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The differential proliferation of AOB and NOB during natural nitrifier cultivation and acclimation with raw sewage as seed sludge†

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Nitrifier immigration from sewers to wastewater treatment systems is attracting increasing attention for understanding nitrifier community assembly mechanisms, and improving process modeling and operation. In this study, nitrifiers in raw sewage were cultivated and acclimated in a sequencing batch reactor (SBR) for 90 days to investigate the characteristics of the influent nitrifiers after immigration. During the experiment, specific nitrite utilization rate (SNUR) exceeded specific ammonia utilization rate (SAUR) when floc size reached 224 ± 46 μm, and nitrogen loss occurred at the same time. The ratio of nitrite oxidizing bacteria (NOB) to ammonia oxidizing bacteria (AOB) increased from 0.84 to 2.14 after cultivation. The Illumina MiSeq sequencing showed that the dominant AOB was *Nitrosomonas* sp. Nm84 and unidentified species, and the three most abundant *Nitrospira* were *Nitrospira defluvii*, *Nitrospira calida*, and unidentified *Nitrospira* spp. in both raw sewage and cultivated activated sludge. The shared reads of raw sewage and activated sludge were 48.76% for AOB and 89.35% for *Nitrospira*. These indicated that nitrifiers, especially NOB, immigrated from influent can survive and propagate in wastewater systems, which may be a significant hinder to suppress NOB in the application of advanced nitrogen remove process based on partial nitrification in the mainstream.

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Introduction

Nitrification is typically the rate-limiting process in activated sludge water resource recovery facilities (WRRFs). Activated sludge systems are common in large-scale municipal wastewater treatment, however, nitrification failure is a too-frequent occurrence in winter due to significant temperature effects and relatively slow growth rate of nitrifiers.[1] Besides, how to stably and sustainably eliminate nitrite oxidizing bacteria (NOB) is still the main bottleneck in the application of advanced mainstream biological nitrogen removal (BNR) technologies based on partial nitrification.[2,3] Thus, the microorganism community of nitrifiers has attracted more and more attention in the field of biological wastewater treatment design and operation.

To maintain a constant amount of biomass in WRRFs, a small fraction of the biomass is removed daily as surplus sludge. Plants in Xi'an, China, which have relatively low temperatures, have a relatively long solids retention time (SRT) of 10–20 days, where 5–10% of the biomass is removed each day. In order to counterbalance this removal in the system, the number of organisms must increase at a rate of 1/SRT per day. This increase is generally considered to be caused by the net growth of bacteria in the bioreactor.

Relatively high abundances of microbes have been confirmed to exist in the influent of WRRFs.[4,5] Due to their constant and effective immigration, the incoming cells might be abundant in the activated sludge community, despite possibly being inactive.[6] Jauffur *et al.* investigated three WRRFs located near Montreal during winter and suggested that the nitrifiers in the influent were active and likely seeded activated sludge bioreactors since the most abundant operational taxonomic units (OTUs) in the influent and mixed liquor were the same.[4] Saunders *et al.* investigated three WRRFs in Denmark and showed that the similar relative abundance of *Nitrospira* and *Nitrotoga* in the activated sludge and wastewater influent may indicate these organisms are acting as a seed for selection in the plants.[6] Moreover, the immigration of influent nitrifiers into activated sludge systems has been shown to enhance the local nitrifier community and function in a lab-scale study.[5] These works test the hypothesis that influent organisms are another important source of biomass addition, it also has significant implications regarding the role of influent populations in the construction of activated sludge communities.

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There is no consensus on approaches to analyzing immigration, and the assembly of bacterial communities in open biological systems, such as activated sludge systems, has long been considered chaotic and unpredictable.[7,8] Therefore, the current best practice for biological wastewater treatment modeling, such as the Activated Sludge Models (ASMs) which are recognized by the International Water Association (IWA), is to assume that there is no active nitrifying biomass in municipal wastewaters at the entrance of treatment facilities.[9] However, the neglection of the impact of natural nitrifier immigration may influence the design of biological wastewater treatment facilities, especially the size of aerobic bioreactors performing nitrification. Meanwhile, a continuous supply of nitrifiers, especially NOB, immigrated from raw sewage is very likely to have an adverse effect on achieving partial nitrification in the mainstream application.[10] Therefore, immigration from sewers to wastewater treatment systems is attracting increasing attention for understanding community assembly mechanisms and improving process modeling and operation. While so far, almost all of the studies previously discussed were based on the investigation of the similarity of the influent and activated sludge nitrifier communities. Comparatively few experiments have been designed directly to understand how the influent nitrifiers grow and persist in environmental conditions prevalent in WRRFs.

In this study, influent nitrifiers from the 2nd WRRF (without primary settler) in Xi'an were cultivated and acclimated in a sequencing batch reactor (SBR) for 90 days. The floc size, nitrification performance, and nitrifier community were investigated to explore the process of the survival and reproduction of the influent nitrifiers in activated sludge bioreactors and evaluate the impact of nitrifier immigration from influent on nitrifier community assembly. Using this basis, we provided new insight into achieving full nitrification in cold northern regions and meanwhile proposes a great challenge of suppressing NOB in the application of advanced BNR process based on partial nitrification in the mainstream.

Methods

Experimental set-up and operation

A lab-scale SBR with a working volume of 4 L was set-up and operated for 90 days. Throughout, the reactor temperature was maintained at 20 ± 1 °C. The processes of influent, effluent and sludge discharge were controlled by a programmable logic controller (PLC) using the peristaltic pumps. In addition, airflow rate was kept constant and average dissolved oxygen (DO) concentration during an operating cycle was above 2.5 mg L^{-1}. pH was maintained at 7.5–8.0 by manually adding 1.0 M $NaHCO_3$ to maintain the alkalinity required for nitrification.

