Influence of attapulgite addition on the biological performance and microbial communities of submerged dynamic membrane bioreactor

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ABSTRACT

A submerged dynamic membrane bioreactor (sDMBR) was developed to test the influence of attapulgite (AT) addition on the treatment performances and the microbial community structure and function. The batch experimental results displayed the highest UV_{254} and dissolved organic carbon (DOC) removal efficiencies with 5% AT/mixed liquid suspended solids addition dosage. The continuous sDMBR results showed that the removal efficiencies of chemical oxygen demand, NH_4^+-N, total nitrogen and total phosphorus significantly increased in the AT added sDMBR. Excitation emission matrix analysis demonstrated that the protein-like peaks and fulvic acid-like peaks were significantly decreased in both in the mixed liquid and the effluent of the AT added reactor. The obligate anaerobes were observed in the sDMBR with AT addition, such as *Bacteroidetes* and *Gamma proteobacterium* in the dynamic membrane, which played an important role in the process of sludge granulation. Bacterial community richness significantly increased after AT addition with predominated phyla of *Proteobacteria* and *Bacteroidetes*. Similarly, species abundance significantly increased in the AT added sDMBR. Further investigations with cluster proved that AT was a favorite biological carrier for the microbial ecology, which enriched microbial abundance and community diversity of the sDMBR.

Key words | 16S rRNA gene cloning, attapulgite, bacterial community, dynamic membrane bioreactor, polymerase chain reaction-denaturing gradient gel electrophoresis

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INTRODUCTION

Membrane bioreactor (MBR) is a promising wastewater treatment technology, as it provides a series of advantages of reliable effluent quality, small land-area occupation, and less sludge production (Holakoo *et al.* 2007; Williams & Pirbazari 2007). However, shortages such as membrane fouling, membrane price and high-energy consumption limit the wide application of MBR (Meng *et al.* 2009). The membrane used in MBR, generally flat or hollow fiber, is of a small pore size (typically 0.10–0.22 μm), which is relatively expensive and easily subjected to membrane fouling and high energy is needed to maintain the constant flux (Krauth & Staab 1993; Brindle & Stephenson 1996).

Recently, the dynamic membrane technology, regarded as a substitute to the conventional MBR, has received great attention in sewage treatment (Artiga *et al.* 2005a; Fu & Lin 2008; Duan *et al.* 2011b; Fu *et al.* 2014). Instead of small pore-size membrane materials in MBR, the dynamic MBR generally applies relatively larger pores as support materials for forming dynamic membrane (DM), which is the key to reject particular materials and supply a high quality effluent (Duan *et al.* 2011b; Xiong *et al.* 2014). The

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DM, mainly composed of sludge flocs, has the advantages of cost-effectiveness, reduced energy consumption and excellent effluent quality (Fan & Huang 2002). For instance, Fan & Huang (2002) used a mesh filter as membrane modules, but replaced the suction pump by using a gravity head to provide trans-membrane pressure for membrane filtration. With further developments, nonwoven fabric filters were applied due to their obvious cost advantages compared with other membrane modules (Seo *et al.* 2003). These developments significantly reduce the cost of membrane separation and energy consumption. In particular, a submerged hybrid MBR has recently become a very hot topic of wastewater treatment technology (Artiga *et al.* 2005b; Liu *et al.* 2010). The comparison of an MBR and hybrid MBR in performance was conducted for the treatment of synthetic water containing a toxic compound (Lesage *et al.* 2008). The hybrid MBR confirmed the favorable condition of membrane fouling control (Liu *et al.* 2010).

To improve the removal efficiency and stability of the bioreactor, various additives have received great attention in the sewage treatment, especially for the bio-carrier of attapulgite (AT) (Huang *et al.* 2007; Duan *et al.* 2013; Zhang *et al.* 2014). AT clay is a crystalline hydrated magnesium silicate with a fibrous morphology, large specific surface area and moderate cation exchange capacity, which is beneficial for the adsorption of heavy metals from solution (Chen & Wang 2009).

Based on the aforementioned technologies, a novel submerged dynamic membrane bioreactor (sDMBR) with the addition of AT was developed for monitoring the treatment performances of municipal wastewater with comparison to a non-AT-addition sDMBR. Furthermore, in order to understand the mechanisms of how AT addition influences the sDMBR treatment performances, the variations of microbial community structure and function after adding AT were investigated by using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). As a prerequisite for a large-scale popularization and application, these fundamental tests could provide detailed information of microbial community structure and the theoretical basis of sDMBR.

MATERIALS AND METHODS

Experimental setup and operation conditions

Two laboratory-scale sDMBR systems with the same type of membrane modules and identical reactor volumes were constructed (Figure 1). These bioreactors consisted of two parts, including a reactor tank in which a DM filter (polyethylene (PE) non-woven filter module) was situated and

