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Novel Start-up for Moving Bed Biological Reactors for Nitrogen Removal with High C/N Influent by Heterotrophic Nitrification and Aerobic Denitrification

Manisha Berde*, and Domènec Jolis

San Francisco Public Utilities Commission, San Francisco, California, USA

***Corresponding author:** Manisha Berde, San Francisco Public Utilities Commission, San Francisco, California, USA, E-mail: mberde@sfwater.org

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Abstract

A novel start-up strategy for MBBR systems was implemented that achieved removal rates of ammonia and total nitrogen of more than 90% and 70%, respectively, with influent C/N ratio of nine. The strategy consists of operating the MBBR with non-nitrified secondary effluent (C/N=2) until ammonia removal is sustained and gradually increase the C/N ratio of the feed to the target value of nine, when the MBBR system is fed primary effluent. Before using the start-up sequence, the MBBR system treating primary effluent (C/N=9) showed negligible ammonia removal after 120 days of operation, indicating the absence of nitrifying bacteria. It was assumed that heterotrophs were outcompeting autotrophs, and the C/N ratio was decreased to allow autotrophs to proliferate. Genomic analysis indicated the presence of bacterial and fungal populations, which can perform nitrification and denitrification. The most abundant heterotrophs observed were *Proteobacteria* at 31%, *Bacteroidetes* at 22%, and *Actinobacteria* at 8%. The most abundant fungal population belonged to *Ascomycota* (10%), *Basidiomycota* (10%), and *Mucoromycota* (1%). Heterotrophic nitrification and aerobic denitrification were found to play an important role in nitrogen removal at C/N=9. Autotrophs may have proliferated at lower C/N ratio as total nitrogen removal was low, but at higher C/N a shift in microbial population should have taken place as indicated by the dominance of heterotrophs.

Keywords: Moving bed bioreactor; Nitrification; Denitrification; Carbon to nitrogen ratio

Introduction

Removal of nitrogen from wastewater treatment plants is important to protect receiving waters to avoid any detrimental effects to the aquatic system. A Conventional Activated Sludge process (CAS) is typically used to remove nitrogen through Simultaneous Nitrification and Denitrification (SND) but requires larger foot print and longer Solids Retention Time (SRT). Their removal efficiency maybe impacted at lower temperatures, low Carbon to Nitrogen Ratio (C/N), low pH, and low dissolved oxygen [1]. Advanced technologies have been developed to overcome the disadvantages associated with the CAS such as Moving Bed Bioreactors (MBBR), and Integrated Fixed Film Activated Sludge (IFAS) system.

MBBR and IFAS processes improve reliability, simplify operation, and require less space than conventional nitrification/denitrification systems. These technologies employ thousands of polyethylene biofilm carriers operating in mixed motion within aeration tanks but differ in the usage of secondary clarifiers in IFAS. Each individual biofilm carrier provides protected surface area to support the growth of heterotrophic and autotrophic bacteria within its cells in high- density populations and allow for the much higher SRT values necessary for ammonia removal in a reduced volume. In addition, through the control of bulk dissolved oxygen concentration and due to the mass transfer limitations inherent in biofilms, simultaneous nitrification and denitrification can be achieved. MBBR technology offers process reliability and ease of operation as the biofilm attached to the mobile carriers effectively responds to load fluctuations and makes for a cost-effective treatment solution. The growth of biofilms on the free moving media has shown to be successful for SND process because various microbial species coexist in the biofilm clusters, thereby enhancing their resilience to the varying environmental conditions [2].

The hybrid IFAS enables activated sludge systems to achieve gains in biomass inventory without increasing Mixed Liquor Suspended Solids (MLSS) levels in the process. By doing so, IFAS systems deliver COD and ammonia removal while reducing the solids impact on clarification processes. The IFAS process shares the ease of operation of the MBBR process but can sustain greater pollutant loads due to the lesser mass transfer limitations for suspended versus attached biomass and because the different ecological niches within the system select for different bacterial consortia in mixed liquor and biofilms. However, it requires more space for secondary clarifiers and adds complexity of operation of the suspended activated sludge biomass and clarification processes.

In large urban treatment facilities, land is at a premium and the use of MBBR would be preferred if the process can be shown to be effective, and resilient to flow and load fluctuations. MBBR systems have been often used for the removal of ammonia from wastewater

effluent with low C/N ratios since large organic carbon loads interfere with the growth of nitrifiers in the biofilm consortium as heterotrophs consume most of the available oxygen [3]. However, large C/N influent ratios are required for effective denitrification to occur [4] and to achieve low total nitrogen effluent concentrations. In this project, a novel start-up sequence for MBBR systems is developed to overcome this C/N ratio problem. An acclimation period of the system biomass to influent with low C/N ratio is used to develop a mature consortium of microbial species capable of simultaneous nitrification and denitrification at variable influent C/N ratios.

