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Pyrosequencing analysis of ammonia oxidizing bacteria in municipal activated sludge system

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Abstract: Nitrification is a critical step that depends on the diversity and functions of nitrifiers in the activated sludge treatment systems. Optimum and efficient nitrification hinges on in-depth understanding of the structure and dynamics of the nitrifying community structure within the wastewater treatment systems. In this study, high-throughput pyrosequencing was employed in profiling ammonia-oxidizing bacteria (AOB) in activated sludge system treating municipal waste. During the investigation, the average temperature was 16.5 ± 2.1^{o}C and 22.8 ± 2.7^{o}C during winter and summer respectively. During this study, the dissolved oxygen level in the plant was constantly lower than the optimum (0.6 ± 0.3 and 0.6 ± 0.1 mg/l winter and summer respectively). The plant treated wastewater with influent ammonia concentration between 24.4 and 31.6 mg/l. The average influent flow rate (ML/Day) was 96.81 during this period. The diversity of AOB, the nitrifiers involved in the first and the rate-limiting step of ammonia removal had correlation with nitrification efficiency and temperature. Pyrosequencing revealed significant differences in the AOB population, which was 6 times higher during summer compared to winter. The AOB sequences related to uncultured bacterium and uncultured AOB showed an increase of 133% and 360% respectively when the season changed from winter to summer. This study suggests that vast population of novel, ecologically significant AOB species that remain unexploited, still inhabit the complex activated sludge communities.

Key words: 454-pyrosequencing; AOB; WWTP; Nitrification

1. INTRODUCTION

Understanding the complex microbial community in wastewater treatment plants (WWTPs) is important in designing functionally stable and effective treatment systems. The advent of molecular techniques has revealed the inadequacies of traditional microbiological methods of identifying and quantifying microbes in wastewater (Xia et al., 2010). The different Sanger-sequencing based molecular approaches including; polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE), terminal restriction fragment length polymorphism (T-RFLP), temperature-gradient gel electrophoresis (TGGE) and cloning have been used in profiling the microbial diversity harboured in WWTPs (McMahon et al., 2009; Gomez-Silvan et al., 2010). Fluorescent in situ hybridization among others have also been used in microbial diversity profiling of engineered wastewater treatment environments with some degrees of success (Awolusi et al., 2015). More recently, it has been shown that the traditional Sanger sequencing is yet, grossly underestimating the communities of the complex environmental samples due to the hundreds or thousands of important sequences that go unnoticed when employing this method (Shokralla et al., 2012). A major shortcoming of this technique is that it requires in vivo amplification of DNA fragments in bacterial hosts (cloning) prior to sequencing. Cloning is labour-intensive, tediously long and subject to bacterial host bias (Morozova and Marra, 2008).

Due to the thousands of template DNA usually present in wastewater samples there is need for technique that is capable of simultaneous reading from different DNA templates (Shokralla et al., 2012). The Next generation sequencing (NGS) approach offers a speedy, and extensive data production with the opportunity of investigating the microbial ecology on a larger scale and with more details (Ju and Zhang, 2015). Next generation sequencing offers the advantage of direct sequencing from environmental samples without prior cloning into a bacterial host before

sequencing, as obtained in the traditional Sanger approach. The NGS has revolutionized the genomic and metagenomic research with different platforms being commercially available. In this study, variation in community structure of the aerobic nitrifying activated sludge over the winter and summer was evaluated using the pyrosequencing techniques.

2. MATERIALS AND METHODS

2.1 Wastewater treatment plant operation

The full-scale WWTP selected for this study is situated in the midlands of KwaZulu-Natal province, South Africa. The plant receives a discharge of 82880 ± 20832 m^3/d (average dry weather flow), including 90% domestic and 10% industrial wastewaters. The plant was designed based on the criteria of a modified Johannesburg (JHB) configuration, which offered anaerobic, anoxic, and aerobic biological processes (Daims and Wagner, 2010). As shown in Fig. 1, the effluent from primary settling tank is distributed to the pre-anoxic and anaerobic tanks. The pre-anoxic basin is enriched with return activated sludge from the bottom of a final settler, whilst effluent from anaerobic tank is discharged into the anoxic unit. An internal recycle is pumped from the last part of aerobic units to the anoxic zone. The mixed liquor, containing activated sludge, flows from the aerobic zone to a secondary settler, where it is separated under a quiescent condition into treated wastewater and return activated sludge. Wastewater characteristics and operational parameters for the plant are shown in Table 1.

