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Quantitative Analysis of the Abundance and Composition of Nitrifying Bacteria on Simultaneous Nitrification and Denitrification in SBBR

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Research Article

ABSTRACT

To reveal the effect of dissolved oxygen (DO) on microorganism community structure during simultaneous nitrification and denitrification (SND) in sequencing biofilm batch reactor (SBBR), the molecular biological techniques of denaturing gradient gel electrophoresis (DGGE), cloning and real-time PCR were applied. DGGE sequence analysis showed some kinds of populations had been highly preponderance consistently, and cloning and phylogenetic tree of AOB gene revealed *Nitrsospira* was dominant species. The real-time PCR analysis indicated that the amount of the AOB and NOB both had most copy number in the lower surface, and the quantity relation of AOB and NOB population between three samples was "lower surface＞upper surface＞internal layer". The result proved that DO concentration directly affected the proportion of aerobic fraction and anoxia fraction in the biofilm, which led to an effect on efficiency of SND.

INTRODUCTION

Sequencing biofilm batch reactor (SBBR) was used to achieve simultaneous nitrification and denitrification (SND) in current study. With SBBR, microorganisms are immobilized on fillers as biofilm, which results in high biomass hold up and enables the process to be operated at high liquid throughputs and organic loading rates [1]. Compared with sludge-based systems, SBBR is generally less energy intensive and more resistant to shock loads to which wastewater treatment systems are frequently subjected [2]. Besides, SBBR is particularly useful where high hydraulic loading variations occur and where slowly growing microbes with special metabolic capacities can be protected from washout[3]. However, several studies approved that simultaneous nitrification and denitrification (SND) can complete biological nitrification coupled to denitrification in one structure [4-6]. At first, ammonia is oxidized to nitrite by ammonia-oxidizing bacteria (AOB) and, subsequently, nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB) [7]. Finally, denitrification involves the reduction of nitrate via nitrite and nitric oxide, to nitrous oxide or dinitrogen gas [8].

Aimed at the achievement of simultaneous nitrification and denitrifycation (SND) in SBBR, the biofilm needs stable aerobic zone and anaerobic zone with right operation parameters, such as dissolved oxygen, temperature, pH, inhibitors, and intermediates [9-11]. Yuan and Blackall[12] stressed that optimization of microbial community structure and functioning should be a major objective in the design and operation of a treatment system. Although developments in reactor designs together with the conventional monitoring of chemical (e.g., NH_4^+-N, NH_3-N, dissolved oxygen, pH) and physical (e.g., flow rate, temperature) variables may enable engineers to enhance the process safety and to optimize the biological reactions in short time periods, a consistent long-term

performance can only been performed when the microbial community within the biofilm optimized its functions. For this purpose, questions about community structure, activity and the population kinetics have to be answered by means of molecular monitoring tools [12], which allows identifying and quantifying the microorganisms present in the wastewater treatment plant.
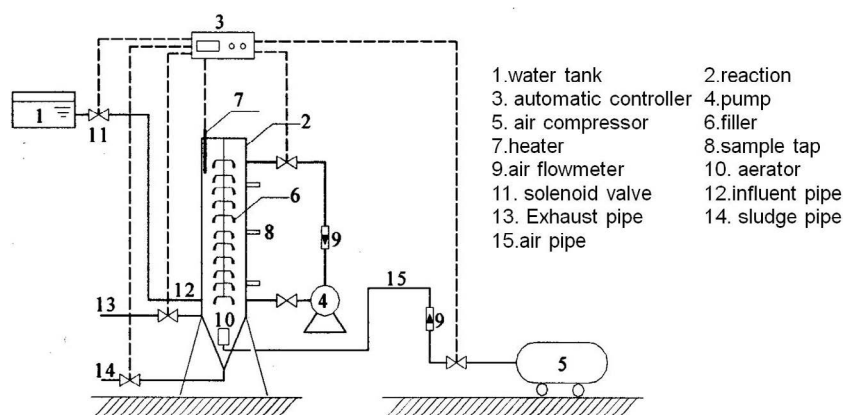
The key to efficient and robust biological wastewater treatment relies on knowing the microorganisms involved and how they respond to different operating conditions [13]. Several microbial diversity studies of activated sludge and biofilms based on 16Sr RNA gene libraries have been reported in the last decade. Denaturant gradient gel electrophoresis (DGGE) has been used to separate amplified 16S rRNA genes and determine the effects of ammonia and dissolved oxygen concentrations on community compositions of nitrifiers [14-16]. However, commonly used 16S rRNA primers for AOB studies have limited specificity, and the high similarity among 16S rRNA genes of AOB makes it impossible to resolve and identify closely related AOB species. Alternatively, the functional gene encoding the alpha subunit of ammonia monooxygenase (AMO), the enzyme responsible for the conversion of ammonia to hydroxylamine found in all AOB, has been used as a specific molecular marker in environmental studies of AOB using DGGE[17-20] and real-time PCR [21].