During start-up, SBR was filled with 4 L real influent sewage from the 2nd WRRF in Xi'an and aerated for six days to cultivate biomass (phase I). The characteristics of the 2nd WRRF and influent sewage were described in ESI (Tables S1 and S2†). On the sixth day, once the ammonium was completely degraded, the reactor began operation for 90 days. It was operated in 48 h cycle (phase II, days 7–8, 15.06 g N per m^3 per day), 24 h cycle (phase III, days 9–13, 30.11 g N per m^3 per day), 12 h cycle (phase IV, days 14–22, 60.23 g N per m^3 per day), 8 h cycle (phase V, days 23–27, 90.35 g N per m^3 per day), 6 h cycle (phase VI, days 28–33, 120.46 g N per m^3 per day), after which the cycle time was gradually reduced to 4 hours (phase VII, days 34–90, 180.69 g N per m^3 per day) with a volumetric exchange rate (VER) of 50%. Each cycle consisted of feeding (5 min), aeration (depending on the operating cycle time of different phases), settling (40 min), decanting (5 min) and idle time (10 min). Once the mixed liquor volatile suspended solids (MLVSS) is close to the value of the 2nd WRRF in Xi'an (2.35 g L^{-1}), a specific volume of activated sludge was discharged prior to the end of the aeration phase to keep the SRT at 15–20 days.

Feed medium

The synthetic influent was used as feed to mimic the average composition and quality of domestic wastewater (the effluent of aerated grit chamber, that is, the influent of the bioreactor) in the 2nd WRRF after the sixth day. The composition was as follows: 13 mg L^{-1} NH_4Cl, 17 mg L^{-1} peptone, 79 mg L^{-1} NaAc, 122 mg L^{-1} starch, 116 mg L^{-1} low fat milk powder, 52 mg L^{-1} yeast, 92 mg L^{-1} urea, 23 mg L^{-1} KH_2PO_4, and 5.8 mg L^{-1} $FeSO_4 \cdot 7H_2O$. The feed was autoclaved at 121 °C for 1 h and allowed to cool down prior to feeding the reactors. A mixture of trace elements was then added to the feed and comprised of the following: 0.770 mg L^{-1} $Cr(NO_3)_3 \cdot 2H_2O$, 0.536 mg L^{-1} $CuCl_2 \cdot 2H_2O$, 0.108 mg L^{-1} $MnSO_4 \cdot H_2O$, 0.336 mg L^{-1} $NiSO_4 \cdot 6H_2O$, 0.100 mg L^{-1} $PbCl_2$, 0.208 mg L^{-1} $ZnCl_2$. The total nitrogen (TN) concentration, and COD of the formulated influent recipe were approximately 60.23 mg L^{-1}, and 385 mg L^{-1}, respectively.

Analytical methods

Morphology observation of activated sludge in raw sewage and SBR was performed using a microscope (ECLIPSE Ti-S, NIKON, JAPAN). DO and pH were monitored by a DO and pH meter (Mettler Toledo, Switzerland). All other physical and chemical parameters, including sludge volume index (SVI), mixed liquor suspended solids (MLSS), MLVSS, chemical oxygen demand (COD), NH_4^+–N, NO_2^-–N, NO_3^-–N were measured according to the standard methods.[11]

The microscopic digital images and floc size of the sludge samples were obtained by an electron microscope (50i, Nikon, Japan). The values of the equivalent diameter (Deq) were calculated using the eqn (1).

$$\mathrm{Deq} = 2\sqrt{A/\pi} \quad (1)$$

where Deq is the equivalent diameter of flocs (μm), and A is the area of microscopic images which is calculated by the software of Image-pro Plus 7.0 (μm^2).

All samples harvested from raw sewage and reactor were investigated with oxygen uptake rates (OUR) for nitrifier activity. The detailed measurement methods of the specific ammonia uptake rate (SAUR) and specific nitrite uptake rate (SNUR) referred to the previous study.[5] Fluorescence *in situ* hybridization (FISH) was performed on day 1 (initial start-up period) and

day 70 (stable operation period) with the same primers as recorded previously.[5] Concretely, the rRNA-targeted oligonucleotide probes used in FISH were EUB_{mix} (EUB338 + EUB338 II + EUB338 III), AOB_{mix} (Nso1225 + Nsv443 + Nsm156 + NmV), and NOB_{mix} (Ntspa662 + NIT3 + Ntcoc206 + Ntspn693). A confocal laser scanning microscope (CLSM) was used for image acquisition (Leica TCS SP8, Leica Microsystems, Germany).

DNA extraction and PCR amplification

The samples of raw sewage and mixed liquor were collected for community structure analysis on day 1 and day 70, respectively. All samples were pelleted by centrifugation for 5 min at 10 000 rpm. Genomic DNA was extracted from 0.25 g of centrifuged wet solids using the TIAN amp Soil DNA Kit (DP336, TIANGEN Biotech, China) according to the protocol provided by the manufacturer. DNA concentration and purity were determined by analysis with a microvolume spectrophotometer (NanoDrop 2000, USA). And the extracted genomic DNA samples were stored at −20 °C until further analysis.

The PCR primers used in this study are shown in Table 1. Each 50 μL of PCR mixture containing 1 μL of 10 μmol L^{-1} forward primer, 1 μL of 10 μmol L^{-1} reverse primer, 1 μL of 20 ng mL^{-1} DNA template, 25 μL of 2.5 units per μL 2× Taq MasterMix (CWBIO, China), and 22 μL of UltraPure™ Distilled Water. The thermocycling conditions for PCR amplification included a prior denaturation at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s, and extension at 72 °C for 10 min. To reduce the deviation, the PCR reaction per sample was performed in triplicate.

Quantitative PCR (qPCR)

Real-time PCR was performed to quantify the copy numbers of ammonia oxidizing bacteria (AOB), *Nitrobacter* spp., *Nitrospira* spp., and *Nitrotoga* spp. by ABI 7500 systems (Applied Biosystems, USA). And an SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio., Dalian, CO., LTD) was used to quantify nitrifiers. Each diluted plasmid, ranging from 10^1 to 10^5 copies per microliter, was used for standardization for qPCR assays. Each tube was loaded with 2 μL DNA sample, followed by 10 μL 2× SYBR Green Master Mix reaction solution (Invitrogen, China), 0.5 μL of 10 μmol L^{-1} forward primer, 0.5 μL of 10 μmol L^{-1} reverse primer, and 7 μL UltraPure™ Distilled Water. A two-stage amplification protocol was performed as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and simultaneous annealing and extension at 60 °C for 40 s.