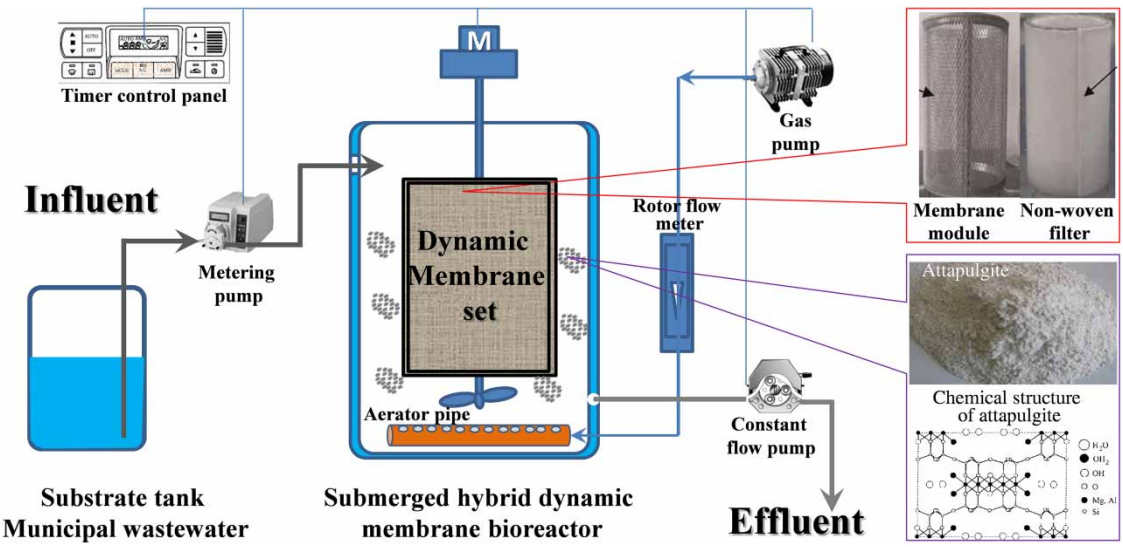

Figure 1 | Schematic diagram of the submerged hybrid DM bioreactor system.

an automatic control system. The total and working volumes of the bioreactor tank were 25 and 20 L, respectively. The DM filter was a double-sided effective filtration area of 0.15 m^2. The non-woven filter module was made of a 5-mm thick nonwoven polyester fabric with a 100 mm nominal pore size and a specific weight of 0.70 kg/m^2. The diameter of the cylindrical support was 18 cm. The system employed two peristaltic pumps (Enertech ENPD-100 Optima, India), one each for intermittently feeding the influent and for withdrawing permeate from the filter module (Figure 1). The two laboratory-scale bioreactors were operated in parallel at a hydraulic retention time (HRT) of 6 hours and solids retention time (SRT) of 30 days, which were achieved by discharging the mixed liquor from the bioreactor once a day. The membrane flux was set at around 62 $L/m^2/h$.

An aeration unit was placed below the filter module serving the aeration of the activated sludge. The total aeration rate was 2.6–4.5 L/min and the dissolved oxygen (DO) concentration was between 2 and 4 mg/L. A temperature control system was installed and temperature fluctuation ranged between 20 and 22 °C according to ambient conditions. The gained values were run automatically in sequencing-flow mode, in which the influent, anaerobic, aerobic and effluent time were 30, 150, 150 and 30 min, respectively. The bioreactors were firstly inoculated with 6–7 g/L seed activated sludge from a local municipal wastewater treatment plant (China, Nanjing). AT particles with an equivalent diameter of 5–10 mm were supplied to the bioreactor during the cultivation stage to reach a concentration of about 0.5 g/L in the reactor tank. The influent to the experimental setup was real municipal wastewater from a local municipal wastewater treatment plant. The characteristics of the wastewater and the operating conditions of sDMBR system are listed in Table 1.

Analytical methods

Chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP) and ammonia (NH_4^+-N) were analyzed according to the Chinese State Environmental Protection Agency Standard Methods (Nepa 2002). DO concentration in the bioreactor was measured by a DO meter (Model YSI 58, YSI Research Inc., OH, USA). Dissolved organic carbon (DOC) was measured by a total organic carbon (TOC) analyzer (TOC-5000A, Shimadzu, Japan) and UV absorbance (at 254 nm) was measured by a Shimadzu UV-2550 spectrophotometer representing the total amount of dissolved organic matter (DOM) in the soluble sample. To obtain the soluble samples in the bioreactor, the mixed liquid suspended solids (MLSS) were centrifuged at 4,000 rpm for 10 min followed by filtration through 0.45 μm filter paper. The filtrate was defined as the soluble fraction in the bioreactor. The soluble fraction of the

Table 1 | Municipal wastewater characteristics and the operating conditions of sDMBR system

Quality of municipal wastewater used in this study

Analysis items	COD (mg/L)	NH_4^+-N (mg/L)	TN(mg/L)	TP(mg/L)	pH
Values	173 ~ 506	36.9 ~ 79.1	50.7 ~ 93.4	1.57 ~ 6.43	7 ~ 8
Average	294	57.2	72.8	3.57	7.5
Operating conditions of the sDMBR system					
Flux ($L\ m^{-2}\ h^{-1}$)				60	
Temperature (°C)				20–22	
HRT (h)				6	
SRT (days)				30	
Membrane flux ($L\ m^{-2}\ h^{-1}$)				62	
Reactor volume (L)				25	
Effective filtration area (m^2)				0.15	
DO concentration ($mg\ L^{-1}$)				2–4	

DMBR effluent was directly obtained from the effluent of the DMBR. All samples were analyzed in duplicate, and the results were given as an average value. A series of ultra-hydrogel 250, 500, and 2,000 columns, heated to 40 °C and maintained by thermostatic control, were used in this work with deionized water as eluent at a flow rate of 1.0 mL min^{-1}. The detection was carried out at 35 °C with a diode array UV detector at 254 nm and simultaneously with a refractive index detector.

The advance in spectroscopic techniques provides an alternative to traditional approaches for characterizing DOM in aquatic ecosystems. Excitation emission matrix (EEM) fluorescent spectra can collect a series of emission spectra over a range of excitation wavelengths based on the dissolved matters characteristics. Reliable information about the source, redox state, and biological reactivity of DOM can be gained by EEM, which is becoming a powerful method of tracing the DOM variation of compositional changes related to the optical properties (Miller & McKnight 2010). Generally, the EEM spectra of DOM fractions can be divided into five regions: Region I (ex = 200–250 nm, em (emitted fluorescence) = 280–330 nm) and Region II (ex = 200–250 nm, em = 330–380 nm) are related to simple aromatic proteins (APs). Region III (ex = 200–250 nm, em = 380–550 nm) represents fulvic acid-like organics. Region IV (ex = 250–400 nm, em = 280–380 nm) and Region V (ex = 250–400 nm, em = 380–550 nm) (Chen *et al.* 2003). Complex DOM fractions in the wastewater treatment process can be categorized into simplification of carbohydrates, proteins, humic acids, and fulvic acids (Her *et al.* 2003).

