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Influence of growth manner on nitrifying bacterial communities and nitrification kinetics in three lab-scale bioreactors

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Abstract The effects of growth type, including attached growth, suspended growth, and combined growth, on the characteristics of communities of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) were studied in three lab-scale Anaerobic/Anoxic$_m$-Oxic$_n$ (AmOn) systems. These systems amplified activated sludge, biofilms, and a mixture of activated sludge and biofilm (AS-BF). Identical inocula were adopted to analyze the selective effects of mixed growth patterns on nitrifying bacteria. Fluctuations in the concentration of nitrifying bacteria over the 120 days of system operation were analyzed, as was the composition of nitrifying bacterial community in the stabilized stage. Analysis was conducted using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and real-time PCR. According to the DGGE patterns, the primary AOB lineages were *Nitrosomonas europaea* (six sequences), *Nitrosomonas oligotropha* (two sequences), and *Nitrosospira* (one sequence). The primary subclass of NOB community was *Nitrospira*, in which all identified sequences belonged to *Nitrospira moscoviensis* (14 sequences). *Nitrobacter* consisted of two lineages, namely *Nitrobacter vulgaris* (three sequences) and *Nitrobacter alkalicus* (two sequences). Under identical operating conditions, the composition of nitrifying bacterial communities in the AS-BF system demonstrated significant differences from those in the activated sludge system and those in the biofilm system. Major varieties included several new, dominant bacterial sequences in the AS-BF system, such as *N. europaea* and *Nitrosospira* and a higher concentration of AOB relative to the activated sludge system. However, no similar differences were discovered for the concentration of the NOB population. A kinetic study of nitrification demonstrated a higher maximum specific growth rate of mixed sludge and a lower half-saturation constant of mixed biofilm, indicating that the AS-BF system maintained relatively good nitrifying ability.

Keywords Ammonia-oxidizing bacteria (AOB) · Nitrite-oxidizing bacteria (NOB) · Nitrifying bacterial community · Growth manner

Abbreviations

AOB	Ammonia-oxidizing bacteria
NOB	Nitrite-oxidizing bacteria
AmOn	Anaerobic/Anoxic$_m$-Oxic$_n$
AS-BF	Activated sludge and biofilm
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
WWTPs	Wastewater treatment plants
AOA	Ammonia-oxidizing archaea
HRT	Hydraulic retention time
SND	Simultaneous nitrification and denitrification
SRT	Sludge retention time
R	Volumetric substrate conversion rate (NH_4^--N and TN)
R_{NH_3}	Volumetric NH_4^--N conversion rate
R_{TN}	Volumetric TN conversion rate
S_0	Influent substrate concentration

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S_{out}	Effluent substrate concentration
Q	Influent flow rate
V	Volume of reactors
MLSS	Concentration of mixed liquor-suspended solid
q_N	Specific substrate conversion rate
S	Substrate concentration
K_S	Half saturation substrate concentration
$q_{N,\ max}$	The maximum specific substrate conversion rate
TN	Total nitrogen
K_{s,NH_3}	Half saturation substrate concentration of ammonia oxidizing
K_{s,NO_2^-}	Half saturation substrate concentration of nitrite oxidizing
q_{max,NH_3}	Maximum specific degradation rate of ammonia
q_{max,NO_2^-}	Maximum specific degradation rate of nitrite
r	Correlation coefficient

Introduction

The removal of nitrogenous contaminants is an important goal in wastewater treatment plants (WWTPs). This goal is generally achieved through biological nitrification and denitrification. Biological nitrification is the major restricting step in the process of nitrogen removal because of the slow growth rate of the relevant organisms. A complete biological nitrification process consists of the oxidation of ammonia into nitrite and the oxidation of nitrite into nitrate, carried out by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively [18]. Nitrifying bacteria are mostly aerobic autotrophic bacteria. They have a slow growth rate and a long generation time. Recent reports have indicated that some ammonia-oxidizing archaea (AOA) can also carry out nitrification. However, the role of AOA in wastewater treatment has not been completely ascertained [25].

According to some researchers' work, nitrifying bacteria are considered to be sensitive to many environmental parameters in wastewater treatment systems, such as temperature, concentration of dissolved oxygen (DO), hydraulic retention time (HRT), and inhibitors. These and other factors can impact the composition of the nitrifying bacterial community [7, 14, 19], and further influence the efficiency of nitrification and denitrification. However, the nitrifying bacteria community also showed resistance to change of the operational condition in some previous studies. In Xia's report, AOB communities were found similar in different tanks at the similar condition; and even at varying addition amounts of adding flocculants and sludge returning rates, the difference in AOB microbial structures were smaller than the detection resolution of DGGE [40]. In another long-term SBR, both community composition and the number of bands in DGGE profiles were almost identical between sequencing the batch reactor and the duplicate one. This also proved the repeatability of DGGE method; the identical community structure could be achieved at the same operation conditions [1].