Illumina MiSeq sequencing and sequence data analysis

The 16S rRNA gene fragments of nitrifiers were amplified *via* the PCR with the above primer sets (Table 1). The PCR products were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using QuantiFluor™-ST (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina MiSeq PE300 platform (Illumina, Inc., CA, USA). The library construction and sequencing were performed by Allwegene Co., Ltd. (Beijing, China).

The extraction of high-quality sequences was performed with the Quantitative Insights Into Microbial Ecology (QIIME) package (v1.2.1). Raw sequences were selected based on sequence length, quality, primer, and tag. The raw sequences were selected and low-quality sequences were removed. This included any raw reads shorter than 110 nucleotides, any truncated reads that were shorter than 50 bp, reads containing ambiguous characters were removed. Only sequences with an overlap of longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded.

The unique sequence set was classified into OTUs under the threshold of 97% identity using UCLUST. Chimeric sequences were identified and removed using Usearch (version 8.0.1623). The taxonomy of each 16S rRNA gene sequence was analyzed by UCLUST against then database using a confidence threshold of 90%. All the raw data have been archived at NCBI Sequence Read Archive (SRA) database with accession number of SRR2106467.

Results

Reactor performance

The nitrogen concentrations of the SBR influent and effluent over the 90 day monitoring period are displayed in Fig. 1a. During phase I (days 0–6), the SBR was filled with raw sewage from the 2nd WRRF in Xi'an and aerated without feeding. The effluent NH_4^+–N concentration gradually decreased from 43.14 mg L^{-1} (day 0) to 3.97 mg L^{-1} on day 3 and was less than

Table 1 List of PCR primers used in this study

Target gene	Primer[a]	Sequence (5′–3′)	Reference
Ammonium monooxygenase (*amoA*)	*amoA-1F*	GGGGTTTCTACTGGTGGT	12
	amoA-2R	CCCCTCTGCAAAGCCTTCTTC	
nxrB genes of *Nitrospira*	*nxrB-169F*	TACATGTGGTGGAACA	13
	nxrB-638R	CGGTTCTGGTCRATCA	
16S rRNA *Nitrobacter* sp.	*FGPS-1269′*	CTAAAACTCAAAGGAATTGA	14
	FGPS-872	TTTTTTGAGATTTGCTAG	

[a] Primer's short name used in the reference.

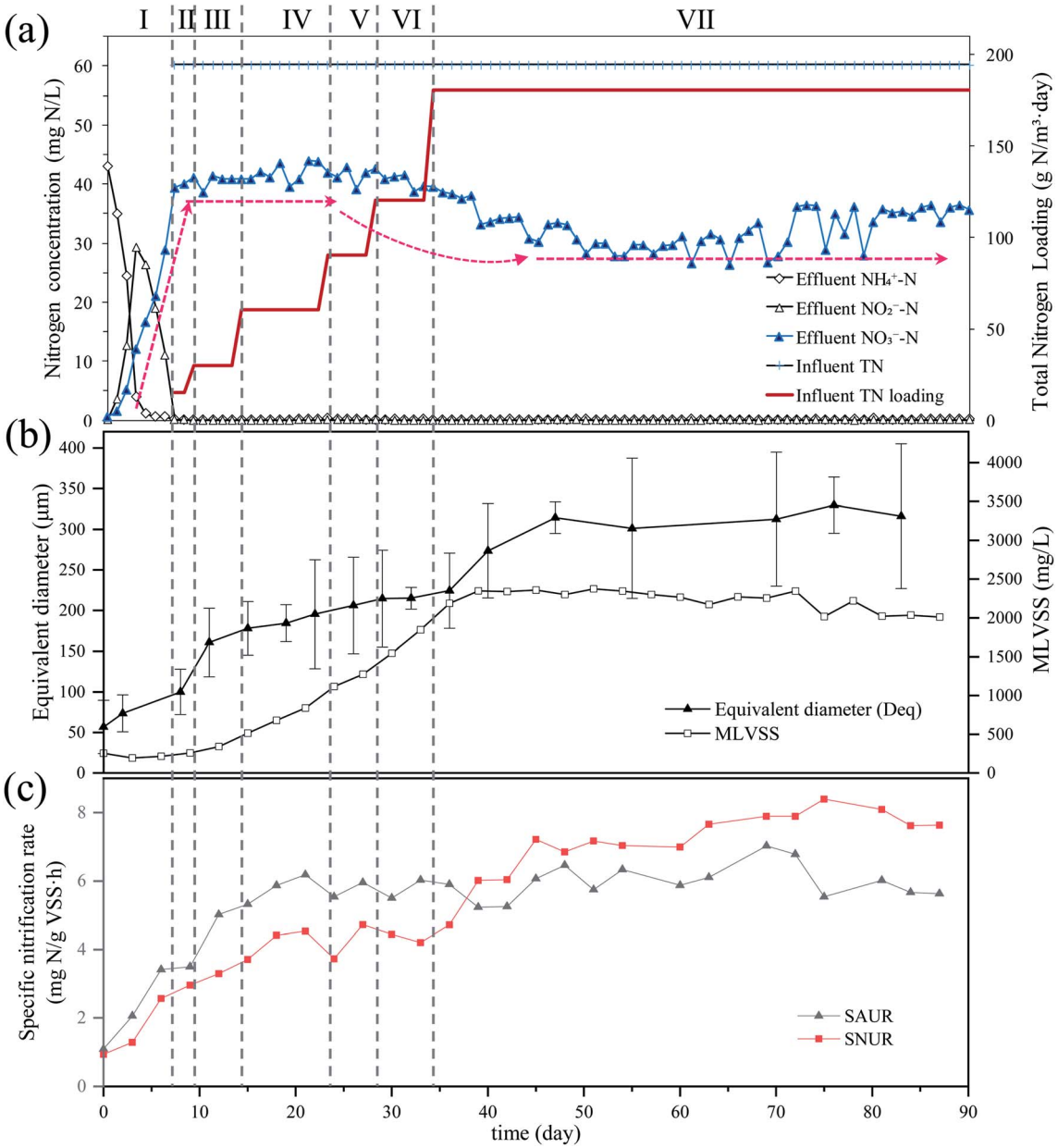

Fig. 1 Nitrification performance of SBR for 90 days. (a) TN loading in the influent and nitrogen contents in the effluent; (b) the values of MLVSS and the equivalent Diameter (Deq); (c) profiles of specific nitrification rate of the activated sludge.