Objective

The main objective of this study was to demonstrate that MBBR can sustain nitrogen removal from a high C/N ratio influent after acclimation of the biomass to feed with secondary effluent and subsequently to influent with medium C/N ratio. Additionally, the study evaluated the microbial diversity in biofilm to gain insight into nitrogen removal under test conditions.

Methods

Pilot system description and operation

The study was conducted using a Sequencing Batch Reactor (SBR) simulating the plant's activated sludge process. Figure 1 presents a schematic diagram of the SBR pilot setup, which is comprised of a reactor with an area of 30.5 cm in diameter and 122 cm in height with a total capacity of 90 L.

The operating volume during the study was 69 L. The reactor was equipped with a coarse bubble diffuser for air sparging of the wastewater. Dissolved Oxygen (DO) in the wastewater was monitored with a dissolved oxygen (DO) probe (Emerson Process Management, Irvine, CA, USA) inserted in the reactor. Airflow adjusted via a combined flow meter/control valve (Sierra Instruments, Monterey, CA) controlled DO levels in the wastewater. The DO setpoint was controlled with a Programmable Logic Controller (PLC). pH was monitored using a pH sensor inserted in the reactor (Rosemount, St. Louis, MO). The water level in the reactors was monitored with a Pulsar Model dBi3 ultrasonic level sensor (Niceville, FL, USA).

The feed was fed into the reactor using a peristaltic pump (Masterflex, L/S, 07523-80) from a feed tank having 208 L capacity. The reactor was filled with K1 carrier plastic media (Evolution Aqua, Wigan, UK) with following dimensions: Diameter: 10.5 mm, Height: 8 mm, Protected Surface Area: 500 m^2/m^3. The media fill volume in the reactor was 50%.

The SBR was programmed to run at 2 cycles of 12 hours per day to simulate the activated sludge process. Each cycle comprised of fill mode, 60 min anaerobic, 170 min aerobic, 435 min of settle mode, and final decant mode. The anaerobic mode simulated the anaerobic selector upstream of aerated reactors of the SEP used to control filamentous bacteria. The Dissolved Oxygen (DO) in the reactor was maintained between 4-5mg/L. The wastewater temperature during the experiment experiments ranged from 18 to 21°C.

Experiments

The study conducted four experiments described in Table 1 to determine the nitrogen removal from the various feeds in these batch reactors. Experiment 1 aimed at showing ammonia removal from an MBBR system with a high C/N ratio feed. Experiments 2 and 3 included the two acclimation steps of the microbial biomass to low to medium C/N ratio feeds. Finally, Experiment 4 was designed to confirm the MBR system's low nitrogen effluent potential after the proposed start-up (Table 1).

Analytical Methods

Samples were collected three times a week to analyze for influent and effluent Total Suspended Solids (TSS), Chemical Oxygen Demand (COD), Ammonia (NH_3-N), Nitrate (NO_3-N), Nitrite (NO_2-N). COD and TSS analyses were performed using Standard Methods. NH_3-N, NO_2-N, NO_3-N were measured using Hach's DR3900 spectrophotometer (Hach, Loveland, Colorado) using following methods:

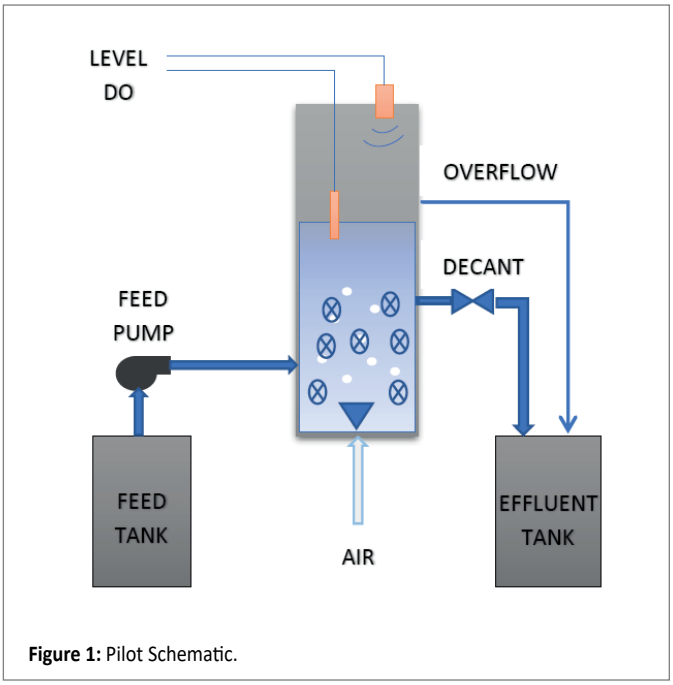

Figure 1: Pilot Schematic.

Table 1: Experiments at different C/N Ratio Feed.

Experiment	Duration	Feed	Feed C/N ratio	Ammonia removal
1	120 days	Primary effluent	9	Negligible
2	150 days	Secondary effluent	2	80%
3	60 days	50:50 PE:SE blend	6	90%
4	45 days	Primary effluent	9	93%

Table 2: Summarizes the feed characteristics for the different experiments.

