Microbial Community Composition in a Simultaneous Nitrification and Denitrification Bioreactor for Domestic Wastewater Treatment

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Abstract. Traditional domestic wastewater treatments rely on aerobic processes followed by anaerobic processes. The aerobic step in which ammonium and organic carbon are oxidized, calls for large oxygen input, while the anaerobic process often requires extra carbon input. The challenge of synchronizing both processes is to maintain an active nitrifiers sludge under low dissolved oxygen (DO) condition. In this study, a membrane bioreactor was established and operated stable with low DO of 0.1-0.4 mg L⁻¹. Chemical indicators were determined daily, and bacterial community was checked by qPCR and 16S rDNA sequencing every month. After 2 months incubation, the bioreactor reached to a stable removal rate of total nitrogen around 50% and total organic carbon around 90% with the retaining time of 12 h. The sludge showed enrichment of low DO nitrifiers (Nitrosomonadaceae, Chitinophagaceae, and Nitrospiraceae) which were different from sludge in other regular wastewater treatment plants with aerobic and anaerobic cycles.

1. Introduction
Traditional domestic wastewater treatments rely on an aerobic process followed by an anaerobic process. In aerobic process, organic carbon is oxidized to carbon dioxide and release from water to atmosphere. Meanwhile, ammonium is oxidized to nitrite and then nitrate via nitrification process. The aerobic step calls for certain amount of oxygen input, which turns to energy consumption during wastewater treatment. In the anaerobic process, nitrate is reduced to nitrogen gas (N₂) via denitrification process or anaerobic ammonium oxidation (anammox). Since organic carbon is consumed within aerobic process, the anaerobic process often requires extra carbon input [1, 2].
Therefore, by setting up a bioreactor that combines the aerobic and anaerobic processes, both issues from less oxygen input in the aerobic process and less carbon input in the anaerobic process can be solved.

The challenge of setting up a simultaneous nitrification and denitrification bioreactor is to maintain an active sludge with both active aerobes and anaerobes. Aerobic ammonium oxidizing-bacteria (AOB), nitrite-oxidizing bacteria (NOB), and complete ammonia oxidation (comammox, species Nitrospira) are known as the key players in an active aerobic sludge for nitrification [3-7]. Ammonium oxidation archaea (AOA) can also contribute to a high level of ammonium oxidation in the ocean system [8], but they are difficult to be cultured in wastewater treatment system. In an active anaerobic sludge, there are a wide taxonomic diversity of denitrifiers [9]. Another nitrogen removal pathway, anammox, which can use the nitrite came from partial ammonium oxidation by AOB, were usually coupled with AOB and be operated in some wastewater treatment plants recently [10, 11]. All of known anammox bacteria species belongs to Planctomycetes phylum perform [12, 13]. In a full-scale wastewater treatment plant, anammox process usually work as a pre-treatment or side-treatment process [11].

A range of operational parameters or wastewater characteristics, such as pH, temperature, dissolved oxygen (DO) and carbon/nitrogen ratio, drive the nitrification and denitrification performance, as well as the microbial communities in wastewater treatment system [14, 15]. Among all these factors, DO is considered to be the key factor and has significant effect on microbial community structure and nitrification rate. For instance, lab-scale bioreactor operated with high and low DO concentration could have different bacterial community structure [16]. Linking the environmental properties and bacterial communities based on 14 wastewater treatment plants, DO concentration significantly shaped microbial community structure of sludge [14].

Thus, the objective of our research is to create a low DO bioreactor to support both nitrifiers and denitrifiers and satisfy the N removal efficiency. The change of microbial community structure under low DO operation is highlighted in this study to better understand the mechanisms of the bioreactor.

2. Materials and Methods

2.1. Sludge collection and bioreactor set-up

Activated sludge samples were combination of a full-scale wastewater treatment system and a lab-scale bioreactor. Both of the systems were operated with same process of anaerobic/anoxic/aerobic (A2O).

A 3 L membrane bioreactor with a DO probe was established and operated for 6 months. An air pressure was designed to maintain the low DO. When DO was lower than 0.1 mg L\(^{-1}\), the air pressure would open and air could come into the bioreactor. When DO reached 0.4 mg L\(^{-1}\), the air pressure would close and stop the air coming in. The bioreactor maintained at 0.1-0.4 mg L\(^{-1}\) DO for 60 days. After that, the bioreactor was maintained at DO with 1-1.2 mg L\(^{-1}\) for another 60 days.

