Int. J. Environ. Sci. Technol. (2014) 11:1845–1854
DOI 10.1007/s13762-013-0380-3

ORIGINAL PAPER

Microbial community analysis for aerobic granular sludge reactor treating high-level 4-chloroaniline wastewater

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Received: 16 January 2013 / Revised: 21 June 2013 / Accepted: 9 September 2013 / Published online: 30 October 2013

Abstract A laboratory-scale sequencing airlift bioreactor continuously treating high-level 4-chloroaniline (4-ClA) wastewater was used for studying the effect of 4-ClA on the characteristics and microbial community of aerobic granular sludge. The granulation of aerobic sludge and efficient pollutant removal performance were developed via shortening sludge settling time and gradually increasing influent 4-ClA concentration to around 400 mg L^{-1}. However, the granular sludge reactor deteriorated with the 4-ClA loading rate above 0.8 kg m^{-3} d^{-1}. Denaturing gradient gel electrophoresis and real-time quantitative PCR were applied to investigate the microbial community succession during the start-up and recovery of bioreactor. The results showed that the performance of granular reactor was significantly influenced by the microbial community of aerobic granule, and stable aerobic granule was dominated with *β-Proteobacteria* (61.28 %), *Flavobacteriales*, *Planctomycetales*, *Clostridiales,* and *Acidobacteria*. Since *Thauera* (21.55 %) related to the genus *β-Proteobacteria* was abundant in the stable 4-ClA-degrading granular sludge, it was speculated as the main 4-ClA-degrading bacteria. Under high chloroaniline level, the sludge granulation may maintain the stability of the bioreactor via adjusting the composition of microbial community and abundance of functional microorganism. This paper provided useful information for better understanding the change of microbial community characteristics under high-level toxic organic pollutants and process optimizing.

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Keywords Aerobic granular sludge · 4-Chloroaniline (4-ClA) · Microbial community · Succession · *Thauera* sp.

Introduction

Chloroaniline is a class of carcinogenic, mutagenic, and teratogenic persistent organic pollutants that has been used widely in the dye, pesticide, pharmaceutical, and preservative production in the world and threaten the ecosystem safety and human health. Therefore, many biological treatment processes have been established to improve their removal efficiency, such as anaerobic granular sludge technology, biofilm reactor, and immobilized microorganism technology (Boon et al. (2003) and Bathe et al. (2009)). For example, Boon et al. (2003) reported that addition of the *Comamonas testosteroni* I2 strain isolated from an activated sludge reactor could enhance the 3-ClA degradation. However, loss of the functional bacteria occurred, and the performance of the reactor finally deteriorated (Boon et al. 2002). It is clear that enriching and maintaining functional microorganisms are important for the efficient treatment of toxic organic wastewater (Zhang et al. 2011).

Aerobic granules are self-immobilized and mixed-culture microbial aggregates and have excellent potential for the application in the areas of dewatering improvement, residual sludge minimization, simultaneous biological nitrogen and phosphorus removal, and toxic organic compound degradation. Since Morgenroth et al. (1997) found the aerobic sludge granulation in a sequence bioreactor, researchers have conducted numerous studies on the formation and characteristics of aerobic granule, affecting factors of sludge granulation, mass transfer and model simulation, extracellular polymeric substances (EPS), and

formation mechanism (Beun et al. 2002; Liu et al. 2006; Wan et al. 2009; de Kreuk et al. 2010; Ni and Yu 2010; Gao et al. 2011, Uan et al. 2013). However, the aerobic granular sludge system is still not stable, and the application of this technology is faced with big bottlenecks and challenges (Winkler et al. 2011; Chen et al. 2007; Adav et al. 2008; Liu and Tay 2008).

It is well known that the microbial communities of activated sludge and biofilms affect the performance of biological wastewater treatment systems, and molecular biology techniques, such as PCR, denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH), have been extensively applied in the related fields (Muyzer et al. 1993; Deng et al. 2012). Boon et al. (2003) compared the microbial communities of activated sludge from fourteen Belgian sewage treatment plants using PCR–DGGE analysis and found that the plants were dominated with *β-Proteobacteria* and *Acidobacterium*, and the community diversity was associated with the value of sludge volume index (SVI). Liu et al. (2006) characterized the enhanced biological phosphorus removal systems using FISH and showed that the efficient phosphorus removal performance was associated with the enrichment of *Candidatus Accumulibacter phosphatis*. Deng et al. (2012) demonstrated that under high antibiotic level, the wastewater treatment system was stability through adjusting the bacterial, archaeal, and eukaryal compositions.

