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Effect of solids retention time on antibiotics removal performance and microbial communities in an A/O-MBR process

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ABSTRACT

The effect of solids retention time on reactor performance and microbial community composition in anoxic/aerobic membrane bioreactors (A/O-MBR) were investigated in this study. Experimental results showed high removal efficiencies of conventional pollutants. Antibiotics removal efficiencies were obviously affected by SRT changes. Longer SRT (above 30 days) was proved to be suitable operational condition for antibiotics removal. Denaturing gradient gel electrophoresis (DGGE) and clone library analysis revealed that bacteria belong to *Betaproteobacteria* and *Gammaproteobacteria* were the dominant species during wastewater treatment and antibiotics removal. SRT significantly influenced the relative numbers of nitrifying bacteria. Removal efficiency of total nitrogen (TN) decreased when SRT was 3 days, because nitrogen loading exceeded the denitrification ability of the reactors. Unlike *tet* C and *tet* E genes, sulfa antibiotics resistance gene presented a decreasing tendency with the decrease of SRT, and finally affected sulfa antibiotics removal efficiencies.

1. Introduction

Antibiotics are an important group of pharmaceuticals in medicine. In addition to the treatment of human infections, they are also used in veterinary medicine. Unlike many other pollutants present in the environment, antibiotics have a direct biological action on microbes. Many of these antibiotics are not completely metabolized or eliminated in the body, between 30% and 90% are excreted unchanged into the waste system (Hirsch et al., 1999). Antibiotics and other pharmaceuticals, including their precursor compounds and transformation products, are usually discharged into the environment after passing through wastewater treatment processes (via wastewater treatment plants, or domestic septic systems), which often are not designed to remove them from the effluent (Ternes et al., 2004). After entering natural waters, these antibiotics could lead to ecological damage and potential adverse effects on various organisms. As suggested recently by some researchers (Kuehn, 2007; Rysz and Alvarez, 2004), the genes of antibiotic-resistant bacteria themselves could be considered to be "pollutants and superbugs" as their wide-spread dissemination is clearly undesirable. Thus, methods to improve antibiotic removal efficiencies in wastewater treatment systems are drawing increased attention.

Previous studies have been conducted on the removal of antibiotics which involved in different wastewater treatment techniques, such as sorption (Figueroa et al., 2004), reverse osmosis, oxidation (Adams et al., 2002), activated sludge process (Kim et al., 2005), and membrane bioreactors (MBRs) (Clara et al., 2005), etc. Most of the treatment techniques have limited effect on preventing the discharge of pathogenic microorganisms with increased antibiotic resistance from the activated sludge mixed liquor into the environment. Through membrane separation processes, MBRs can produce much higher quality effluent by efficiently retaining microorganisms such as the fast-growing heterotrophs and the slow-growing nitrifiers in the system, and meanwhile prevent the harmful pathogenic microorganisms being discharged into the environment. In the MBR process, solids retention time (SRT) has a significant influence on pollutant removal efficiencies (Cicek et al., 2001; Duan et al., 2009). MBRs operated at longer or shorter SRT frequently have serious problems, including raised aeration demand and increased mixed liquor viscosity, which leads to decreased mass transfer efficiency and serious membrane fouling. Thus, SRT is an important parameter in MBRs. In addition, a better understanding of the structure and dynamics of microbial communities at different SRTs will be helpful for optimizing the operating conditions of MBRs. However, limited studies have been conducted on the performance of MBRs on the treatment of wastewater containing antibiotics and the microbial communities associated with it. Previous studies looking at microbial

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communities as a function of SRT have mostly focused on municipal wastewater treatment (Xia et al., 2010; Zhang et al., 2006).

The goal of this research was to understand how microbial community structure changes in an anoxic/aerobic-MBR (A/O-MBR) treating wastewater that contains antibiotics, and how this relates to reactor performance. The effect of four SRTs (3, 10, 30 and 60 days) on antibiotic removal and microbial community structure was studied. The selected compounds belong to three antibiotic structural classes that are widely used for the medication of humans and livestock. The changes of microbial population were monitored with polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and 16S rRNA clone libraries. Finally, total bacterial 16S rRNA genes, nitrifying, denitrifying bacteria genes and antibiotic resistance genes (ARG) copies were quantified by Real-time quantitative PCR.

2. Methods

2.1. MBR and operations

As shown in Fig. 1, the lab-scale A/O-MBR (6 L) used in this study consisted of an anoxic unit (AN, 2 L) and an aerobic unit (AO, 4 L). A hydrophilic polyvinylidene fluoride (PVDF) hollow fiber membrane module was used in the AO unit. The mean pore size of the membrane module was 0.02 μm (Litree, China), and the effective filtration area was 0.1 m^2. Aeration was supplied by means of diffusers at the bottom of the reactors to assure a homogeneous oxygen concentration in the mixed liquor and reduce membrane fouling via hydrodynamic scouring. Three peristaltic pumps (Longer, China) performed the feeding, the permeation and the activated sludge backflow of the MBR. Intermittent filtration was selected (10 min filtration and 2 min pause) using a permeation peristaltic pump. A manometer was situated before the permeate pump, in order to clean the membrane when transmembrane pressure (TMP) was above 50 kPa. The membrane modules were physically back washed using cleaned water every 5 days by removing the sludge cake deposited on the membrane surfaces, and chemically cleaned every 20–35 days by mixed solution of NaClO and NaOH solutions (effective Cl was 3000 mg/L). Liquid level in the bioreactor was monitored with a water level sensor and kept constant by feeding with a peristaltic pump. Sludge used for seeding in this study was obtained from the aeration tank in Quyang sewage treatment plant, Shanghai, China.