To reach a low DO stage, the hydraulic retention time (HRT) of the bioreactor was set as 24 h for 15 days, decreased to 12 h for another 15 days, then further decreased to 6 h for a longer operation situation. The ammonium, total nitrogen (TN) and total organic carbon (TOC) from both influent and
effluent were determined every day. The bacterial community was checked by real time quantitative PCR (qPCR) assay and 16S rDNA amplicon sequencing every month.

2.2. qPCR assay

Sludge DNA was extracted according to the manual of the Fast DNA spin kit (Bio 101, Qbiogene Inc. CA, USA). The qPCR experiments were carried out using a StepOne Plus (Agilent). Total bacteria were represented via quantification of the 16S rRNA genes, using primer sets 1055f/1392r [17] (Table 1). For the detection of denitrifiers, denitrifying functional genes (narG, nirS, nirK, and nosZ, ) were quantified with primer sets NarG1/NarG3, NirS3/NirS5, NirK1/NirK5 and NosZ-1f/NosZ-2 [18-21] (Table 1). For the detection of the bacterial amoA gene, primer set amoA1f/amoA2r [22] was used. The nitrite oxidizing gene nxrB was detected by nxrB169F/nxrB638R [23] (Table 1). Anammox bacteria were detected by primer set Amx368f/Amx820r [24] (Table 1). Each PCR mixture (20 μL) was composed of 10 μL of SYBR Premix Ex TaqTM II (2×), 0.8 μL 10 nM of each forward and reverse primers, 0.4 μL ROX Reference Dye II (50×)*3, 6.0 μL ddH2O and 2.0 μL of template DNA (TaKaRa Biotechnology, Japan). The PCR program was initiated with 30 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, 30 s at specific annealing temperatures (Table 1), and 30 s at 70°C. Each assay contained a standard using a serial dilution of plasmids containing specific target genes, independent triplicate templates for each soil sample, and triplicate no template controls (NTC). A melt curve was obtained at the end of each PCR reaction, with the protocol of 1 min at 95°C, 30 s at 55°C and 30 s at 95°C . The single peak of the dissociation curve indicated the specificity of the PCR products.

| Primer  | Target gene | Sequence (5' - 3') | Annealing temperature |
|---------|-------------|---------------------|-----------------------|
| 1055f   | 16S rRNA    | ATGGCTGTCGTCAGCT    | 57                    |
| 1392r   | 16S rRNA    | ACGGGGCGGTGTTGAC    | 57                    |
| NarG1   | narG        | GACAAACCTTCGCAACGGi | 61                    |
| NarG3   | narG        | TCACCCAGGAGCTGTTTC  | 61                    |
| NirS3   | nirS        | CCTCATC(T)TGCCCGCA(G/CA/AG)GT  | 57                   |
| NirS5   | nirS        | GCGCCCGT(C/A/GTG(A/C/G)AGGA A  | 57                    |
| NirK1   | nirK        | GCGATGTTGC(G/T)CC(G/C)TGCGA  | 57                    |
| NirK5   | nirK        | GCCGATCGGT(A/G)T(A/G)TG  | 57                    |
| NosZ-1f | nosZ        | TGGGAATCAGCCGAAAGG  | 57                    |
| NosZ-2r | nosZ        | AAAACCCCTCTACTATGCGCC  | 57                   |
| amoA1f  | AOB amoA    | GGGTTTCTACTATGCTG  | 59                    |
| amoA2r  | AOB amoA    | CCCCTCAGAAAAACCTTCTC  | 59                   |
| nxrB169F| nxrB        | TACATGTGGTGGAACA   | 55                    |
| nxrB638R| nxrB        | CCGTTCTGCTGCTA      | 55                    |
| Amx368F | nxrB        | TGGGTATGGCCGAAAGG   | 57                    |
| Amx820R | nxrB        | GGGGACTAAGTAGGGGGTTT | 57                  |

2.3. 16S rRNA gene amplicon sequencing

Total DNA of sludge was extracted using CTAB/SDS method. The 16S rRNA gene of V4-V5 was amplified with the barcode by Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products was mixed in equidensity ratios. Then, mixture PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo
Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated. The 16S rRNA gene amplicon sequencing was run by Novogene Co., China.

2.4. Data Analyses
Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were split and assembled by FLASH (V1.2.7)[25]. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags [26] according to the QIIME(V1.7.0) [27] quality controlled process. The tags were compared with the reference database (Gold database) using UCHIME algorithm (UCHIME Algorithm) [28] to detect chimeric sequences, and then the chimeric sequences were removed [29]. Then the Effective Tags finally obtained.