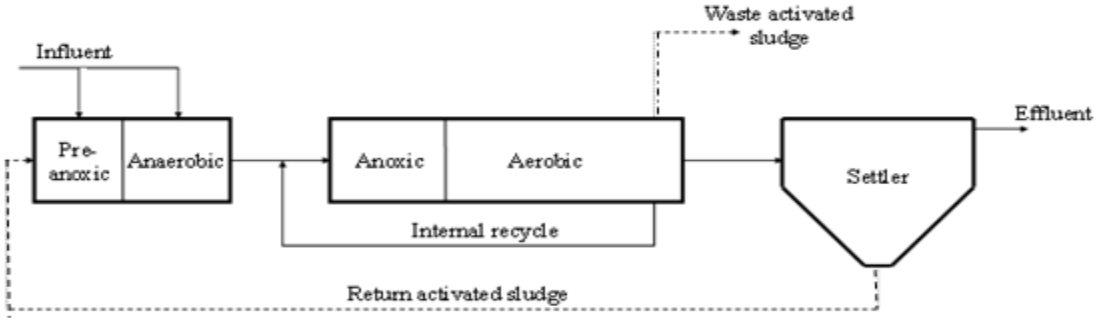

Figure 1. Schematic representation of the wastewater treatment plant.

Table 1. Average (winter and summer) wastewater characteristics and operational parameters of the selected plant as observed during the study period

Parameter	Phase-1	Phase-2
	Winter	Summer
Rainfall (mm)	26.0 ± 18.6	116.8 ± 32.0
Temperature	16.5 ± 2.1	22.8 ± 2.7
pH	7.3 ± 0.2	7.2 ± 0.1
DO (mg/l)	0.6 ± 0.3	0.6 ± 0.1
MLSS (mg/l)	6157 ± 783.3	4728 ± 1282.0
Chemical oxygen demand (mg/l)	1156 ± 976.1	684.7 ± 258.9
Ammonia (mg/l)	31.6 ± 6.0	24.4 ± 4.5
Flow Rate (ML/Day)	62.0 ± 2.2	96.8 ± 14.5
HRT (h)	6.3 ± 0.2	4.3 ± 1.0
OLR (kg-COD/m^3.d)	4.0 ± 1.1	4.5 ± 1.8
ALR (g-NH_4/m^3.d)	121 ± 22.0	144 ± 29.0
F/M (g-COD/g-MLSS.d)	0.6 ± 0.1	0.9 ± 0.3
COD removal (%)	97.5 ± 1.3	94.1 ± 2.6
Ammonia Removal (%)	60.0 ± 18.0	83.0 ± 13.0

COD: chemical oxygen demand; HRT: hydraulic retention time; OLR: organic loading rate; ALR: ammonia loading rate; F/M: food to micro-organisms ratio

Composite sludge samples (from the aeration tank), influent and effluent water samples were collected fortnightly for a period of 237 days (May - July 2012 and November 2012 - March 2013). Sterile sampling bottles were used in collecting the samples and all samples were maintained at 4 ºC while in transit to the lab. Temperature, dissolved oxygen (DO) concentrations and pH measurements were done using a portable YSI meter (YSI 556 Multiprobe System). The plant operational parameters were obtained from the plant operators.

2.2 Sample preparation and DNA extraction for seasonal community structure analysis in the activated sludge

The diversity of ammonia oxidizing bacteria was assessed using high-throughput pyrosequencing approach. Samples taken between the 1st and 78th day represented the winter period (May - July 2012) whilst the samples from 79th through 237th day (November 2012 - March 2013) represented summer. The extracted DNA samples for the winter months were pooled together in equimolar quantities to make up the winter template DNA sample, whilst the same was done for the summer months. These resulted in two separate template DNA samples that used for pyrosequencing analysis. The freeze-thaw DNA extraction was according to modified Briese (2002) protocol.