1 mg L^{-1} during days 4–7. Corresponding to the reduction of NH_4^+–N, the NO_2^-–N concentration gradually increased from 0 mg L^{-1} at the beginning of the observation period to 29.38 mg L^{-1} on day 3, and then gradually decreased to 0.14 mg L^{-1} by day 7. The NO_3^-–N concentrations gradually increased from 0 mg L^{-1} at the beginning of the observation period to 39.39 mg L^{-1} by day 7. During phase II–VII, the concentration of NH_4^+–N and NO_2^-–N in effluent were all lower than the minimum detection limit, while the effluent NO_3^-–N concentration was 41.36 ± 1.42 mg L^{-1} during days 7–30. It is, however, worth noting that the effluent NO_3^-–N concentration decreased gradually during days 30–44 until reaching and maintaining 30.58 ± 2.14 mg L^{-1} from day 45 until the end of the observation period.

As shown in Fig. 1b, the MLVSS in SBR gradually increased with the influent nitrogen loading rate. On day 39, the MLVSS reached 2349 mg L^{-1}, which was close to the MLVSS in the bioreactor of the 2nd WRRF in Xi'an (2.35 g L^{-1}). The excess sludge was then discharged to keep the SRT of the SBR at 15–20 days and to maintain the MLVSS at 2279 ± 96 mg L^{-1} during days 40–90.

The Deq of flocs in raw sewage was 57 ± 33 μm and then quickly increased to 161 ± 42 μm during days 0–11 (Fig. 1b). During days 11–36, the increasing rate in Deq was slow, with the value increasing from 161 ± 42 μm to 224 ± 46 μm. The floc size

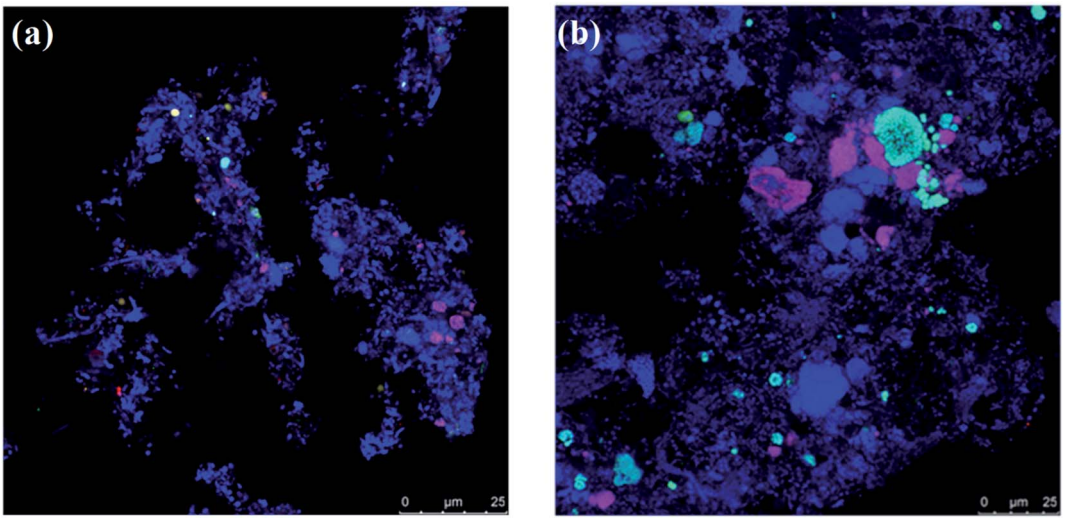

Fig. 2 Confocal laser scanning microscope images. (a) Raw sewage, and (b) activated sludge samples were hybridized with FLUOS-labeled AOB_{mix} (green + blue = cyan), Cy3-labeled NOB_{mix} (red + blue = purple) and Cy5-labeled EUB (blue).

increased quickly again from 224 ± 46 μm to 314 ± 19 μm during days 36–47. After day 47, the floc size stabilized at 315 ± 62 μm until the end of the observation period.

Nitrifier activity

Respirometric assays were used to evaluate the activity of nitrifiers. Fig. S1† showed that the AOB and NOB in raw sewage required approximately 0.5 h after ammonia addition and 2 h after nitrite addition, respectively, to acclimatize to the new environment. The maximal OUR of AOB and NOB were 17.06 ± 0.20 mg O_2 per L and 8.10 ± 0.21 mg O_2 per L, respectively. After the conversion of mg O_2 per L to mg N (g VSS h)$^{-1}$, the maximum SAUR and SNUR of raw sewage in the experiment were 1.09 ± 0.02 mg N (g VSS h)$^{-1}$ and 0.93 ± 0.07 mg N (g VSS h)$^{-1}$, respectively. And the SNUR/SAUR of raw sewage was 0.85.