Sequences analysis were performed by Uparse software (Uparse v7.0.1001) [30]. Sequences with \( \geq 97\% \) similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, the GreenGene Database [31] was used based on RDP classifier (Version 2.2) [32] algorithm to annotate taxonomic information. In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples(groups), multiple sequence alignments were conducted using the MUSCLE software (Version 3.8.31) [33]. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences.

Alpha diversity was performed basing on this output normalized data and applied in analyzing complexity of species diversity for a sample through 6 indices, including Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage. All this indices in our samples were calculated with QIIME (Version 1.7.0) [27] and displayed with R software (Version 2.15.3).

3. Results and Discussion

3.1. Bioreactor performance and operational conditions
After 3 months incubation, the performance of the bioreactor was maintained with relatively stable pH about 7, HRT around 12 h, and DO from 0.1 to 0.4 mg L\(^{-1}\). TOC removal efficiency was 86.7%-96.4%, and the average TOC concentration in the effluent was 10.2 mg L\(^{-1}\). TN in the effluent ranged from 35 to 50 mg L\(^{-1}\), while the NH\(_4\)^+-N concentration was between 27 and 45 mg L\(^{-1}\). The average removal efficiencies of TN and ammonium were 52.8% and 54.9%, respectively (Figure 1). There were similar removal efficiencies after raising DO to 1 mg L\(^{-1}\). TOC removal was around 83.5%-98.1% and the average TOC concentration in the effluent was 8.2 mg L\(^{-1}\). The average removal efficiencies of TN and ammonium were 47.1% and 50.2%, with the average 38.7 mg L\(^{-1}\) TN and 25.2 mg L\(^{-1}\) NH\(_4\)^+-N in the effluent, respectively.

These results were similar to the other lab-scale bioreactor with A\(^2\)O process, HRT around 8 h, biological oxygen demand (BOD) and TN removal rate of 94% and 61%, respectively [15] Full-scale wastewater treatment plant can have more variable influent of chemical oxygen demand (COD) 200-1200 mg L\(^{-1}\) and TN 20-120 mg L\(^{-1}\) because of operation situation, plant size, surrounding
population, processes, etc.[34]. Considering the low DO demand of our bioreactor, the energy input can be much less than those lab-scale bioreactors or wastewater treatment plants.

![TOC and TN concentration](image)

**Figure 1.** The TOC and TN concentration when bioreactor maintained stable

### 3.2. Bacterial abundance by qPCR assay

When the bioreactor maintained at low DO of 0.1-0.4 mg L$^{-1}$ for 60 days, the total bacterial abundance (shown by 16S rRNA gene copies) increased from 13.0 to 32.0 copies mL$^{-1}$. Meanwhile, the functional gene structure had changed. The *nirS*, *nirK*, and *nxrB* gene copies decreased, while *narG*, *nosZ*, and *amoA* gene copies increased (Table 2). The number of anammox bacteria was under detection. These results indicated that AOB would take responsible for nitrification during this stage, while nitrite oxidizing process may be inhabited.
When the bioreactor maintained at DO around 1 mg L\(^{-1}\), the total bacterial abundance showed similar increase trend as low DO level. Nevertheless, the bacterial structure changed. Both \(\text{amoA}\) and \(\text{nxrB}\) increased, suggesting that full nitrification process would be enhanced during this stage. In terms of denitrifying genes, \(\text{nirS}\), \(\text{nirK}\) and \(\text{nosZ}\) showed a decrease trend, while \(\text{narG}\) kept the similar level. These results suggested that denitrification would be inhibited during this stage.

| Table 2. Functional gene abundance by qPCR assay |
|-----------------------------------------------|
| DAY | 16S rRNA \(\times 10^8\) | \(\text{narG}\) \(\times 10^7\) | \(\text{nirS}\) \(\times 10^6\) | \(\text{nxrB}\) \(\times 10^6\) | \(\text{nosZ}\) \(\times 10^6\) | \(\text{amoA}\) \(\times 10^6\) |
| DO 0.1-0.4 mg L\(^{-1}\) | | | | | |
| 0 | 13.02 | 2.34 | 16.34 | 12.34 | 3.15 | 2.75 | 4.87 |
| 20 | 27.21 | 6.87 | 13.32 | 3.21 | 9.18 | 5.51 | 1.54 |
| 40 | 32.01 | 6.95 | 5.64 | 2.23 | 9.35 | 5.90 | 1.42 |
| DO 1.1-1.2 mg L\(^{-1}\) | | | | | |
| 0 | 13.24 | 5.10 | 6.77 | 1.98 | 6.35 | 1.82 | 1.23 |
| 20 | 27.45 | 15.34 | 22.34 | 7.34 | 8.01 | 4.15 | 6.87 |
| 40 | 34.34 | 13.23 | 23.54 | 5.34 | 4.85 | 5.42 | 8.31 |
| 60 | 38.54 | 16.34 | 14.34 | 2.13 | 3.80 | 5.72 | 8.09 |