	Experiment 1 Average ± S.D. mg/L	Experiment 2 Average ± S.D. mg/L	Experiment 3 Average ± S.D. mg/L	Experiment 4 Average ± S.D. mg/L
COD	280 ± 80	85 ± 60	255 ± 70	380 ± 120
TSS	70 ± 17	15 ± 6	52 ± 14	85 ± 20
NH_3-N	35 ± 10	42 ± 9	42 ± 11	42 ± 16
NO_3-N	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2
NO_2-N	0.06 ± 0.03	0.04 ± 0.04	0.1 ± 0.1	0.1 ± 0.1

Nitrite: Low Range (HachTNT839-Method 10207), High Range (HachTNT840-Method10237).

Nitrate: HachTNT835-Method10206

Ammonia: HachTNT832-Method 10205

Samples were sent to IEH Laboratories and Consulting Group, Lake Forest, WA for Metagenomic analysis. Samples were analyzed for 16S Bacterial and ITS Fungal Metagenomics. The IEH Laboratories conducted 16S Bacterial and ITS Fungal Metagenomic analysis by following protocol developed by Illumina Technology (Illumina Inc, San Diego, CA). The protocol for 16s Bacterial analysis includes the primer pair sequence for the V3 and V4 region that create a single amplicon of approximately ~460bp.The protocol also includes overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters. Illumina sequencing adapters and dual-index barcodes are added to the amplicon target by amplifying V3 and V4 region and using a limited cycle PCR. The ends of Sequence on MiSeq-Using paired 300-bp reads, and MiSeq V3 reagents reads are overlapped to generate high-quality, full-length reads of the V3 and V4 region in a single 65 hours run. A taxonomic classification is performed using the Green genes database showing genus or species level classification.

The Fungal Metagenomic Sequencing is performed by amplifying the ITS1 (Internal Transcribed Spacer) region using a limited cycle PCR, and then adding Illumina sequencing adapters and dual-index bar codes to the amplicon target. Sequencing with 2 × 150 cycles and 15,000-100,000 reads per sample is conducted to provide high classification resolution and accuracy. A taxonomic classification is performed using the UNITE database [5] showing classification across all taxonomic levels.

Results

During Experiment 1, the MBBR system did not remove ammonia from the feed, as shown in figure 2. The observed minimum or no removal efficiency of ammonia could be due to the dominant heterotrophs outcompeting slow-growing autotrophs for nutrients and oxygen due to their high metabolic rate; hence, organic loadings should be kept as low as possible to promote and maintain nitrification in MBBR [6].

In Experiment 2, the MBBR system was fed an influent with an average C/N ratio of 2, and, as can be seen in figure 3, it reached almost 90% of operation before ammonia removal was observed. A steady increase in ammonia removal was apparent over the next several weeks, with steady, the removal rates of about 80% during the last three weeks.

An increase in effluent nitrate concentration combined with a decrease in alkalinity (Figure 4) was a sure indication of nitrification in progress. Alkalinity was added in the form of sodium bicarbonate to support the process.

During Experiment 3, the feed to the MBBR system had an average C/N ratio of 6, and figure 5 shows the ammonia observed in this phase of the study. After the initial 30 days of operation, when some fluctuations in ammonia were observed, the movement efficiency was steady at 90%, while the effluent ammonia concentration was consistently measured below 5 mg/L.

Figure 6 shows a temporary presence of nitrite in the effluent and a steady increase in effluent nitrate concentration after four weeks of operation, suggesting balanced nitrification and denitrification occurring in the MBBR system. Nitrogen removal at the end of Experiment 3 was about 70%. Alkalinity addition was continued throughout this experiment.

