Sludge bulking impact on relevant bacterial populations in a full-scale municipal wastewater treatment plant

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ABSTRACT

We have investigated the changes of microbial community structures and the concomitant performance in two biological wastewater treatment systems (conventional and inverted A²/O processes) over a whole cycle of sludge bulking. A low level of filament abundance was observed during non-bulking period, with types 0092 and 0041 as the dominant filamentous bacteria. With the increase of the sludge volume index values from 76 (73) to 275 (300) mg/L, the filament abundance estimated by microscopic examination increased from 1 (few) to 5 (abundant), with *Microthrix parvicella* becoming the dominant filament bacteria. Sludge bulking resulted in a significant shift in bacterial compositions from *Proteobacteria* to *Actinobacteria* dominance, characterized by the significant presence of filamentous *M. parvicella* (from not detected to higher than 60% of clones) and decrease of the dominant Betaproteobacterial population (from higher than 40% to less than 1%). Important relevant bacterial populations including polyphosphate-accumulating organism (PAO, *Candidatus Accumulibacter phosphatis*), ammonia-oxidizing bacteria (AOB, *Nitrosomonas*), nitrite-oxidizing bacteria (NOB, *Nitrospira*) and denitrifying bacteria (*Thauera*) were absent under the serious bulking condition. Accumulation of nitrite and ammonia was observed during serious bulking, while the phosphorus removal performance was not decreased because *M. parvicella* could behave as a PAO.

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1. Introduction

The phylum *Proteobacteria* has been found to be the dominant bacterial group (35–65%) in conventional municipal wastewater treatment plants (WWTPs) [1], suggesting the importance of this group for pollutant removal. Among the *Proteobacteria*, *Betaproteobacteria* is the most abundant class, playing a key role in nitrification, phosphorus removal and the removal of many organic pollutants [2]. The betaproteobacterial *Candidatus Accumulibacter phosphatis*, the representative polyphosphate-accumulating organism (PAO) in full-scale systems, was found to be present in a range from 4 to 22% [3]. The genus *Nitrosomonas* in *Betaproteobacteria* has been found to be mainly responsible for nitrification in municipal WWTPs [4]. Denitrifying bacteria like *Thauera* spp. were

normally detected in WWTPs [5]. In a word, many *Betaproteobacteria* members play a key role in the functioning of municipal WWTPs while few filamentous bacteria, such as *Sphaerotilus natans* and Type 1701, belong to this class.

Sludge bulking, with the overgrowth of filamentous bacteria as the main reason, is a frequently encountered problem in WWTPs, particularly for those with the function of enhanced nutrient removal [6]. Although the distribution of filamentous microorganisms varies in different geographical areas, *Microthrix parvicella* and Types 0092 and 0041/0675 have been considered as the major morphotypes responsible for the bulking events observed in municipal WWTPs [7]. The excessive growth of filamentous bacteria could result in the deflocculation of activated sludge and the decrease of non-filamentous bacteria, making these non-filamentous bacteria susceptible to being washed out from the WWTPs. A previous investigation on dynamics of bacteria communities of a sewage treatment plant using PCR-DGGE showed that *Betaproteobacteria* could not be detected when serious sludge bulking occurred [8]. Our previous study has found that *M. parvicella* possibly played a role in phosphorus removal when *Candidatus Accumulibacter phosphatis* was washed out due to sludge bulking [9]. However, knowledge of

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the sludge bulking impact on the main functional bacteria has been very limited. Since sludge bulking can normally continue for several months, deterioration of the performance of WWTPs due to the washout of the non-filamentous relevant bacteria could become of great concern. Our preceding study [9] has revealed that the phosphorus removal performance would not be adversely affected by sludge bulking because *M. parvicella* could act as a PAO when *Candidatus Accumulibacter phosphatis* is not available. However, the nitrogen removal performance may be affected because the roles of AOB and NOB could not be easily replaced by the filamentous bacteria. In this study, we tracked the filament levels as well as the performance of two parallel full-scale municipal wastewater treatment systems over a period covering a whole cycle of a sludge bulking event. In order to reveal the potential impacts of sludge bulking on microbial community structures, four biomass samples were taken from each system to construct four clone libraries representing bacterial compositions at the non-bulking, initial bulking, serious bulking and recovery phases, respectively. The results of this study provide valuable information for better management of WWTPs.