After inoculation and start-up, the system was operated and monitored continuously at different SRTs and HRTs to investigate their influences on microbial community characteristics and antibiotic removal. The detailed operating conditions of the MBR during the course of the study are listed in Table 1. Inoculated sludge and MBR samples obtained from the AN and AO units at HRT of 6 h during different phases were collected for investigating the diversity of microbial community.

2.2. Raw wastewater and analytical determinations

Artificial wastewater containing antibiotics was prepared every 2 days with tap water, glucose (169 mg/L), cane sugar (150 mg/L), amylum (80 mg/L), NH_4Cl (75 mg/L), KH_2PO_4 (4.4 mg/L), and K_2HPO_4 (16.8 mg/L) as main nutrient sources for microorganism, and Tetracycline (TC, 500 μg/L), Oxytetracycline (OTC, 500 μg/L), Chlortetracycline (CTC, 500 μg/L), Sulfamethoxazole (SMX, 500 μg/L), Sulfadiazine (SDZ, 500 μg/L) and Ampicillin (AMP, 500 μg/L) as main antibiotic pollutants. Mineral components and trace element solution were prepared every 2 days as described previously and spiked into the artificial antibiotics contained wastewater (Jia et al., 2011). The initial chemical oxygen demand (COD) and total nitrogen (TN) concentration were determined as approximately 350–520 mg/L and 17–30 mg/L, respectively. The ratio of COD:N:P was set at 100:5:1. The pH value of the synthetic wastewater was controlled at 7.8 ± 0.2 by the addition of $NaHCO_3$.

Raw wastewater and MBR permeate were collected and analyzed everyday, and the data was average value of 5 days. COD and ammonia-nitrogen (NH_4^+-N) were measured using Hach Method 8000 and 8008, respectively, according to manufacturer's instructions. Total organic carbon (TOC) and TN were analyzed with a TOC/TN analyzer (TOC-V cpn, TNM-1, Shimadzu Co., Japan). Analysis of antibiotics was performed by liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) (Thermo

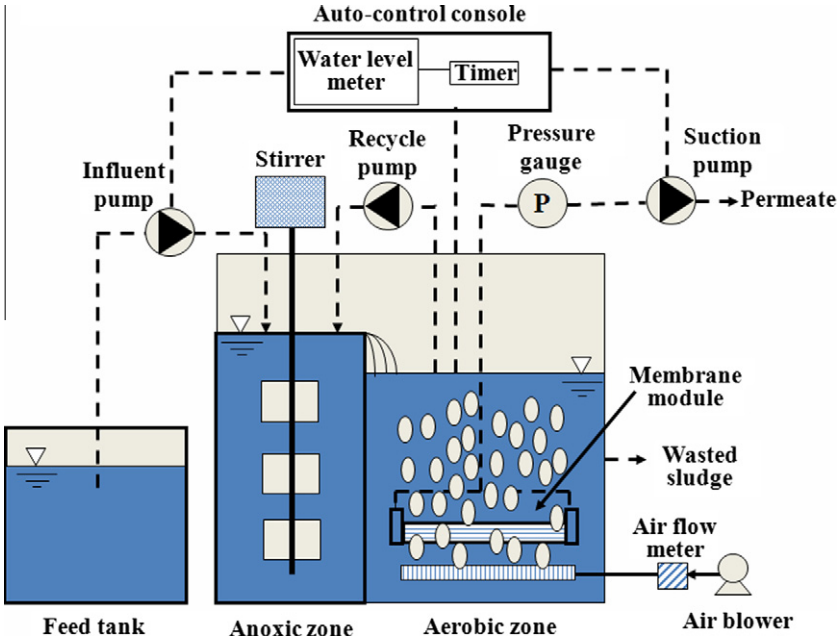

Fig. 1. Schematic structure of anoxic–oxic membrane bioreactor (A/O-MBR).

Table 1
Operating schedule for A/O-MBR.

Phase	SRT (days)	HRT (h)	MLSS (mg/L) (range)	Working temperature (°C)	Duration (days)
1	60	24 12 6	6307 (5497–8562)	25 ± 1	180
2	30	24 12 6	3363 (2893–3771)	25 ± 1	180
3	10	24 6	1529 (1365–1759)	25 ± 1	90
4	3	24 6	312 (246–389)	25 ± 1	70

Electron, San Jose, CA, USA) as described previously (Jia et al., 2011).