2.3 Sequencing and analysis

The amoA locus of the AOB in the activated sludge was amplified using the primer set: amoA-1F/amoA-2R. The PCR was performed in a total reaction volume of 50 µl containing 10 ng of DNA template. The final concentrations of the different components in the reaction mix (200 µM of DNTPs, 1.5 mM of MgCl [Taq buffer with initial MgCl concentration of 20 mM], 2.5 U of Taq DNA polymerase [Thermo Scientific, Lithuania] and 0.5 µM of each primer) were according to modified protocols from Degrange and Bardin (1995). The PCR products were purified and end-repaired. The amoA amplicons generated from the PCR were sent to Inqaba Biotechnical Industries (Pty), South Africa, where the composition of the amplicon of amoA targeted locus was then determined by pyrosequencing. The barcodes for multiplexing were incorporated between the forward primers (amoA-1F) and the 454-adpter sequence for pyrosequencing using Roche 454 FLX Titanium sequencing platform (Roche, USA) (Schloss et al., 2009). After sequencing, the unique tags obtained were aligned with the 16S rRNA database with the aid of the BLASTN programme (Sekar et al., 2014). The tag redundancy was eliminated and sequences were assigned into OTU based on similarities of greater than 90%. With the aid of MEGA6 software, representative sequences were aligned using the ClustalW programme. The neighbour-joining method was employed for the phylogenetic analysis (Tamura et al., 2013). The raw pyrosequencing .sff file has been deposited into the NCBI sequence Read Archive.

2.4 Short-read archive accession numbers

The raw reads for the pyrosequencing have been deposited into NCBI Sequence Read Archive under the accession numbers SRP053412.

3. RESULTS AND DISCUSSION

3.1 Ammonia oxidizing bacteria profiling based on amoA gene using high throughput pyrosequencing

Ammonia oxidation has been identified as the rate-limiting step in nitrification hence, the diversity of the AOB in this system was examined to understand their role in nitrification

efficiency. The AOB diversity for the different seasons was revealed by 454-pyrosequencing using the amoA-1F and amoA-2R primer sets (with multiplex barcodes inserted between the forward primers and the 454 adapter sequence). With the aid of MEGA6 software, representative sequences were aligned using the ClustalW programme and the neighbour-joining method was employed for the phylogenetic analysis (Figs. 2-3). A total record of 212 and 1192 effective sequences were obtained from the winter and summer samples respectively. After comparing them with the NCBI database, substantial percentages of the read from the two samples (72 % in winter and 78 % in summer samples) returned no hits and could not be assigned to any phylum (Fig. 4).

The identified AOB populations during the study were related to uncultured ammonia oxidizing bacteria, uncultured bacterium, *Nitrosomonas oligotropha,* and Uncultured *Nitrosospira sp.* (Figs. 2-4). Among these identified AOB populations, the uncultured ammonia oxidizing bacteria was the most dominant throughout the study with 97% and 95% during summer and winter seasons respectively, whilst the uncultured bacterium was 2% and 5% during the summer and winter respectively. The *Nitrosomonas oligotropha* was about 1% of the AOB population during the summer season, however, it was not detected during the winter (Fig. 4). Figs. 2-3 indicated that the AOB diversity during winter were not related to that detected in summer. Moreover, Uncultured *Nitrosospira sp.* were only detected during the summer (Fig. 2). The AOB diversity was 6 times higher during summer than winter (Fig. 4) when a higher NH_3 removal rate and temperature were recorded (Table 1). The AOB sequences related to uncultured bacterium and uncultured AOB showed increase of 133% and 360% respectively when the season changed from winter to summer (Fig. 4).