2. Materials and methods

2.1. Description of the investigated WWTP

The investigated municipal wastewater treatment plant in northern China consists of a conventional A²/O system (anaerobic/anoxic/aerobic) and an inverted A²/O system (anoxic/anaerobic/aerobic) (Fig. 1), each of which has a treatment design capacity of 200,000 m³/day. Since the two systems were fed with the same sewage, one system could be used as the reference for another system. The sludge recycling ratio was approximately 100% for both the systems, while the mixed liquor recirculation ratio was 250% for the conventional system and none for the inverted one. In the inverted system, the influent was distributed into the anoxic and anaerobic tanks, respectively, at a ratio of 7:3. In the past 4 years, each system had received mean wastewater flows of 225,000 m³/day and had exhibited sludge bulking from late December to early May, with the inverted A²/O system exhibiting more serious bulking [8]. The operating conditions of the two systems, including sludge retention times (SRT, 12.7 ± 6.6 and 10.9 ± 5.8 days for the conventional and inverted A²/O systems, respectively), mixed liquor suspended solids (MLSS, 3009–5021 and 3010–4810 mg/L, respectively)

and food/microorganism (F/M, 0.09 ± 0.03 and 0.11 ± 0.04 kg BOD/kg MLSS/day, respectively) were kindly provided by the plant operators. The determination of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total phosphorus and soluble orthophosphate concentrations was done according to the Standard Methods of Water and Wastewater Monitoring [10]. Two week average values for each sampling time were used for evaluating the performance of the two systems under different sludge conditions, and are shown in Table 1.

2.2. Activated sludge samples and DNA extraction

In total, four activated sludge samples were taken from the end of each system's aerobic unit: one in September when the systems were in normal state (SVI = 76 and 73 mL/g for the conventional and inverted A²/O systems, respectively); one in January when significant sludge bulking occurred (SVI = 231 and 246 mL/g, respectively); one in March when serious sludge bulking occurred (SVI = 275 and 300 mL/g, respectively); one in July when sludge bulking began to disappear (SVI = 150 and 131 mL/g, respectively). The samples were centrifuged at 10,000 rpm for 10 min at 4 °C, and 0.25 g (wet weight) of activated sludge was used for DNA extraction using a FastDNA SPIN kit for soil (MP Biomedicals, USA) in accordance with the instructions provided by the manufacturer. The final volumes of the DNA solutions were 50 µL, which were then quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Triplet genomic DNA was extracted from these samples and then pooled.

2.3. Cloning and sequencing of 16S rRNA gene

The 16S rRNA genes were amplified using bacterial universal primer 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3') [11] for bacteria. The conditions for PCR amplification were as follows: initial 95 °C for 10 min, followed by 35 cycles of 95 °C for 60 s, 55 °C for 60 s, and 72 °C for 90 s, with a final extension step of 10 min at 72 °C. The PCR products were confirmed by electrophoresis in 1.0% (w/v) agarose gel. Three separate reactions were conducted for each sample to minimize PCR bias in subsequent cloning steps, and all PCR products were purified using TIAPure Mini Plasmid kit (Tiangen, China). The 16S rRNA amplicons were cloned into TOPO TA cloning vector pCR2.1, with TOP10 *Escherichia coli* transformants further selected according to the manufacturer's instruction (Invitrogen, China). The transformants were selected by blue-white selection on Luria-Bertani (LB) agar plates containing ampicillin (100 µg/mL). The cloned inserts were amplified from lysed colonies by PCR with plasmid-vector-specific primers M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3'). Positive clones were sequenced with an ABI 3730 automated sequencer (Invitrogen, Shanghai, China).