The activities of nitrifiers in SBR were analyzed every three days (Fig. 1c). The SAUR increased quickly until day 12 and kept relatively stable at 6.30 ± 0.44 mg N (g VSS h)$^{-1}$ during the rest of the experiment period. On the contrary, the SNUR increased more slowly than SAUR and reached a relatively stable value of 7.89 ± 0.29 mg N (g VSS h)$^{-1}$ after day 45; the SNUR/SAUR was 1.25. It was worth noting that the SNUR was lower than the SAUR during days 0–39, while higher than SAUR during days 40–90. Similar results that the average SNUR was higher than SAUR in the investigation of 10 full-scale WRRFs in Xi'an, China, were also reported by Yao and Peng.[15]

Nitrifier community

Fluorescence *in situ* hybridization (FISH). Confocal laser scanning microscope images before and after natural cultivation by FISH are shown in Fig. 2. These images showed the *in situ* spatial organization of the nitrifiers (AOB + NOB) and heterotrophic bacteria in the samples. The relative amounts of the targeted bacteria species were calculated as the percentage of total bacteria (EUB_{mix}). The fraction of nitrifiers (AOB + NOB) to EUB_{mix} in raw sewage was 3.10 ± 0.80%, among them AOB_{mix}/EUB_{mix} was 1.62 ± 0.43% and NOB_{mix}/EUB_{mix} 1.49 ± 0.39%. And the ratio of NOB_{mix} to AOB_{mix} was 0.92. After 90 days of culture in the reactor, nitrifiers accounted for 9.15 ± 4.96% of total bacteria in activated sludge, which far exceeds the average nitrifying bacteria account (5.29 ± 2.11%) in 10 full-scale WRRFs in Xi'an, China as described in Yao and Peng.[15] Concretely, AOB_{mix}/EUB_{mix} was 3.93 ± 2.00% (>1.27%), and NOB_{mix}/EUB_{mix} was 4.80 ± 3.13% (>4.02%). NOB/AOB was 1.22. In summary, after natural nitrifier cultivation and acclimation with raw sewage as seed, the ratio of NOB to total bacteria was relatively larger than that of AOB.

qPCR. Relative quantifications of AOB and NOB (*Nitrospira* and *Nitrobacter*) were also determined by qPCR to study the

Table 2 Relative quantification of AOB and NOB determined by qPCR

Sample source	AOB (copies per L)	*Nitrospira* spp. (copies per L)	*Nitrobacter* spp. (copies per L)	NOB/AOB[a] (cell/cell)
Raw sewage	3.70×10^6	1.56×10^6	1.43×10^3	0.84
Activated sludge	1.06×10^8	1.13×10^8	2.11×10^4	2.14

[a] Cell/cell: the ratio of cell number per liter, cells per L = copies per L ÷ (gene copy number per cell). Assumed gene copy number per cell is 1 for *Nitrospira* 16S rDNA, 1 for *Nitrobacter* 16S rRNA, and 2 for *amoA* gene.[16]

Table 3 The numbers of final reads and alpha diversity indexes of the sequencing of nitrifying bacteria[a]

Nitrifier	Samples	Final reads	Shared reads (%)	OTUs	Shared OTUs	Chao1	Shannon
AOB	RS[b]	22 454	48.76	175	20	176.15	3.86
	AS[c]	14 282		80		80.41	3.85
NOB (*Nitrospira*)	RS	33 787	89.35	206	29	207.03	3.41
	AS	45 663		46		51.47	1.82

[a] Shared reads (%) $= \left(\frac{\text{no. of reads of shared OTUs from raw sewage}}{\text{total no. of reads from raw sewage}} + \frac{\text{no. of reads of shared OTUs from activated sludge}}{\text{total no. of reads from activated sludge}}\right) / 2 \times 100\%$. [b] RS, raw sewage. [c] AS, activated sludge.

nitrifying population within the raw sewage and cultured activated sludge (Table 2). The qPCR results showed that the average copies number of AOB and NOB in raw sewage was 3.70×10^6 copies per L and 1.56×10^6 copies per L, respectively, while the copies number increased to 1.06×10^8 copies per L of AOB and 1.13×10^8 copies per L of NOB in cultured activated sludge. Compared to *Nitrospira*, the copies number of *Nitrobacter* was relatively lower in NOB in either raw sewage or activated sludge (*i.e.*, *Nitrospira* is the dominant NOB). The number of AOB and NOB cells per liter were calculated from copies per liter using several assumptions regarding gene copies per cell.[16] Among of them, *Nitrospira* and *Nitrobacter* were assumed to contain 1 copy 16S rDNA per cell, and one cell of AOB was assumed to contain 2 copies *amoA* gene. Thus, the cell number ratio of NOB/AOB (cell/cell) was 0.84 in the raw sewage and gradually increased to 2.14 in the cultured activated sludge.

Illumina MiSeq sequencing. The bacterial community of the samples was analyzed by Illumina MiSeq sequencing to evaluate whether there were significant shifts in the composition of the bacterial communities throughout the cultivation. *Nitrobacter* was not detected due to its poor abundance as described in Table 2. OTUs were defined as sequences with 97% similarity. As shown in Table 3, for AOB sequences, 175 OTUs were observed in the raw sewage sample, and 80 OTUs in the activated sludge sample. OTUs were detected for the NOB (*Nitrospira*) sequences, with 206 OTUs in the raw sewage sample, and 46 OTUs in the activated sludge sample. No matter AOB or NOB, it is obvious that the OTUs in raw sewage samples was higher than that in activated sludge after acclimation. Similarly, Jauffur *et al.* investigated the community structure of nitrifying bacteria in the influent and activated sludge systems of three WRRFs in Canada.[4] The results showed that 371 OTUs in the influent samples and 236 OTUs in the mixed liquor samples for the AOB sequences, 99 OTUs in the influent and 83 OTUs in the mixed liquor samples for the NOB (*Nitrospira*) sequences. Both AOB and NOB sequencing revealed that raw sewage had a higher number of OTUs than activated sludge in bioreactors of three WRRFs. Besides, an explicit comparison of the sequence reads showed a different level of sharing between the nitrifying AOB and NOB OTUs in raw sewage and activated sludge, which were 48.76% for AOB and 89.35% for NOB (*Nitrospira*), respectively (Table 3).

The Chao1 index and Shannon index were used to evaluate the species richness and diversity of nitrifiers, respectively. As shown in Table 3, the Chao1 and Shannon index of raw sewage all were higher than these of cultured activated sludge. This implied that raw sewage had higher species richness and diversity of AOB and NOB.