2.3. DNA extraction and PCR amplification

The sludge samples obtained from the AN and AO units at different phases were taken simultaneously for bacterial analysis. Prior to DNA extraction, cell pellets were rinsed twice with TENP buffer (50 mM Tris, 20 mM EDTA, 100 mM NaCl, 0.01 g/mL PVP, pH 10) to remove humic substances, and re-suspended in a sodium phosphate buffer (120 mM, pH 8.0) and washed twice. Total genomic DNA was extracted using a Fast DNA spin kit (MP Biomedicals, LLC, France) according to the manufacturer's instructions (Xia et al., 2010). To minimize DNA variations as a result of the extraction process, the DNA templates were prepared by mixing the DNA extracted in triplicate for each sample. The concentration of extracted DNA was determined using a UV spectroscopic method at 260 nm.

For denaturing gradient gel electrophoresis (DGGE) analysis, a two-step nested PCR was performed on the purified DNA. In the first step, the universal primers 27f and 1492r were used for amplification (Mobarry et al., 1996). The PCR reaction was completed in 50 μL with a DNA thermo-cycler (Thermo, USA). After an initial denaturing step at 94 °C for 5 min, the PCR amplification conditions were as follows: 30 cycles of denaturing (94 °C for 45 s), annealing (55 °C for 1 min), and extension (72 °C for 90 s), and a final extension at 72 °C for 7 min. The second step was performed with the primers F357-GC and R518 (Mobarry et al., 1996), and the PCR products obtained from the first step was used as DNA templates. The amplification program was amended as 32 cycles and the annealing temperature was 57 °C. After analysis on 0.7% agarose gel electrophoresis, the PCR products were directly used for DGGE analysis.

2.4. DGGE analysis

The PCR products from the second PCR were directly used for DGGE analysis on 8% (w/v) polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) using a denaturing gradient ranging from 30% to 55% (100% denaturant was 7 M urea and 40% formamide in the 1× TAE buffer). Gels were run first at 200 V for 5 min and then at 80 V for 13 h at 60 °C. Then the gels were stained for 30 min with SDNA-Nucleic Acids Stain Dye (BIO BASIC Inc., Canada), washed and visualized with a UV transilluminator (FR-980A, Furi Tech. Ltd., Shanghai, China). Specific DGGE bands were manually excised from the gel, re-amplified, cloned and sequenced as described previously (Xia et al., 2008). All nucleotide sequences were determined by Map biotech Co. Ltd. (Shanghai, China). DGGE images were processed with Smartview software.

2.5. 16S rRNA gene-cloning and phylogenetic analysis

Clone libraries of 16S rRNA were constructed from DNA extracted from the inoculum and MBR sludge. Nearly complete 16S rRNA gene fragments were amplified in triplicate with primers 27f and 1492r as described previously (Duan et al., 2009). The amplicons were pooled to minimize bias, purified with a QIA-quick PCR purification kit (Qiagen), and cloned into pTG19-T vector by TA Cloning Kit (Generay, China). Randomly selected clones from the library were determined by colony PCR (with primer combinations 27F/M13-47 and 27F/RV-M) (Wan et al., 2011) for their rRNA gene inserts orientation and then sequenced forwardly using vector primers M13 in accordance with the same program for 16S rRNA amplification described above. Chimeric sequences were identified and excluded from subsequent analysis. For representatives of the operational taxonomic units (OTUs, defined as groups in which sequences similarity was more than 97%) that comprised two or more clones, nearly complete 16S rRNA gene sequences were obtained by a second sequencing run starting from the opposite side of the vector with the corresponding vector-primers. All the sequences were compared to known sequences by phylogenetic analysis. Phylogenetic trees were constructed using a neighbor-joining method with the Clustal X software package. One thousand bootstraps were performed to estimate the confidence of tree topologies. The 16S rRNA gene sequences from this study have been deposited in National Institutes of Health (NIH) genetic sequence database (GeneBank) under accession numbers JF808727 to JF809022.

2.6. Real-time quantitative PCR assays

To investigate how changes in nitrifying and denitrifying bacteria populations as well as the antibiotic resistance gene (ARG) quantities varied with of the process performance, Real-time quantitative PCR was carried out using a Rotor-Gene 3000 Real-time PCR systems (Corbett research, NSW, Australia). Quantification of the 16S rRNA gene of total bacteria was performed using the primers 1055F and 1392R (Ferris et al., 1996). The primers listed in Table S1 were used to quantify ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and denitrifying bacteria (DNB), respectively (Supplementary material). For ARG detection, the primers are listed in Table S2 (Supplementary material).

Each PCR mix (total volume of 25 μL) for all triplicate Real-time PCR assays consisted of 2 × SYBR Premix TaqTM (Takara, China) (12.5 μL), forward and reverse primers (0.625 μmol), DNA template (approximately 5 ng) and PCR-grade sterile water. The PCR program was: 95 °C predegeneration 1 min; 40 cycles of denaturing 5 s at 95 °C, annealing and extension 30 s at 60 °C. For ARG detection, the annealing and extension temperatures are listed in Table S2 (Supplementary material). Standards were prepared using serially diluted plasmid DNA with 10^4–10^{10} gene copies/mL. The standard curves were constructed by using standards ($R^2 > 0.99$). All PCR runs included a negative control reaction and a positive control reaction using PCR-grade H_2O without template and a previously amplified template, respectively. The efficiency of PCR amplification for each gene was between 90% and 100%.