2.4. Phylogenetic analysis

After editing and trimming manually using BioEdit, the bacterial 16S rRNA gene sequences were searched against the GenBank database using BLASTN [12]. The most similar reference sequences were downloaded and aligned with the sequences of the present study using CLUSTALX [13]. Four 16S rRNA gene libraries were constructed for each system: AN, AB, ASB and AR for A²/O samples taken in non-bulking, significant bulking, serious bulking and recovery states, respectively, and IN, IB, ISB and IR for corresponding inverted A²/O ones. The operational taxonomic unit number was determined using the software MOTHUR by defining the sequences sharing 97% or greater similarity as one OTU [14]. The sequences obtained in the present study were deposited in the NCBI GenBank under accession numbers KJ807835–KJ808573. OTU

Fig. 1. Flow chart of the anaerobic/anoxic/aerobic process (conventional A²/O system) and aerobic/anaerobic/aerobic process (inverted A²/O system).

Table 1
Operating conditions and performance of the conventional and inverted A²/O systems.^a

SVI (mg/L)	Conventional A ² /O system				Inverted A ² /O system			
	76	231	275	150	73	246	300	131
COD removal efficiency (%)	89.0 ± 3.7	89.8 ± 1.4	90.1 ± 1.1	89.7 ± 2.0	89.0 ± 3.6	89.9 ± 1.3	89.3 ± 1.5	89.9 ± 1.9
Statistical significance ^b	–	P>0.05	P>0.05	P>0.05	–	P>0.05	P>0.05	P>0.05
Total phosphorus removal efficiency (%)	96.8 ± 0.6	96.1 ± 1.2	96.0 ± 1.3	96.7 ± 2.3	95.9 ± 1.8	96.0 ± 2.3	96.3 ± 4.4	97.0 ± 1.8
Statistical significance ^b	–	P>0.05	P>0.05	P>0.05	–	P>0.05	P>0.05	P>0.05
Soluble orthophosphate removal efficiency (%)	96.7 ± 2.0	96.9 ± 2.0	97.6 ± 0.8	97.9 ± 1.1	97.2 ± 0.6	97.3 ± 0.8	97.4 ± 1.0	97.6 ± 1.3
Statistical significance ^b	–	P>0.05	P>0.05	P>0.05	–	P>0.05	P>0.05	P>0.05
TN removal efficiency (%)	75.5 ± 4.1	78.2 ± 3.6	83.6 ± 2.9	73.6 ± 5.4	66.5 ± 2.5	68.5 ± 3.2	76.0 ± 7.2	72.5 ± 6.1
Statistical significance ^b	–	P>0.05	P<0.01	P<0.01	–	P>0.05	P<0.01	P>0.05
NH ₄ ⁺ -N removal efficiency (%)	94.9 ± 7.5	97.2 ± 1.6	93.5 ± 3.1	97.9 ± 1.7	98.0 ± 0.7	95.3 ± 2.2	90.8 ± 7.2	96.3 ± 2.9
Statistical significance ^b	–	P>0.05	P<0.01	P<0.01	–	P<0.01	P<0.05	P<0.05
NO ₂ -N _{eff} (mg/L)	0.33 ± 0.51	0.31 ± 0.44	5.63 ± 0.91	0.24 ± 0.55	0.21 ± 0.38	0.41 ± 0.55	9.11 ± 1.85	1.02 ± 0.88
Statistical significance ^b	–	P>0.05	P<0.01	P<0.01	–	P>0.5	P<0.01	P<0.01

^a Two-week average related to sludge sampling.

^b Paired-samples *t*-test was used to assess the significant differences between the efficiencies of adjacent two periods on *P*-values.

richness values, as well as the Shannon diversity index (*H*), were calculated using MOTHUR. Evenness (*E*) indices were calculated as follows: $E = H/\ln n$, where *n* is the number of OTUs [15].

2.5. Microscopic examination and filament identification

Sludge samples were observed immediately after sampling (within 2 h). Fifty microliter well mixed samples were dropped on a microscope slide. Microscopic observation of filamentous bacteria was carried out under contrast at 100× and 1000× magnification after the slide was covered with a glass cover slip using an Olympus BX51 microscope, and the micrographs of sludge samples were taken via a charge-coupled device connected to the microscope. A subjective scoring system (0, none; 1, few; 2, some; 3, common; 4, very common; 5, abundant; 6, excessive) was used to determine filament abundance [6]. In all samples, the microscopic examinations were conducted independently by three observers to confirm the filament abundance.

