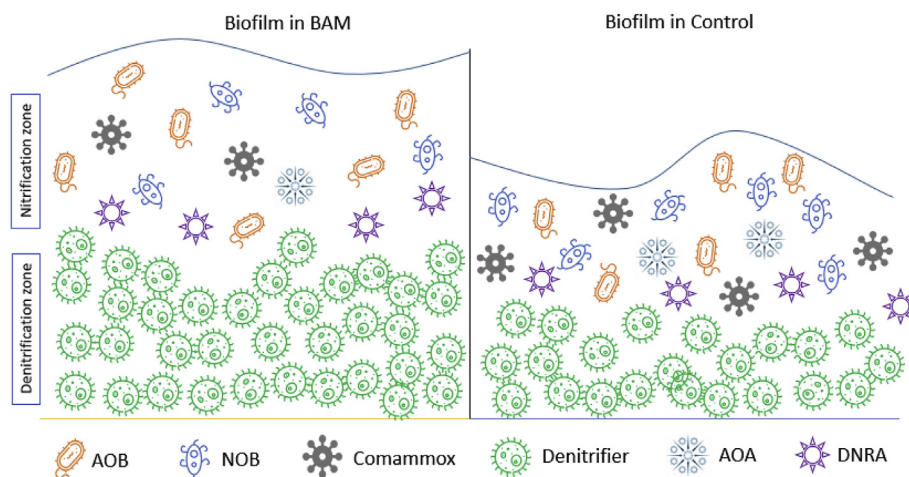


Fig. 13. Microbial community in BAM and Control biofilm. Comparison of microbial density in biofilm in BAM and Control RIB.

## References

- Annavaiah, M.K., et al., 2018. Comammox functionality identified in diverse engineered biological wastewater treatment systems. *Environ. Sci. Technol. Lett.* 5, 110–116.
- Arriagada, C., et al., 2017. NOB suppression and adaptation strategies in the partial nitrification–Anammox process for a poultry manure anaerobic digester. *Process Biochem.* 58, 258–265.
- Berndt, M.P., et al., 2014. Water quality in the upper floridan aquifer and overlying surficial aquifers, southeastern United States, 1993–2010. In: Naugle, K.P. (Ed.), USGS Science Publishing Network.
- Bollag, J.-M., Henninger, N.M., 1978. Effects of nitrite toxicity on soil bacteria under aerobic and anaerobic conditions. *Soil Biol. Biochem.* 10, 377–381.
- Bouchard, D.C., et al., 1992. Nitrate contamination of groundwater: sources and potential health effects. *J. Am. Water Works Assoc.* 83, 85–90.
- Bru, D., et al., 2007. Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. *Appl. Environ. Microbiol.* 73, 5971–5974.
- Cao, S., et al., 2019. Denitrifiers in mainstream Anammox processes: competitors or supporters? *Environ. Sci. Technol.* 53, 11063–11065.
- Chang, N.-B., et al., 2010. New performance-based passive septic tank underground drainfield for nutrient and pathogen removal using sorption media. *Environ. Eng. Sci.* 27, 469–482.
- Chang, N.-B., 2011. Making a progress to speed up the nitrification and denitrification processes in novel biosorption activated media: can Archaea be in concert with Anammox? *Journal of Bioprocessinf & Biotechniques* 1, 1–5.
- Chang, N.-B., 2018. Bio-sorption Activated Media for Nitrogen Removal in a Rapid Infiltration Basin—Monitoring Project. pp. NS003 FDEP Project Report.
- Chang, N.-B., et al., 2019. Comparison of biological nutrient removal via two biosorption-activated media between laboratory-scale and field-scale linear ditch for stormwater and groundwater Co-treatment. *Water, air, & Soil Pollution* 230, 151.
- Cormier, J.A., Duranceau, S., 2019. Comparison of a Modified and Traditional Rapid Infiltration Basin for Treatment of Nutrients in Wastewater Effluent. *Water Environment Research*, pp. 1–14.
- Costa, E., et al., 2006. Why is metabolic labour divided in nitrification? *Trends Microbiol.* 14, 213–219.
- Cotto, I., et al., 2020. Long solids retention times and attached growth phase favor prevalence of comammox bacteria in nitrogen removal systems. *Water Res.* 169, 115268.