Our previous studies showed that aerobic 4-ClA-degrading granules could be cultivated under the condition of high chloroaniline loading rates (Zhu et al. 2008, 2011). In this study, the impacts of high-level 4-ClA on wastewater treatment communities are revealed via investigating the succession of microbial community in aerobic granular sludge reactor, and the community structures during the start-up and recovery stages of bioreactor are characterized using PCR-DGGE and real-time quantitative PCR. The purpose of this study is to provide useful information for understanding the characteristics of microbial community under high-level toxic organic pollutants and process optimizing.

Materials and methods

Experimental setup and sludge sample

A 5-L sequencing airlift bioreactor (SABR) used in this study (8 cm of down-comer I.D., 64 cm of riser height, and 5 cm of riser I.D.) was established in a temperature-controlled room at 25 ± 2 °C and operated in 12-h cycle consisting of 20 min of influent filling, 685–690 min of aeration, 5–10 min of settling, and 5 min of effluent withdrawal. For the aeration, fine air bubbles were supplied through a dispenser at the reactor bottom at a superficial gas velocity of 2.4 cm s^{-1}. Effluent was discharged at a volumetric exchange ratio of 70 %, equivalent to a hydraulic retention time of 17.2 h. The abiotic loss of 4-ClA in the SABR was negligible under identical operational conditions.

Inoculated sludge consisted of activated sludge from the aeration tank of the Sibao Wastewater Treatment Plant in Hangzhou, which was dark yellow or brown in color and flocculent in shape with a mixed liquor suspended solid (MLSS) value of approximately 2.15 g L^{-1} and an SVI of approximately 120 mL g^{-1}. The compositions of synthetic wastewater and trace mineral solution were analyzed according to the method reported by Zhu et al. (2011). The experimental parameters were adjusted based on the operational conditions of the reactor, as shown in Table 1.

The target of pollutant of the aerobic granular sludge bioreactor was 4-ClA. The influent substrates contained readily biodegradable organics, such as glucose and acetate. The COD/N/P ratio was maintained constantly at 100:5:1.

Table 1 Operational conditions of aerobic granular sludge reactor

Operational parameter	Stage		
	I (1–15 days)	II (16–90 days)	III (91–150 days)
Cycle time (h)	12	12	12
Influent (min)	20	20	20
Aeration (min)	685	690	690
Settling (min)	10	5	5
Effluent withdrawal (min)	5	5	5
Influent COD (mg L^{-1})	500–1,200	1,000–1,800	700–1,800
Influent 4-ClA (mg L^{-1})	0–10	10–450	180–400
Superficial air velocity (cm s^{-1})	2.4	2.4	2.4

Analysis methods

Conventional chemical analysis

Raw samples from the SABR were periodically analyzed for COD, pH, MLSS and volatile suspended solids (VSSs), and SVI in accordance with standard methods in APHA (1998). 4-ClA was analyzed using HPLC, and the analytical method is detailed in Zhu et al. (2011). All samples were analyzed in triplicate.

DNA extraction and determination

The extraction and purification of total DNA from sludge samples were performed using the 3S DNA isolation kit for environmental samples V2.2 from Biocolor BioScience and Technology (Shanghai). The integrity of the extracted DNA was determined using 1 % agarose gel electrophoresis. The DNA concentration was determined in triplicate using a SHIMADZU 2401PC UV spectrophotometer and then was stored at −20 °C.

PCR amplification and DGGE PCR amplification was performed on an iCycle thermal cycler (Bio-Rad). The bacterial 16S rRNA V3 variable regions were amplified by PCR using the following universal bacterial primers: GC-PRBA338f and PRUN518r synthesized by TaKaRa (Dalian, China). The PCR was carried out in a total volume of 50 μL containing: 5 μL of 10× PCR buffer (15 mM Mg^{2+}), 4 μL of dNTP mixture (2.5 mM each), 1 μL each of primers (25 μM), 0.5 μL of Taq DNA polymerase, approximately 15 ng of DNA template, and DEPC-treated water (RNase-free). The cycling parameters were as follows: one cycle of denaturation at 94 °C for 10 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. PCR amplification products were stored at −20 °C after confirmation by 1.5 % agarose gel electrophoresis.