Fixed growth (such as occurs in biofilm) and suspended growth (such as occurs in activated sludge) are the two biological wastewater treatment methods by which almost all biological wastewater treatment systems can be classified. These two processes are selective for microorganisms, and it has been widely accepted that the activated sludge process is fit for the enrichment of bacteria with shorter generation times and can offer a relatively high mass transfer efficiency. Biofilm processes are believed to enrich bacteria with relatively long generation times and are considered fit for the growth of bacteria with different oxygen demands, such as aerobic, anoxia, and anaerobic conditions. Biofilm processes also offer a certain degree of simultaneous nitrification and denitrification (SND) [3, 4, 15, 38, 39]. The mixed-system pattern can theoretically offer various microenvironments for multiple microorganisms and reduce required reactor volume. We have performed studies evaluating mixed-system designs in Anaerobic/Anoxic$_m$-Oxic$_n$ (AmOn) before, and we found them to affect the removal of carbon, nitrogen, and phosphorus simultaneously within a single reactor [22, 43].

Current reports on nitrifying bacteria have mainly focused on sole growth patterns. The effects of AS-BF systems on nitrifying bacteria have not been fully studied. The influence of altered growth patterns on the composition of nitrifying bacterial communities and the kinetic characteristics of nitrification remain particularly mysterious. Thus, based on AmOn reactors that we have studied previously, we investigated the effects of the AS-BF system on biological nitrifying performance by measuring the fluctuations of nitrification kinetic constants. We also studied the effects and selectivity of this AS-BF system on the composition of the nitrifying community by analyzing the variations in nitrifying bacteria density and community composition. We also carried out an analysis of the correlation between community characteristics and nitrifying ability. The results of this study may foster better understanding of the mechanisms involved in nitrifying communities in wastewater treatment systems.

Materials and methods

Reactor design and operation

This experiment took place at the Shanghai East Region WWTP, which mainly disposes of the municipal sewage

from neighboring regions. Three identical lab-scale AmOn reactors (reactors A, B, and C) were operated in parallel for 120 days. Reactor A adopted a suspended carrier process (carriers), reactor B employed a traditional completely stirred activated sludge process, and reactor C was inoculated with 1:1 sludge:carrier in accordance with biomass (MLSS). Activated sludge and carriers were sampled from the WWTP's long-term operating aeration tank and carrier tank, respectively. The three reactors each had a volume of approximately 20 l, and were fed the same municipal sewage with 60 l day^{-1}as influent, with an HRT of 8 h. The sludge retention time (SRT) of activated sludge in reactors B and C was controlled at about 10 days, and the biomass concentration of the suspended carrier was 1.8–2.0 g l^{-1}. Reactor A had a carrier packing percentage of about 50% (volume ratio) and a biomass concentration of 1.8–2.0 g l^{-1}. The concentration of activated sludge in reactor B was about 2.0 g l^{-1}. The experiment was designed so that reactor C would experience a 30% (volume ratio) packing percentage, and a 1.1–1.2 g l^{-1} biomass concentration, and a 1.0 g l^{-1} activated sludge concentration were measured there. To guarantee total nitrification, sufficient aeration was provided to all three reactors, giving a DO concentration of over 4.0 mg l^{-1}.

The sketch of AmOn bioreactor applied in Fig. 1. Experimental influent is fed into bioreactor bottom alone the inlet line. Reacting room could be divided into two parts as aerators were designed to locate at mid-level of reactor; the upper half and lower half of the reactor acted as aerobic zone and anoxic/anaerobic zone, respectively. During reaction, the influent was fully mixed with biomass (sludge and biofilm) after spurting into the reactor. After this, influent flowed along the anaerobic/anoxic and aerobic zone in sequence. The effluent was discharged through a diversion cofferdam and sludge was returned to the reactor along the sedimentation slot. The ratio of aerobic zone and anoxic/anaerobic zone was alterable by adjusting the aeration mode, which is why it is called (Anaerobic/Anoxic)$_m$-Oxic$_n$. Suitable utilization of carrier also gave the potential for SND in one reacting room [22, 43].

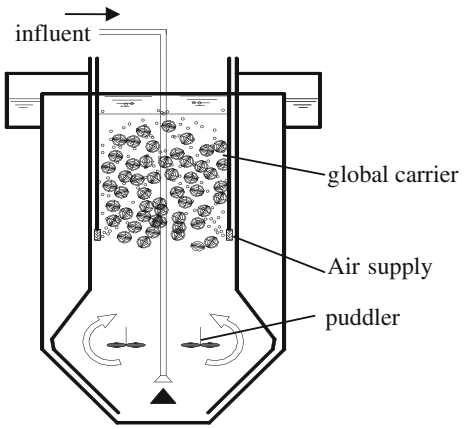

Fig. 1 Structural diagram of AmOn reactors used in this experiment

Sampling and total DNA extraction

Sampling and DNA extraction were carried out on day 1, day 60, and day 120. For reactors A and C, biofilm samples were obtained by scraping the surface of suspended carriers. For reactors B and C, 10 ml of mixed liquor was extracted. The sludge sample was then collected after 5 min of centrifugation (6,000 rpm, 4°C). Genomic DNA extraction was performed as described previously [41].