DNA extraction and nested PCR

The seed activated sludge, mixture liquid and DM of sDMBR with and without AT addition were prepared for DNA extraction. Genomic DNA was extracted from the pellets using a Fast DNA spin kit (Bio101, Qbiogene Inc., Carlsbad, CA) with a small modification at the initial step: 1 mL of sodium phosphate buffer solution was added and mixed with the sample by a hand-held blender, and then the tube was sonicated for 30 s on ice. The remaining steps followed the manufacturer's instructions. The product from DNA extraction was verified by electrophoresis in 0.7% (w/v) agarose. To minimize the variation in DNA extraction, the templates used for nested PCR quantification were prepared from the mixture of DNA, which was extracted in triplicate for a sample.

DGGE fingerprints and statistical analysis

The PCR-amplified DNA fragments were separated on polyacrylamide gels (8%, 37.5:1 acrylamide-bisacrylamide) in 0.5TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM Na2EDTA, pH 7.4) using a denaturing gradient ranging from 30 to 60% (100% denaturant contains 7M urea and 40% (vol/vol) formamide). The amplicons were purified with Wizard PCR preps (Promega), and then aliquots (2 mL) of purified amplicons were quantified densitometrically. For DGGE, 100 ng of purified amplicons was used. DGGE was performed by using a D-Code system (Bio-Rad Laboratories, Inc., Tokyo Japan) Electrophoresis was initially at 60 °C for 20 min at 100 V, and thereafter for 5 h at 200 V. Following electrophoresis, the gel was soaked for 15 min in SYBR Gold (Molecular Probes; Eugene, OR, USA) and then visualized with a UV transilluminator (302 nm).

The nested PCR amplicons were separated on polyacrylamide gels (8%, 37.5:1 acryl amide–bisacrylamide) with a 35–55% linear gradient of denaturant (100% denaturant = 7 M urea plus 40% formamide). The gel was run for 7 h at 150 V in 1 × TAE buffer (40 Mm Tris–acetate, 20 mM sodium acetate, 1 m MNa_2EDTA, pH 7.4) maintained at 60 °C. Denaturing gradient gels were poured and run by using the DGGE-2001 System (C.B.S. Scientific, Del Mar, CA, USA) after electrophoresis, silver-staining and development of the gels were performed as described elsewhere, air-dried and scanned (Stamper *et al.* 2003). The gel images were analyzed with the software Quantity. DGGE fingerprints were manually scored by the presence of bands with consideration of the band brightness intensity. This was carried out at least three times to ensure constant results.

The scanned gel images were analyzed using Quantity One imaging devices (Bio-Rad, Hercules, CA, USA). Additionally, species richness and equitability index were calculated by the following equation (Stamper *et al.* 2003):

$$H = -\sum [P_i \times ln(P_i)]$$

where P_i is the relative probability of the bands in a gel lane.

H was calculated on the basis of the bands on the gel lane that were applied for the generation of the dendrograms by using the intensities of the bands as judged by peak heights in the densitometric curves.

The P_i was calculated as:

$$P_i = \frac{n_i}{N}$$

where n_i is the height of a peak and N is the sum of all peak heights in the densitometric curve.

The dissimilarity index (D) was adopted to show the difference among the communities (Hiraishi *et al.* 1991):

$$\mathrm{D(i,\,j)} = \frac{1}{2}\sum_{k=1}^{n}|x_{ik} - x_{jk}|$$

where $\sum_{ik}^{x} = \sum_{jk}^{x} = 1.00$ and x_{ik} and x_{jk} represent the ratio of relative band intensity for sample i and j in the location of band number k. Dissimilarity indices were expressed numerically within a range of 0 (perfectly similar) to 1.00 (completely dissimilar). We detected consistent and significant changes among our treatments in the 16S rRNA gene libraries composition using Unifrac tests, P-tests, and principal coordinate analyses (PCoA).

RESULTS AND DISCUSSION

Biological removal performance

The single factor analysis of AT doses was conducted in series batch experiments. UV_{254} and DOC removal efficiencies before and after AT addition were compared to investigate the optimum AT dosage. Following the increase of the AT doses from 3 to 7%, the removal efficiencies of both UV_{254} and DOC were increased to a maximum value and then decreased with an optimum dosage range. Five per cent of AT/MLSS (g/g) addition owned the best removal efficiencies for both of the UV_{254} and DOC (Table 2).

The comparison of treatment performances between the sDMBRs with and without AT addition during lengthy operation is summarized in Table 3. The COD removal efficiencies of sDMBR with and without AT addition were 95.7 and 96.3%, while NH_4^+-N removal efficiencies were 97.7 and 98.8%, respectively. A significant increase of the TN and TP removal efficiencies was accounted for with the AT added sDMBR compared to sDMBR only. TN removal efficiency was 51% in sDMBR compared to 77% in AT added sDMBR, meanwhile TP removal was 50% in sDMBR compared to 75% in the AT added sDMBR. The granular sludge formatted in the sDMBR after AT addition contributed to the high TN removal efficiency. Microbial community could form *zoogloeas* on AT particles, and subsequently aggregate functions of microorganisms, through microbial capsules and surface mucus.