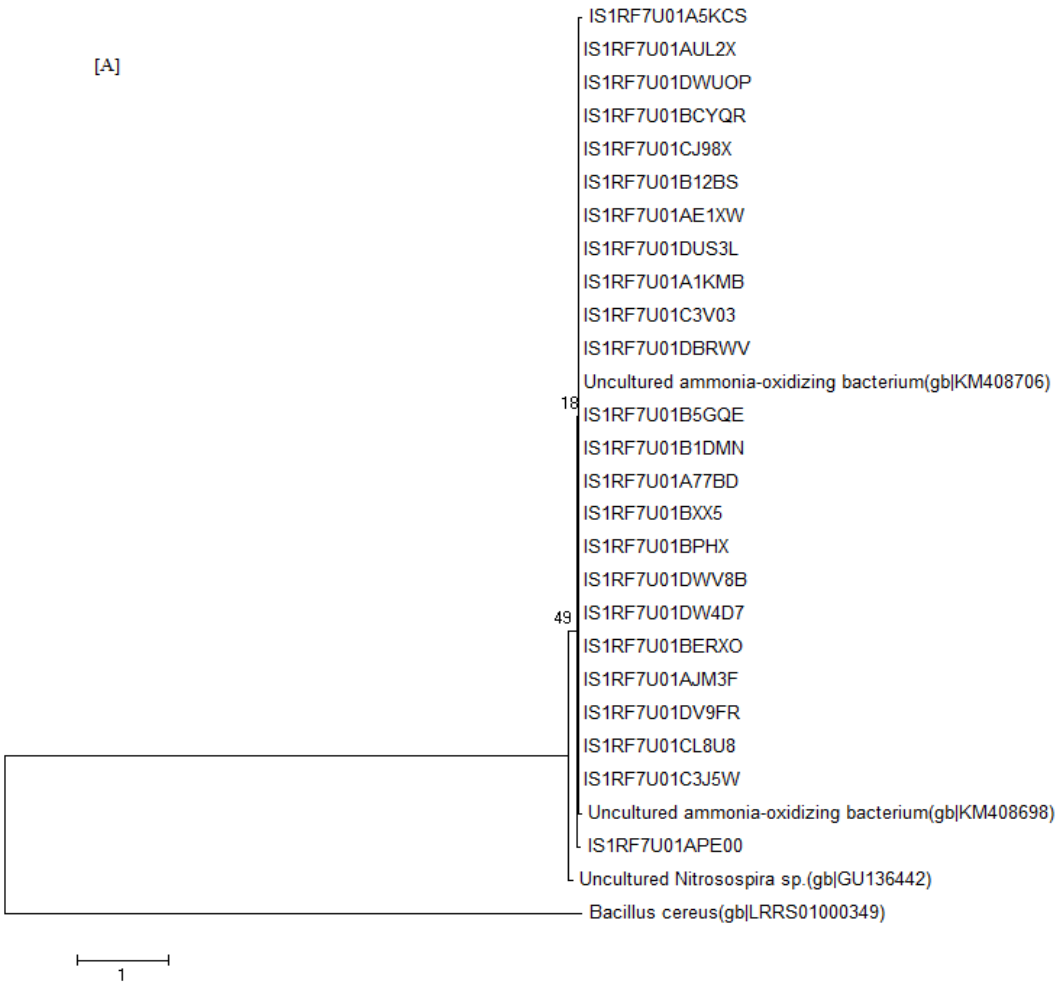

Figure 2. Phylogenetic tree of some selected ammonia oxidizing bacteria OTUs based on amoA gene locus pyrosequencing reads using BLASTN and MEGAN for samples collected during summer.