PCR amplification and DGGE analysis

PCR amplification methods used for DGGE analysis are listed in Table 1. A nested PCR method was used in this experiment for the amplification of the 16S rDNA fragments of target bacterial communities: *Nitrospira* (AOB) and *Nitrobacter* (NOB). Amplification products were confirmed by agarose gel electrophoresis (1% agarose).

DGGE analysis was performed using Bio-Rad's D-Code electrophoresis system. Mass concentration of denaturant (urea) used for AOB and *Nitrospira* was 25–50%, for *Nitrobacter*, 45–65%. Conditions for electrophoresis were selected as described previously [41].

After the electrophoresis, the gel fraction with targeted DNA was cut-off and transferred to a microcentrifuge tube. Then, 30 μl DI water was added and the product was stored overnight at 4°C. The product was used as a PCR template and amplified. After that, amplification products were sequenced in Shanghai Boya Biotechnology Engineering Company. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [34].

Quantification of nitrifying bacteria population

Real-time PCR analysis based on SYBR Green I Assay was performed in Rotor-Gene 3000 (Corbett Research, Australia). The 25-μl reaction system adopted in this experiment referred to previous research [42]: 2.5 μl 10× PCR buffer, 2.5 μl 10× SYB Green I, 2.0 mmol $MgSO_4$, 10 nmol dNTP, 10 nmol of each primer, and either 1 μl of template or 10^5–10^{10} copies of the standard 16S rDNA. A standard calibration curve was generated for the detection of each bacterial community density using corresponding PCR products of each given strains' fragments. The given strains used in this experiment for the determination of bacterial density included *E. coli* DH5 for *Bacteria*, *Nitrosomonas europaea* (ATCC 19718) for AOB, *Nitrospira marina* (ATCC 43039) for *Nitrospira*, and a *Nitrobacter* sp. separated and purified from sludge in lab for *Nitrobacter*.

Table 1 Primers and programs used for PCR amplification

Target	Primer	PCR conditions[a]	Expected size of the amplifications (bp)	Reference(s)
DGGE analysis				
First round of amplification				
AOB	CTO189fAB/ CTO189fC, CTO653r	95°C, 1 min; 63°C, 1 min; 72°C, 2 min	470	[29]
Nitrospira	P63f, NSR1264r	95°C, 1 min; 60°C, 1 min; 72°C, 2 min	1,215	[12, 26]
Nitrobacter	P338f, Nb1000r	95°C, 1 min; 58°C, 1 min; 72°C, 2 min	676	[11, 26]
Second round of amplification				
All three communities	P338f-gc, P518r	95°C, 1 min; 60°C, 1 min; 72°C, 2 min	233	[20, 35]
Real-time PCR				
AOB	CTO189f A/B, CTO189f C, RT 1r	95°C, 1 min; 63°C, 1 min; 72°C, 2 min	115	[17, 28]
Nitrospira	NSR1113f, NSR1264r	95°C, 1 min; 60°C, 1 min; 72°C, 2 min	165	[26, 42]
Nitrobacter	Nb1000f, 1387r	95°C, 1 min; 62°C, 1 min; 72°C, 2 min	394	[26, 42]

[a] Before each cycle, the temperature was maintained at 95°C for 10 min and after each run, the temperature was maintained at 72°C for 10 min. There were 35 cycles altogether

Reactor physical–chemical parameters and measurement of nitrification kinetic constants

Concentrations of ammonium, nitrite, nitrate and total nitrogen (TN) were measured according to the standard methods [2]. Concentrations of ammonia nitrogen, nitrate nitrogen, and nitrite nitrogen were measured each day to evaluate nitrification and nitrogen removal efficiency. The volumetric substrate conversion rate (R) was calculated as follows in Eq. (1):

$$R = \frac{(S_0 - S_{\text{out}}) \cdot Q}{V \cdot \text{MLSS}} \quad (1)$$

Here, R is the volumetric substrate (NH_4^--N and TN) conversion rate, S_0 is the influent substrate concentration, S_{out} is the stable effluent substrate concentration, Q is the influent flow rate, V is the volume of reactor, and MLSS is the concentration of mixed liquor-suspended solids.

The Monod model is the most popular means of describing the kinetics of pollutant biodegradation. After 120 days, the nitrification kinetic parameters (including ammonia oxidation and nitrite oxidation) were determined using a nitrification Monod equation (Eq. 2) :

$$q_N = \frac{q_{N,\max} \cdot S}{S + K_s} \quad (2)$$

Here, q_N is the specific substrate conversion rate, S is the substrate concentration, K_S is the half saturation substrate concentration, and $q_{N,\max}$ is the maximum specific substrate conversion rate.