Aimed to reveal the effect of DO on microorganism community structure during SND in SBBR, the analysis need to show how the microorganisms change with different operating conditions. The primary objectives of the current study were to examine the reliability of bacterial diversity assessment using PCR-DGGE analysis and to quantify ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in a sequencing biofilm batch reactor (SBBR). DGGE provides information about sequence variation in a mixture of PCR fragments of identical length based upon differential mobility in an acrylamide gel matrix of increasing denaturant concentration [15]; it can be used conveniently to infer differences in the composition of microbial communities [22,23]. Combined with sequence analysis, the major environmental bands were excised, re-amplified, and sequenced to investigate their identities further. And the quantitative real-time PCR is used to analyze and compare the quantification of nitrifying bacteria during the simultaneous nitrification and denitrifycation (SND) periods.

MATERIAL AND METHODS

Experimental setup and sample preparation

A schematic representation of the sequencing biofilm batch reactor (SBBR) is given in **Figure 1.** In this study, the sludge collected from the secondary sedimentation tank in a local municipal wastewater treatment plant was seeded with 59.6% NH_4-N removal efficiency. Artificial imitated sewage wastewater was used as the influent for SBBR systems, with about 300 mg/L COD, 25 mg/L NH_4-N and 4.5 mg/L total phosphorus during the acclimation and operation periods, respectively[24]. During the aeration, the DO in bulk solution was maintained at 2.5-4.0 mg/L. The mixed liquor suspended solids and mixed liquor volatile suspended solids (excluding the biofilm) in SBBRs were about 1.0 and 0.6 g/L, respectively. The pH was maintained at 7.0, Hydraulic retention time (HRT) was 7 h and the temperature was 20 ± 2°C.

1.water tank   2.reaction
3. automatic controller   4.pump
5. air compressor   6.filler
7.heater   8.sample tap
9.air flowmeter   10. aerator
11. solenoid valve   12.influent pipe
13. Exhaust pipe   14. sludge pipe
15.air pipe

Figure 1. Schematic representation of the SBBR.

The experimental set-up of SBBR is built, and the formation process of biofilm is about 25 days. While the biofilm formation was finished successfully, the sludge samples were collected each hour in a cycle, which was selected randomly.

DNA extraction

A sludge sample (0.5 g) was collected from three depth of a biofilm, which was called upper surface, internal layer and lower surface, respectively. The sample schematic was showed in **Figure 2.** DNA was extracted by using 3S DNA Isolation Kit V2.2 for environmental samples (Shanghai, ShenergyBiocolor Co.) as described by the manufacturer. Extracted DNA was dissolved in 50 μl of TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 7.5) [25] and quantified by measuring its absorbance at 260 nm with a spectrophotometer. The quality of the extracted DNA was analyzed by electrophoresis on a 1.0% agarose gel.

PCR and DGGE

All the used primers and PCR conditions are listed in **Table 1.** For bacteria population analysis, the primers were used to

directly amplify above samples by adding 1 µl of extracted DNA to 25 µl PCR master-mix (Beijing, Tiangen biotech Co.). PCR cycling was carried out in a thermocycler (S1000, Bio-Rad, USA) under the certain conditions, respectively. After PCR amplification, 3 µl of the PCR product was electrophoresed on 1.2% (w/v) agarose gel, and then checked with ethidium bromide staining.