3. Results and discussion

3.1. Antibiotics and conventional pollutants removal performance

The lab-scale MBR was operated at different SRTs and HRTs to investigate the removal performance of antibiotics during MBR process. As described previously, no significant differences were observed for antibiotics removal at different HRTs (Jia et al.,

2011). General performance of the MBR at different SRTs is summarized in Table 2. Average dissolved influent antibiotic concentrations were 500 μg/L. As listed in Table 2, the removal efficiencies of antibiotics were not influenced when the SRT was decreased from 60 days (phase 1) to 30 days (phase 2). These results were similar to or higher than those previously reported for municipal and industrial activated sludge systems (Lindberg et al., 2005; Prado et al., 2009b). However, when the SRT was adjusted to 10 or 3 days, the removal efficiencies of antibiotics decreased, especially for Tetracycline. This phenomenon may be due to the fact that there is lower MLSS levels present in the reactor. Previous studies have shown that Tetracycline Antibiotics was removed mainly by biosorption in activated sludge systems (Kim et al., 2005; Prado et al., 2009a). Reduced SRT led to decreased biomass concentration, an increased floc particle size, and a decreased specific surface area, as shown in Table 1. This change favors less sorption and less removal. Therefore, it is tempting to conclude that SRT is an important variable. For sulfa-based antibiotics and AMP, microbial degradation is one of the main mechanisms of removal in the aquatic environment. No biosorption removal was detected for sulfa-based antibiotics (Ingerslev and Halling-Sorensen, 2000; Perez et al., 2005). Thus, the decrease of biomass concentration and the changes of microbial community structure with changing SRT may lead to a decrease in removal efficiency.

As shown in Table 2, the A/O-MBR process had a high removal percentage for COD and TOC. No obvious decrease in removal efficiencies for the above pollutants was found at different SRTs in this study. This finding was consistent with previous research (Duan et al., 2009). In that work, the MBR process was capable of achieving high removals efficiency for both COD and NH_4^+-N, and SRT did not significantly affect nitrification in the MBR systems when the SRT was above 10 days, due to complete suspended solid retention and also because of retention of protein and polysaccharides from the sludge supernatant. But with SRT decreasing, antibiotics, organic and nitrogenous compound loading increased, and this decreased TN and NH_4^+-N removal efficiencies, as shown in Table 2. Similar results were reported by Holakoo et al. as SRT decreased from 40 to 20 days (Holakoo et al., 2007).

3.2. Microbial community structures analysis

3.2.1. DGGE analysis

The microbial community of the inoculation sludge and the mixed liquor in MBR were analyzed by PCR-DGGE at different SRTs. Triplicate DGGE profiles from each operation condition showed good reproducibility. For clarity, only one of the triplicates is shown in this paper.

According to the DGGE profile and clustering analysis (Fig. 2), the inoculation sludge communities were significantly different from the activated sludge mixed liquor communities in the MBR. The microbial population diversity and richness of the inoculation sludge were higher than in the MBR. The bacterial community in the AN units showed a greater similarity (nearly 100%) to that in the AO units at different SRT conditions. The suspended bacterial community in phase 1 showed 0.72 similarities with phase 2, while the other two phases indicated 0.63 similarities. The similarity index between the first two and the subsequent phases is 0.54. As the SRT decreased from 60 to 3 days, the microbial population diversities decreased, whereas the brightness of dominant bacteria bands increased contrarily. Band 2 was consistently present at all conditions although its intensity varied in different periods. Some bands such as 1, 3 and 4, which represent dominant species in column A1 and A2 became weak or even almost invisible in other operating conditions. Similar phenomenon was observed for bands 5, 7, 9, 10, 11 and 12, which represent dominant species in column B1 and B2. Based on these changes, it could be concluded that these bacteria species, which were represented by these bands, were accustomed to the environment of longer SRT. While other bands are acting differently, bands 6, 8, 13 and 14 appeared or became more intense at short SRT conditions; the bacteria species represented by these bands may require more substrates or are more resistant to antibiotics loadings increasing. So it seems that the SRT affects the capabilities and performance of biological treatment systems by influencing the dominant microbial species.

The bands were excised and amplified for nucleotide sequence analysis. Results showed that *Betaproteobacteria* (band 4, 7 and 11) was frequently detected in nucleotide sequence analysis. Uncultured *Burkholderia* sp. (band 4) became weak or invisible in phase 2–4. *Leptothrix* sp. (band 7) and *Rhodocyclales* sp. (band 11) which also belonged to *Burkholderiales* were detected, and both of these two bacteria have resistance to antibiotics (Kanagawa et al., 2000), and could adsorb antibiotics. Subsequent cloning library analysis also showed frequent detection of these bacteria. *Thiothrix* sp. (band 2), which belongs to *Gammaproteobacteria*, was detected in all MBRs and inoculation sludge, but the band intensity of this bacterium in inoculation sludge was weaker than those in MBR. This bacterium is known for its ability to degrade sulfur containing compounds, and may have played a role in the biodegradation of sulfa-based and penicillin antibiotics in the MBR process. Affiliation analysis showed very high similarities (97%) between DNA sequence represented by band 8 and *Denitrifying bacterium*. This suggests that the denitrification was favorable in the reactor at SRTs of 10 and 30 days, which was consistent with the results in Table 2. When SRT was 3 days, there existed higher

Table 2
Performance of the MBR operated at different SRTs.