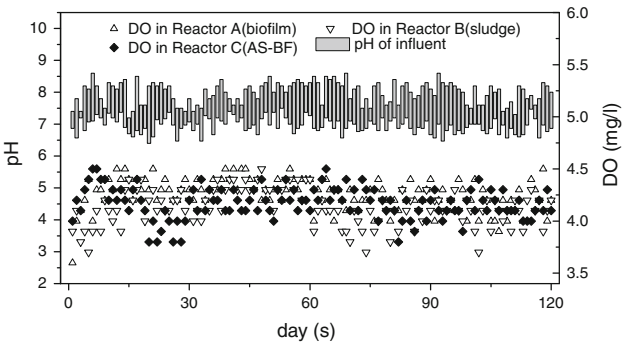

Fig. 2 Change of influent pH and mean DO in bioreactors over time (120 days)

Results

Process and nitrification performance

Influent quality was weak alkaline according to mean pH value of 6.8–8.0 (Fig. 2). To start the system quickly, suspended carriers and activated sludge from stable carrier and aeration tanks, respectively, were used as inoculants. This ensured each reactor a relatively sufficient amount of

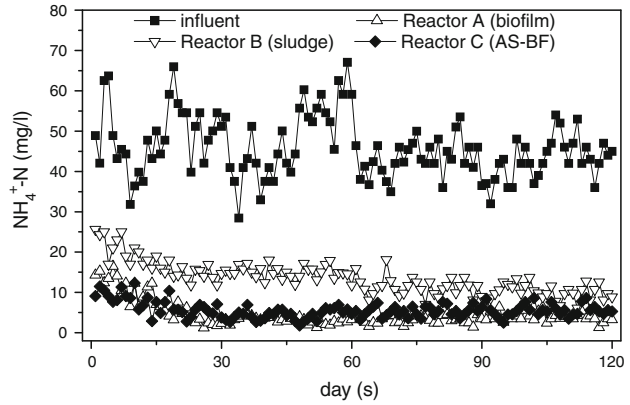

Fig. 3 Change of influent and effluent NH_4^+-N over bioreactors running (120 days)

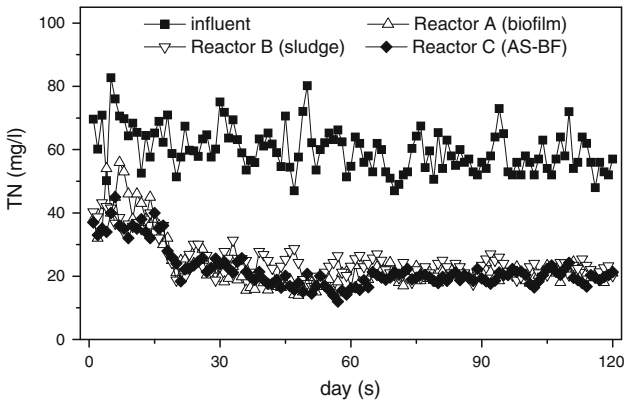

Fig. 4 Change of influent and effluent TN over bioreactors running (120 days)

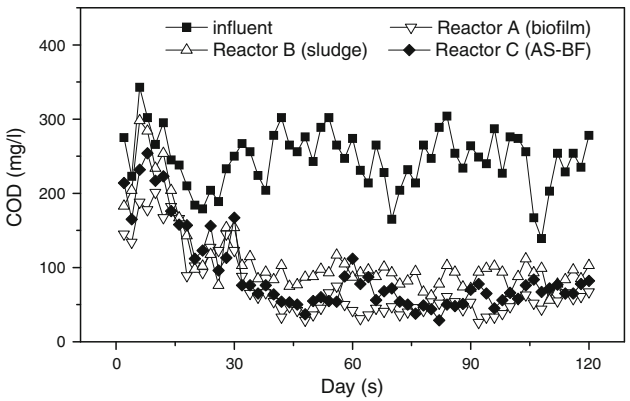

Fig. 5 Change of influent and effluent COD over bioreactors running (120 days)

starting biomass. After a short period of fluctuation (about 2 weeks), nitrogen pollutant removal efficiency reached stable levels (Figs. 3, 4), while for COD, removal stability was achieved after about 30 days (Fig. 5). Aeration level was controlled evenly in three bioreactors referring to mean DO ranged from 4.0 to 4.4 mg l^{-1} (Fig. 2). Most of the nitrogenous contaminants in the influent were ammonia nitrogen and nitrate nitrogen. Average concentrations of nitrite nitrogen in both influent and effluent were under 0.1 mg l^{-1}, indicating that the nitrifying reactions in all reactors were capable of complete nitrification.

The statistical results of nitrogen removal efficiency in reactors A, B, and C under identical influent conditions (day 20 to day 120) are listed in Table 2. These results showed that the removal rates of ammonia nitrogen and TN in reactor A were 93 and 67%, respectively. The removal rates in reactor B were the lowest, 74 and 62%, respectively. The removal rate and R (NH_4^--N and TN) in reactor C were lower than those of reactor A but higher than those of reactor B.

To compare the nitrifying efficiency of different reactors, the kinetic parameters of ammonia oxidation and nitrite oxidation were measured, including the maximum specific conversion rate and substrate half saturation constant (Table 3). For both ammonia oxidation and nitrite oxidation, the q_{max} in two types of biofilms were clearly higher than those in activated sludge. K_{s,NH_3} was also higher in biofilms, but K_{s,NO_2^-} was similar in biofilms and sludge. The activated sludge in reactors B and C had q_{max,NH_3} values of 0.154 and 0.192 day^{-1}, respectively, indicating that the mixture process increased ammonia conversion in activated sludge. The K_{s,NH_3} of biofilm in reactor C showed a decrease relative to that in reactor A, with 2.46 and 1.42 mg l^{-1} in reactors A and C, respectively. Based on kinetic constants in reactor C, it was found that the suspended carrier contributed more to nitrification than activated sludge. From this, we concluded that mixed cultivation might affect or change the characteristics of ammonia oxidation in biofilms and activated sludge.