A:The horizontal direction of Biofilm   B:The vertical direction of one fibre
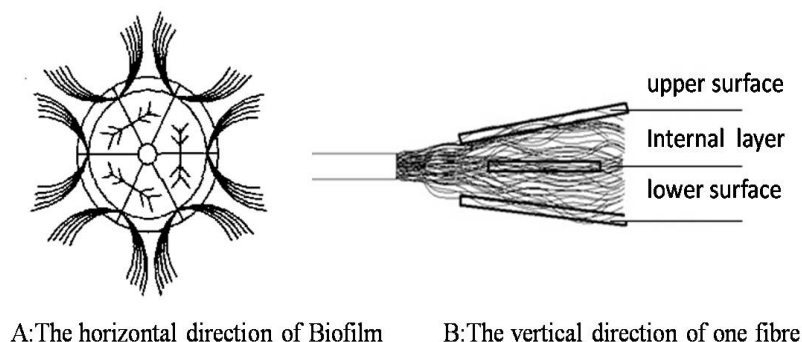
Figure 2. Sample Schematic.

Table 1. Primers and parameters of PCR. [a]Primer's short name used in the reference; [b]A mixture of CTO 189fA/B and CTO 189fC at the weight ratio of 2:1 was used as the forward primer; [c]DGGE primers were used for DGGE analysis, R-PCR primers were used for real-time PCR analysis.

Group	Primer[a]	Sequence (5'-3')b	Temperature proguram	Reference
V6-rDNA (DGGE)[c]	B1401R B968F	CGGTGTGTACAAGACCC AACGCGAAGAACCTTAC	94°C × 5min; 94°C × 30s, 56°C × 30s,72°C × 1min, 35cycles;	[27] [27]
AOB (DGGE)	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CTAAAACTCAAAGGAATTGA	94°C × 5min; 94°C × 30s, 62°C × 30s (-0.5°C/cycle), 72°C × 1min, 20cycles;	[28] [28]
NOB (DGGE)[c]	NOB-F NOB-R	TTTTTTGAGATTTGCTAG GTTTGCAGCGCTTTGTACCG	94°C × 5min; 94°C × 30s, 50°C × 30s, 72°C × 1min, 35cycles;	[29] [29]
Denitrify bacteria (DGG[E])c	Cd3aF R3cd	AACGYSAAGGARACSGG GASTTCGGRTGSGTCTTSAYGAA	94°C × 5min; 94°C × 30s, 58°C × 30s, 72°C × 1min, 35cycles;	[30] [30]
AOB (R-PCR)[c]	CTO 189f A/B[b] CTO 189f C[b] RT1r	GGAGRAAAGCAGGGGATCG GGAGGAAAGTAGGGGATCG CGTCCTCTCAGACCARCTACTG	95°C × 3min; 95°C × 15s, 61°C × 30s,72°C × 1min, 45 cycles;	[15] [15] [15]
NOB (R-PCR)[c]	NSR1113f NSR1264r	CCTGCTTTCAGTTGCTACCG GTTTGCAGCGCTTTGTACCG	95°C × 5min; 95°C × 15s, 60°C × 30s, 72°C × 1min, 45 cycles;	[31] [31]

DGGE of the PCR product was performed in a polyacrylamide gel (8% (w/v)) containing denaturing gradients (urea and formamide) at 35% to 65% using a D-code DGGE system (BIORAD Laboratories, USA). Electrophoresis was conducted using a 1x TAE butter at 75 V and 60°C for 14 h. After electrophoresis, the gel was stained with GelRed for 30 min, followed by rinsing with Milli-Q water. The gel was scanned on a UV transillumination table (Bio-Rad's Polaroid Gel Documentation System) to acquire the DGGE band image and analysis with Quantity One software [26].

Sequence analysis

The selected bands were excised from the DGGE gel and eluted in 20 µl tissue culture water at 4°C overnight. Three microliters of the eluted DNA was re-amplified by PCR following the program described above. Each reaction mixture was also subjected to DGGE analysis to confirm the melting behavior of the band recovered. Subsequently, idiographic sequences were attained by the Beijing Genomics Institute (BGI). Finally, Sequence identities were determined by BLAST GenBank database searches [32].The nucleotide alignments were used to create a phylogenetic tree using Clustalx 1-83 software program [33].

Real-Time PCR Assays

Real-time PCR quantification was carried out using an Applied BiosystemsStepOne™ Real-Time PCR System. The primers for AOB and NOB **(Table 1)** were produced by Shanghai Sangon Biological Engineering Technology and Services Company (Shanghai, China), and each tenfold dilutions of the DNA extracted from the samples were prepared.

