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Nitrifier characteristics in submerged membrane bioreactors under different sludge retention times

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ABSTRACT

Three submerged membrane bioreactors (MBRs) were operated continuously for 230 days by feeding with synthetic inorganic wastewater ($\text{NH}_4^+\text{-N}$, 100 mg L^{-1}) under different solids retention times (SRTs. $M_{30\text{d}}$, 30 days; $M_{90\text{d}}$, 90 days; M_{infinite} , no sludge purge) to examine the influence of SRT on nitrification performance and microbial characteristics. All the reactors could oxidize $\text{NH}_4^+\text{-N}$ to $\text{NO}_3^-\text{-N}$ effectively without accumulation of $\text{NO}_2^-\text{-N}$. $M_{30\text{d}}$ with the shortest SRT showed significantly higher specific ammonium oxidizing rate (SAOR, $0.22 \text{ kg NH}_4^+\text{-N kg}^{-1} \text{ MLSS day}^{-1}$) and specific nitrate forming rate (SNFR, $0.13 \text{ kg NO}_3^-\text{-N kg}^{-1} \text{ MLSS day}^{-1}$) than the other two MBRs ($0.12\text{--}0.14 \text{ kg NO}_3^-\text{-N kg}^{-1} \text{ MLSS day}^{-1}$ and $0.042\text{--}0.068 \text{ kg NO}_3^-\text{-N kg}^{-1} \text{ MLSS day}^{-1}$, respectively). Short SRT led to low extracellular polymeric substances (EPS) concentration and long operating cycle. The nitrite oxidizing bacteria (NOB) ratios by both the fluorescence in situ hybridization (FISH) (3.6% for $M_{30\text{d}}$ and 2.1–2.2% for $M_{90\text{d}}$ and M_{infinite}) and MPN ($1.4 \times 10^7 \text{ cells g}^{-1} \text{ MLSS}$ for $M_{30\text{d}}$ and 6.2×10^5 and $2.7 \times 10^4 \text{ cells g}^{-1} \text{ MLSS}$ for $M_{90\text{d}}$ and M_{infinite}) analyses showed that $M_{30\text{d}}$ favored the accumulation of NOB, which was in accordance with the SNFR result. However, the ammonia oxidizing bacteria (AOB) ratios (3.5%, 3.2% and 4.9% for $M_{30\text{d}}$, $M_{90\text{d}}$ and M_{infinite}) were not in accordance with the SAOR result. PCR-DGGE, clone library and FISH results showed that the fast-growing *Nitrosomonas* and *Nitrobacter* sp. were the dominant AOB and NOB, respectively for $M_{30\text{d}}$, while considerable slow-growing *Nitrospira* sp. existed in M_{infinite} , which might be an important reason why M_{infinite} had a low SAOR and SNFR.

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1. Introduction

The most important point of membrane bioreactors (MBRs) is that this technology permits biological wastewater treatment under desirable solids residence times (SRTs), which allows the systems to keep a sufficient amount of slow-growing microbes, such as ammonia oxidizing bacteria (AOB) and those specializing in degrading refractory compounds, by choosing a relatively long SRT (Stamper et al., 2003; Xing et al., 2000). A long SRT, at the same time, will permit

a stable treatment and low excess sludge production (Fan et al., 2005; Howell et al., 2003). The higher biomass concentration also increases shock tolerance, which is particularly important where feed is highly variable (Bin et al., 2009). However, too long an SRT may result in membrane fouling due to the accumulation of large amount of EPS in the MBR systems (Djamila et al., 2008). How to choose a proper SRT to achieve high water quality while prevent serious membrane fouling is therefore an important issue for the operation of MBRs.

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On the other hand, activated sludge is a complicated ecological system where different types of microbes cooperate or compete with each other. Our previous study has shown that a large amount of heterotrophic bacteria coexisted with AOB and nitrite oxidizing bacteria (NOB) even in an MBR fed with inorganic ammonia-bearing wastewater (Hongyan et al., 2005b). It was considered that these heterotrophs live mainly on EPS and dead AOB and NOB. Different microbes have different growth rates. Therefore, the selection of SRT may affect microbial community structures as well as bioactivities in the biological systems.