Analysis on the concentration of nitrifying bacteria

The primers that we used in real-time PCR mainly targeted *β-proteobacteria* AOB and two subclasses of NOBs, *Nitrospira* and *Nitrobacter*. The results are displayed in Fig. 6. For NOB, the population size of *Nitrospira* was an order of magnitude higher than that of *Nitrobacter*. Therefore, *Nitrospira* can be called the dominant NOB population. However, the concentrations of AOB and NOB (the sum of *Nitrospira* and *Nitrobacter*) were on the same order of magnitude.

Throughout the experiment, the AOB population sizes in the two types of biofilm were clearly higher than those in activated sludge, indicating good AOB enrichment in biofilm. Biofilm alone had the highest AOB concentration (7.65×10^{10} cells l^{-1}), 3.8 times higher than that in activated sludge alone (2.03×10^{10} cells l^{-1}), and 2.2 times

Table 2 Effluent concentrations of nitrogenous contaminants and conversion rate (statistical results from day 20 to day 120, 100 points)

Pollutant	Reactor	Influent substrate concentration, S_0 mg l^{-1}	Effluent substrate concentration, S_{out} mg l^{-1}	Volumetric substrate conversion rate, R_{NH_3} or R_{TN} g N day^{-1} g^{-1} MLSS
NH_4^+-N	Reactor A (biofilm)	48.4 ± 5.6	3.2 ± 1.5	0.073
	Reactor B (sludge)		12.4 ± 2.3	0.054
	Reactor C (AS-BF)		5.3 ± 1.9	0.069
TN	Reactor A (biofilm)	62.7 ± 8.4	20.4 ± 2.7	0.069
	Reactor B (sludge)		23.6 ± 3.2	0.059
	Reactor C (AS-BF)		19.5 ± 24	0.067

Table 3 Nitrification kinetic constants of sludge and biofilm

Measured carrier/ sludge	Ammonia nitrogen oxidation			Nitrite nitrogen oxidation		
	Maximum specific degradation rate, q_{max,NH_3} day^{-1}	Half-rate constant, K_{s,NH_3} mg l^{-1}	Correlation coefficient r^2	Maximum specific conversion rate, q_{max,NO_2^-} day^{-1}	Half-rate constant, K_{s,NO_2^-} mg l^{-1}	Correlation coefficient r^2
Reactor A (biofilm)	0.213	2.46	0.95	0.342	2.4	0.94
Reactor B (sludge)	0.154	0.98	0.95	0.267	2.5	0.97
Reactor C (biofilm)	0.227	1.42	0.94	0.335	3.0	0.93
Reactor C (sludge)	0.192	0.86	0.94	0.274	2.7	0.96

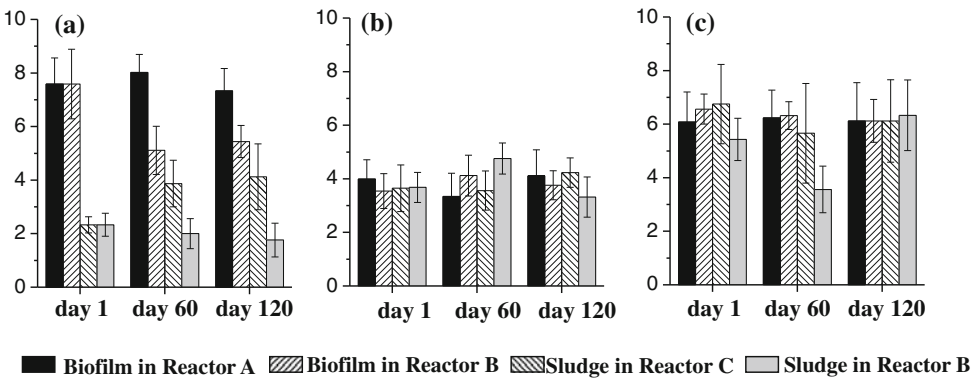

Fig. 6 Comparison of concentrations of AOB and NOB in three reactors: **a** AOB ($\times 10^{10}$ cells l^{-1}); **b** *Nitrospira* ($\times 10^{10}$ cells l^{-1}); **c** *Nitrobacter* ($\times 10^9$ cells l^{-1})

of that in mixed sludge (3.44×10^{10} cells·l^{-1}). However, the size of the AOB population in biofilm and activated sludge in the AS-BF system showed adverse trends over time. It decreased from 7.59×10^{10} to 5.44×10^{10} cells l^{-1} in biofilm, whereas that of activated sludge increased from 2.33×10^{10} to 4.12×10^{10} cells l^{-1} over the course of the 120-day experimental run. The distribution of NOB was more even; *Nitrospira* and *Nitrobacter* population sizes remained stable among reactors for 120 days with a ratio over 5.2. The effects of mixed cultivation on NOB density were less dramatic than on AOB; fewer fluctuations were observed in sludge and biofilm.