SRT (d)	60	30	10	3
Antibiotics influent (μg/L)	500	500	500	500
TC removal efficiency (%)	93.6	92.6	89.7	83.6
CTC removal efficiency (%)	82.9	84.4	81.5	77.6
OTC removal efficiency (%)	88.6	87.9	84.4	79.7
SMX removal efficiency (%)	99.5	99.3	96.9	88.5
SDZ removal efficiency (%)	99.7	99.6	97.5	93.8
AMP removal efficiency (%)	99.9	99.9	99.6	94.4
COD influent (mg/L)	422 ± 198	410 ± 107	409 ± 86	422 ± 68
COD effluent (mg/L)	10.5 ± 10.5 (97.5%)	10.6 ± 10.4 (97.3%)	13.4 ± 8.6 (96.6%)	14.8 ± 6.2 (96.4%)
TOC influent (mg/L)	103 ± 25	101 ± 23	102 ± 21	103 ± 25
TOC effluent (mg/L)	4.9 ± 2.9 (95.2%)	5.1 ± 2.5 (94.9%)	5.1 ± 1.9 (94.9%)	5.6 ± 2.3 (94.4%)
NH_4^+-N influent (mg/L)	20.5 ± 8.5	18.8 ± 4.5	18.5 ± 3.2	19.8 ± 3.7
NH_4^+-N effluent (mg/L)	0.59 ± 1.77 (97.1%)	0.45 ± 1.36 (97. 5%)	0.57 ± 3.52 (96.9%)	0.61 ± 1.37 (96.9%)
TN influent (mg/L)	23.3 ± 6.8	22.5 ± 7.0	23.7 ± 4.5	25.0 ± 5.0
TN effluent (mg/L)	2.48 ± 1.45 (89.0%)	1.88 ± 1.96 (91.5%)	1.73 ± 1.58 (92.5%)	3.79 ± 1.16 (84.1%)

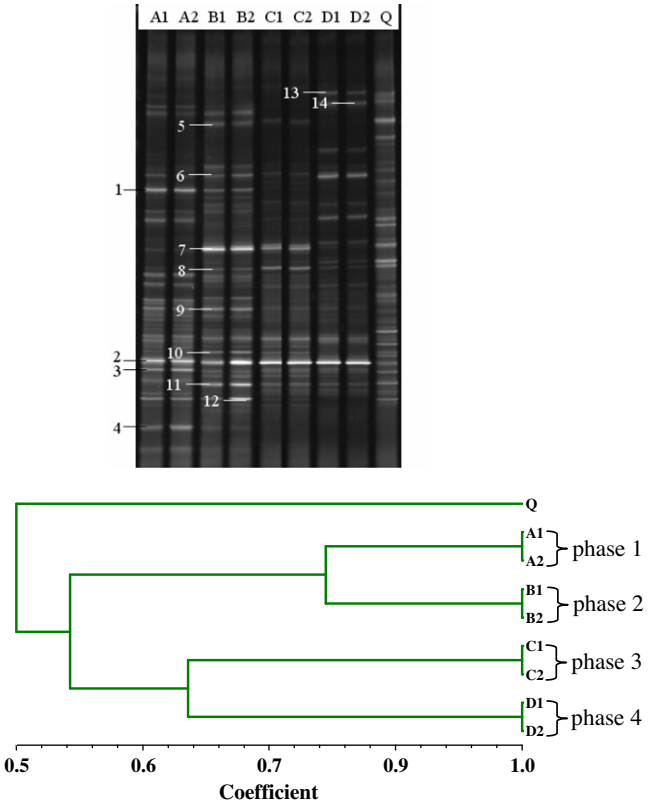

Fig. 2. DGGE fingerprints and cluster analysis of mixed liquor samples at different SRTs. A1, A2 – the anoxic and aerobic sludge samples (SRT 60 days); B1, B2 – the anoxic and aerobic sludge samples (SRT 30 days); C1, C2 – the anoxic and aerobic sludge samples (SRT 10 days); D1, D2 – the anoxic and aerobic sludge samples (SRT 3 days), Q – inoculation sludge sample.

antibiotics sludge loadings in the bioreactor, and a comparison revealed that bacteria of bands 13 and 14 were affiliated with *Arenimonas* sp. and *Iron-reducing* sp., respectively. *Betaproteobacteria* and the *Arenimonas* sp. were also considered to play important roles in sulfa-based and penicillin antibiotics due to its high resistance to antibiotics (Goni-Urriza et al., 2000).

The sequences of bands 1, 5, 6, 9, 10 and 12 were affiliated with *Bacteriodetes*; these bands became weak or disappeared when SRT was adjusted to lower conditions. This suggests that *Bacteroidetes* was more vulnerable to SRT and antibiotics loading changes, and wasn't the dominant species in the MBR process when treating antibiotics containing wastewater.

3.2.2. 16S rRNA clone library analysis

Three clone libraries were constructed with 100, 96 and 100 randomly selected clones to identify potentially dominant species from the inoculation sludge sample (Q) and the MBR treated antibiotics containing wastewater (B2: SRT = 30 days, and D2: SRT = 3 days). All clones were grouped into different OTUs on the basis of more than 97% sequence similarity within an OUT (Xia et al., 2010). 49, 45 and 51 OTUs were obtained from Q, B2 and D2 clone library, respectively. Phylogenetic trees of Q, B2 and D2 are shown in Fig. S1 (Supplementary material).