Denaturing gradient gel electrophoresis analysis for PCR products was performed on a Bio-Rad DCode System according to the following procedure: 8 % polyacrylamide gel (acrylamide/bis-acrylamide 37.5:1) was mixed with 80 μL of 10 % ammonium persulfate and 18 μL of TEMED, and the denaturing gradient range was set at 30–60 % (100 % denaturing agents contained 7 M urea and 40 % deionized formamide). The samples were loaded onto the gel with 50-μL micropipette, and the gel was prerun at 20–40 V for 30 min followed by 150–160 V at 60 °C for 5–6 h in 1× TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM Na_2 EDTA). After electrophoresis, the gel was stained with Goldviewna II, purchased from BioDev-Tech (Beijing, China), for 20 min in the dark and then was subsequently washed with dH_2O for 5 min. The DGGE gel image was collected using the GelDoc2000 gel imaging system (Bio-Rad), and the fingerprint was analyzed using Quantity One 1-D Analysis Software v4.5 (Bio-Rad).

DNA cloning and sequence analysis

The target bands on DGGE gel were carefully excised with a sterile scalpel, transferred to sterile centrifuge tubes, and washed with sterile water three times. Gels were then cut into small pieces and put in 50 μL of TE (pH 8), and gel slices were incubated at 4 °C for 16 h. After incubation at 37 °C for 2 h, the samples were placed at 4 °C for 30 min. The supernatant was collected after centrifugation at 10,000 rpm for 1 min, and the recovered DNA was used as a template for the PCR amplification with the primers PRBA338f and PRUN518r. The PCR conditions refer to the above.

Cloning and sequencing were performed by Invitrogen (Shanghai) as follows: The PCR product was recovered using a gel extraction kit, and the size of the product was confirmed by agarose gel electrophoresis of 5 μL of the recovered DNA product; the purified product was ligated into the pMD18-T vector and transformed into *Escherichia coli* strain DH5α; positive colonies were selected using blue/white screening and inoculated into culture medium; plasmids were extracted using a plasmid purification kit and identified by PCR with the M13F(-47) and M13R(-48) primers; the plasmids were further confirmed by EcoRI and SalI double digestion; and the identified samples were sequenced. The obtained sequences were compared with nucleotide sequences in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). Related strains highly homologous to the obtained clones were selected for the construction of a phylogenetic tree. The sequences acquired from GenBank and the sequences obtained in this study were input into DNAStar, and the maximum parsimony method was used to construct a phylogenetic tree. The comparison of some 16S rRNA sequences in the GenBank database (NCBI) was performed using ClustalW v1.8 for sequence comparison and genetic analysis, and the neighbor-joining method in DNAStar was used for the construction of a phylogenetic tree (Neighbor-Joining/UPGMA, version 3.6).

PCR and quantitative real-time PCR

Four pairs of primers were designed for the detection of total bacteria, *β-Proteobacteria, Thauera* sp, and ammonia-oxidizing bacteria (AOB). Amplification was performed with the SYBR Green fluorescence quantitation kit

Table 2 RTQ-PCR primer and operational parameters

Species	Primer	Sequence	Operational parameters
Eubacterium	P338f	5′-ACT CCT ACG GGA GGC AG-3′	94 °C 5 min; 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, 35 cycles
	P518r	5′-ATT ACC GCG GCT GCT G-3′	
β-Proteobacteria	27f-GC	5′-GAG AGT TTG ATC CTG GCT CAG-3′	94 °C 5 min; 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, 35 cycles
	bP365r	5′-GCG CCC ATT GTC CAA A-3′	
Thauera sp.	–	5′-GAG AGT TTG ATC CTG GCT CAG-3′	94 °C 5 min; 94 °C 30 s, 57 °C 30 s, 72 °C 60 s, 35 cycles
		5′-CTA CGG CTA CCT TGT TAC GA-3′	
AOB	P189f	5′-GGA GRA AAG CAG GGG ATC G-3′	94 °C 5 min; 94 °C 30 s, 57 °C 30 s, 72 °C 60 s, 35 cycles
	P654r	5′-CTA GCT TTG TAG TTT CAA ACG C-3′	

Table 3 Relevant parameters of RTQ-PCR standard curve

Species	R^2	Standard curve	Solute peak (°C)
Eubacterium	0.997	Ct = −4.233 lgC + 37.632	79.2
β-Proteobacteria	0.985	Ct = −3.926 lgC + 32.108	81.3
Thauera sp.	0.994	Ct = −3.265 lgC + 18.694	85.5
AOB	0.983	Ct = −3.379 lgC + 25.579	82.4

on a Bio-Rad IQ5 quantitative PCR machine (PCR was performed to determine the optimal conditions and parameters for each sample, shown in Table 2).