The relative abundances of the microbial communities at the species level are shown in Fig. 3a. It could be seen that the dominant AOB species in both raw sewage and activated sludge were *Nitrosomonas* sp. Nm84 and unidentified species. The immense ecological significance of this particular group of bacteria contrasts with our limited knowledge about them because most species that inhabit activated sludge are still uncultured and unidentified.[17] Similar to the AOB community profiles, the dominant *Nitrospira*-related NOB species identified

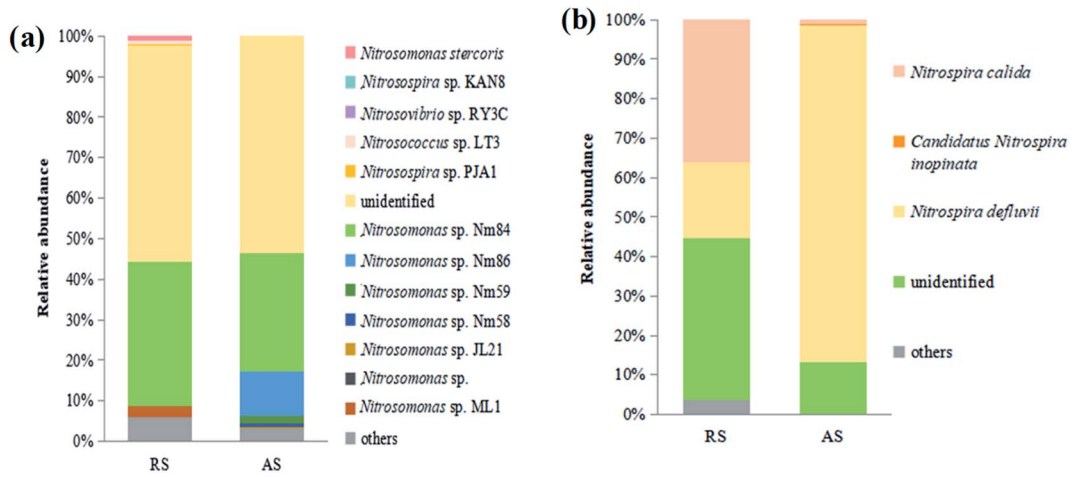

Fig. 3 Relative abundances of microbial community of two samples (raw sewage, RS; activated sludge, AS) at species level. (a) AOB; (b) NOB (*Nitrospira*).

in the activated sludge, are the same as those detected in raw sewage, namely *Nitrospira defluvii*, *Nitrospira calida*, and unidentified (Fig. 3b). These have previously been reported to be important NOB species in sewage treatment.[18]

The AOB populations and the NOB (*Nitrospira*) populations were classified under lineages as shown in Fig. 4. Among the AOB populations, four lineages were found in the raw sewage, namely *Nitrosomonas oligotropha*, *Nitrosomonas communis*, *Nitrosomonas europaea*, and *Nitrosospira* lineages. But only *Nitrosomonas oligotropha* lineage and *Nitrosomonas communis* lineage were found to be dominant in the activated sludge, and these are common AOB present in activated sludge of WRRFs.[19] Among the NOB (*Nitrospira*) populations, *Nitrospira* lineage I, *Nitrospira* lineage II, and *Nitrospira* lineage VI were found in both the raw sewage and activated sludge samples. This is in agreement with Saunders *et al.* who detected a relatively high abundance of *Nitrospira* in influent and activated sludge of WRRFs.[6]

Discussion

Additional nitrite produced by nitrite loop urges the increase of NOB/AOB

Due to mass conservation law, the concentration of NO_2^-–N cannot be higher than that of NH_4^+–N in the complete nitrification pathway. Besides, the yield coefficient of AOB was higher than that of NOB,[20] thus it seems almost inevitable that SNUR will be lower than SAUR in the sewage treatment systems. This was exactly the case during the initial 39 days of the experiment, however, there was an unexpected turn that SNUR > SAUR after day 40 (Fig. 1c). Furthermore, the floc size gradually increased in natural cultivation and acclimation (Fig. 1b). When the floc size reached 224 ± 46 μm (day 36), the effluent NO_3^-–N concentration decreased significantly and the nitrogen loss occurred as shown in Fig. 1a. Almost at the same time (day 40–50), the effluent NO_3^-–N concentration, the Deq of the flocs, and SNUR all tended to be stable.

Besides, it is known that in full-nitrification, NOB generates only two electrons from the oxidation of nitrite to nitrate, which is three times lower than the number of electrons generated by AOB during oxidizing of ammonium to nitrite.[21,22] Due to ammonium activated by the ammonia monooxygenase (AMO) in the AOB metabolic pathway, these two electrons are not available for energy generation. Thus, it is expected that the biomass yield of NOB is about two times lower than that of AOB per unit of nitrogen. This implies a theoretical NOB/AOB ratio of 0.5.[23] However, the FISH and qPCR results both showed the same trend that the ratio of NOB/AOB increased after cultivation and acclimation with raw sewage as seed. Concretely, the NOB/AOB ratio (the ratio of cell number per liter) in the raw sewage was 0.84, relatively close to the theoretical value, however, it increased to 2.14 in the cultured activated sludge (days 90) (Table 2). Winkler *et al.* also showed higher NOB/AOB ratios (3–4) in aerobic granular sludge samples.[23] This has corresponded to the increase of SNUR/SAUR with the floc size as shown in Fig. 1.