Fig. 3 shows the taxonomic breakdown at the bacterial class level for the three clone libraries. Overall, the Q bacterial communities contained sequences from more than 10 bacterial classes. The majority of the sequences belonged to *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, with the remainder spread among *Firmicutes*, *Actinobacteria*, *Chlorobi*, *Streptococcus*, *Prosthecobacter*, *Gemmatimonas*, and other minor groups. As shown

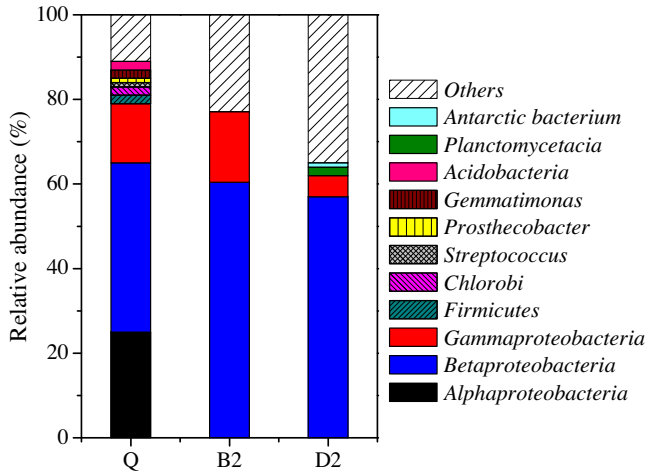

Fig. 3. Phylogenetic distribution of the OTUs in the three clone libraries.

in Fig. 3, a certain quantity of the clones in the three clone libraries exhibited the highest levels of similarity to *Gammaproteobacteria* (Q: 14%, B2: 16.67%, D2: 5%). After comparison on-line, these clones for sample Q were similar to *Thermomonas* sp. (3%), *Pseudoxanthomonas* sp. (3%), *Dokdonella* sp. (5%), *Aquimonas* sp. (1%), and others belonged to uncultured *Gammaproteobacterium* (2%). Different from the comparison results of sample Q, only three bacterial species were detected for sample B2, which were *Tolumonas* sp. (6.25%), *Dokdonella* sp. (1.04%), *Aeromonas* sp. (9.38%), respectively. In sample D2, *Tolumonas* sp. (5%) was detected as shown in Fig. S1. To conclude from the results described above, the bacteria belong to *Gammaproteobacteria* was the dominant species in the three cloning libraries.

The relative abundance of *Betaproteobacteria* was markedly higher in MBRs fed with antibiotics contained wastewater than in the inoculation sludge sample (Q). Fig. 4 shows the microbial structures and changes of *Betaproteobacteria* in different clone libraries. Fig. 4a shows the relative proportions of the most abundant bacterial genera in the clone library. The members of *Methylibium* sp. were highly enriched in sample Q, but not in the MBRs. The second most abundant genera was *Aquabacterium* sp., *Methylibium* sp., *Aquabacterium* sp. and *Nitrosomonas* sp. were reported to play an important role in nitrogen compound removal (Iasur-Kruh et al., 2010). *Leptothrix* sp., a typical filamentous bacterium was detected in this clone library, which indicated that sludge bulking phenomenon existed in the inoculation sludge. Other minor lineages detected in the sample are *Sterolibacterium* sp., *Dechloromonas* sp., etc.

In the reactor operated at SRT 30 days, the bacteria belongs to *Proteobacteria* was also the dominant species (77.08% of the total population). 60.41% of the total population was *Betaproteobacteria* as shown in Fig. 3 and Fig. 4b. Unlike Fig. 4a, *Dechloromonas* sp. (19.79%) was the dominant species in MBR operated at SRT 30 days which was reported as a NO_3^- reducer (Frankenberger and Zhang, 2007). And this is compatible with a high denitrification in this study. Fig. 4b also shows that the antibiotics and SRTs altered the microbial communities significantly compared with the inoculation sludge sample. The content of *Comamonas* sp., which was considered as antibiotic resistance gene carrier (Mormile et al., 2007), increased from undetectable to 7.29% when antibiotics were present, while *Methylobacillus* increased to 5.21%. Even more significant increase was observed among the group of *Rhodocyclales* (*Dechloromonas* sp., *Denitratisoma*, *Perchlorate-reducing* and *Azospira* sp.), which became the most abundant genus (32.29%) in the presence of antibiotics. This suggests that *Rhodocyclales* played an important

role in organic pollutant and nitrogen compound removal when SRT was 30 days.