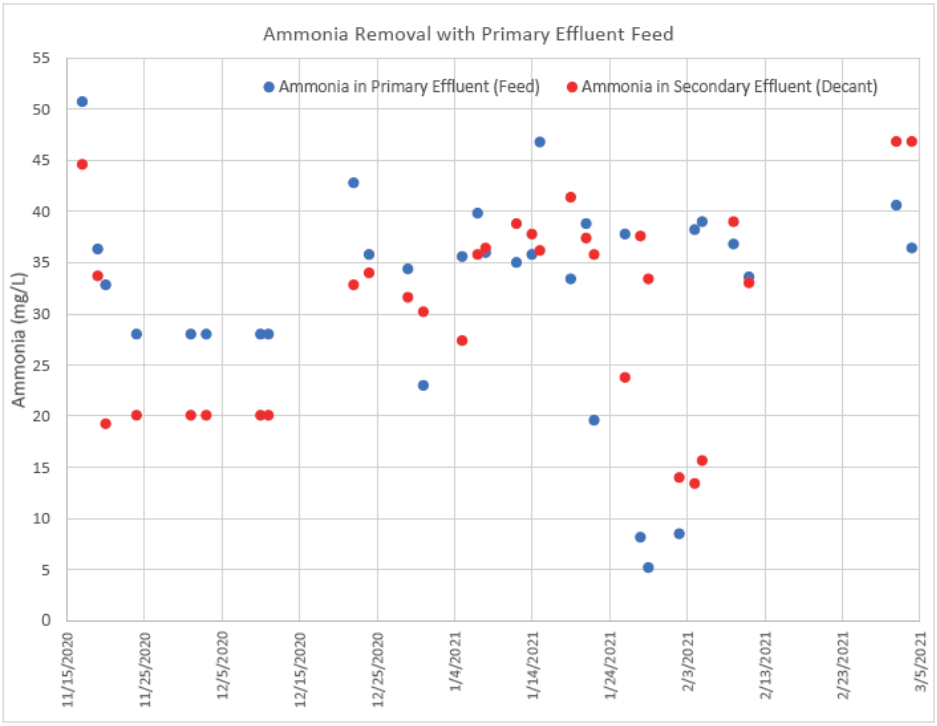

Figure 2: Ammonia removal during Experiment 1.

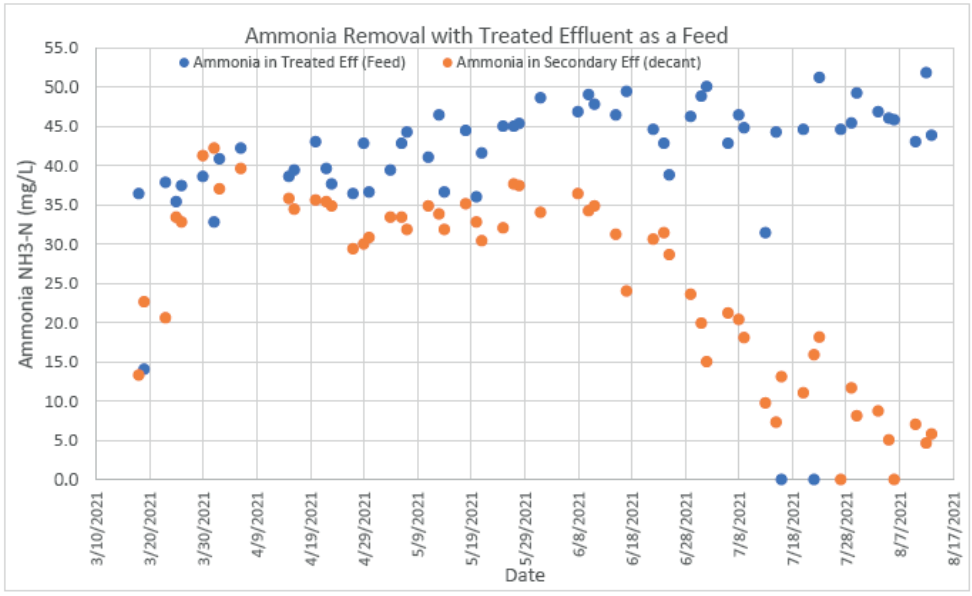

Figure 3: Ammonia removal during Experiment 2.

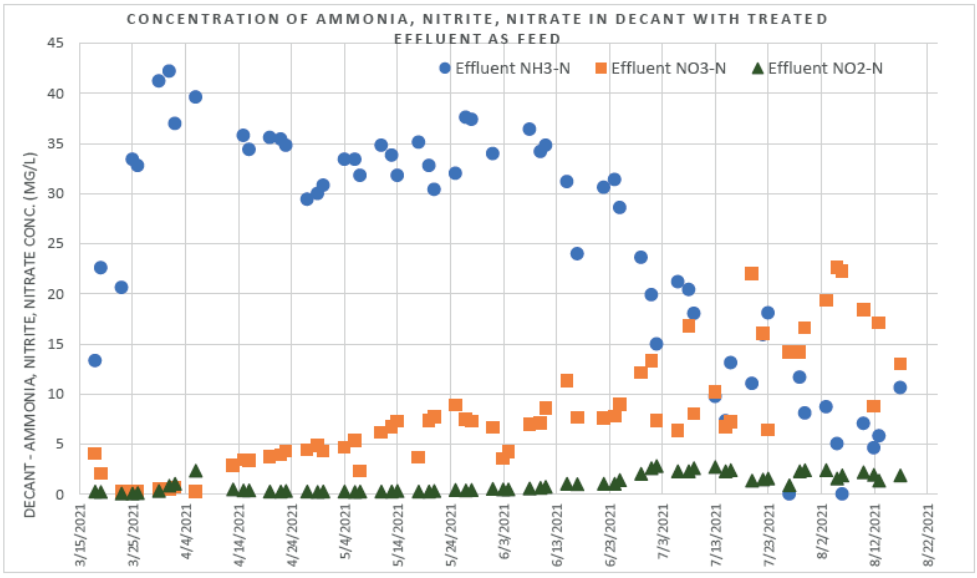

Figure 4: Ammonia, alkalinity, nitrate and nitrite concentrations in MBBR effluent during Experiment 2.