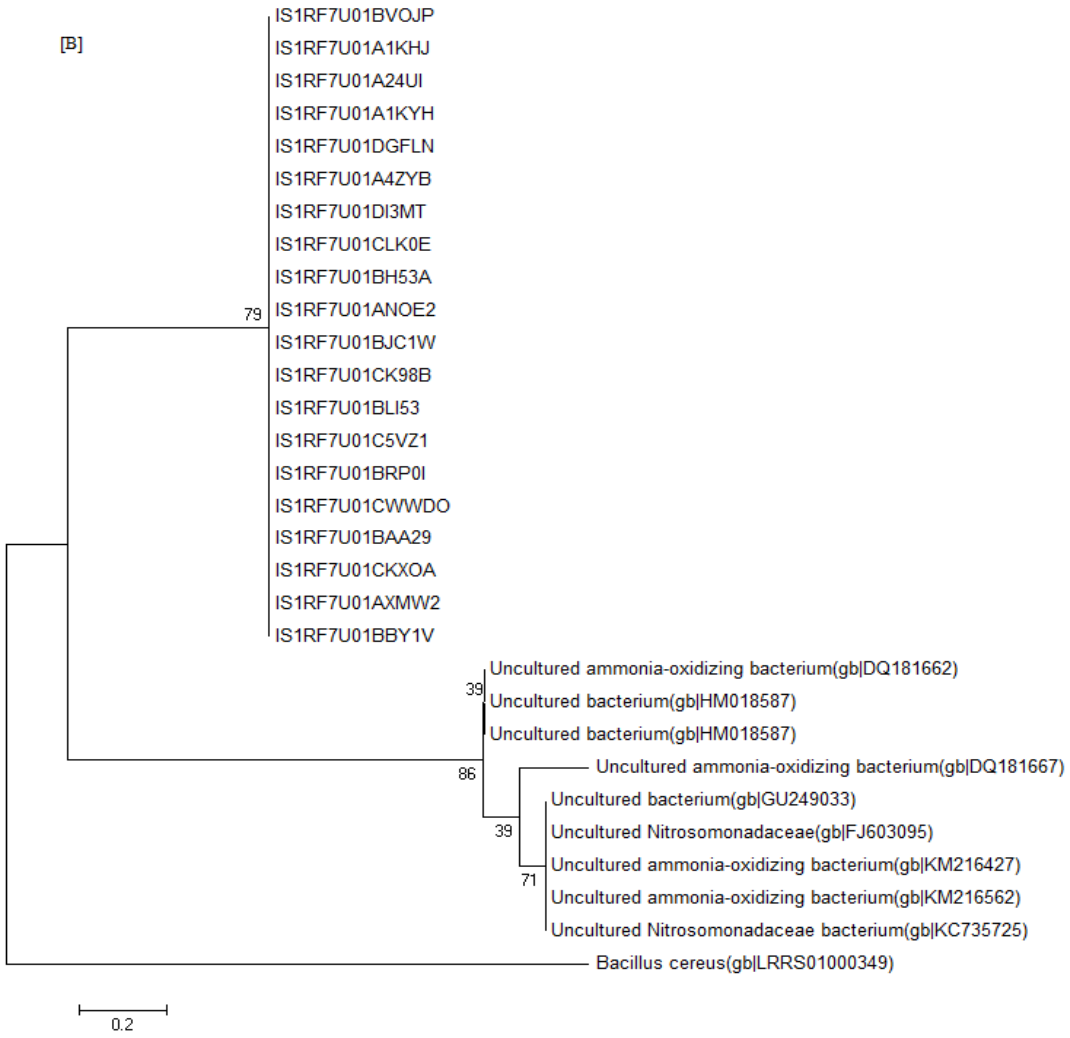

Figure 3. Phylogenetic tree of some selected ammonia oxidizing bacteria OTUs based on amoA gene locus pyrosequencing reads using BLASTN and MEGAN for samples collected during winter.

The plant had significant variation in the influent wastewater compositions, prevailing operational and environmental parameter (Table 1) which correlated with variations in the microbial community structure of the reactor. This variation in microbial diversity affects the planted seasonal nitrification performance as earlier reported (Rowan et al., 2003; Miura et al., 2007). The highest percentage ammonia removal efficiency was recorded during summer when the greatest AOB diversity was found in the reactor (Table 1). It indicates that AOB diversity could be a contributory factor to efficient ammonia removal in activated sludge.

Based on the pyrosequencing analysis, the higher AOB diversity was observed in summer when there was a comparatively higher ammonia removal efficiency (Table 1). The *Nitrosomonas oligotropha* was only detected in the summer sample which indicated a possible higher diversity during summer compared to the winter period (Fig. 4). Furthermore, the AOB diversity was 6 times higher during summer than winter when a higher NH_3 removal rate and temperature were recorded. Using pyrosequencing, Zhang et al. (2015) also observed higher AOB diversity in summer compared to winter when monitoring three different WWTP. Niu et al. (2016) also reported a significant decrease in bacterial amoA genes copy numbers during winter, in a water purification plant. In this study, the AOB sequences related to uncultured bacterium and uncultured AOB showed increase of 133% and 360% respectively when the season changed from winter to summer. *Nitrosomonas oligotropha*-like sequence that were detected from summer samples (1%) were absent in winter samples (Fig. 4). Earlier research efforts have documented similar trend in different environment. Temperature has a major influence on AOB diversity and population structure with

lower species richness correlating to lower temperatures (Urakawa et al., 2008). Similarly, Ju et al. (2014) observed a higher abundance of *Nitrosomonas* in activated sludge during summer. Faulwetter et al. (2013) also reported seasonal impact on AOB community structure in constructed wetland with higher diversity during summer compared to winter. A higher diversity of amoA gene was also recorded during summer in the tidal wetland investigated by Zheng et al. (2013).