When the SRT was adjusted to 3 days, the phylogenetic relationship changed as shown in Fig. S1 of the Supporting information compared to 30 days of SRT. About 13 species of *Betaproteobacteria* were detected as SRT decreased to 3 days (Fig. 4c). *Nitrosomonas* sp. was not detected in this clone library. *Leptothrix* sp., which was detected in DGGE analysis, constituted 10% of the whole microbial community. *Sphaerotilus* sp. (7%) and *Leptothrix* sp. belonging to the genus of *Burkholderiales* which previously reported to have multiple antibiotics resistance (Mormile et al., 2007), were also present in the sample. Their presence ensured conventional organic pollutant removal at SRT 3 days condition. *Dechloromonas* sp. was detected in all the three clone libraries. Other species that belonged to *Betaproteobacteria* are listed in Fig. 4c. As relative antibiotics loading increased with the decrease of SRT, the quantity of *Burkholderiales* including *Leptothrix* sp., *Sphaerotilus* sp., *Ideonella* sp., and *Pelomonas* sp. etc. increased from 10.42% (30 days SRT) to 33% (3 days SRT) due to the higher antibiotics resistance. Based on the comparison of different clone libraries in Fig. 4, it can be concluded that the SRT and the amount of antibiotics in the influent greatly affected the dominant species selected in the MBR, which would have further influence on the removal performance of antibiotics and conventional pollutants of the MBR.

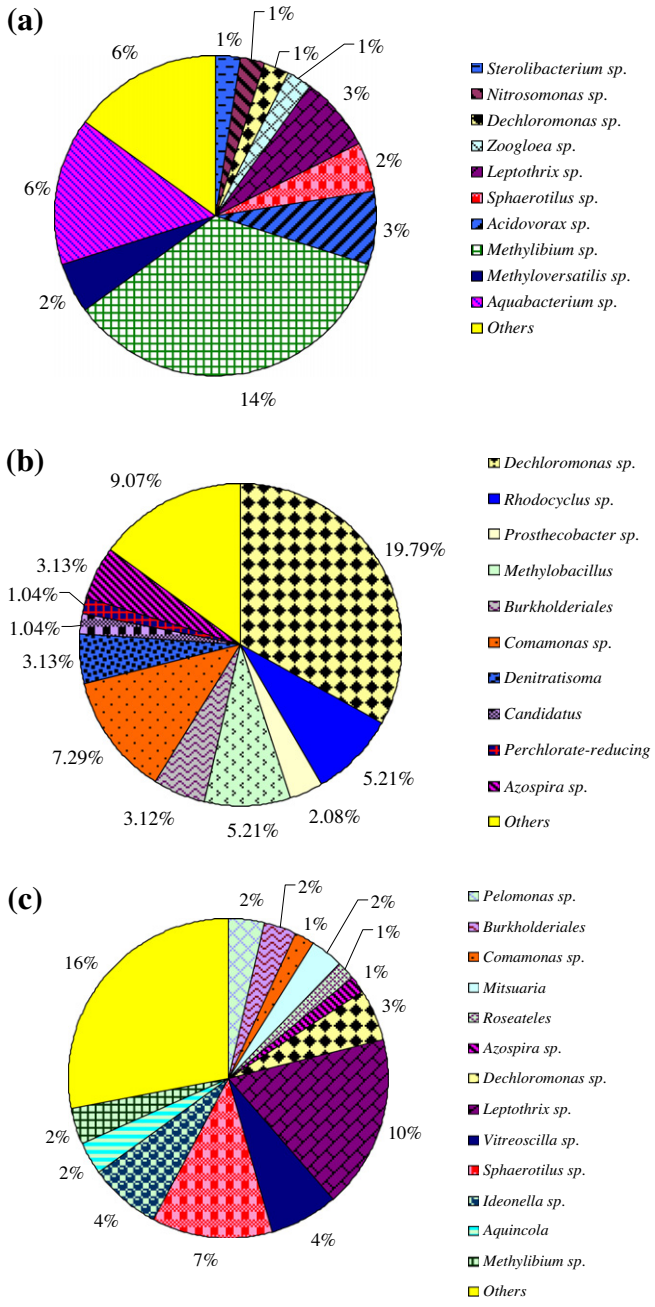

Fig. 4. The microbial structures and changes of *Betaproteobacteria* in the clone libraries of the inoculation sludge sample Q (a) and the MBR sludge samples B2 (b) and D2 (c).

3.2.3. Real-time quantitative PCR

Real-time quantitative PCR was used to examine dynamic changes in total bacterial 16S rRNA gene copies (Fig. S2), nitrifying, denitrifying bacteria gene copies (Fig. 5) and antibiotic resistance gene copies (ARG) (Fig. 6) in the MBR. Fig. S2 shows the dynamic changes of the total bacterial 16S rRNA gene copies. The microbial cells concentration was detected as approximately were 1.13×10^{11} copies/mL (biomass) in the inoculation sludge sample, and the numbers of bacterial cells decreased with SRT, which apparently led to the MLSS changes in the MBR (Table 1).