It is considered that almost no excess sludge will be generated if an MBR is operated without sludge purge. However, it is not known if such an operation mode will adversely affect the bioactivity and performance of the MBR system. So in the present study, three reactors (M_{30d} , M_{90d} and $M_{infinite}$) fed with inorganic ammonia-bearing wastewater were established, and the reactor performance, bioactivity and microbial community structures of the MBRs were investigated under different SRTs (M_{30d} , 30 days; M_{90d} , 90 days; $M_{infinite}$, no sludge purge) for 230 days.

2. Materials and methods

2.1. Experimental system

Fig. 1 shows the schematic diagram of the experimental system consisting of three identical MBRs (M_{30d} – $M_{infinite}$). A submerged hollow fiber membrane module (Mitsubishi Rayon Co. Ltd., Tokyo) with an average pore size of $0.4\ \mu\text{m}$ and surface area of $0.2\ \text{m}^2$ was used for each MBR. The working volume of each reactor was 12 L and the HRT was controlled at 16 h. Air was supplied at a rate of $1\ \text{L}\ \text{min}^{-1}$ to maintain dissolved oxygen (DO) at $3\text{--}5\ \text{mg}\ \text{L}^{-1}$. The pH in the three reactors was $7.5\text{--}8.0$ and the temperature was kept at $20 \pm 1\ ^\circ\text{C}$. The permeate flux was suctioned by a metric pump controlled with a timer (Hongyan et al., 2005a). The operational cycle included 4 min of suction and 1 min break. M_{30d} and M_{90d} were operated at an SRT of 30 days and 90 days, respectively, and $M_{infinite}$ was operated without sludge purging. The three systems were operated in parallel for 230 days. The membranes were rinsed with water when the operating pressure suddenly increased.

Fig. 1 – Schematic diagram of membrane reactors.

Seed sludge was taken from one sewage treatment plant in Beijing, and inoculated to MBRs with an average MLSS of $4300\ \text{mg}\ \text{L}^{-1}$. Synthetic inorganic wastewater (Hongyan et al., 2005a) with an influent $\text{NH}_4^+\text{-N}$ of approximately $100\ \text{mg}\ \text{L}^{-1}$ was fed to the MBRs soon after the inoculation of seed sludge to give a volumetric loading rate of $0.15\ \text{kg}\ \text{NH}_4^+\text{-N}\ \text{m}^{-3}\ \text{day}^{-1}$. Influent and effluent samples were taken from each reactor to analyze $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, $\text{NO}_3^-\text{-N}$ and EPS, and sludge samples were taken to analyze MLSS, specific ammonium oxidizing rate (SAOR), specific nitrate forming rate (SNFR) and microbial community structures. SAOR and SNFR were used to determine the activities of activated sludge in oxidizing ammonia and nitrite, respectively. Detailed methods have been described previously (Hongyan et al., 2005a). EPS was extracted according to the methods proposed by Frolund et al. (1996). The content of polysaccharides was measured according to a phenol-sulphuric acid method and that of protein was determined with the Lowry method.

2.2. Most probable number (MPN) enumeration and plate counting

The MPN was used for the enumeration of AOB and NOB, respectively, together with the FISH method (Hongyan et al., 2005a). 1 mL sample taken from a reactor was dispersed by sonication. Serial 10-fold dilutions of the inoculum sources were prepared in sterile distilled water and 1 mL portions were transferred to MPN tubes containing 9 mL of the enumeration medium. Five replicate tubes were prepared per dilution for enumeration. The MPN tubes were incubated for 30 days at $28\ ^\circ\text{C}$. Production and oxidation of nitrite in each tube were examined by colour determination after adding zinc powder and Griess reagent, respectively. The colony-forming unit (CFU) was used for the heterotrophs by depositing 0.1 mL serial dilutions on LB agar plates as inoculum. After incubating for 24 h under $37\ ^\circ\text{C}$, the numbers of the colonies on the plates were counted.