Dominant nitrifier sequences and community diversity

DGGE analysis of the compositions of the AOB and NOB communities was carried out (Fig. 7). The dominant NOB populations in wastewater are reported to be *Nitrospira* and *Nitrobacter*, which were both detected from reactors in this experiment. There were fewer dominant *Nitrobacter* sequences than dominant *Nitrospira* sequences. Five bands (Nb.1–Nb.5) were found to be *Nitrobacter* sequences through sequencing, of these, Nb.1, Nb.3, and Nb.4 were observed in all samples, although the band density of Nb.3 weakened significantly in the mixed cultivation sample.

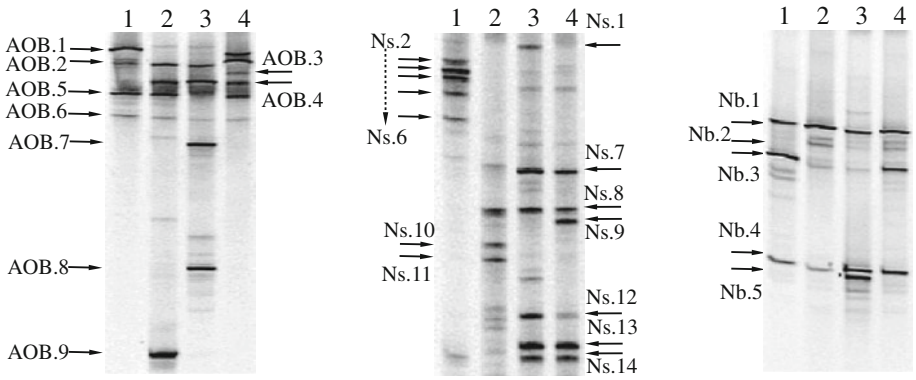

Fig. 7 Analysis of DGGE patterns in nitrifying communities. *Lane 1* biofilm in reactor A; *lane 2* biofilm in reactor C; *lane 3* sludge in reactor C; *lane 4* sludge in reactor B

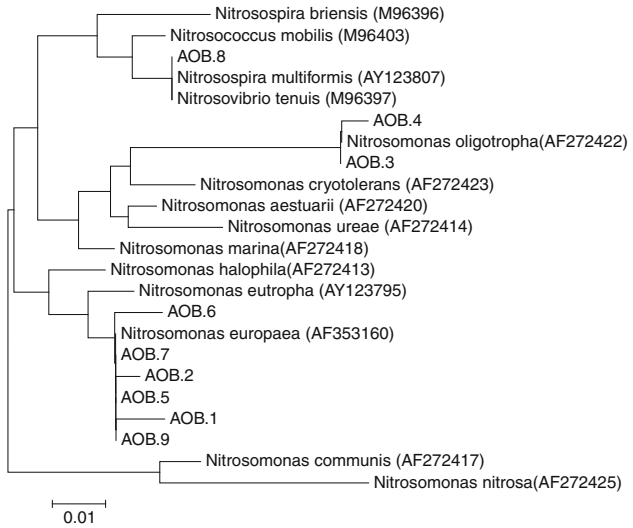

Fig. 8 Phylogenetic relationships between AOB bands detected in the experiment and typical AOB strains

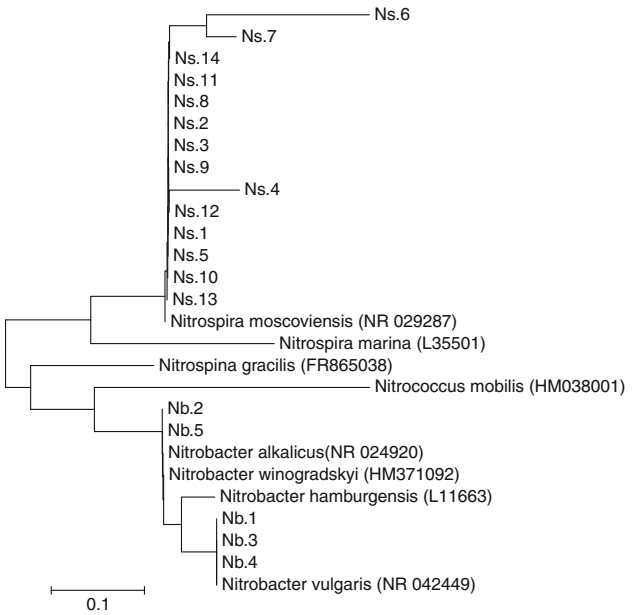

Fig. 9 Phylogenetic relationships between NOB bands detected in the experiment and typical NOB strains

The dominant sequence, Nb.5, was detected specifically in mixed-cultivation activated sludge. Fourteen sequences of *Nitrospira* (Ns.1–Ns.14) have been determined through sequencing. Of these, Ns.2, Ns.3, Ns.4, Ns.5, Ns.6, and Ns.9 have been found in sole cultivation sample. Ns.1, Ns.10, and Ns.11 were only observed in the AS-BF bioreactor.