2.6. Statistical analysis

The similarities and diversities in bacterial compositions between different samples were determined using MOTHUR software [16]. All other statistical analyses were performed using the SPSS software, version 18.0.

3. Results and discussion

3.1. Changes of activated sludge properties in a yearly cycle

Fig. 2 shows the changes of SVI values and water temperature over a period of 1 year. SVI, which is defined as the volume (mL) occupied by 1 g of sludge after 30 min settling, is typically used to monitor the settling characteristics of activated sludge and other biological suspension [17]. The sludge settleability was normal from September to the first half of December for the two systems as indicated by the low SVI values (<150 mL/g). The microscopic examination of sludge samples in September and October showed that the filament abundance was 1 (few) (Fig. 3 and Table S1). The flocs were compact and their sizes were in general greater than 150 μm. Filamentous bacteria were only observed in occasional cases with the dominant ones as types 0041 and 0092. The SVI value began to be greater than 150 mL/g from the second half of December, showing the beginning of sludge bulking [18,19]. The floc size decreased to a range of 100–150 μm, then to smaller than 50 μm when the most serious bulking (SVI>250 mL/g [20]) occurred. Filamentous bacteria could be observed in almost all flocs of the initial bulking sludge samples with *M. parvicella* as the dominant filamentous

bacteria in both systems. The filament abundance increased to 4 (very common) and 5 (abundant). It is clear that sludge bulking was accompanied with significant increase of the abundance of *M. parvicella*, which was accordance with our previous study [9] conducted 1 year earlier.

It is clear that the changes of SVI were closely associated with the water temperature. Sludge bulking began to occur when the temperature was below 20 °C, which was in accordance with an investigation on a laboratory-scale plant [21]. Serious sludge bulking appeared at the lowest temperature (13–15 °C). With a low maximum specific growth rate, high storage capacity, and low maintenance energy requirement, *M. parvicella* has a significant advantage over other microorganisms prevailing in the extended aeration activated sludge systems, especially under low temperature conditions. Previous studies show that *M. parvicella* are more hydrophobic than most other activated sludge bacteria [22], which may allow them to easily access the hydrophobic substrates such as lipids for growth at low temperature [23]. The whole sludge bulking continued for almost 5 months. It is interesting that the morphology of *M. parvicella* was quite different between the periods of sludge bulking and recovery. As shown in Fig. 4, a large number of extended filaments stretching out from the flocs could be observed in the sludge samples in the initial bulking period and were very much longer than in the recovery sludge samples. The longest extended filaments were observed in February and March, when serious sludge bulking appeared. Previous studies have indicated that filaments extending from the flocs correspond to a high SVI [24], and short *M. parvicella* do not have a negative effect on sludge settleability [21].

3.2. Dynamics of bacterial community structures

Four 16S rRNA gene libraries were constructed for each system: AN (SVI = 76 mL/g), AB (SVI = 231 mL/g), ASB (SVI = 275 mL/g) and AR (SVI = 150 mL/g) for A²/O sludge samples taken in non-bulking, initial bulking, serious bulking and recovery states, respectively, and IN (SVI = 73 mL/g), IB (SVI = 246 mL/g), ISB (SVI = 300 mL/g) and IR (SVI = 131 mL/g) for corresponding inverted A²/O ones. In total, eight 16S rRNA gene libraries were obtained with 739 sequences and 324 OTUs, as shown in Fig. 5 and Table S2.

The majority of sequences (57.8 and 68.1%) in the non-bulking samples (AN and IN) were affiliated with the phylum *Proteobacteria*, primarily including classes *Betaproteobacteria* (42.2 and 48.4%), *Alphaproteobacteria* (3.3 and 13.2%) and *Gammaproteobacteria* (12.2 and 6.6%), followed by the phylum *Actinobacteria* (4.4 and 1.1%), *Bacteroidetes* (8.9 and 3.3%), *Chloroflexi* (10.0 and 12.1%) and *Nitrospirae* (6.7 and 5.5%). This was in accordance with previous studies [25], showing that bacteria belonging to the phylum

Fig. 2. Variations of SVI and temperature in conventional and inverted A²/O systems.