The 16S rRNA genes from total bacteria, *β-Proteobacteria, Thauera* sp., and AOB cloned into pMD18-T served as quantitative standards. Positive control recombinant plasmids were prepared using the TA cloning method. After enzymatic digestion, plasmids were confirmed by agarose gel electrophoresis and sequencing, and the corresponding fragments were quantitated to calculate the copy number per unit volume. Standards were serially diluted tenfold, and 0.5 μL was used as a template for quantitative PCR to generate standard curves. The recombinant plasmids were diluted and quantitated, and the dilutions that were closest to the following values were selected: 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 copies mL^{-1}. Standard curves for each species of bacteria were obtained using the RT-qPCR results from a standard with a known starting copy number. Results are shown in Table 3.

The extracted genomic DNAs from environmental samples were used for RT-qPCR amplification with the four pairs of primers using the method described above, and four C_t values were obtained. Total copy numbers of 16S rRNA in total bacteria, *β-Proteobacteria*, AOB, and *Thauera* sp. were confirmed according to the standard curve. Because the copy number of 16S rRNA varies between different species of bacteria, the average 16S rRNA copy numbers of total bacteria, *β-Proteobacteria, Thauera* sp., and AOB, according to the data provided by http://rrndb.cme.msu.edu, were used as conversion factors to calculate the percentage composition of the four species in the original samples. All samples were analyzed in triplicate.

Results and discussion

Performance of aerobic granular sludge reactor

The bioreactor was started up with a 4-ClA loading rate of 20 g m^{-3} d^{-1} and a chemical oxygen demand (COD) of 1.0–2.4 kg m^{-3} d^{-1}. After 6 days of operation, the 4-ClA removal efficiency reached 99 % (shown in Fig. 1). The minimum settling velocity of the sludge increased from 1 to 8.1 m h^{-1} after 15 days' operation, and the average sludge diameter was 0.38 mm and the SVI decreased below 100 mL g^{-1}. Since then, the sludge settling time was reduced to 5 min, and the loading of 4-ClA gradually increased to 0.8 kg m^{-3} d^{-1}. After 65 days of operation, the 4-ClA removal efficiency gradually increased to 99.9 %, and the COD removal efficiency remained above 90 % (Fig. 2). With the further increase in the influent 4-ClA loading and sludge concentration in the reactors to 8.53 g L^{-1}, the average granular diameter reached

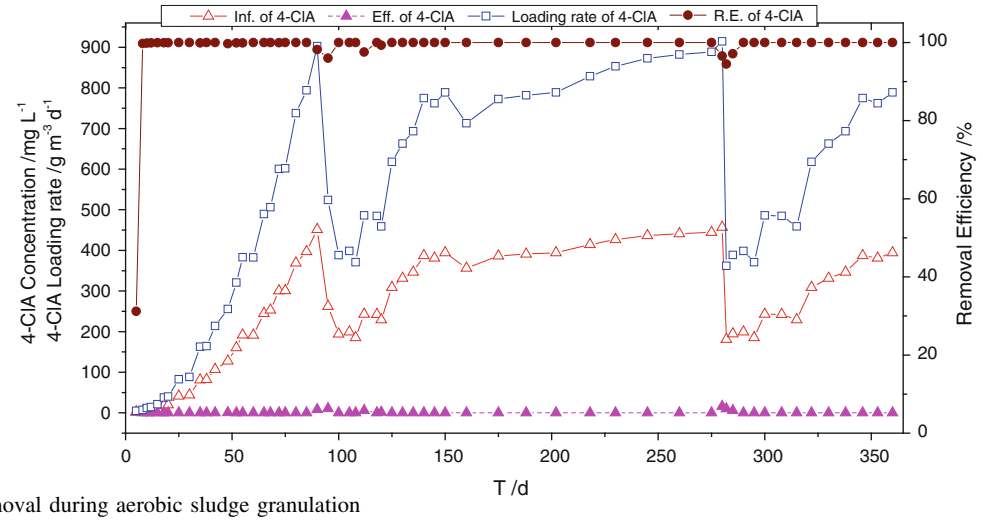

Fig. 1 4-ClA removal during aerobic sludge granulation

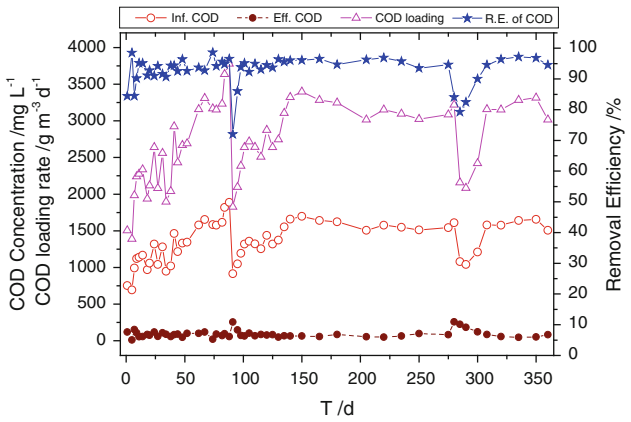

Fig. 2 COD removal during aerobic sludge granulation

1.19 mm. After 88 days of operation, the influent 4-ClA loading reached 0.9 kg m^{-3} d^{-1}, and the performance of the reactor deteriorated within 1 week. The sludge SVI increased from 50 to 120 mL g^{-1}, and the removal efficiencies of 4-ClA and COD decreased to 98.2 and 77.5 %, respectively.