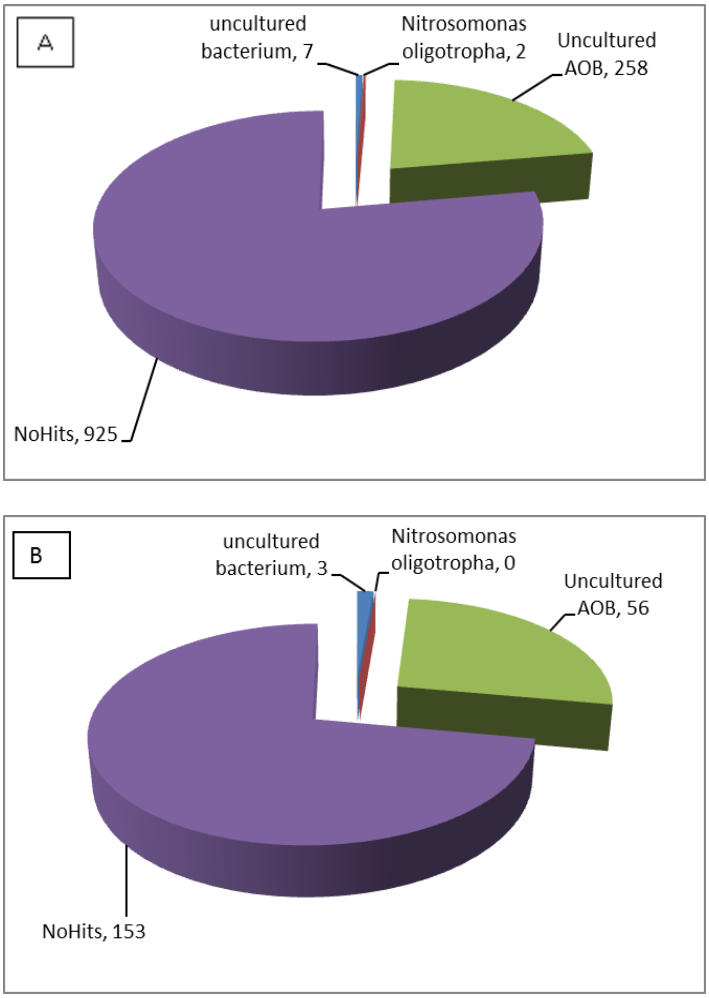

Figure 4. (a) AOB diversity as revealed by pyrosequencing during summer, (b) AOB diversity as revealed by pyrosequencing during winter.

Overall, the summer period harboured larger AOB diversity compared to winter as revealed in this study. A significant proportion of the effective sequences (72% in winter and 78% in summer sample) termed "no hits" could not be successfully classified into any known bacterial 16S rRNA sequences since they showed no similarity to the available sequences in NCBI database. This suggests yet unidentified populations and vast unexploited AOB diversity. This corroborates a recent report (Abe et al., 2017) in which phylogenetic analysis of the pure strains isolated from a domestic WWTP revealed a deeply branching, previously unknown lineage and diversity within the genus *Nitrosomonas*. Yang et al. (2011) earlier on had reported that unassigned or unclassified bacteria usually consists higher proportion in activated sludge compared to other environments such as soil, because activated sludge have a more complex microbial communities. In a study by Shi et al. (2013) the occurrence of unclassified bacteria sequences in chlorination and clear water tanks were detected. They noted that these unclassified OTUs from the different samples were closely clustered in the phylogenetic tree. Therefore, there is need for more study in order to identify these ecologically significant diversity of novel AOB species which makes up the complex activated sludge communities.

4. CONCLUSIONS

Pyrosequencing reveals higher diversity of AOB in the reactor during summer that was characterized by higher temperature. Furthermore, *N. oligotropha* and Uncultured *Nitrosospira sp.* were only identified during summer. This indicates that higher temperature elicited increased AOB diversity.

The AOB diversity was 6 times higher during summer than winter when a higher NH_3 removal rate and temperature were recorded. The AOB sequences related to uncultured bacterium and uncultured AOB showed increase of 133% and 360% respectively when the season changed from winter to summer. This suggests that higher AOB diversity resulted in increased nitrification in activated sludge.

The finding suggests that vast diversity of novel, ecologically significant AOB species, which remain unexploited still inhabit the complex activated sludge communities

Future research should target characterization of the nitrifying populations in wastewater as pyrosequencing revealed a large percentage of the microbial community that did not match any of the know sequences on the existing database.

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