In Experiment 4, the feed C/N ratio was again 9, and the performance of the MBBR for ammonia removal is shown in figure 7. The average ammonia removal efficiency for this experiment was over 90%, whereas the nitrogen removal efficiency was almost 75%.

Alkalinity was supplemented by about 50 mg/L during all experiments to avoid limiting removal rates. Figure 8 presents the alkalinity concentrations in the MBBR effluent throughout the study.

Microbial Diversity

Metagenomic analysis was conducted to determine 16S bacterial and ITS fungal sequencing. The samples for this analysis were conducted to determine the distribution of the microbial population towards the end of Experiment 4, operated with 100% PE. There were 89,000 reads for 16S bacterial reads, whereas ITS fungal reads were 178,000.

The most predominant phyla within the bacterial groups were *Proteobacteria* at 31%, *Bacteroidetes* at 22%, and *Actinobacteria* at 8%, and the remaining Phyla: *Acidobacteria, Nitrospirae, Ignavibacteriaecae, Verrucomicrobia, Firmicutes, Chloroflexi, Plantomycetes, Plantomycetota, Patescibateria, and Gemmatimnadetes* comprised 8%. The unclassified bacterial phyla contained 31%.

The most common classes of proteobacteria present were Betaproteobacteria (18%), *Alphaproteobacteria* (9%), and

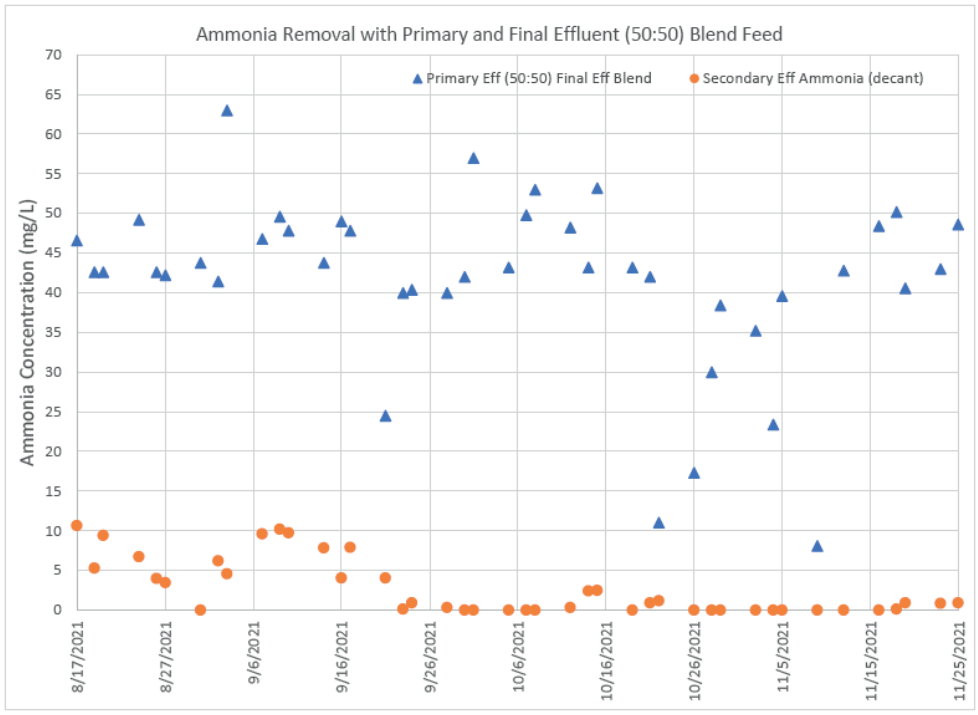

Figure 5: Ammonia removal during Experiment 3.