Copy numbers of 16S rRNA gene of AOB and NOB bacteria of the samples from SBBR were determined by quantitative real-time PCR analysis using a Bioanalyzer UV spectrophotometer Q3000 (Quawell Technology Inc., San Jose, CA, USA). Each PCR mixture (25 ml) was composed of 12.5 µl of 1 × SYBR Green PCR master mix (Applied Bio-systems), 1 µl of each forward and reverse primers, either 1 µl of template DNA or 10-fold diluted extracted of plasmid DNA[34]. A melting curve analysis for SYBR Green assay was done after amplification to distinguish the targeted PCR product from the non-targeted PCR product.

For preparation of bacterial standards, the 16S rRNA gene of AOB and NOB were PCR-amplified from extracted DNA with the specific primer **(Table 1)**, and the PCR products were purified by the purification kit (Biodev-Tech Co., Ltd., Beijing, China), followed by cloning using the pGEM-T Easy Vector (Takara Biotechnology, Japan). Plasmids from the proper insert clones of the target gene were extracted and used as standards for calibration curve.
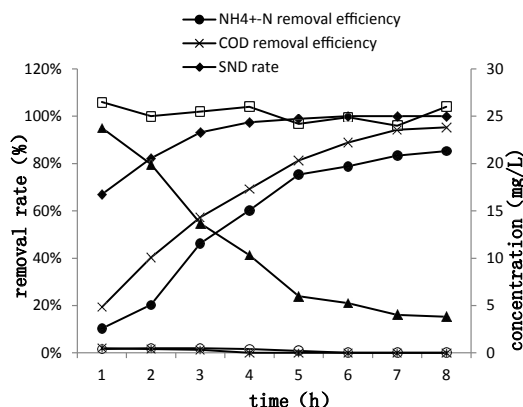
Results

Reactor performance

The rate of SND (η_{TN}) may be an indicator of the simultaneous nitrification and denitrifycation (SND). The estimates of SND rate were based on the concentration of nitrate, nitrite or NH_4-N of influent and effluent, respectively.

$$\eta_{TN} = \left[1 - \frac{\rho\ (NO_x^- - N)_{effluent} - \rho\ (NO_x^- - N)_{influent}}{\rho\ (NH_4^+ - N)_{influent} - \rho\ (NH_4^+ - N)_{effluent}} \right] \times 100\%$$

In the formula, is the numbers of base pairs, $\rho(NO_x^- - N)_{effluent}$ and $\rho(NO_x^- - N)_{influent}$ is the sum of the concentration for the nitrate and nitrite, respectively in the effluent and the influent. And $\rho(NO_4^+ - N)_{effluent}$ and $\rho(NO_4^+ - N)_{influent}$ is the concentration for the NH_4-N of the effluent and the influent, respectively.

Figure 3 illustrates that NH_4-N concentration of effluent had decreased quickly as the aeration time gone on, and the removal rate of the system to ammonium had achieved 85% after the 8th hour. The SND rate showed in **Figure 3** of the system had gone over 90% in the 3rd hour, and kept stable SND rate (90%~100%) until the cycle finished. The stable effluent with high SND rate (≥ 90%) indicated the system of SND was achieved successfully.


Figure 3. Water quality monitoring results in stable operation condition.

DGGE analysis of the bacterial community in biofilm

The DGGE profiles of three samples amplified with different special primers for V6-rDNA, AOB, NOB and denitrifying bacteria (listed in **Table 1**) was shown in **Figure 4.** The three samples collected from different depth (shown in **Figure 2**) of a biofilm, while the SND was achieved in stable operation condition. It can be seen that the bacteria community structure of the upper surface, internal layer and lower surface were very similar, hardly without any difference. And the variation of Shannon diversity index also was little (result was not shown). Our previous study found that SND in SBBR was caused by anoxic microenvironment which existed in the biofilm under aerobic conditions. DO concentration directly affected the proportion of aerobic fraction and anoxia fraction in the biofilm, which led to an effect on efficiency of nitrification and denitrification [36]. So the aerobic bacteria may grow abundantly in the upper and lower surface, and the anaerobic bacteria could only live in the internal layer. However, the DGGE result hadn't agreed with expectation, because of the highly sensitive of DGGE for the genetic diversity between different bacteria. The DO concentration of different depth in one biofilm maybe only the quantity, but not the communities composition of the microbes[23]. DGGE can't reflect the change of community structure, and the quantitative real-time PCR is used to analyze and compare the quantification of nitrifying bacteria in different depth of the biofilm.