EEM fluorescent characteristics of DOM

As is known, the formation of membrane bio-fouling caused by DOM, particularly the high concentration of protein, is mainly contributed by the production of extracellular polymeric substances (Flemming 2002). Increases in the pore blocking and cake layer on the membrane surfaces of membrane add to the fouling. The DOM in the reactor can be partly reflected to the member condition. The EEM fluorescence spectra for the raw water and the mixed liquid and effluent collected from the two bioreactors are represented by contour lines (see Figure 2). Each EEM image provided spectral information about the DOM compositions

Table 2 | Impact of AT dosages on the UV254 and DOC removal efficiency

	AT dosages (g/gMLSS)	3%	4%	5%	6%	7%
UV_{254}	Influent (cm^{-1})	0.246	0.256	0.242	0.250	0.252
	Effluent (cm^{-1})	0.236	0.227	0.230	0.234	0.242
	Removal efficiency (%)	4.1%	5.0%	5.3%	6.4%	4.0%
DOC	Influent (mg/L)	54.25	43.86	49.67	50.84	52.81
	Effluent (mg/L)	27.21	24.91	23.24	25.22	27.04
	Removal efficiency (%)	49.84	43.2	53.2	50.4	48.8

Table 3 | Comparisons of pollutants removal efficiencies in sDMBR with and without AT addition, all of the values represent mean ± standard deviation ($n = 22$)

	sDMBR without AT addition		sDMBR with AT addition	
Parameters	**Effluent**	**Removal efficiency (%)**	**Effluent**	**Removal efficiency (%)**
COD (mg L^{-1})	12.64 ± 0.47	95.7 ± 2.2	10.88 ± 6.53	96.3 ± 2.6
NH_4^+-N(mg L^{-1})	1.32 ± 0.08	97.7 ± 0.4	1.12 ± 0.14	98.8 ± 0.4
TN (mg L^{-1})	35.67 ± 6.26	51 ± 8.6	16.74 5.23	77 ± 7.2
TP (mg L^{-1})	1.78 ± 0.12	50 ± 6.2	0.94 ± 0.14	75 ± 3.8

which were divided into five regions. Measurements of EEM were carried out repeatedly and the results were similar in the entire parallel tests.

There were significant differences of the DOM fractions in the DMBR before and after AT addition. The peaks of I, II and III disappeared compared to the influent with AT addition. The peak V composed of microbial products and humic acid-like organics significantly increased compared to the influent and the reactor without AT addition. Meanwhile the peak V of the effluent in the sDMBR with AT addition was high, indicating that the microbial products accumulated in the reactor after AT addition. Moreover, the representative complex organics based on the characteristic peak are summarized in Table 4. The two dominating peaks could be divided among the detected contour lines. Shapes of sample spectra from the sDMBRs with and without AT addition were significantly different. A peak that could be attributed to a protein-like substance observed at Ex/Em = 270/320 nm dominated in the case of mixed liquid in the sDMBR, whereas peaks that could be attributed to humic acid-like substance at Ex/Em = 330/400 nm and Ex/Em = 270/450 nm became apparent in the case of mixed liquid in the sDMBR. However, relative dominance of the peak of protein-like substances differed in the descending order of sDMBR without AT addition < sDMBR with AT addition. The results also proved that the AT added sDMBR could obtain a steady performance with the fluorescent intensity reduced by 16 ~ 35% compared to the sDMBR without AT addition. Moreover, the decreasing number of conjugated bonds in the protein-like structure was observed in DOM during the sDMBR treatment process, indicating a potential of EEM fluorescence spectra as an effective analytical tool for DOM (Duan *et al.* 2011c). The DOM removal efficiency clarified that the AT addition played an important role in the DOM removal.

Community diversity and abundance analyses

PCR-DGGE was employed to investigate the microbial communities of the seed activated sludge, mixture liquid and DM of sDMBR with and without AT addition. Phylogenetic spectrum revealed that the bacterial communities among the samples were nearly identical with the similar locations of bars, and that the predominant microorganisms exhibited a high homology. However, the population distribution was different between the two reactors. The diversity of microbial communities was determined by Shannon index for the DGGE analysis (Moura *et al.* 2009). The Shannon diversity index of the community detected in the reactor (see Figure 3) showed that both the mixed liquid in the AT added reactor and DM were higher than the seed sludge and the reactor without AT addition. The total DNA concentrations of the G2 and G3 with AT addition reached 300 and 250 ng/μL, respectively, which were significantly higher than the seed sludge and the reactor without AT addition. Moreover, MLVSS/MLSS in the sDMBR with AT addition both for the mixture liquid and the DM were higher than in the sDMBR without AT addition. It was also proved that the AT addition made the process more stable and enriched the microorganisms. The impact of AT addition on the reactor is shown in Figure 4. DGGE profiles of bacteria distribution demonstrated that higher diversity was obtained by adding AT into the reactor. The unweighted pair group method with arithmetic mean (UPGMA) similarity analysis of the microbial community (Lozupone & Knight 2005) exhibited that the G2 and G3 with AT addition were clustered together, while G4 and G5 were clustered