Fig. 5 illustrates the changes of AOB, NOB and DNB genes under different operating conditions. No significant difference of the number of gene copies were found between AN and AO units at the same operation conditions regardless of the differences of total bacterial 16S rRNA gene in different units (Fig. S2). Similar proportion occurred at SRTs of 30 and 60 days, as well as at 3 and 10 days. AOB and NOB genes at 3 and 10 days, were an order of magnitude or more less than at higher SRTs. This suggests that SRT had a significant influence on AOB and NOB contents. Although AOB and NOB populations varied under different conditions, good removal performance of NH_4^+-N was maintained in MBRs (Table 2), which has also been described by other researchers (Ng and Hermanowicz, 2005). It is also noted that the DNB gene content remained stable throughout the test. But considering the result that the total bacterial 16S rRNA gene decreased with decreasing SRT, it can be concluded that SRT also had a greater influence on DNB gene content. Due to the fact that the DNB received sufficient carbon to replicate and decompose pollutants, their relative content was maintained with a decrease of SRT, which in turn insured the organic pollutant and nitrogen compounds removal efficiencies of the MBR process. When SRT was 3 days, relative nitrogen load-

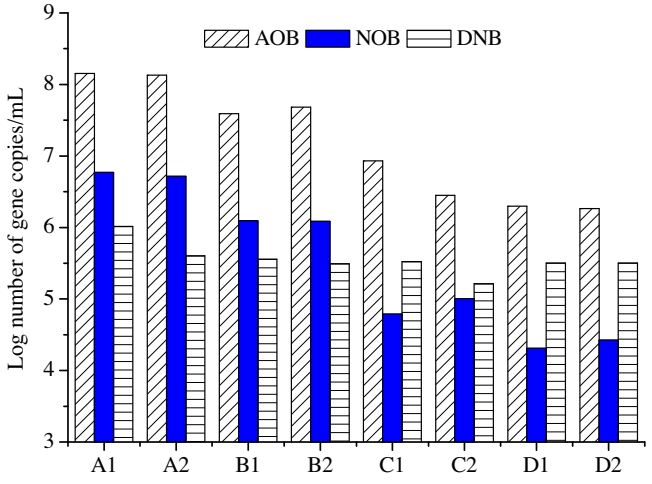

Fig. 5. Nitrifying and denitrifying bacteria gene copy number changes in MBR at different SRTs.

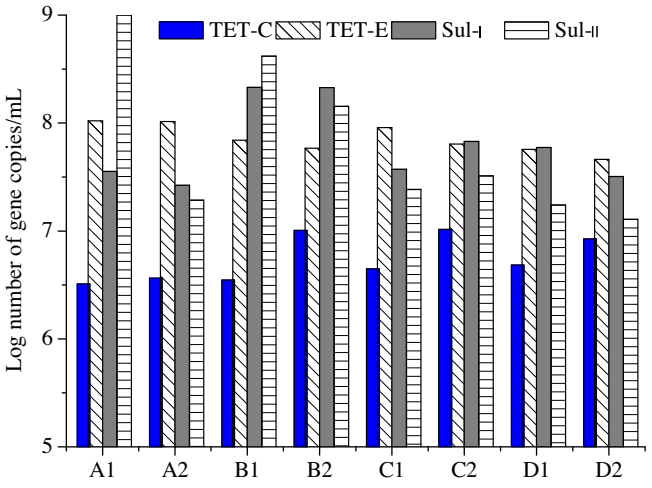

Fig. 6. Antibiotic resistance gene copy number changes in MBR at different SRTs.

ing was beyond the DNB's denitrifying ability, which led to an apparent decrease of TN removal efficiency in MBRs (Table 2).

The MBR process achieves good solid–liquid separation with a membrane, and prevents outflow of antibiotic-resistant bacteria, which are considered as "pollutants". This played an important role in organic and antibiotic removal. Fig. 6 shows how the ARG copy number changes under different operating conditions. For *tet* C, the gene copy number increase from 3.23×10^6 copies/mL (biomass) at an SRT of 60 days to 1.03×10^7 copies/mL at an SRT of 3 days. The proportion of total bacterial 16S rRNA genes rose to 4.7%. This suggests that antibiotic loading in the influent relative to the MLSS increased and that *tet* C gene carrier was highly enriched in the MBR after long-term operation. Similar to *tet* C, *tet* E was not appreciably affected by changes in SRT. But its gene copy numbers in the MBR under different operating conditions were maintained on a higher level (above 1.17×10^7 copies/mL); this demonstrated that bacteria with the *tet* E gene were highly enriched in the MBR. These ARG carrier bacteria which were kept in the MBR ensured a higher antibiotics removal efficiency (above 77%) especially for tetracyclines antibiotics. *Sul* I and *Sul* II showed a decreasing trend as SRT decreased. Hamscher reported that sulfa antibiotics could be biodegraded through anaerobic fermentation process, but the bioreactor needed to be operated for longer periods of time (Hamscher et al., 2009). This suggested that sulfa antibiotic degrading bacteria had a slower growth rate and mainly existed in the AN unit. In this study, the decrease of SRT affected the environment and growth trend of the sulfa antibiotics degrading bacteria, and this led to lower sulfa antibiotics removal efficiencies as shown in Table 2.

4. Conclusions

This study compared the performance and microbial community composition in MBR treating wastewater contained antibiotics. The results showed that the MBR was able to achieve good conventional pollutant removal efficiencies and a longer SRT (above 30 days) was suitable for antibiotics removal. Bacterial community analyses suggested that bacteria belongs to *Betaproteobacteria* and *Gammaproteobacteria* were the dominant species and played an important role in wastewater treatment that contains antibiotics. Nitrifying, denitrifying and antibiotic resistance genes were affected by the change in SRT. Sulfa antibiotics resistance genes tended to decrease with decreasing SRT, and presumably affected the sulfa antibiotics removal efficiencies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.11.112.

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