Comparative sequence analysis of DGGE bands

Several separated bands we were interested in were excised from the acrylamide gel, re-amplified with the special primers, and attained the sequences of the bands. Using the BLAST search algorithm, low similarity values were found among the general bacteria. These clones were clustered into three distinct groups by sequence analysis. Sequencing of DGGE bands from AOB gene fragments showed that they had high similarity values among the bands located relatively close **(Figure 5).** Several of the clone sequences might differ by only a few bases [37]. By analyzed AOB phylogenetic tree results, group A and group B were affiliated with amoA gene and almost all had high sequence similarity to uncultured bacterium. In group C, the OUT12 had 98% sequence similarity to *Nitrosospira*, and the sequences of OUT13 related to bacterium and *Nitrsospira*, which similarity were 80% and 92% respectively. The result obtained from the AOB phylogenetic tree showed the presence of some dominant species, such as *Nitrsospira*, which contained the largest number of clones and was present throughout the column [38,39].
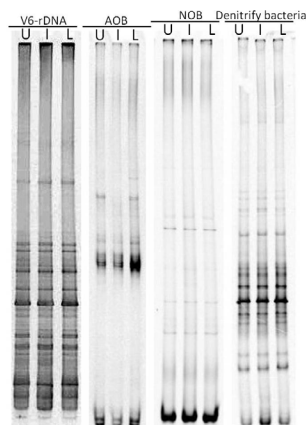
Lanes U\I\L: sludge sample collected on the upper surface, internal layer and lower surface, respectively.

Figure 4. DGGE patterns of V6-16S rDNA, AOB, NOB and denitrify bacteria fragments.

Quantification of AOB and NOB by Real-time PCR

In real-time PCR analyses, quantification is based on the threshold cycle (Ct), which is inversely proportional to the logarithm of the initial gene copy number. The threshold cycle values obtained for each sample should be compared with a standard curve to determine the initial copy number of the target gene. Because the rationale behind the real-time PCR assay was to compare quantification data obtained with a range of different primer sets to only one standard series of only one primer set, a PCR efficiency, which is similar among the different reactions, was required to allow comparison between real-time results obtained with different primer sets[40,41].

Real-time PCR was used to quantify the copy number of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing (NOB) in the different depth of a biofilm during the stable SND condition, and the result is listed in **Figure 5.** According the data from real-time PCR, the quantity of the AOB and NOB both had most copy number in the lower surface, and the copy number of AOB is 4.79×10^9 copies/ng and NOB is 7.43×10^4 copies/ng, respectively **(Figure 6).** Basic on the copy number of AOB and NOB population, the quantity relation was "lower surface＞upper surface＞internal layer". The lack of oxygen leads to the low quantity of AOB and NOB in the internal layer of biofilm.
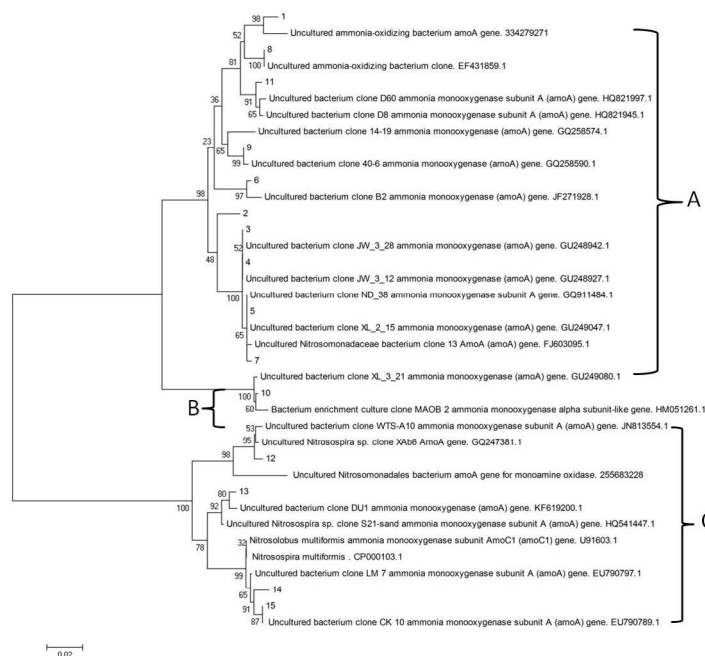
**Figure 5.** AOB phylogenetic tree in biofilm in stable operation condition.