To investigate the recovery and stability of aerobic granular sludge reactor after the instability, influent 4-ClA concentration was reduced to approximately 180 mg L^{-1}. The 4-ClA and COD removal efficiencies recovered within 10 days to above 99 and 90 %, respectively, and the reactor operated stably for 5 months under the high 4-ClA loading (119–278 days). After 280 days, the granular reactor became unstable again under high 4-ClA loading approximately 0.9 kg m^{-3} d^{-1}, and 4-ClA removal efficiency decreased from 99 to 96 %. After the adjustment of influent 4-ClA loading and related parameters, the performance of the reactor recovered and stabilized, and the removal efficiencies of 4-ClA, COD, and total nitrogen remained above 99.9, 93, and 70 %, respectively. The progress of 4-ClA degradation conformed to Haldane inhibition kinetics with the Haldane equation fitting analysis. The degradation rate increased with the initial concentration and speeded up and declined due to the inhibition of pollutants under high concentration with the kinetic parameters of a 41.72 mg g VSS^{-1} h^{-1} Vmax, a 267.32 mg L^{-1} Ks, and a 787.34 mg L^{-1} Ki.

Microbial community change during the aerobic sludge granulation

Based on the above operational condition and performance of the reactor, sludge samples were obtained from several specific stages for the microbial community analysis, including the start-up period, stable period, and instability period. The PCR–DGGE results are shown in Fig. 3. Thereinto, lanes 1–6 are sludge samples from 0, 10, 25, 95, 280, and 360 days, respectively. The similarity and cluster analysis of the DGGE profile are shown in Fig. 3.

Results showed that the microbial community of sludge at different stages maintained relatively abundance, with approximately 16–23 bands per lane (shown in Fig. 3). However, the community structure changed significantly. During the start-up of the reactor, most of the original bands from inoculated sludge disappeared, and new bands appeared. The changes in band profiles were associated with community succession during sludge granulation and performance recovery. After 10 days of reactor operation, the morphology and color of the sludge changes obviously, and its community

similarity to the inoculated sludge was 34.8 %. During the sludge granulation, disintegration, and recovery, the microbial community succession of sludge was significant, with less than 32.4 % similarity, indicating that the change in sludge morphology correlated with the community structure. Compared with the two instability periods of the reactors (lanes 4 and 5 in Fig. 3), the similarity of sludge microbial structure reached 64.6 %, suggesting that the bands that disappeared together may be associated with the dominant microorganism.

For the dominant microorganism analysis of different sludge samples, bands 1–13 from the gel (Fig. 3) were recovered, cloned, and sequenced. The sequences of clones approximately 190 bp in length were compared with those in GenBank using the BLAST program, and the highest

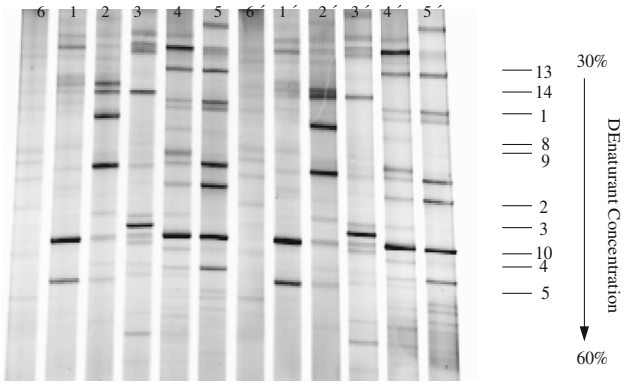

Fig. 3 DGGE profiles of 16S rRNA fragments obtained from aerobic granules (1,1′:0 days; 2,2′:10 days; 3,3′:25 days; 4,4′:95 days; 5,5′:280 days; 6,6′:360 days)

sequence similarity analyses are listed in Table 4. In the bioreactor using 4-ClA as the target pollutant, the stable aerobic granules were dominated with *β-Proteobacteria*, *Flavobacteriales*, *Planctomycetales*, *Clostridiales*, and *Acidobacteria*. The diverse microbial composition of the sludge at different stages may be associated with the influent composition and operational conditions of the reactor.