2.3. FISH analysis

Following sonication on ice for about 3 min, activated sludge samples were fixed for 3 h with 4% paraformaldehyde at $4\ ^\circ\text{C}$ and stored in a 1:1 mixture of phosphate-buffered saline (PBS, pH 7.4) and ethanol at $-20\ ^\circ\text{C}$ (Amann et al., 1995). $3\ \mu\text{L}$ samples were placed in wells of the slides and immobilized for 3 h. Then the slides were dehydrated for 5 min using 50%, 80%, 98% ethanol, respectively. The hybridization and washing procedures was the same with the protocol previously described by Amann et al. Fluorescent hybridized cells were analyzed with an epifluorescence microscope (Axioskop2 mot plus, Zeiss, Germany) equipped with a cooled CCD camera (AxioCam MRm, Zeiss Corp., Germany). The proportion of the nitrifiers was calculated with the software provided by Zeiss (Axio Vision 4.1). At least 40 views were obtained for each sample to give an average result.

Probes NSO190 (5'-CGA TCC CCT GCT TTT CTC C-3'), NIT3 (5'-CCT GTG CTC CAT GCT CCG-3') and EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') were used for AOB, NOB and active bacteria, respectively (Mobarry et al., 1996).

2.4. PCR-DGGE analysis

A procedure described in a previous paper was adopted (Muyzer et al., 1993). DNA was extracted from 0.5 mL of mixed liquor samples. 16S rRNA gene fragments were amplified from the extracted total DNA from the samples with Taq DNA polymerase by using bacterial primer sets GC-341F (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG G-CCT ACG GGA GGC AGC AG-3') and 534R (5'-ATT ACC GCG GCT GCT GG-3'). The conditions used for the PCR were as follows: 5 min of initial denaturation at 94 °C and 25 cycles of 30 s at 94 °C, 30 s at 62.5 °C and 40 s at 72 °C, the annealing temperature was decreased by 0.3 °C from 62.5 °C to 65 °C every cycle, followed by 10 cycles of 30 s at 94 °C, 40 s at 55 °C and 40 s at 72 °C. A final extension step was conducted at 72 °C for 10 min prior to cooling at 4 °C.

The AOB DNA fragments of 465 bp in length were acquired using a specific primer pair of two forward primers CTO189fA/B-GC (5'-CCG CCG CGC GGC GGG CCG GGC GGC GGC ACG GGG GGA GRA AAG CAG GGG ATC G-3') and CTO189fC-GC (5'-CGC CCG CCG CGC GGC GGG CCG GGC GGC GGC ACG GGG GGA GGA AAG TAG GGG ATC G-3') in a ratio of 2:1, and a reverse primer CTO654r (5'-CTA GCY TTG TAG TTT CAA ACG C-3'). The PCR condition was as follows: 95 °C for 5 min followed by 35 cycles of 45 s at 94 °C, 45 s at 57 °C, 45 s at 72 °C, and a final extension of 10 min at 72 °C. All the PCR products were electrophoresed on a 1% agarose gel.

DGGE was performed by using a Dcode system (Bio-Rad Co., USA). The PCR-amplified DNA fragments were separated on polyacrylamide gels (10%, acrylamide:bisacrylamide = 37.5:1) in 1 × TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM EDTA, PH 7.4) with a denaturing gradient ranging from 35% to 55%. Electrophoresis was initially run for 30 min at 60 V and subsequently for 8 h at 120 V. Then, the gel was soaked for 30 min in ethidium bromide and photographed on a UV transillumination table with a Gel Documentation Systems (BIO-RAD Laboratories-Segrade, Milan, Italy). The images were analyzed with Quantity One Software (Bio-Rad, USA). Prominent bands were excised and put in 50 µL sterilized water at 4 °C overnight. 5 µL of the eluate was used as template DNA for a PCR performed with the primers 534R and 341F without GC clamp, and conditions described above for environmental samples. The remaining PCR products were sequenced with an ABI 3730 automated sequencer (Invitrogen, Shanghai, China).

The obtained DGGE patterns were subsequently analyzed with the BioNumerics software version 6.0 (Applied Maths, Kortrijk, Belgium). During this processing, different lanes were defined, the background was subtracted, differences in the intensity of the lanes were compensated, and the correlation matrix was calculated. Clustering was done with Pearson correlation and the UPGMA method (Possemiers et al., 2004). The correlation between day x and day x – 30 was plotted for day 50, 80, 110, 140, 170, 200 and 230 to evaluate the community stability within the groups of AOB and general bacteria in reactors M_{30d} , M_{90d} and $M_{infinite}$ using the principle of moving window correlation.