- Crites, R.W., 1981. Rapid Infiltration Land Treatment: A Recycle Technology. U.S. Environmental Protection Agency Water Operations Municipal Construction Division.
- Daims, H., et al., 2015. Complete nitrification by Nitrospira bacteria. *Nature* 528, 504.
- Dang, H., Chen, C.-T.A., 2017. Ecological energetic perspectives on responses of nitrogen-transforming chemolithoautotrophic microbiota to changes in the marine environment. *Front. Microbiol.* 8, 1246.
- Denizman, C., 2018. Land use changes and groundwater quality in Florida. *Applied Water Science* 8, 1–17.
- Dionisi, H.M., et al., 2001. Quantification of Nitrosomonas oligotropha-like ammonia oxidizing bacteria and Nitrospira spp. from full-scale wastewater treatment plants by competitive PCR. *Appl. Environ. Microbiol.* 68, 245–253.
- EPA, U. S. E. P. Agency, 2003. Rapid infiltration land treatment. In: Municipal Technology Branch.
- Friedl, J., et al., 2018. Dissimilatory nitrate reduction to ammonium (DNRA), not denitrification dominates nitrate reduction in subtropical pasture soils upon rewetting. *Soil Biol. Biochem.* 125, 340–349.
- Goldstein, R., Smith, W., 2002. Water & sustainability. U.S. Electricity Consumption for Water Supply & Treatment—The Next Half Century, vol. 4 EPRI.
- Henry, S., et al., 2004. Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. *J. Microbiol. Methods* 58, 327–335.
- Hossain, F., et al., 2010. Nitrification and denitrification in a passive on-site wastewater treatment system with a recirculation filtration tank. *Water Quality, Exposure and Health* 2, 31–46.
- Isobe, K., Ohte, N., 2014. Ecological perspectives on microbes involved in N-cycling. *Microb. Environ.* 29, 4–16.
- Jeyanayagam, S., 2005. True confessions of the biological nutrient removal process. *Florida Water Resources Journal* 38–46.
- Kartal, B., et al., 2011. Molecular mechanism of anaerobic ammonium oxidation. *Nature* 479, 127–130.
- Koch, H., et al., 2019. Complete nitrification: insights into the ecophysiology of comammox Nitrospira. *Appl. Microbiol. Biotechnol.* 103, 177–189.
- Liao, X., et al., 2019. Subsurface transport and potential risk of phosphorus to groundwater across different land uses in a karst springs basin, Florida, USA. *Geoderma* 338, 97–106.
- López -Gutiérrez, J.C., et al., 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. *Journal of Microbiol. Methods* 57, 399–407.
- Marshall, A., et al., 2018. Primer Selection Influences Abundance Estimates of Ammonia Oxidizing Archaea in Coastal Marine Sediments.
- Mueller, D.K., Helsel, D.R., 2017. Nutrients in the nation's waters—too much of a good thing? National water-quality assessment (NAWQA) program, U.S. Geol. Surv. Circular 1136.
- Mustafa, A., 2009. Nutrient Removal with Integrated Constructed Wetlands School of Engineering Vol. Doctro of Philosphy. The University of Edinburgh, pp. 255.
- National Academy of Engineering, 2018. Manage the Nitrogen Cycle.
- O'Reilly, A.M., et al., 2012a. Cyclic biogeochemical processes and nitrogen fate beneath a subtropical stormwater infiltration basin. *J. Contam. Hydrol.* 133, 53–75.
- O'Reilly, A.M., et al., 2012b. Nutrient removal using biosorption activated media: preliminary biogeochemical assessment of an innovative stormwater infiltration basin. *Sci. Total Environ.* 432, 227–242.
- Putz, M., et al., 2018. Relative abundance of denitrifying and DNRA bacteria and their activity determine nitrogen retention or loss in agricultural soil. *Soil Biol. Biochem.* 123, 97–104.
- Qin, W., et al., 2017. Influence of oxygen availability on the activities of ammonia-oxidizing archaea. *Environmental Microbiology Reports* 9, 250–256.
- Qiu, Y., et al., 2010. Nitrogen and phosphorus removal in municipal wastewater treatment plants in China: a review. *Int. J. Chem. Eng.* 1–10.