3.3. Diversity of bacterial communities

After filtering the low quality reads and trimming the adapters, barcodes and primers, there were 46401 effective reads. There sequences were grouped into a total of 411 OTUs, with a 3% sequence dissimilarity cut-off. The number of OTUs was relatively low compared to the number of OTUs within typical bioreactors (2513-3878)[15] or wastewater treatment plants (2176-4123)[34]. This means that our bioreactor had a low bacterial diversity after 6 months incubation. The index of bacterial diversity was shown in Table 3. The abundant OTUs (with > 0.1%) accounted for 18.7% of the total number of OTUs but represented 93.4% of all reads, which mean that some bacterial group would dominate the activated sludge.

| Table 3. The diversity of bacterial communities |
|-----------------------------------------------|
| No.of OTUs | Observed species | Shannon | Simpson | Chao1 | ACE |
| 411 | 411 | 4.493 | 0.835 | 447.0 | 434.9 |

At the phylum level, *Proteobacteria* represented the most abundant phylum, accounting for an average of 81.7% of the total sequences. *Bacteroidetes* was the second most abundant phylum (6.5%), followed by *Acidobacteria* (2.7%), *Planctomycetes* (1.2%), *SHA-109* (1.1%) and *Cyanobacteria* (1.1%). Within *Proteobacteria*, *Alphaproteobacteria* (49.9%) and *Betaproteobacteria* (27.9%) were the most dominant subdivision, followed by *Grammaproteobacteria* and *Deltaproteobacteria*. The population of *Bacteroidetes* was mainly composed of two classes: *Sphingobacteria* and *Cytophagia*. 
The bacterial diversity at the phylum level and class level showed typical activated sludge communities [7, 14, 15, 34].

At the order level, the bacterial diversity of this study was quite similar as other activated sludge [7, 14, 15, 34]. We also observed an enrichment of *Nitrosomonadales* (top 12, 0.96%) and *Nitrospirales* (top 13, 0.93%) in our bioreactor, indicating an accumulation of nitrifiers after long-term low DO incubation. At the family level, a total of 100 families were observed. This number was quite low compared to typical bioreactors or full-scale wastewater treatment plants, although most of them showed the same families [7, 34]. The abundant (>0.1%) families were illustrated in Figure 2. At the genus level, a total of 140 genera were obtained, compared to usually more than 300 genera in full-scale wastewater treatment plants [7, 14, 34]. The low numbers of order, family and genus of our bioreactor showed that low DO changed the bacterial community structure to a low diversity but suitable for less oxygen input group.

![Figure 2](image)

**Figure 2.** Abundant microbial community of sludge (> 0.1%) at the family level

3.4. **Diversity of N-related bacterial communities**

The N-related bacterial communities has been enriched. Among the abundant (>0.1%) families, N-related bacteria accounted for 51.8% of the total bacterial sequences (Figure 2). Among them, AOB group took part of 5.8%, containing families from *Saprospiraceae*, *Nitrosomonadaceae*, *Chitinophagaceae*, *Sphingomonadaceae*, and *Flavobacteriaceae*. NOB mainly belonged to *Nitrospiraceae* (1.2%). Anammox, which was shown as *Planctomycetaceae*, comprised 1.4%. Denitrifiers (43.4%) came from families including *Comamonadaceae*, *Rhodocyclaceae*, *Alcaligenaceae*, *Cytophagaceae*, *Caulobacteraceae*, *Xanthomonadaceae* and *Bradyrhizobiaceae*. 
4. Conclusions

In this study, we set up and operated a bioreactor at a low DO level of 1 mg L\(^{-1}\). After 2 months operation, the bioreactor reached a stable removal rate of TN and TOC around 50% with the HRT of 12 h. The microbial community structure showed enrichment of low DO nitrifiers (Nitrosomonadaceae, Chitinophagaceae, and Nitrospiraceae), denitrifiers, and anammox bacteria groups. These results confirmed that simultaneous nitrification and denitrification were occurring in our bioreactor.

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