2.5. Clone library construction

The total extracted DNA was used as the DNA template in the PCR reaction with the primer *amoA1f* (5'-GGG GTT TCT ACT

GGT GGT-3') and *amoA2r* (5'-CCC CTC (C/T)G (G/C)A AAG CCT TCT TC-3'). Primers *amoA1f* and *amoA2r* were used to amplify a 491-bp fragment of the *amoA* gene, according to the protocol described by You and Chen (2008). The PCR products were then used for cloning, in accordance with the manufacturer's protocol (TA cloning kit, Invitrogen). Colonies with the correct inserts were then screened using PCR with another plasmid-vector specific primers M13f and M13r (95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 90 s, annealing at 47 °C for 45 s and extension at 72 °C for 2 min, and to be completed after 5 min at 72 °C). The positive *amoA* PCR products were then used for screening the genetic diversity by using restriction fragment length polymorphism (RFLP) screening with *HaeIII* (GG/CC) restriction enzyme digestion reactions (mixed with 10 µL of the PCR product, 1 µL of each restriction enzyme (10 units per µL), 2 µL of 10× buffer and 7 µL of ddH₂O, at 37 °C for 6 h). Positive clones were sequenced with an ABI 3730 automated sequencer (Invitrogen, Shanghai, China).

2.6. Phylogenetic analysis

After editing and trimming manually using BioEdit, bacterial 16S rRNA gene and *amoA* gene sequences obtained in this study were searched against the GenBank database using the BLASTN program (Altschul et al., 1997). The most similar reference sequences were retrieved and aligned with the sequences of this study using CLUSTALX (Thompson et al., 1997). Phylogenetic trees were constructed using MEGA version 4 by the neighbour-joining algorithm and the Jukes-Cantor distance estimation method with bootstrap analyses for 1000 replicates (Kumar et al., 2004).

2.7. Nucleotide sequence accession numbers

The 16S rRNA nucleotide sequences as well as *amoA* gene sequences of this study were deposited in the GenBank database under the accession numbers GU121104–GU121133, GU121134–GU121151, respectively.

3. Results and discussion

3.1. Reactor performance

Because of the relatively low volumetric loading rate (0.15 kg NH₄⁺-N m⁻³ day⁻¹), all of the three MBRs showed stable nitrification performance, the effluent NH₄⁺-N and NO₂⁻-N were generally below 1.0 mg L⁻¹ and 0.5 mg L⁻¹, and the average effluent NO₃⁻-N concentrations of M_{30d} , M_{90d} and $M_{infinite}$ were 96.8 mg L⁻¹, 93.7 mg L⁻¹ and 94.8 mg L⁻¹, respectively. Fig. 2(a)–(c) show the changes of MLSS, EPS and transmembrane pressure (TMP) of the three reactors, respectively. The three reactors began to show significant differences in biomass concentration soon after the start of the experiment, and the average MLSS values of M_{30d} , M_{90d} and $M_{infinite}$ were 1354 mg L⁻¹, 3131 mg L⁻¹ and 4897 mg L⁻¹, respectively, from day 60. The MLSS values of $M_{infinite}$ were in accordance with our previous results acquired with an NH₄⁺-N-feeding MBR operated without sludge purge (5000 mg L⁻¹, Mengchun et al., 2004), but were significantly lower than those acquired for MBRs fed

Fig. 2 – (a) The changing course of MLSS of the three MBRs. (b) The operation pressure of three MBRs (c) Change of EPS in the MBRs.

on organic wastewater without sludge purge (18,000–20,000 mg L⁻¹, Darren et al., 2006; Rosenberger et al., 2002).

As shown in Fig. 2(b), a sudden increase of operating pressures appeared due to the accumulation of sludge on the membrane surface after the reactors were run for several months. The filtration performance of the membranes was recovered after sludge on the surface was removed physically in clean water. The length of the first cycle was 105 days, 90 days and 83 days, respectively, for M_{30d} – $M_{infinite}$, and that for the second cycle was 88 days, 72 days and 63 days, showing that long SRTs led to short operating cycles.