Given all that, there is probably another route by which NOB could contact more NO_2^-–N. In this study, the floc size gradually increased during cultivation. The increasing floc size produced greater mass transport resistance, and the diffusion of oxygen in the inner flocs would be limited. The aerobic region was thus gradually confined to the surface layer, and then an anoxic zone would occur in the inner part of the flocs. Numerous studies have shown experimentally as well as by mathematical modeling that oxygen penetration is restricted to the outer rim of the flocs (<100 μm).[24] Therefore, anoxic denitrification should occur along with the increase of the floc size after day 36, which is corresponding to the decrease in effluent NO_3^-–N concentration during days 30–44. Based on the fact that nitrite is an intermediate compound in both nitrification and denitrification steps,[25] Winkler *et al.* adopted a conceptual "nitrite loop" model to describe the bacterial growth balance in a nitrifying community (Fig. 5).[23] Nitrite oxidation is coupled with nitrate reduction. According to the nitrite loop theory, the

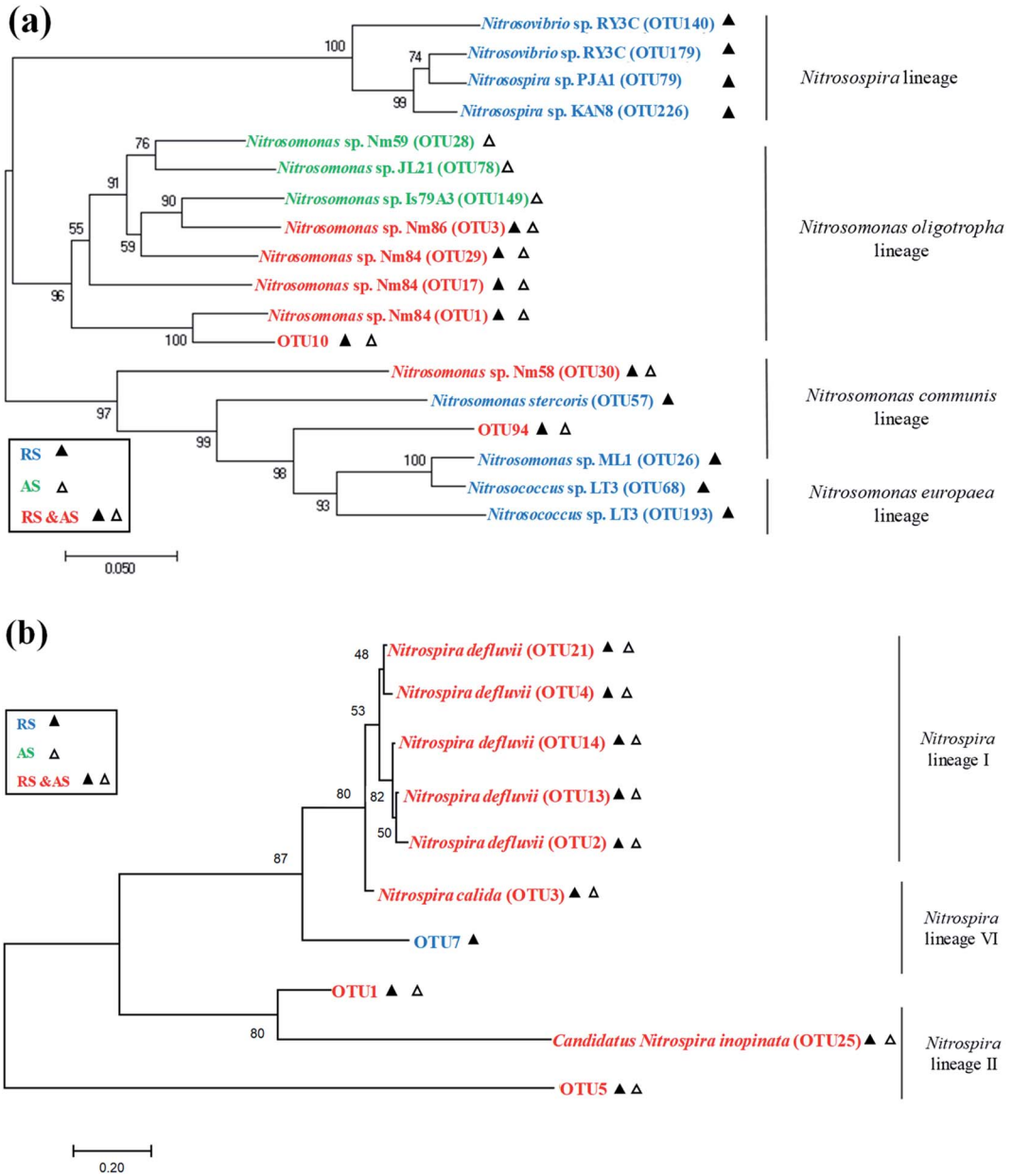

Fig. 4 Phylogenetic trees showing the position of the most abundant AOB and NOB (*Nitrospira*) of two samples (raw sewage, RS; activated sludge, AS). (a) AOB; (b) NOB (*Nitrospira*).

additional nitrite produced in the denitrification pathway may transfer to the oxic zone and be reoxidized to nitrate by NOB. Therefore, it is possible for NOB to receive a larger amount of NO_2^-–N than that of NH_4^+–N received by AOB, with the result that the SNUR will be higher than SAUR after day 36 when the average floc size has increased to 224 ± 46 μm.

The effect of nitrifier immigration on the community of activated sludge system

Raw sewage obviously had a certain nitrifier activity that required several hours to recover as shown in Fig. S1.† Meanwhile, the recovery time of AOB in raw sewage significantly is shorter than that of NOB (*i.e.*, AOB activity relatively is easier to recover). Similar results were also obtained by Yu *et al.* and Jauffur *et al.*[4,5] As the measured recovery time of nitrifier activity is shorter than the conventional SRT of activated sludge systems in WRRFs (15–20 days), nitrifiers immigrated from raw sewage are likely to achieve full induction in biological treatment systems.[5] This is confirmed by the qPCR results that the copies number of AOB and NOB in cultured activated sludge were significantly larger than that in raw sewage (Table 2). Therefore, nitrifier immigrated from raw sewage should not be neglected in biological treatment systems.