Fig. 3. Changes of the filament abundance in relation to SVI in conventional and inverted A²/O systems.

Fig. 4. Phase contrast micrographs of flocs (original magnification 100 $\times$). (A) The flocs of conventional A²/O system during non-bulking period (SVI = 76 mL/g). (B) The flocs of conventional A²/O system during initial sludge bulking period (SVI = 231 mL/g). (C) The flocs of conventional A²/O system during serious bulking period (SVI = 275 mL/g). (D) The flocs of conventional A²/O system during recovery period (SVI = 150 mL/g). (E) The flocs of inverted A²/O system during non-bulking period (SVI = 73 mL/g). (F) The flocs of inverted A²/O system during initial sludge bulking period (SVI = 246 mL/g). (G) The flocs of inverted A²/O system during serious bulking period (SVI = 300 mL/g). (H) The flocs of inverted A²/O system during recovery period (SVI = 131 mL/g).

Fig. 5. Abundance distribution of bacteria in conventional and inverted A²/O systems. *The clones with a fraction of less than 5% are grouped into "others".

Proteobacteria, particularly the class *Betaproteobacteria*, play the key role in pollutant removal.

Proteobacteria was still the dominant phylum when sludge bulking occurred in January, accounting for 57.1 and 52.2% in the AB and IB libraries, respectively, with *Betaproteobacteria* as the major class (43.9 and 40.2%). The numbers of clones affiliated with phylum *Actinobacteria*, however, increased significantly (4.4 vs. 18.4% and 1.1 vs. 23.9%), with the filamentous *M. parvicella* as the major group (12.2% and 16.3%, respectively). The increase of *M. parvicella* was in accordance with the filament abundance (=4) and sludge settleability (SVI=231 and 246 mL/g, respectively).

Drastic change of bacterial population structures was observed when serious sludge bulking (SVI, 275 and 300 mL/g) occurred in March. The vast majority of the clones (72.7 and 75.0%) were affiliated with the phylum *Actinobacteria* in ASB and ISB libraries, among which, *M. parvicella* was the dominant bacteria (60.6 and 65.6%). The remaining clones were mainly classified into the phyla *Proteobacteria* (mostly *Alphaproteobacteria*, 16.2 and 10.4%) and *Firmicutes* (9.1 and 12.5%). Only one clone (1%) affiliated with *Betaproteobacteria* was found in the ASB clone library from

the conventional A²/O system, which was in accordance with a previous study using PCR-DGGE [8].

When the sludge settleability was recovered (SVI=150 and 131 mL/g, respectively) in May, clones associated with *Betaproteobacteria* appeared again (14.4 and 8.0% for the AR and IR, respectively). However, the *M. parvicella* population (44.9 and 41.7%) was still dominant in the two systems, showing that the recovery of the bacterial community to the normal state requires a long period. Although there were more filamentous bacteria present in the samples related to the recovery phase (AR and IR), their SVI value was lower than initial bulking samples (AB and IB). As mentioned above, the microscopic examination of sludge samples showed that the morphology of *M. parvicella* was obviously different between these two periods (Fig. 4). The short extended filaments of *M. parvicella* had a lower impact on sludge settleability [21].

Table 2 shows Shannon and evenness indices calculated from the clone library data. It is understandable that the lowest diversity was observed in the samples with the most serious sludge bulking, as indicated by the low Shannon (2.79 and 2.49) and evenness

Table 2

Coverage and diversity indices of bacterial 16S rRNA gene libraries for conventional and inverted A²/O systems.