It has been reported that EPS consisting of polysaccharides and proteins was a key factor for membrane pollution in MBRs (Lee et al., 2003). It was observed that the amount of EPS per unit of biomass with MBR was approximately 30–80 mg g⁻¹ MLSS in some studies (Ahmed et al., 2007; Fangang et al., 2006; How et al., 2005). Our previous results (Mengchun et al., 2004) showed EPS in MBR increased from 20 to 160 mg g⁻¹ MLSS under different volumetric loading rate (0.18–1.30 kg NH₄⁺-N m⁻³ day⁻¹). The EPS levels in this study were within the above reported ranges: the EPS levels in M_{90d} and $M_{infinite}$ increased slowly from 40 to 55 mg g⁻¹ MLSS, while that in M_{30d} remained a constant value of 40 mg g⁻¹ MLSS. The relatively low EPS level in M_{30d} might be related with its relatively long operating cycle.

3.2. Bioactivity

Fig. 3 shows that the values of SAOR and SNFR in all of the three reactors did not change much for the duration of 230 days. It is clear that the bioactivities of the MBRs were affected by SRT markedly. M_{30d} with the shortest SRT showed the highest average SAOR (0.22 kg NH₄⁺-N kg⁻¹ MLSS day⁻¹) and SNFR (0.13 kg NO₃⁻-N kg⁻¹ MLSS day⁻¹) among the three MBRs.

While M_{90d} and $M_{infinite}$ showed a similar SAOR value (0.12–0.14 kg NH₄⁺-N kg⁻¹ MLSS day⁻¹), their SNFR values were different (0.068 and 0.042 kg NO₃⁻-N kg⁻¹ MLSS day⁻¹).

3.3. FISH and cell number counting

Probes EUB338, NSO190 and NIT3 were used for the detection of bacteria, AOBs in the β subclass of *Proteobacteria* (including *Nitrosomonas*, *Nitrosococcus mobilis*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus*) (Purkhold et al., 2000; Hesselsoe et al., 2001; Persson et al., 2002) and *Nitrobacter* sp., respectively, and the results are shown in Fig. 4. Fig. 4(c) shows the changes of the ratio of active bacteria (specific to probe EUB) to total microbes (DAPI stained particles). The EUB/DAPI ratios in all of the three MBRs decreased with the increase of operation time, possibly due to the accumulation of inactive substances in the MBRs. In comparison with M_{90d} and $M_{infinite}$ where the EUB/DAPI ratio decreased from 56% to 28%, M_{30d} showed a relatively high active bacterial percentage (from 56% to 42%). As shown in Fig. 4(a), the AOB/DAPI values varied in a relatively wide range, suggesting some inaccuracy in detecting AOBs with the FISH technique. Despite this, however, it is still clear that the AOB/DAPI ratio in $M_{infinite}$ was much higher than those in the other two systems (the average ratios were 3.5%, 3.2% and 4.9% for M_{30d} , M_{90d} and $M_{infinite}$), showing that no sludge purge strategy led to the increase of AOB ratios. It is interesting why $M_{infinite}$ with the highest AOB ratio showed the lowest ammonia oxidizing rate. On the other hand, as shown in Fig. 4(b), the NOB/DAPI ratios were quite stable from day 60, with an average value of 3.6% in M_{30d} contrast to 2.1–2.2% in the other two reactors, which was in accordance with the SNFR result. Since NIT3 was only sensitive to *Nitrobacter* sp., this result means that the short SRT in M_{30d} favored the accumulation of the fast-growing NOB. The changes of the ratios of nitrifiers (the sum of AOBs and NOBs) to active bacteria (EUB) are shown in Fig. 4(d). The nitrifier ratio in $M_{infinite}$ increased from 5% to 25%, while those in the other two MBRs increased from 5% to 15%, showing that the nitrifier ratios significantly increased

Fig. 3 – (a) The specific ammonium oxidizing rate (SAOR) of the three MBRs. (b) The specific nitrate forming rate (SNFR) of the three MBRs.