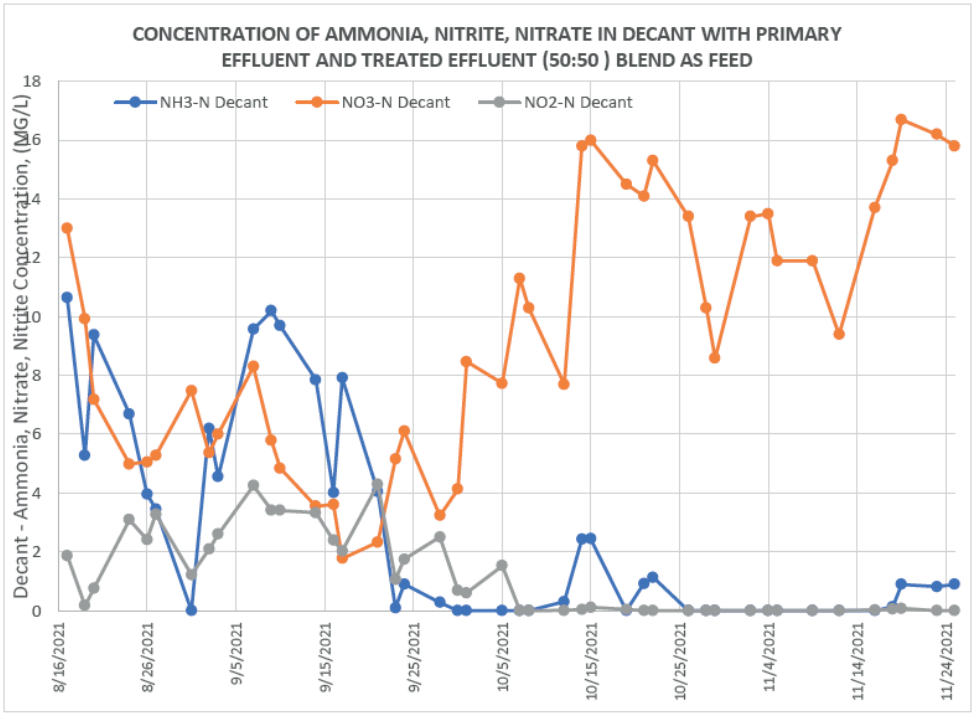

Figure 6: Ammonia, alkalinity, nitrate and nitrite concentrations in MBBR effluent during Experiment 3.

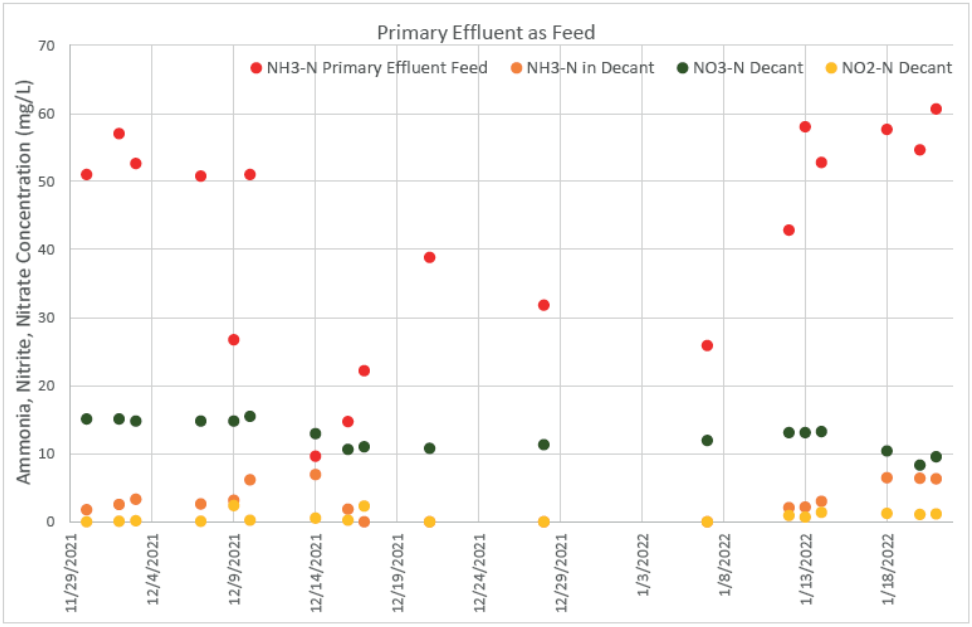

Figure 7: Ammonia, nitrate and nitrite concentrations in MBBR effluent during Experiment 4.

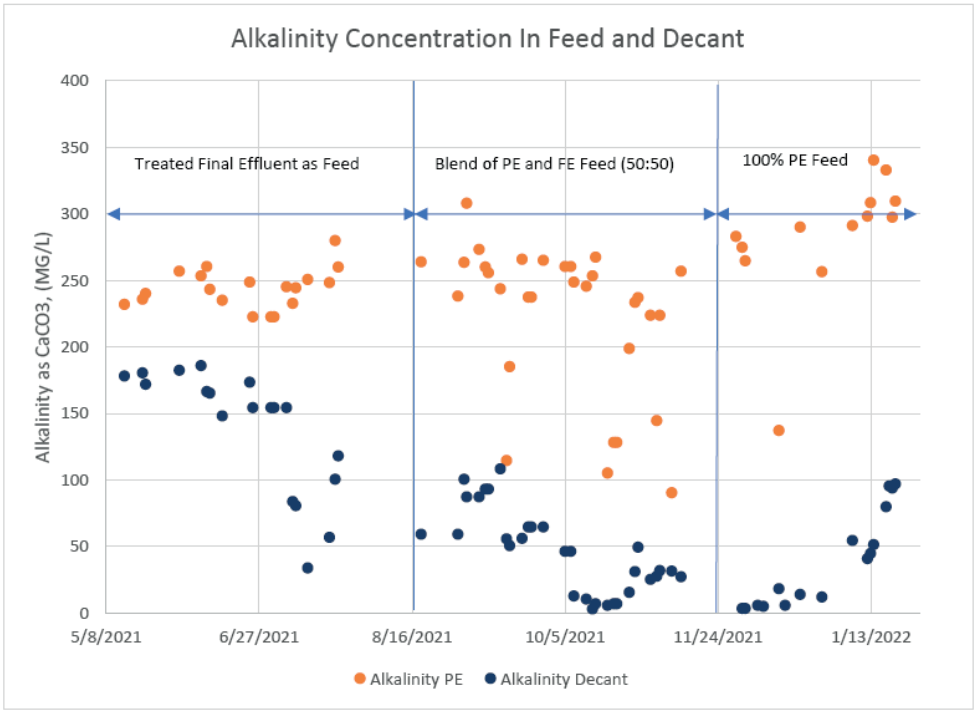

Figure 8: Alkalinity concentrations in MBBR influent and effluent during Experiments 2,3 and 4.