DISCUSSION

The study developed and combined real-time PCR and PCR-DGGE analysis to analyze the differences in the composition of microbial communities, with the samples collected in different depth of one biofilm.

Combined with clone libraries, the conclusion obtained from the AOB phylogenetic tree showed the presence of some dominant species, such as *Nitrsospira*, which contained the largest number of clones and was present throughout the column.

However, the result wasn't in line with our previous study by DGGE gel pure culture[42], which found that most of the DGGE bands of various sewage treatment plants were closely related to *Nitrosomonas spp.*, not to *Nitrosospira spp.*[23]. Stephen et al. [43] had conducted a preliminary phylogenetic survey of ammonia-oxidizing β-proteobacteria, using 16S-rRNA gene libraries prepared by selective PCR and DNA, and revealed that most enrichments contained novel *Nitrosomonas*-like sequences whereas novel *Nitrosospira*-like sequences were more common from gene libraries. The conclusion helped to explain the contradiction between our two studies.
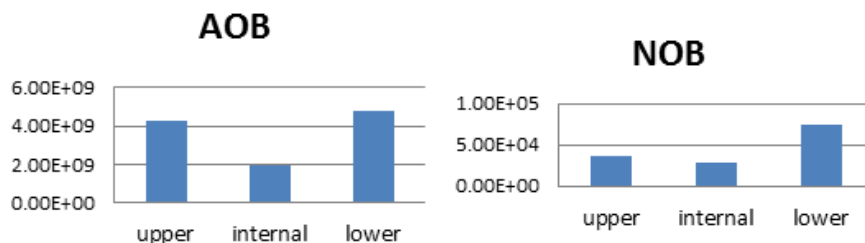
Figure 6. AOB and NOB quantity of mirco-zone samples in biofilm.

The most important factor that has to be controlled in the reactor was a stable microbial community composition. The sequence analysis of AOB created a reliable way to monitor the general microbial community shifts. We can control the parameters corresponding to the characterization of the microorganisms as closely as possible to enhance the effect of SND. For example, choosing suitable carbon or nitrogen sources will be available in later study. Meanwhile, the sequence analysis indicated that the *Nitrosospira* bacteria were not the exclusive community members in the reactor, and there were some other Pseudomonas, which might interfere with the growth of nitrite bacteria and affect the SND. Therefore, we can add some reagent to inhibit these useless bacteria. This approach will help the achievement of SND and improve the effluent quality.

The real-time PCR assay, targeting AOB and NOB, shows the copies numbers of different depth in a biofilm during SND. The quantity relation of AOB and NOB population between three samples was "lower surface ＞ upper surface ＞ internal layer". It showed that the internal layer in the biofilm had formed the anaerobic environment which inhibits the aerobic bacteria, such as AOB and NOB. The mechanism of SND in SBBR was analyzed on the basis of DO diffusion model within biofilm. It was found that SND in SBBR was caused by anoxic microenvironment which existed in the biofilm under aerobic conditions. DO concentration directly affected the proportion of aerobic fraction and anoxia fraction in the biofilm, which led to an effect on efficiency of nitrification and denitrification[44,45]. With increase of DO concentration, the speed transfer efficiency of oxygen was enhanced to make microenvironment convert from anoxia environment to aerobic environment. Microenvironment inclined to convert from aerobic environment to anoxia or anaerobic environment with decrease of DO concentration [36,46].

The amount of the AOB in the upper surface close to the lower surface, and the NOB of the lower surface was double of the upper. Moreover, the quantity of NOB in the upper surface close to the copy number in the internal layer. According to the structure of SBBR, the aerator was at the bottom of the reaction so the lower surface exposed to oxygen firstly. Aeration plays an important role and the velocity of bubble rising in water lead to the DO in the lower surface higher than in the upper. What's more, the oxygen saturation coefficient of NOB was 1.2~1.5 mg/L, and the AOB was 0.2~0.4 mg/L[44]. For the reasons above, the low concentration of DO inhibit the activity of NOB without affecting the growth rate of AOB in the upper surface. The result was in accord with theory, which showed that the copy number of NOB in the lower surface was double of the upper, but the AOB had almost the same quantity. And it also revealed that the dissolved oxygen (DO) present in SBBR was an important factor in achieving a higher degree of SND.

ACKNOWLEDGEMENT

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