Fig. 4 – (a) Composition of AOB for probe NSO190. (b) Composition of NOB for probe NIT3. (c) Composition of active bacteria for probe EUB338. (d) Composition of Nitrifiers/EUB.

with the increase of operation time. But still, the majority of bacteria were heterotrophs.

At the same time, culture-based cell counts of AOB, NOB (by MPN enumeration) and heterotrophs (by plate counting) were conducted on day 180. The density of AOB for M_{30d}–M_{infinite} was 1.6×10^7 , 3.4×10^7 and 1.5×10^7 cells g⁻¹ MLSS, respectively, that of NOB was 1.4×10^7 , 6.2×10^5 and 2.7×10^4 cells g⁻¹ MLSS, and that of heterotrophs was 0.8×10^8 , 1.2×10^7 , 5.3×10^7 cells g⁻¹ MLSS. The culture-based result also showed that the ratio of NOB in M_{30d} was higher than those of the other two reactors. Therefore, both the FISH and MPN results showed that shorter SRT resulted in a higher NOB density, which led to the higher SNFR. At the same time, the AOB ratio in M_{30d} was in the same order with those in other two reactors. Together with the FISH result, it is speculated that the AOB species with lower bioactivities might have accumulated in M_{infinite} because of the extremely long SRT.

3.4. PCR-DGGE

16S rRNA gene fragments for bacteria and AOB were investigated by PCR-DGGE method. It is interesting that the bacterial communities in the three reactors kept changing in spite of the stable operating conditions and treatment performance during the period. In all of the three MBRs, the diversity of bacteria decreased with the increase of operation time, with M_{infinite} showing the highest diversity in the end (The S values for M_{30d}–M_{infinite}: 0.39, 0.43 and 0.52). On the other hand, the AOB communities in the three reactors were relatively stable, with M_{infinite} showing the highest diversity.

All of the apparent bands were excised from the gels and sequenced, and a neighbour-joining tree, which graphically depicts the homologies of aligned sequences, was constructed for both bacteria and AOB as shown in Figs. 6 and 7. The three MBRs only shared one common bacterium (DM11, DM21, and

DM31 in Fig. 6) which was assigned to uncultured *Acidobacteria* bacterium. Bacteria in the phylum *Acidobacteria* are abundant in soils, and can be isolated from soils using low-nutrient medium (Stephanie et al., 2007). Although most of AOB are assigned to *Nitrosomonas* sp. for all of the three reactors, *Nitrospira* sp. was also found in M_{infinite} with a bright band. On the other hand, although *Nitrobacter* sp. was found to exist in all of the three reactors abundantly by FISH, they were not detected in DGGE. We have not been able to identify *Nitrobacter* sp. in a previous study (Hongyan et al., 2005a,b), either. The bands related to *Nitrospira* sp. for NOB were found in M_{90d} and M_{infinite}, but not in M_{30d}. It has been reported that *Nitrospira* sp. possessed a slow growing rate and a low NO₂⁻-N oxidizing rate (Magdalena et al., 2008; Okabe et al., 1999), while *Nitrobacter* sp. represent a fast growing rate and a high NO₂⁻-N oxidizing rate. Thus, the relatively low SNFR values with M_{90d} and M_{infinite} might be attributed not only to the low NOB ratios, but also to the composition of NOB.

In the moving window correlation approach, the evolution of the similarity over time is depicted (Wittebolle et al., 2008; Marzorati et al., 2008). By plotting the correlation coefficients between day x and day x – 30 for each reactor against the time, graphical representations of the evolution of stability over time for AOB and general bacteria in the three MBRs were obtained (Fig. 5). The similarity of AOB in M_{30d} became relatively stable after reaching the lowest value of 38%. On the other hand, the changes of AOB similarity in M_{90d} and M_{infinite} were quite similar: sharp decrease of the similarity value occurred on days 140 and 170, respectively. The above result can be explained by the differences of AOB community structures: the fast-growing *Nitrosomonas* sp. grew quickly and became the dominant AOB with a relatively short period in M_{30d}, while in M_{infinite} and perhaps M_{90d}, it took a much longer

Fig. 5 – Moving window correlation for reactors M_{30d}, M_{90d} and M_{infinite}. Variability between day x and day x – 30 was calculated from DGGE patterns of AOB (a) and general bacteria (b).