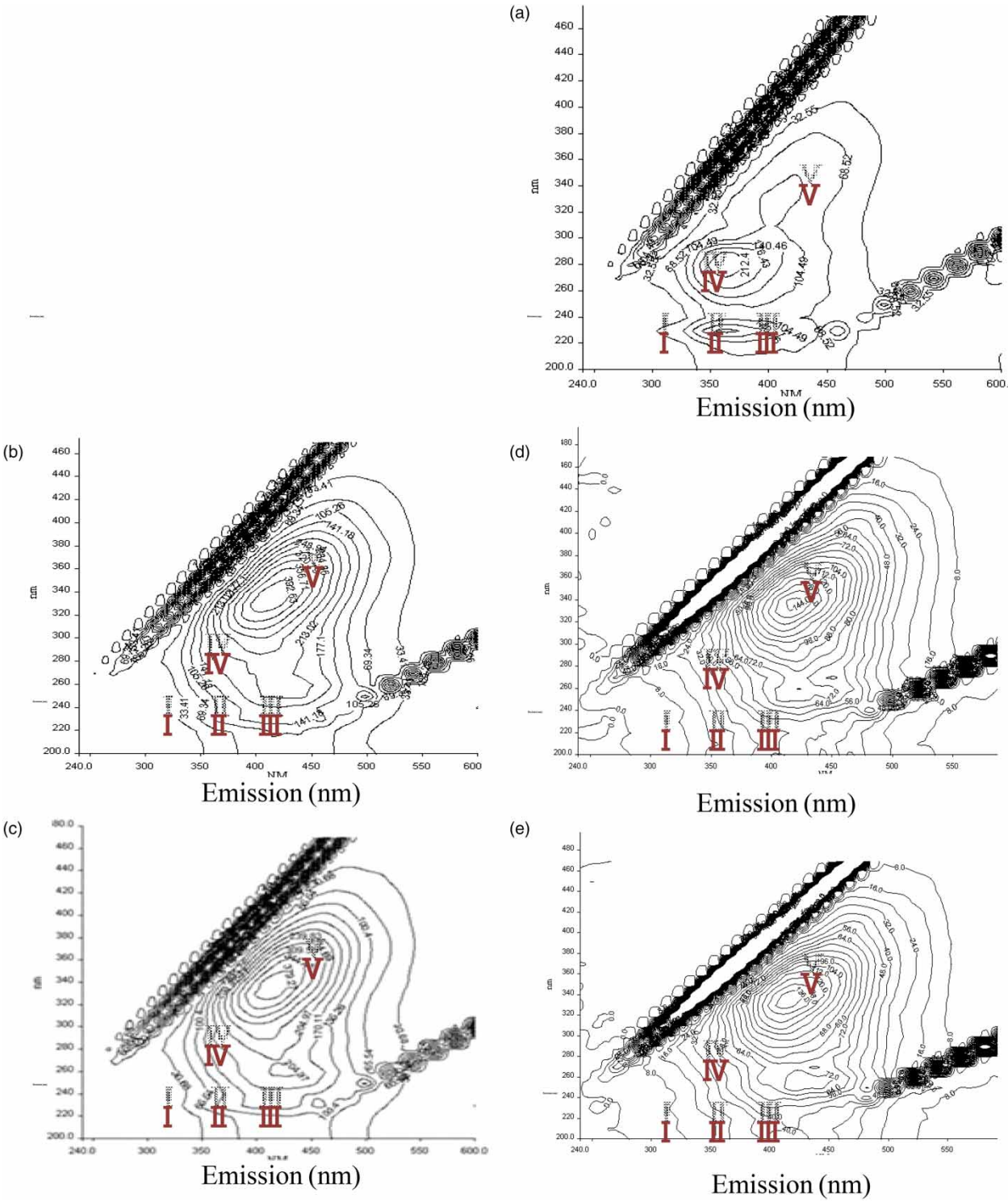

Figure 2 | The EEM fluorescence spectra of DOM in (a) the raw water in sDMBR, (b) mixed liquid in sDMBR without AT addition, (c) effluent in sDMBR without AT addition, (d) mixture liquid in the AT added sDMBR. (e) Effluent in AT added sDMBR. Parts of the data were reanalyzed from Duan *et al.* (2011a).

Table 4 | Characteristics of EEM fluorescent intensity (FI) for specific peaks in sDMBR with and without AT addition

	Protein like-peak		Fulvic acid-like peak	
Samples	**λex/λem**	**FI**	**λex/λem**	**FI**
Influent	230/363 280/363	940.3 1,204.6	330/405	568.1
Mixture liquid in sDMBR without AT addition	260/420	235.8	340/417	427.0
Effluent in sDMBR without AT addition	260/425	211.5	340/410	413.2
Mixture liquid in sDMBR with AT addition	260/423	80.21	340/419	146.96
Effluent in sDMBR with AT addition	260/427	65.01	340/420	111.59

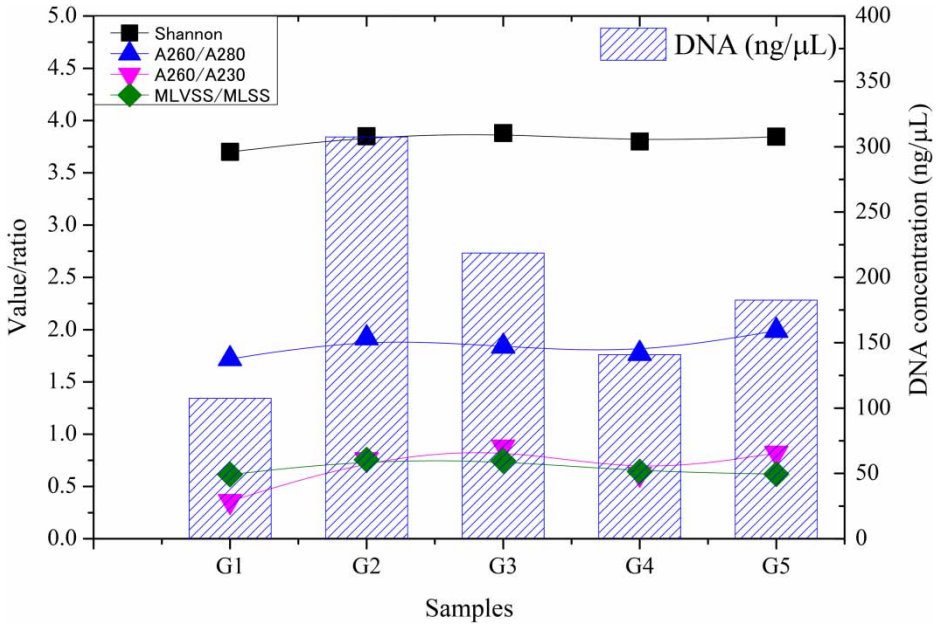

Figure 3 | Change of Shannon diversity index of (G1) seed activated sludge, (G2) mixed liquid in AT added sDMBR, (G3) DM of AT added sDMBR, (G4) mixed liquid in sDMBR, (G5) DM of sDMBR.

together in which the samples were taken from mixed liquid and DM without artificial AT addition (Figure 4).