Structure and function of microflora in microbial aggregates, especially aerobic granules, affect sludge morphology and performance (Boon et al. 2003; Liu et al. 2006). Intensive study on the diverse microbial colonies and high retention of functional microorganisms of aerobic granules, and molecular biology techniques are used for analyzing the relationship between the structure and function of microflora and the stability of biological wastewater treatment systems, which can provide the optimization of process operating conditions and the improvement of system performance (Seviour et al. 2009). Sludge samples were obtained from several specific stages, including the start-up, stable, deterioration, and recovery periods, and the microflora changes in aerobic granular sludge reactor were investigated. According to the results of experiment, the microbial population diversity was relatively rich during the sludge granulation, and the succession of microbial community in the reactor was also significant with the influent 4-ClA concentration, removal efficiency, and sludge morphology. According to the 16S rRNA sequencing results of representative bands, stable aerobic granules degrading high-level 4-ClA were dominated with *β-Proteobacteria* (61.28 %), *Flavobacteriales*, *Planctomycetales*, *Clostridiales*, and *Acidobacteria* in the bioreactor. The constructed cometabolic

Table 4 Sequences of relevant DGGE fragments from aerobic granules

Band	Database match with accession no. in parentheses	Similarity (%)	Phylogenetic group
1	*Thauera* sp. Al7 (AY570693)	100	*β-Proteobacteria*
2	*Thauera* sp. B4P (AJ315678)	99	*β-Proteobacteria*
3	*Azoarcus* sp. mXyN1 (X83533)	99	*β-Proteobacteria*
4	*Bacterium* CYCU-0216 (DQ232380)	100	Unknown
5	Uncultured *Cytophagales* OPB56 (AJ630296)	91	*Cytophagales*
6	Uncultured *planctomycete* clone T-RF20-23 (AY555682)	90	*Planctomycetales*
7	Uncultured *planctomycete* clone LiUU-9-3 (AY509495)	93	*Planctomycetales*
8	*Thauera* sp. DNT-1 (AB066262)	99	*β-Proteobacteria*
9	*Thauera selenatis* (Y17591)	100	*β-Proteobacteria*
10	Uncultured *Flavobacteriales bacterium* clone LiUU-3-107 (AY509268)	98	*Flavobacteriales*
11	*Anoxynatronum sibiricum* (AF522323)	89	*Clostridiales*
12	*Xanthomonas* sp. PG-07 (AY566580)	99	*γ-Proteobacteria*
13	Uncultured *Acidobacterium* group bacterium clone SBR1013 (AF368180)	91	*Acidobacteria*