Actually, the nitrifier immigration of raw sewage is accompanied by the variation of species richness and the change of nitrifier community occurred in activated sludge systems. The Illumina MiSeq sequencing showed that raw sewage had higher

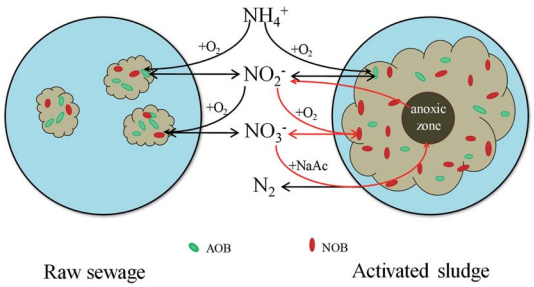

Fig. 5 Schematic view of nitrite loop theory (adapted from Winkler *et al.*[23]). The additional nitrite produced in the denitrification pathway may transfer to the oxic zone and then be reoxidized to nitrate by NOB.

species richness and diversity of AOB and NOB than cultured activated sludge (Table 3). The reasons for lower diversity of activated sludge may be: (i) the activated sludge process operates some kind of selection,[7] (ii) the number of ecological niches in biological treatment process is lower than that in sewer system,[26] or (iii) the environmental conditions prevalent in WRRFs may also exert a sort of selective pressure on the species assembly of the nitrifier population.[27] The real reasons still need to be further studied. In the community structure of nitrifiers, the dominant AOB species in both raw sewage and activated sludge were *Nitrosomonas* sp. Nm84 and unidentified species (Fig. 3a). For NOB, the dominant *Nitrospira*-related NOB species in the activated sludge are *Nitrospira defluvii*, *Nitrospira calida*, and unidentified, which are the same as those detected in raw sewage (Fig. 3b). However, *Nitrospira defluvii* from the raw sewage eventually becomes the most predominant NOB in the acclimated activated sludge. It is worth mentioning that *Nitrospira defluvii* is always be considered as the most predominant nitrite oxidizer in WRRFs.[28] Phylogenetic trees reflected evolutionary relationships among various nitrifiers of raw sewage and activated sludge based upon similarities and differences in their genetic characteristics (Fig. 4). For AOB, only *Nitrosomonas oligotropha* lineage and *Nitrosomonas communis* lineage, common AOB present in WRRFs,[19] were found to be dominant in the activated sludge. However, for NOB, the *Nitrospira* lineage I, II, and VI were all found in the raw sewage and activated sludge samples. These results indicated that the nitrifier communities of raw sewage and activated sludge have a certain similarity.

In brief, raw sewage makes a great contribution in supplying valuable AOB and NOB populations to bioreactor by natural continuous seeding, and then plays an important role in the nitrifier community construction of activated sludge systems. Considering that nitrifiers immigrated from raw sewage could survive and propagated in activated sludge systems, theses nitrifiers could partially compensate for the decreasing nitrifier activity of activated sludge systems in cold northern regions. Actually, nitrifiers were reportedly able to form strong microcolonies in flocs, which are more resistant to high shear forces so that it could be more effectively removed by primary settler.[29] Based on incubation tests and modelling by Duan *et al.*, the primary settler designed in WRRFs exhibited high efficiencies for AOB removal and NOB removal, at 72.3% and 94.2%, respectively.[10] Therefore, primary treatment for raw sewage, *e.g.* primary settler, may be unnecessary for achieving efficiently full-nitrification in cold northern regions.

The intractable challenge for achieving partial nitrification in the mainstream

However, the nitrifiers immigration from raw sewage also may bring bad consequences to BNR process. It can be observed from Table 3 that the percentages of shared reads in raw sewage and activated sludge were 48.76% for AOB and 89.35% for NOB (*Nitrospira*), respectively. Similarly, Jauffur *et al.* reported that the percentage of reads belonging to OTUs that appeared in both influent and mixed liquor of the same WRRF averaged 78% for AOB and 86% for NOB. NOB (*Nitrospira*), by contrast, is more effective than AOB in seeding the activated sludge systems.[4] Thus, it was inferred that the percentage of shared reads could even be used to evaluate the seeding efficiency in the design of biological wastewater treatment modeling (*e.g.* ASMs).

It is known that the key to achieving stable partial nitrification is to sustainably retain AOB while eliminating NOB in the mainstream BNR process.[30,31] However, raw sewage that contains a few nitrifiers, especially the more efficient NOB in seeding as shown in Table 3, continuously inoculates the bioreactor, which might have a devastating effect on achieving stable partial nitrification. Duan *et al.* reported that the continuously seeding of NOB in raw sewage resulted in different extents of ineffective NOB suppression in the mainstream activated sludge systems.[10] Meanwhile, the NOB in the raw sewage could stimulate the NOB community shifts under NOB suppression pressure to develop resistance.

As the municipal sewage has too lower carbon-nitrogen ratio to meet the carbon source requirement in the BNR process, canceling primary settling tanks usually are used to increase the concentration of organic carbon source entering the bioreactors in the engineering, like the 2th WRRF in Xi'an, which ultimately leads to the reduced TN concentration of secondary effluent.[32] It has been proved that the primary settling tank could remove about 94.2% of NOB contained in the raw sewage.[10] Considering that the NOB immigration from raw sewage is definitely a significant hinder to suppress NOB, primary treatment is necessary for ensuring stable mainstream NOB suppression.

To sum up, when the mainstream BNR process in WRRFs is based on traditional nitrification-denitrification process in cold northern regions, if without any special requirements, it may be considered not to set up primary settling tank to achieve efficiently full-nitrification. However, when advanced mainstream BNR process based on partial nitrification was applied in WRRFs, primary settling tanks was necessary for achieving stable partial nitrification process.

Conclusion

(1) In natural cultivation and acclimation with raw sewage as seed sludge, the flocs size gradually increased, which provided

valuable habitats for influent nitrifiers to retain and propagate, and then enough nitrifiers could be cultured.

(2) Nitrogen loss occurred when the flocs size reached 193 ± 46 μm (day 36), and then SNUR unexpectedly exceeded SAUR and the NOB/AOB ratio increased with the floc sizes due to nitrite loop.

(3) The shared reads were 48.76% for AOB and 89.35% for NOB (*Nitrospira*) for raw sewage and activated sludge. Thus, nitrifiers, especially NOB, immigrated from influent can survive and propagate in wastewater systems, which should be benefit for achieving full nitrification in cold northern regions and may be a significant hinder to suppress NOB in the application of advanced BNR process based on partial nitrification in the mainstream.

Conflicts of interest

The authors declare no conflict of interest.

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