The comparison of the microbial diversity and dissimilarity among the samples was conducted based on the DGGE bands. DGGE bands are likely to be derived from one phylogenetically distinct population, all of the band patterns were calculated by Quantity One® (Bio-Rad Technical, USA) (Yang *et al.* 2001). In this study, 41 bands were detected among the samples with seeding sludge of 28 bands, DM of 38 bands and liquid of 37 bands since AT was added and DM of 31 bands and mixture liquid of 30 bands in the reactor without AT addition. The DGGE band pattern for samples displayed similar changes compared to the seed activated sludge, though there was a difference in intensity between bands. The band patterns of DM and mixed liquid had a higher intensity in the reactor without AT addition. The band patterns of both DM and mixture liquid in the reactor with AT addition during the operation showed high diversity. Among the samples, bands 5, 8, 10, 11, 12, 13, 14, 15, 17, 18, 20, 21, 22, 24 and 25 had a high intensity in all of the samples. Particularly, band 5 contributed a similar high intensity of the samples. In the seed activated sludge, bands 5, 10, 20, 21, 23 and 24 were consistently thick throughout the operation

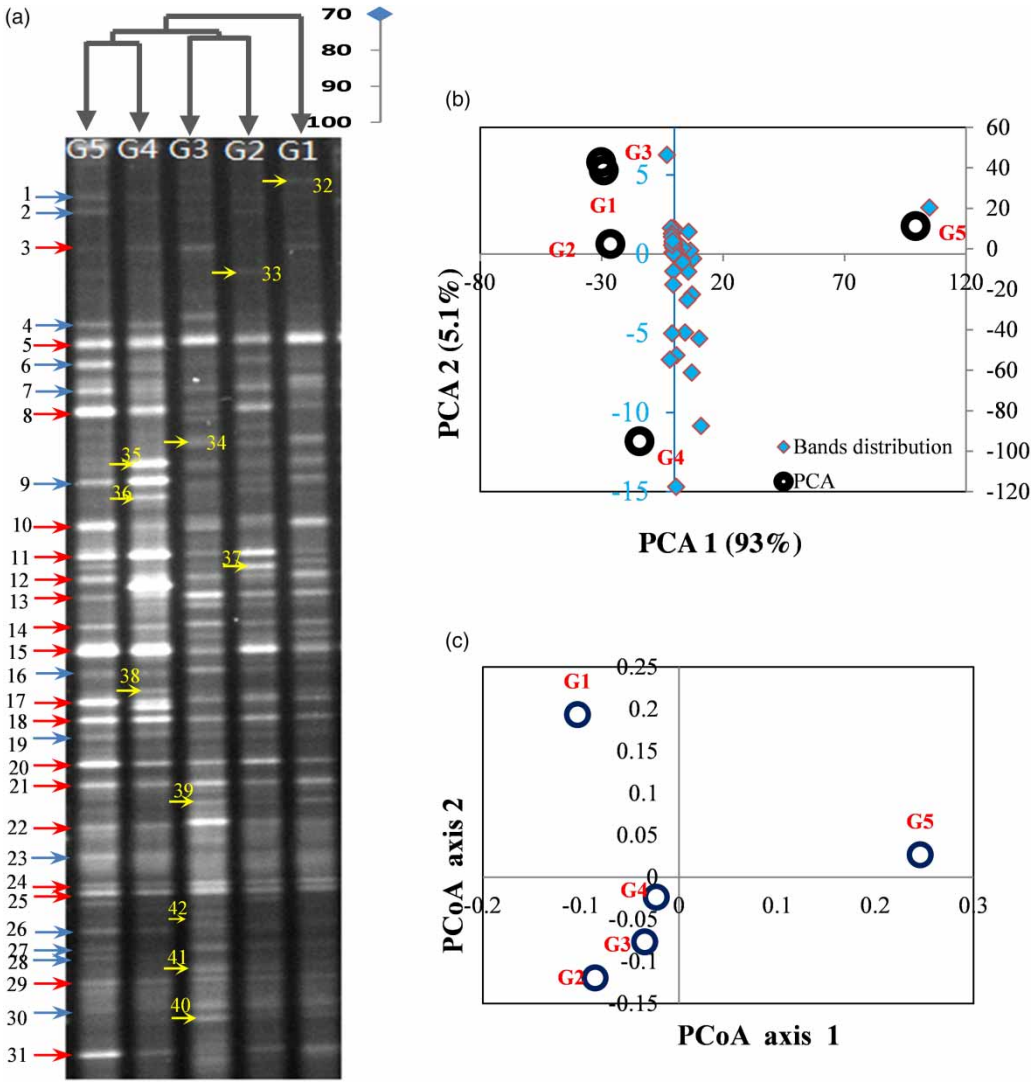

Figure 4 | DGGE profiles of bacteria in the sludge samples (a), PCA analysis of bands (b) and PCoA analysis of bands (c). (G1) seed activated sludge, (G2) mixed liquid in AT added sDMBR, (G3) DM of AT added sDMBR, (G4) mixed liquid in sDMBR, (G5) DM of sDMBR.

and independent on AT addition, while bands 8, 11, 15 and band 31 gradually became prominent. In contrast, band 32 was only present in seed activated sludge while band 32 was only present in the G2 sample. The high intensity of the bands detected in the reactor without AT addition showed high abundance of the microbial community. DGGE bands 35 and 36 were remarkably present in the mixed liquid with no detection in the DM. Bands 6, 7, 10, 20, 21 and 23 exhibited much higher intensity in the G5DM than in the mixed liquid G4 of the reactor without AT addition, while band 9 in G4 samples was much higher than G5 in the reactor. With higher diversity in the sample of G2 and G3, bands 40 and 41 are the new emergence. Bands 11 and 13, as well as band 15 species, in the reactor after AT addition significantly increased with the proof of the enhanced high intensity band.