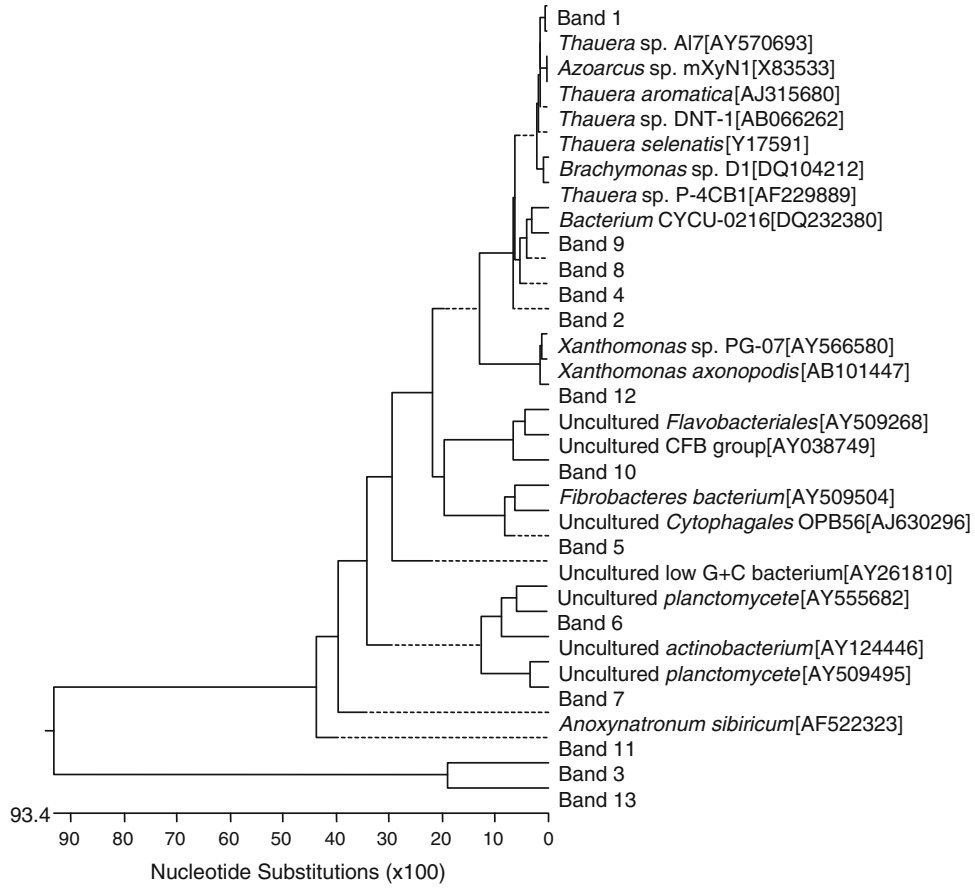

Fig. 4 Phylogenetic tree showing the affiliation-dominant bacteria from aerobic granules for degrading 4-ClA. "*Band*" refers to the DGGE bands in the polyacrylamide gels. *Numbers in parentheses* represent the sequences' accession number in GenBank. The number at each branch point is the percentage supported by bootstrap. *Bar*, 5 % sequence divergence

system in aerobic granules favored the stability of the bioreactor.

Thereinto, the homology between band 1, band 2, band 4, and band 8, and band 9 was 90.8–98.9 % (shown in Fig. 4). The comparison indicated that they belonged to *Thauera* sp. which is related to the genus *β-Proteobacteria*. The homology between band 1 and *Thauera* sp. Al7 (Grabowski et al. 2005) was 99.5 %. The homology of band 2 and band 9 with *Thauera selenatis* (Scholten et al. 1999) was 99.5 %. The homology between band 4 and *Thauera* sp. P-4CB1 (Song et al. 2002) was 97.5 %, and the homology between band 8 and *Thauera* sp. DNT-1 (Shinoda et al. 2004) was 93.5 %. The homology between band 11 and *Anoxynatronum sibiricum* was 88.1 %, whereas the homology between band 12 and *Xanthomonas* sp. PG-07 (Jiang et al. 2004) and *Xanthomonas axonopodis* (Malik et al. 2003) was 99.0 %. Furthermore, the homology between band 5 and the uncultured *Cytophagales* OPB56 was 90.6 %, and the homology between band 6 and the uncultured *Planctomycete* Clone T-RF20-23 was 90.4 %. The homology between band 7 and the uncultured *Planctomycete* Clone LiUU-9-3 was 93.1 %, and the homology between band 10 and the uncultured *Flavobacteriales* bacterium clone LiUU-3-107 was 96.4 %. The sequences of band 3 and band 13 did not match any sequences with greater than 70 % similarity in NCBI GenBank. According to the recommendation by the International Taxonomy Committee, the species boundary is 70 % DNA similarity. Because the above bands 5, 6, 7, and 10 with matched homologies were not found, further studies are needed to verify whether they are indeed new species (Fig. 5).

Quantification of functional microorganism in aerobic 4-ClA-degrading granule

Results of RT-qPCR quantitation of 4-ClA-degrading granule are shown in Table 5. With the operation of the reactor, the proportion of *β-Proteobacteria*, *Thauera* (related to the genus *β-Proteobacteria*), and AOB increased with different degrees. The percentages of AOB

among total bacteria were maintained at 5.02–10.79 %. The percentages of *β-Proteobacteria* among total bacteria were 23.69, 46.65, 51.22, and 61.28 % during aerobic sludge granulation. Thereinto, *Thauera* was barely detectable in inoculated sludge, whereas by 48 days of operation of the reactor and influent 4-ClA loading of 0.16 kg m^{-3} d^{-1}, *Thauera* in granular sludge accounted for 9.12 % of total bacteria. After 100 days of operation and the influent 4-ClA loading of 0.8 kg m^{-3} d^{-1}, the proportion of *Thauera* in sludge reached 18.34 % of total bacteria. After 120 days of reactor operation, *Thauera* accounted for more than 20 % of the total bacteria in the stable granular sludge. Results showed that *β-Proteobacteria*, especially *Thauera,* may highly be enriched along with aerobic sludge granulation.