Fig. 6 – Neighbour-joining tree of partial 16S rRNA sequences recovered from samples with three MBRs using bacterial primers (DM11–DM18 for M_{30d} , DM21–DM23 for M_{90d} and DM31–DM34 for $M_{infinite}$) and CTO primers (CDM11–CDM15 for M_{30d} , CDM21–CDM24 for M_{90d} and CDM31–CDM36 for $M_{infinite}$). The reference sequences are obtained from GenBank. GenBank accession numbers are in parentheses.

time for the slowly-growing *Nitrospira* sp. to become dominant. For general bacteria, on the other hand, the changes of similarity of the three reactors were almost the same: the similarity decreased at first, and then increased. This result shows that in spite of the differences of the community structures of AOBs, the three MBRs possibly possessed similar heterotrophic community structures.

3.5. Clone libraries of *amoA* gene

In order to get more detailed information regarding AOB community diversity in MBRs, three *amoA* gene clone libraries were constructed for the three MBR reactors. 30–60 clones were selected and processed by RFLP for each library, at least one

clone of each pattern was sequenced and 40 sequences were totally acquired for the three MBR reactors (14 for M_{30d} , 9 for M_{90d} and 17 for $M_{infinite}$). All bacteria from the cloned libraries were classified into *Nitrosomonas* sp. and *Nitrospira* sp. which both belong to the Betaproteobacteria (Fig. 7) and are important AOB in activated sludge systems (Dytczak et al., 2008). However, the distribution of them in three MBRs was quite different. The *Nitrosomonas* sp. comprised of 89% of the clones in M_{30d} library, 100% in M_{90d} library, and 57% in $M_{infinite}$ library. Meanwhile, 11% of the total clones in M_{30d} and 43% of the clones in $M_{infinite}$ library were affiliated with *Nitrospira* sp. bacteria.

The *Nitrosomonas* and *Nitrobacter* sp., growing quickly with high concentrations of substrates, are two classic nitrifiers commonly used in engineering texts to describe ammonia and

Fig. 7 – Neighbour-joining tree of partial *amoA* sequences recovered from samples with three MBRs using *amoA* primers. Bootstrap values (based on 1000 replicates) are given at nodes when they exceed 50% of replicates. The reference sequences are obtained from GenBank. GenBank accession numbers are in parentheses (M11–M16 for M_{30d} , M21–M26 for M_{90d} and M31–M36 for $M_{infinite}$).

nitrite oxidation, respectively (Stehr et al., 1995). In contrast, the *Nitrosospira* and *Nitrospira* sp. can survive longer periods of starvation, due to their slower rates (Schramm et al., 1999). It has been reported that in winter when the temperature was below the optimum range for nitrifier growth and the SRT was long, *Nitrosospira* thrived better in municipal wastewater treatment plants (Siripong and Rittmann, 2007). Due to the extremely long SRT, *Nitrosospira* sp. accumulated in $M_{infinite}$, resulting in the high AOB ratio. This is why $M_{infinite}$ showed the lowest SAOR in spite of its high AOB ratio. The relatively low density of AOB in $M_{infinite}$ by the MPN method was perhaps related with the significant presence of the slow-growing *Nitrosospira* sp. No *Nitrosospira* sp. was detected in M_{90d} , perhaps due to the fewer sequence number obtained and the relatively low *Nitrosospira* ratio in the system.

4. Conclusions

The impacts of SRT on reactor performance and microbial community structures of MBRs were investigated and the following conclusions were acquired.

1. Purge of activated sludge at a proper interval permitted higher ammonia and nitrite oxidation rates and lower EPS concentrations.
2. MBR with the shortest SRT (30 days) had the highest NOB ratio while that with the longest SRT (no purge of sludge) had the highest AOB ratio.
3. The fast-growing *Nitrosomonas* and *Nitrobacter* sp. bacteria were the dominant AOB and NOB, respectively in the MBR

with the shortest SRT, while considerable slow-growing *Nitrosospira* and *Nitrospira* sp. bacteria existed in the MBR with the longest SRT.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at [doi:10.1016/j.watres.2010.02.021](https://doi.org/10.1016/j.watres.2010.02.021).

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