The richness (R) for all the DGGE band patterns was calculated by using the respective band data analyzed by the Quantity software. Numerical analysis of microbial community diversity and the dissimilarity among the samples is shown in Table 5. Based on the band patterns, the dissimilarity between seed sludge and the samples with and without AT addition ranged from 0.392 to 0.565. It could be concluded that the microbial communities in the DM samples G3 and

Table 5 | Comparisons of the microbial diversity and dissimilarity of (G1) seed activated sludge, (G2) mixed liquid in AT added sDMBR, (G3) DM of AT added DMBR, (G4) mixed liquid in sDMBR, (G5) DM of sDMBR

Samples	Richness	Microbial diversity (H)	Dissimilarity to the seed sludge (D)	Dissimilarity (D)	Dissimilarity (D)	Dissimilarity (D)
G1	28	2.458				
G2	37	2.970	0.433	0.444		
G3	38	3.230	0.392			
G4	30	2.987	0.492		0.410	
G5	31	2.470	0.516			
DM (G3,G5)						0.565
Suspended (G2,G4)						0.342

G5 significantly changed, while the suspended sludge G2 and G4 with a lower dissimilarity of 0.342 provided the most similar among the samples. The results indicated that the AT addition contributed to the community abundance and structure with a significant change compared to the seed activated sludge. Moreover, compared to G4 and G5, the dissimilarity index (D) of G2 and G3 was almost the same as 0.4, indicating similar microbial diversity. In some instances (Sharp *et al.* 2011), the results proved that the microbial communities in DMBR may be similar. However, the higher diversity in the sDMBR with AT addition both for the DM and mixture liquid indicated that the microbial community was enriched after AT addition.

Bacterial community and phylogenetic analyses

The DGGE analysis method may have specific limitations such as band crowding or malposition during the electrophoresis. The agreed primer of DGGE also can not cover or separate all kinds of bacteria. Occasionally, special classes cannot be discerned due to their migration to identical positions in the gel and thus further qualitative analysis needs to be taken. To understand the detailed community structure, 16s rRNA gene cloning library of the microbial community was established. The effect of the AT addition on the microbial functional diversity was investigated. The phylogenetic tree of the samples taken from the sDMBR with (SEUDM4) and without (SEUDM1) AT addition is shown in Figure 5 and Table 6. In total, there were nine phyla: *α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, δ-Proteobacteria, Firmicutes, Acidobacteria, Bacteroidetes, Nitrospira* and *Planctomycete*. In the phylum of the *Firmicutes*, the *Lactococcus*-like genus which contains *Homofermentors* species with a single product-lactic acid (Buyze *et al.* 1957) were detected in both SEUDM4 (four clones) and SEUDM1 (one clone). In the dominated phylum of *β-Proteobacteria*, 34 and 26 clones were detected inSEUDM4 and SEUDM1, respectively. There were eight clones of *γ-Proteobacteria* detected in SEUDM4 compared to 15 clones detected in SEUDM1. There were three and two clones of *γ-Proteobacteria* detected in SEUDM1 and SEUDM4, respectively. In the phylum of the *Acidobacteria*, two clones of SEUDM4 and one clone of SEUDM1 were detected as *Acidobacteriaceae*. In the phylum of *Bacteroidetes*, six clones were detected as *Trachelomonas*-like and *Sphingobacteria*-like in SEUDM1 and eight clones were detected in SEUDM4. There were 13 clones of uncultured-*Bacterodetes*-like in SEUDM1, but only one clone in SEUDM4 was detected. In total, 22 clones in SEUDM4 and 30 clones were detected in the phyla of *Bacteroidetes*. The nitrite-oxidizing bacteria were detected in both of the samples, which was important for nitrogen removal in the system and these belong to the genus of *Nitrospira* (Juretschko *et al.* 1998). Moreover, two clones of *Planctomycete* were detected in both of the samples. Among the *Planctomycete* (Strous *et al.* 1999), there were some unusual bacteria such as an ammox bacteria which contributed to the nitrogen cycle. Furthermore, the microbial community structure of the SEM proved to have high diversity cellular morphology in our previous study during the reactor start up (Duan *et al.* 2011c). All indices support our observation that microbial communities became more diverse after AT addition. Our previous study showed that a dense layer of porous dynamic film was formed on the non-woven surface with a high density of microbe. Similar results of a variety of