Gammaproteobacteria (3%). Among *Bacteroidetes*, the predominant class was found to be *Saprospiria* at 17%, whereas within *Actinobacteria*, *Acidimicrobiia* was found to be at 6%.

Proteobacteria are soil bacteria involved in the carbon, nitrogen and sulfur cycles [7]. Major autotrophic and heterotrophic bacterial species implicated in nitrogen removal in wastewater treatment belong to this phylum in the alpha-, beta-, and gamma-*proteobacteria* classes, all well represented in the samples from this study. The other two major phyla present in the samples are *Bacteroidetes*, found in the digestive systems of mammals, and *Actinobacteria*, soil bacteria responsible for the decomposition of organic carbon.

The ITS fungal belonged to 3 phyla and 23 genera. The most abundant phylums, *Ascomycota* (10%), *Basidiomycota* (10%), and *Mucoromycota* (1%), were found to be at 21%, and the rest, 71%, were unclassified. *Ascomycota* and *basidiomycota* are important in domestic sewage treatment [8]. Among *Ascomycota*, the predominant class was found to be *Saccharomycetales*, whereas the predominant classes for *Basidiomycota* and *Mucoromycota* were observed to be *Tremellomycetes* and *Mucoromycetes*, respectively. Genus *Trichosporon* belonging to phyla *Basidiomycota* was found in abundance (24%) which implies they play an important role in denitrification and the oxidation of ammonia [8,9].

Discussion

Studies have shown the population ratio of heterotrophs to autotrophs depends on the carbon-to-nitrogen (C/N) ratio in the wastewater [34], which could explain the results of Experiment 1 when the average C/N ratio of the feed was 9. Dominance of heterotrophs is observed at C/N greater than 1, and after organic carbon is depleted and the C/N ratio increases, the available oxygen and nutrients become available for autotrophs to proliferate and the nitrification processes proceeds [10].

The extended time during Experiment 2 before ammonia removal was observed was probably due to the low growth rates of species responsible for ammonia oxidation [11] and the non-ideal C/N feed ratio [1]. This long acclimation period could be shortened with a smaller C/N ratio. Regardless, once established and mature, the microbial consortium showed ammonia removal rates of 90% or better when challenged with feeds with C/N ratios of 6 and 9 during experiments 3 and 4, respectively, and in contrast to results from Experiment 1. In Experiment 1, under initial conditions, heterotrophs out competed ammonia oxidizers for available oxygen, but the high C/N ratios of Experiments 3 and 4 did not negatively affect ammonia removal in the MBBR system. A shift in microbial species must have occurred [12,13] from autotrophic ammonia and nitrite oxidizers to heterotrophic nitrifiers, aerobic denitrifiers, and fungi, as shown by the increased nitrogen removal rates from Experiment 2 to Experiments 3 and 4 presented in Table 3 [4]. Moreover, ratios of 5 [14] to 9-10 [15] have been reported in the literature as thresholds for aerobic denitrification to occur, and this nitrate removal mechanism was therefore unlikely during Experiment 2 when C/N=2. Similarly, fungi require high C/N substrates to proliferate owing to their chemical composition with C/N ratios between 7:1 and 25:1 [16] and could not have thrived during Experiment 2.

Thus, after the proposed novel start-up and acclimation of the MBBR system, sustained nitrogen removal was shown.

Autotrophic nitrification is the most common mechanism for ammonia removal in wastewater treatment. The main autotrophs for conducting nitrification are Ammonia Oxidizing Bacteria (AOB), to which belongs Genus *Nitrosomonas*, and Nitrite Oxidizing Bacteria (NOB), to which belongs Genus *Nitrospira*. These autotrophs are very sensitive to factors like pH, dissolved oxygen, and the carbon-to-nitrogen ratio [17]. AOB and NOB were found to be at very low concentrations during experiment 4. *Nitrosomonas* were found at 1.29% and *Nitrospira* at 1.42%. Overall nitrogen removal increased from 25% in Experiment 2 to 70% or more in Experiment 3 and 4, while feed C/N ratios increased from 2 to 6 and 9, respectively, likely resulting in the shift from autotrophic nitrification to heterotrophic nitrification/denitrificacion as the main mechanism in the MBBR biofilm. Microorganisms possess a wide variety of metabolic pathways and adapt to changing environmental conditions [18]. A recent study demonstrated the influence of C/N on the shift of the microbial population. The decline in AOB and NOB was observed with the COD/TN ratio increasing from 15 to 35, indicating inhibition of organic carbon concentration [19]. Autotrophic nitrification is generally carried out at lower C/N [20]. However, heterotrophic nitrifiers, aerobic denitrifiers, and fungi require higher C/N ratios, and nitrogen removal efficiency is optimal at high C/N ratios [20,21]. Metagenomic analyses of biofilm from Experiments 1-3 would help determine the shift in microbial population. However, the increase in C/N ratio explains the dominance of faster-growing heterotrophic bacteria during Experiment 4, occupying the ecological niche of autotrophic AOB and NOB in Experiment 2 [22].