- Rothauwe, J.-H., et al., 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale Analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63, 4704–4712.
- Shimomura, Y., et al., 2012. Comparison among amoA primers suited for quantification and diversity analyses of ammonia-oxidizing bacteria in soil. *Microb. Environ.* 27, 94–98.
- Spasov, E., et al., 2019. High Functional Diversity Among Nitrospira Populations that Dominate Rotating Biological Contactor Microbial Communities in a Municipal Wastewater Treatment Plant. *bioRxiv*.
- Tao, R., et al., 2017. Response of ammonia-oxidizing archaea and bacteria in calcareous soil to mineral and organic fertilizer application and their relative contribution to nitrification. *Soil Biol. Biochem.* 114, 20–30.
- Tsushima, I., et al., 2007. Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR. *Water Environ. Res.* 41, 785–794.
- Upchurch, S., et al., 2019. Eogenetic Karst in Florida. The Karst Systems of Florida: Understanding Karst in a Geologically Young Terrain. Springer International Publishing, Cham, pp. 1–15.
- van den Berg, E.M., et al., 2017. Role of nitrite in the competition between denitrification and DNRA in a chemostat enrichment culture. *Amb. Express* 7, 91.
- Van Kessel, M.A., et al., 2015. Complete nitrification by a single microorganism. *Nature* 528, 555.
- Wang, H., et al., 2015. Nitrate accumulation and leaching in surface and ground water based on simulated rainfall experiments. *PLoS One* 10, 1–18.
- Wang, Z., et al., 2019. Comammox Nitrospira clade B contributes to nitrification in soil. *Soil Biol. Biochem.* 135, 392–395.
- Wen, D., et al., 2018. Copper toxicity impact and enzymatic cascade effect on biosorption activated media and woodchips for nutrient removal in stormwater treatment. *Chemosphere* 213, 403–413.
- Wen, D., et al., 2019a. Fate and transport processes of nitrogen in biosorption activated media for stormwater treatment at varying field conditions of a roadside linear ditch. *Environ. Res.* <https://doi.org/10.1016/j.envres.2019.108915>.
- Wen, D., et al., 2019b. Evaluation of green sorption media blanket filters for nitrogen removal in a stormwater retention basin at varying groundwater conditions in a karst environment. *Sci. Total Environ.* <https://doi.org/10.1016/j.scitotenv.2019.134826>.
- Wuchter, C., et al., 2006. Archaeal nitrification in the ocean. *103*, 12317–12322.
- Xia, F., et al., 2018. Ubiquity and diversity of complete ammonia oxidizers (Comammox). *Appl. Environ. Microbiol.* 84 (24) e01390-18.
- Xia, S., et al., 2008. Nitrogen removal performance and microbial community structure dynamics response to carbon nitrogen ratio in a compact suspended carrier biofilm reactor. *Ecol. Eng.* 32, 256–262.
- Xu, Z., et al., 2019. Numerical study of groundwater flow cycling controlled by seawater/freshwater interaction in Woodville Karst Plain. *J. Hydrol.* 579, 124171.
- Xuan, Z., et al., 2010. Laboratory-scale characterization of a green sorption medium for on-site sewage treatment and disposal to improve nutrient removal. *Environ. Eng. Sci.* 27, 301–312.
- Yao, H., et al., 2011. Links between ammonia oxidizer community structure, abundance, and nitrification potential in acidic soils. *Appl. Environ. Microbiol.* 77, 4618–4625.
- Yao, Q., Peng, D.-C., 2017. Nitrite oxidizing bacteria (NOB) dominating in nitrifying community in full-scale biological nutrient removal wastewater treatment plants. *Amb. Express* 7, 2–11.
- Yin, Z., et al., 2018. Ammonia-Oxidizing Archaea (AOA) Play with Ammonia-Oxidizing Bacteria (AOB) in Nitrogen Removal from Wastewater. *Archaea*. 2018.
- Yin, G., et al., 2017. DNRA in Intertidal Sediments of the Yangtze Estuary, vol. 122. pp. 1988–1998.
- Zhou, Z., et al., 2018. Practical applications of PCR primers in detection of anammox bacteria effectively from different types of samples. *Appl. Microbiol. Biotechnol.* 102, 5859–5871.