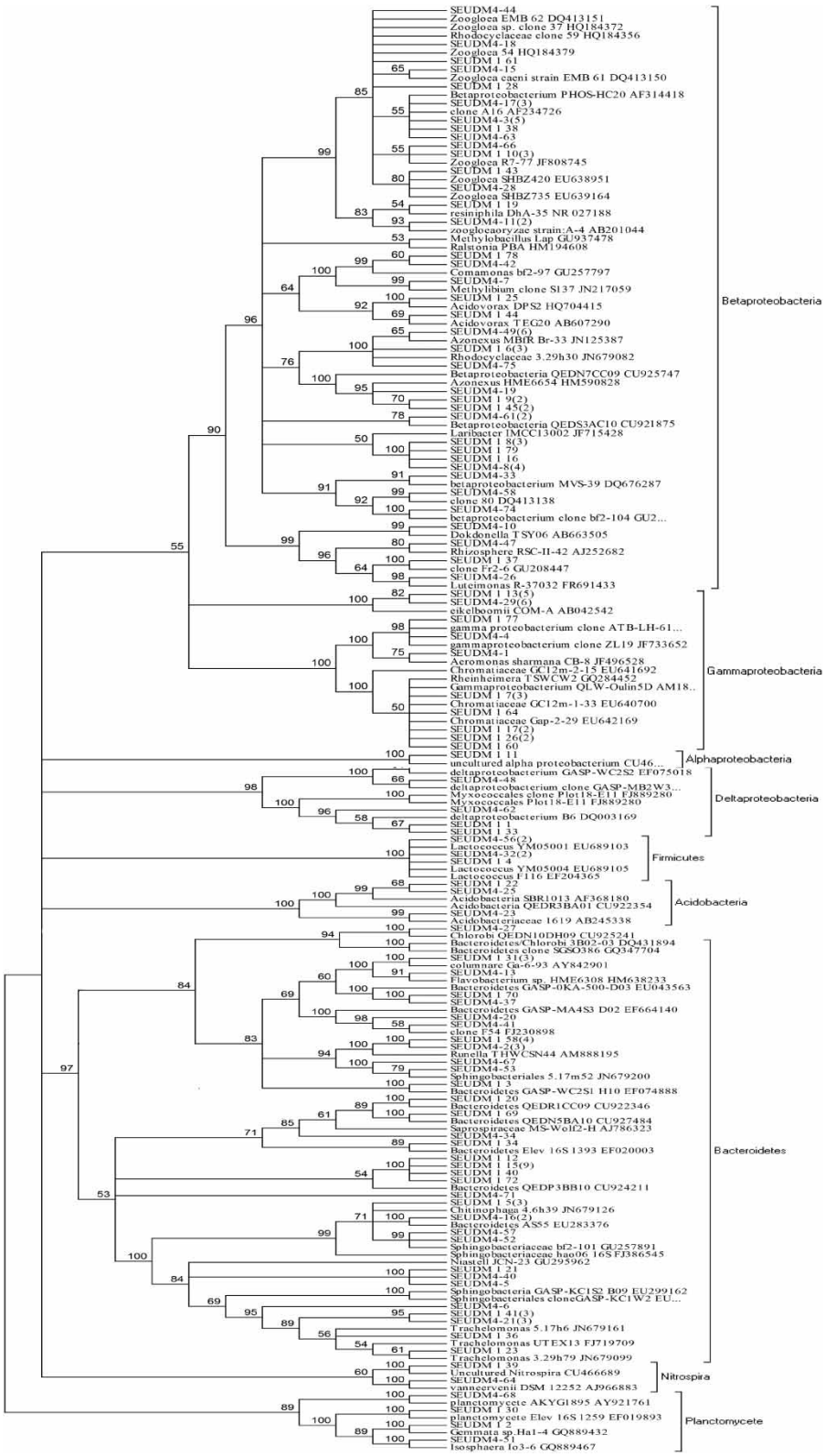

Figure 5 | Phylogenetic tree comparison of the two samples with and without AT addition.

Table 6 | Dominant microbial species in sDMBR with and without AT addition detected by high-density 16S rRNA microarray

	Bands	Name	NCBI	Identity (%)
sDMBR without AT addition	SEUDM1	*Gemmata SP*	GQ889432	99
	SEUDM1	*Planctomycete Elev*	EF019893	97
	SEUDM1	*Uncultured Nitrospira*	CU466689	98
	SEUDM1	*Trachelomonas*	JN679099	98
	SEUDM1	*Chitinophaga*	JN679126	99
	SEUDM1	*Bacteroidetes*	CU924211	98
	SEUDM1	*Columnare*	AY842901	97
	SEUDM1	*Acidobacteria*	CU921213	97
	SEUDM1	*Deltaprotebacterium*	DQ182112	96
	SEUDM1	*Rheinheimera*	GQ284452	98
	SEUDM1	*Gamma proteobacterium*	AM182112	99
	SEUDM1	*Chromatiaceae*	EU640700	98
	SEUDM1	*Acidovorax*	HQ704415	99
	SEUDM1	*Resiniphila*	NR027188	98
	SEUDM1	*Zoogloea*	JF808745	99
sDMBR with AT addition	SEUDM4	*Rhodocyclaceae*	HQ184356	98
	SEUDM4	*Betaproteobacterium*	CU456789	99
	SEUDM4	*Zoogloeaorgzae*	AB201044	97
	SEUDM4	*Comamonas*	GU257797	99
	SEUDM4	*Methylibium*	JN217059	98
	SEUDM4	*Azonexus*	JN125387	99
	SEUDM4	*Dokdonella*	AB663505	99
	SEUDM4	*Rhizosphere*	AJ252682	98
	SEUDM4	*Aeromonas*	JF496528	99
	SEUDM4	*Deltaproteobacterium*	GASP-MB2W	98
	SEUDM4	*Flavobacterium SP*	HM638233	99
	SEUDM4	*Sphingobacteriales*	JN679200	97
	SEUDM4	*Bacteroidetes*	EU283376	99
	SEUDM4	*Vanneervenii*	AJ966883	99
	SEUDM4	*Planctomycete*	AY921761	98
	SEUDM4	*Isosphaera*	GQ889467	99

microbes were found in the DMBR, such as *Coccus, Bacillus, Chainlike Cocci*, *Filamentous* bacteria, etc. (Fan & Huang 2002).

CONCLUSIONS

The removal efficiencies of COD, NH_4^+-N, TN and TP significantly increased in the AT added sDMBR. The protein-like peaks and fulvic acid-like peaks were significantly decreased in both the mixed liquid and the effluent of the AT added reactor. The obligate anaerobes were observed in the sDMBR with AT addition, such as *Bacteroidetes* and *Gamma proteobacterium* in the DM, which played an important role in the process of sludge granulation. Bacterial community richness significantly increased after AT addition with predominated phyla of *Proteobacteria* and *Bacteroidetes*. Similarly, species abundance significantly increased in the AT added sDMBR. The emergence of granular sludge in the DM clarified the high TN removal efficiency with functional community layers in the reactor after AT addition. AT was a favorite biological carrier for the microbial ecology, which enriched microbial abundance and community diversity of the sDMBR.

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