Table 3: Summarizes the water quality of the MBBR effluent for experiments where ammonia removal was observed.

	Experiment 2 Average ± S.D. mg/L	Experiment 3 Average ± S.D. mg/L	Experiment 4 Average ± S.D. mg/L
COD	47 ± 26	35 ± 13	37 ± 9
NH_3-N	26 ± 11	4 ± 3	3 ± 7
NO_3-N	8 ± 6	10 ± 5	12 ± 2
NO_2-N	1.0 ± 0.9	2.0 ± 1.5	0.7 ± 0.8

A recent study [23] demonstrated a reduction in ammonium concentration due to the metabolic activity of heterotrophic nitrifiers and denitrifiers. Under limiting oxygenation conditions and high C/N ratios, bacterial heterotrophic nitrification becomes the dominant process and can achieve up to 60% of total nitrification. *Proteobacteria, bacteriodetes* and *Actinobacteria* are predominantly found in domestic wastewater treatment plants and are involved with heterotrophic nitrification and aerobic denitrification [24,25]. *Proteobacteria* play an important role in C, N, S and P cycles due to their wide diversity and metabolic capacity [26]. Facultative anaerobes such as *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* can perform denitrification. Abundant presence of *betaproteobacteria* is associated with organic matter degradation and the S cycle [27]. A study conducted to characterize different microbial community and their taxonomic diversity at different DO *Gammaproteobacteria* and *Betaproteobacteria* at DO levels ranging from 4.5 to 0.35 mg/L. The population of *betaproteobacteria* decreased with an increase in DO levels, indicating the role of DO concentrations in causing a shift in microbial diversity [28]. Most bacteria capable of carrying out simultaneous nitrification and denitrification by heterotrophic nitrification and aerobic denitrification belong to *Proteobacteria*, *Betaproteobacteria* , at *Alphaprotobacteria* [25].

Published literature indicates that microorganisms such as prokaryotes and fungi can carry out heterotrophic nitrification and denitrification [23]. Nitrification during Experiments 3 and 4 was not inhibited by high C/N ratio, indicating that nitrifiers had heterotrophic metabolism at high organic carbon concentrations [29].

Heterotrophic nitrifiers can sustain low temperatures, have the ability to utilize a wide range of substrates, have faster growth rates, and are capable of maintaining active biomass throughout the treatment. The cell walls are composed of polymers of chitin and melanin [9], making them very resistant to degradation and therefore resistant to environmental changes. Hence, it is advantageous to have heterotrophic nitrification over autotrophic nitrification [30] in wastewater treatment. However, heterotrophs have adaptation problems at low concentrations of carbon, thereby limiting the nitrification process [31].

Ascomycota and *Basidiomycota*, found in this study, are the most abundant fungal phyla in activated sludge and are capable to remove COD, P, ammonia, and TN [32,9] and can metabolize complex organic matter compared to bacteria [16]. This trait, combined with the resilience to change, may allow robust, fungi-centered wastewater treatment systems that are less expensive and simpler to build and operate than current processes. There needs further research to learn the importance of fungal populations in advanced treatment systems although, based on the genomic results of this study, it is evident that bacterial and fungal populations together were carrying out heterotrophic nitrification and aerobic denitrification [33,34].

Conclusion

This study demonstrated that MBBR systems can achieve ammonia removal efficiency above 90% combined with an overall nitrogen reduction of more than 70% after a proposed novel start-up. The MBBR process can be implemented at full scale to remove nitrogen and meet stringent effluent numerical limits to the discharge of reduced nitrogen species (i.e., ammonia and organic nitrogen).

The concept of heterotrophic nitrification and aerobic denitrification is new and gaining popularity due to the ease of operation and optimum nitrogen removal efficiency that could be achieved.

This study indicates the occurrence of heterotrophic nitrification and aerobic denitrification, as the population of autotrophs was found to be at very low concentrations when C/N ratios were high. Genomic analysis confirmed the role of heterotrophs and fungi in the MBBR biofilm. However, autotrophs may have proliferated at lower C/N ratio, but a shift in microbial population to heterotrophic nitrification likely occurred as the C/N ratio increased. Further research is required to understand the role of other factors that promote the shift as well as the role of the fungal community in wastewater treatment, which is not fully explored but holds great promise.

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