Some sequences were observed solely in biofilm (Ns.2, Ns.3, Ns.4, Ns.5, Ns.6, Ns.10, and Ns.11) and others only in activated sludge (Ns.1, Ns.13, and Ns.14). Nine sequences of AOB were detected (AOB.1–AOB.9). Of these, AOB.3 existed solely in the activated sludge bioreactor, and AOB.7, AOB.8, and AOB.9 were present in the AS-FM bioreactor. Generally, the AOB communities in reactors A and B were similar, but AOB.9 and AOB.8 were dominant in mixed-cultivated biofilm and activated sludge, respectively.

Detected DGGE bands were compared to those in the NCBI database, and the results are displayed in Figs. 8 and 9, showing the phylogenetic relationship to typical nitrifier strains. Figure 8 shows that AOB sequences detected in this experiment belong to lineages of *β-proteobacteria* AOB, namely, *Nitrosomonas europaea*, *N. oligotropha*, and *Nitrosospira*. *N. europaea*-like AOB was the largest lineage. Six *N. europaea*-like AOB sequences (AOB.1, AOB.2, AOB.5, AOB.6, AOB.7, and AOB.9) were found to belong to this lineage. Two sequences of *N.oligotropha*-like AOB (AOB.3 and AOB.4) and one sequence of *Nitrosospira*-like AOB (AOB.8) were detected. There was great diversity within the *Nitrospira*-like sequences (fourteen sequences) in NOB. All of these sequences were found to belong to the *Nitrospira moscoviensis*. Two lineages of *Nitrobacter* were observed, namely *Nitrobacter vulgaris* (Nb.1, Nb.3, and Nb.4) and *Nitrobacter alkalicus* (Nb.2 and Nb.5).

Discussion

Dominant nitrifying bacteria and major controlling factors

Dominant AOB sequences observed in DGGE gel fell into *N. europaea*, *N. oligotropha*, and *Nitrosospira* lineages within the *Nitrosomonas* genus. Of these, six *N. europaea*-like sequences made up the largest AOB lineage, which has been found in multiple environments, such as soil, salt water, and fresh water. It is generally considered the most common AOB strain in wastewater treatment systems [16, 21, 27, 32]. Koops and Pommerening-Roser [19] discovered that, unlike other AOB populations, *N. europaea* had a relatively high K_S for ammonia (30–61 μM). Thus, it appears more frequently in environments with moderately high NH_4^+-N concentrations. A stable influent NH_4^+-N concentration around 50 mg l^{-1} might contribute to the dominant presence of *N. europaea*-like AOBs.

Throughout the experiment, the population of *Nitrospira* was found to be more abundant than that of *Nitrobacter*. Some studies have found that *Nitrobacter* prefer to exist in the form of suspended cells, whereas *Nitrospira* have been discovered attached to activated sludge flocs and biofilms [19]. This means that *Nitrobacter* is more likely to be washed out through effluent, and *Nitrospira* is more likely to accumulate in treatment systems [5, 31, 32, 36, 37]. On the other hand, *Nitrobacter* has a higher K_S for nitrite. Therefore, in environments with high concentrations of nitrogen, *Nitrobacter* is frequently found to be the dominant NOB [10]. In our study, the ratio between populations of *Nitrospira* and *Nitrobacter* varied from 5.2 to 13.4; *Nitrospira* showed evident dominance in population concentration compared with *Nitrobacter*, which was identical to many result [8, 31, 36, 37]. This dominance between the two NOB clusters in three bioreactors also confirmed that *Nitrospira* might be a typical K-strategist compared with the r-strategist *Nitrobacter* [32].

In this study, *Nitrobacter* comprised two genera of *Nitrobacter alkalicus* and *Nitrobacter vulgaris*. Sorokin et al. [33] isolated some *Nitrobacter alkalicus* sequences (AN1-AN5) from sediments of soda lakes after enrichment at pH 10 and claimed that *Nitrobacter alkalicus* was discovered to prefer to alkaline environment, even the upper limit for growth and activity of *Nitrobacter alkalicus* might be higher than pH 10. In a constructed nitrite-oxidizing biofilm, *Nitrobacter alkalicus* were identified as one member of NOB at pH of 7.8 ± 0.2 [13]. It was not unexpected that two *Nitrobacter alkalicus*-like sequences appeared in our experimental bioreactors with highest influent pH of 8.6, which was relatively high than ordinary sewage and might resulted from partial industrial wastewater in influent. So *Nitrobacter alkalicus*-like strain could be regarded as a biomarker of alkaline environmental for wastewater treatment plant. Another discover is *Nitrobacter alkalicus*-like bands (Nb.2 and Nb.5) were exclusively presented in combining pattern, this finding might hinted AS-BF system provided more abundant microenvironment for different nitrifying bacteria community with distinct growth requirement. *Nitrobacter vulgaris*, the other *Nitrobacter* found in this study, was reported to be presented in soils, groundwater, fresh and brakish water environments, sewage disposal plants, with a optimum pH values between 7.5 and 8.0 [6]. *Nitrobacter alkalicus* strains were rarely discovered in artificial system such as WWTPs, whereas *Nitrobacter alkalicus*-like sequences appeared both in sludge and biofilm in this study, which might also result from alkaline influent.