Thauera sp. is a large class of denitrifying microorganism, and studies in recent years have confirmed that these species can degrade aromatic compounds under aerobic and anaerobic conditions (Yang et al. 2011). For example, *Thauera* sp. DNT-1 can use toluene as its only carbon source in the presence of nitrate, and *Thauera* sp. P-4CB1 can degrade 4-chlorobenzoate (Shinoda et al. 2004). Liu et al. (2006) found that the percentage of the clones affiliated with the genera *Thauera* and *Azoarcus* was 74 % in the denitrifying reactor and 4 % in the seeding sludge, and the greater abundance of *Thauera* in association with higher efficiency after adaptation suggested that these phylotypes might play an important role in quinoline and COD removal under denitrifying conditions. The abundance of potential denitrifiers in full-scale wastewater treatment plants suggested that the most abundant potential denitrifiers were related to the genera *Aquaspirillum*, *Azoarcus* and *Thauera,* all within the *β-Proteobacteria*. Thereinto, *Thauera* is the most versatile consuming some volatile fatty acids, ethanol, and amino acids. The coexistence of *Aquaspirillum*-, *Azoarcus*-, and *Thauera*-related bacteria in a range of treatment plants with differences in wastewater, design, and operation suggests that the populations ensure a functional stability of the plants by occupying different ecological niches related to the carbon transformation. Furthermore, a wide variety of bacterial species in full-scale wastewater treatment plants were found at the genera and class level, and some potential degraders such as *Thauera* were abundant in all samples, which potentially contributed to the strong catabolic activity observed in Biolog assay (Yang et al. 2011). Liu et al. (2006) found that *Thauera* spp. were important for the formation of acetate-fed granules, accounting for 49 % of the total clones. de Sanctis et al. (2010) also observed that *Thauera* were enriched during operation of a granular process. Interestingly, *Thauera* spp. is also found in activated sludges and produces exopolysaccharides which consists predominantly of *N*-acetyl-α-fucosamine, *N*-acetyl-α-glucosamine, β-rhamnose, and β-galacturonic acid. And the highest amount of eDNA was found in and around the microcolonies of denitrifiers belonging to the genera *Thauera* and *Curvibacter*, the ammonium-oxidizing *Nitrosomonas,* and the nitrite-oxidizing *Nitrospira*, and eDNA was an important structural component in activated sludge biofilms (Dominiak et al. 2011). As also shown in many researches of extracellular polymeric substances (EPS), the highly variable content of EPS, such as protein, polysaccharide, and eDNA, in different bacterial species means that the microbial composition in wastewater treatment plants must affect the properties of microbial aggregates, and thus also drainage and dewatering.

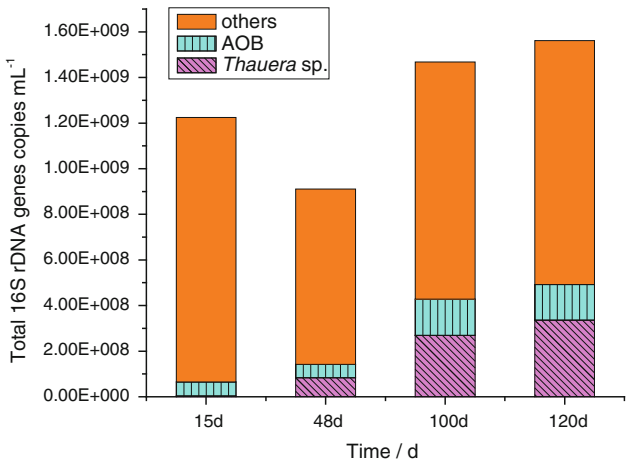

Fig. 5 Abundance of *Thauera* sp., AOB, and other bacteria in aerobic granules

Table 5 16S rRNA gene copies of functional microorganism in aerobic granules

Stage (days)	16S rRNA gene copies (copies mL^{-1})			
	Thauera sp.	AOB	*β-Proteobacteria*	*Eubacterium*
15	3.78×10^6	6.12×10^7	2.89×10^8	1.22×10^9
48	8.31×10^7	5.91×10^7	4.25×10^8	9.11×10^8
100	2.69×10^8	1.59×10^8	7.53×10^8	1.47×10^9
120	3.36×10^8	1.56×10^8	9.56×10^8	1.56×10^9

Conclusion

Results showed that the *β-Proteobacteria* class dominated the aerobic granular sludge and some important functional groups such as *Thauera* related to the performance of wastewater treatment were relatively abundant. Complex microbial community plays a key role in the aerobic sludge granulation, and a thorough knowledge of microbial information about the microbial composition and their EPS production is essential to develop the operating strategies and improve process performance of aerobic granule in the future.

Acknowledgments This work was funded by the National Natural Science Foundation of China (Nos. 51078327 and 51008269) and Zhejiang Environmental Protection R&D Project (No. 2011B10).

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