All of *Nitrospira*-like sequences were found to belong to the *Nitrospira moscoviensis*, which was frequently found dominant in either activated sludge or biofilm [9, 24, 30, 31]. In a drinking-water distribution system, this predominance of *Nitrospira moscoviensis* was characterized by a similar high proportion of population size in both new and old biofilm biomass [24]. In addition, the ratio of *Nitrospira moscoviensis* seemed to be a stable predominant organism in activated sludge [31]. A similar result was detected in this experiment that *Nitrospira moscoviensis* was the dominant species in NOB in respect to *Nitrospira* and *Nitrobacter* concentration, and this predominance almost did not vary during running.

Kinetic constants and microbiological mechanisms of nitrification in activated sludge and biofilm

In this experiment, K_{s,NH_3} ranged from 0.86 to 2.46 mg l^{-1} in all activated sludge and biofilm samples, with the highest value (2.46 mg l^{-1}) achieved in individually cultivated biofilm and the lowest in activated sludge. This could be explained by the different dominant communities. According to DGGE profiles, activated sludge and biofilm shared two sequences of *N. europaea*-like organisms, AOB.2 and AOB.5. The sequences found to resemble *N. oligotropha* (AOB.3 and AOB.4) were not found in biofilm cultivated alone. In accordance with previous reports, *N. oligotropha* were found to have relatively low K_S values for ammonia, around 1.9–4.2 μM [19]. The absence of these two sequences of *N. oligotropha*-like AOB might have caused the high K_s for ammonia. Another reason for the higher maximum specific growth rate observed in the biofilm system was that the density of AOB in biofilm was higher than that in activated sludge. The specific nitrification rate of biofilm calculated by VSS per unit was higher than that of activated sludge.

The q_{max,NO_2^-} was higher in biofilm than in activated sludge. However, no similar differences were observed for

K_{s,NO_2^-}. The composition of the *Nitrospira*-like sequences differed significantly across bioreactors. Many sequences were found to belong to *Nitrospira*. Upon examination of phylogenetic relationships, all of them were found to belong to the *Nitrospira moscoviensis* lineage. Some sequences, such as Ns.4, Ns.6, and Ns.7, were different from the others, as shown by the phylogenetic tree. It was estimated that there may be some variation in ecophysiology within the *Nitrospira* genus, as has been reported in other nitrifier populations [23]. This may have contributed to substrate conversion efficiency.

Response of nitrification kinetic constants to the AS-BF system and mechanism-based analysis of the nitrifying bacterial community

Another objective of this experiment was to investigate changes in the nitrifying reactions in a mixed system. After combining equal biomasses of activated sludge and biofilm, the nitrifying performance of the mixed system was maintained at its original level for 4 months. The q_{max} and K_s of the two kinds of biofilm and two kinds of activated sludge measured in this experiment confirmed a strengthened nitrifying efficiency in AS-BF bioreactor. After 120 days of operation, the q_{max} of the biofilm in the mixed system was found to have remained high, but the K_s for ammonia decreased from 2.46 to 1.42 mg l^{-1}, which suggested an increase in affinity for ammonia. The maximum specific ammonia oxidation rate in the activated sludge in the mixed system (0.192 day^{-1}) was higher than in individually cultivated activated sludge systems. This also indicated an increase in nitrifying capacity.

In terms of the nitrifying bacteria community, this phenomenon could be explained by fragments of biofilm flaking off into and supplementing the activated sludge biomass. Biofilm fragments are relatively large, so they have less chance of being washed out than sludge floc. This supplementation may also have led to the introduction of different AOB species from biofilm to sludge. It has been speculated that this kind of introduction may happen from sludge to biofilm as well as from biofilm to sludge. In AS-BF bioreactors, the nitrifying bacterial communities were found to be more affected by competition for substrates and DO than in other types of reactors. Remarkable differences in nitrifying bacteria community structures were observed in bioreactors containing sludge alone, biofilm alone, or AS-BF mixtures on day 120. Although parameters such as substrate load and DO have generally been close in all three reactors, combinations of biofilm and activated sludge can offer various microenvironments for the nitrifying population. Some specific sequences (Ns.1, Ns.10, Ns.11, AOB.7, AOB.8, and AOB.9) were observed exclusively in the AS-BF system.

Conclusions

After setting identical operating conditions in three AmOn reactors using biofilm, activated sludge, and a combination of the two, distinct nitrifying bacteria communities and dissimilar bacterial concentrations were observed. The presence of some specific nitrifying bacterial strains might be the cause of the variations observed in nitrification efficiency in sludge and biofilm alone. Some dominant AOB and NOB sequences were found to accumulate specifically in the AS-BF system. These were found likely to enhance the nitrifying capacity of activated sludge and realize the maintenance of nitrifying capacity in biofilm. These results provide information that may help researchers understand the mechanisms by which nitrifying bacteria act in wastewater treatment systems.

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