Sample	No. of clones	No. of OTUs	Shannon index	Evenness index
AN	90	64	4.83	1.16
AB	98	73	5.07	1.18
ASB	99	34	2.79	0.79
AR	89	46	3.74	0.98
IN	91	46	3.96	1.03
IB	92	61	4.59	1.12
ISB	96	29	2.49	0.74
IR	84	49	4.04	1.04

indices (0.79 and 0.74) for ASB and ISB, since *M. parvicella* represented over 60% of clones. It is interesting, however, that the highest diversity was observed when sludge bulking began to break out in January (Shannon index, 5.07 and 4.59 for clone libraries AN and IN, respectively). It seems that the bacterial communities in this period were in a transitional state, changing from the *Proteobacteria* dominance to the *Actinobacteria* dominance, and resulting in a higher diversity.

3.3. Bulking impact on bacterial populations related to nutrient removal performance

As discussed above, the bacterial communities changed from *Proteobacteria* to *Actinobacteria* dominance when serious sludge bulking occurred (Fig. 5). Table 3 shows the abundances of some important relevant groups responsible for nutrient removal in different clone libraries. Significant decrease of the abundances of the main relevant bacteria including *Nitrosomonas*, *Candidatus Accumulibacter phosphatis* and *Thauera* belonging to the class *Betaproteobacteria* was observed when serious sludge bulking occurred. In a previous study investigating over 50 Danish WWTPs with nutrient removal, the abundances of these were quite stable in different seasons [26]. Therefore sludge bulking should be the main reason for the significant changes of bacterial communities in the present study.

Candidatus Accumulibacter phosphatis and, occasionally, *Tetrasphaera* spp. are known to be the principal polyphosphate-accumulating organisms (PAOs) [27]. *Candidatus Accumulibacter phosphatis* (3.3–6.6%) and *Tetrasphaera* spp. (0–2.4%) were detected in sludge samples before serious sludge bulking occurred. However, no clone affiliated to *Candidatus Accumulibacter phosphatis* was observed in libraries ASB and ISB representing the most serious sludge bulking samples, and libraries AR for the recovering samples, showing that this important PAO might have been washed out from the systems due to the excess growth of *M. parvicella*. However, the phosphorus removal efficiency was not adversely affected by the significant loss of *Candidatus Accumulibacter phosphatis*, as shown in Table 1. This result was in accordance with our previous study [9] showing that the dominant PAO *Candidatus Accumulibacter phosphatis* decreased sharply when serious sludge bulking occurred (lower than 1%). However, changes of *Tetrasphaera* spp. were not revealed in the previous study, perhaps due to the lower clone numbers for each clone library. On the other hand, no significant change in the numbers

of *Tetrasphaera* spp. (in the range of 0–2.4% in all libraries) was observed in the two systems over the whole period. This group of bacteria, however, was believed to play a key role in phosphorus removal only at much higher abundance (18–30%) [1]. Our previous study showed that *M. parvicella* might have been responsible for the removal of phosphorus during sludge bulking in plants with similar configurations [9,28].

A large part of the clones belonging to the class *Betaproteobacteria*, accounting for 13.3 and 16.5% respectively for the AN and IN libraries, was further grouped into Genus *Thauera* and was affiliated with or closely related to denitrifying bacteria, including *Thauera aminoaromatica* strain S2 (GenBank accession number NR_027211) or strain B4P (GenBank accession number NR_027224). These two strains could reduce nitrate to N₂ [29]. Their high percentage in activated sludge in the non-bulking phase suggests that they might have played an important role in the denitrification in the systems. Their numbers in clone libraries AB and IB decreased to 9.2 and 5.4%, respectively, when sludge bulking occurred, and disappeared when serious sludge bulking appeared.

Nitrospira was the predominant NOB (nitrite-oxidizing bacteria) in the two systems before the appearance of serious sludge bulking (Table 3). This finding was in accordance with the previous study, showing that *Nitrospira* (phylum *Nitrospirae*) often was the dominant nitrite oxidizing bacteria in activated sludge systems [30]. No clone was detected when serious bulking occurred. Significant decrease of the abundances of NOB (*Nitrospira*) might be responsible for the significant accumulation of NO₂-N (5.63 ± 0.91 and 9.11 ± 1.85 mg/L for the conventional and inverted A²/O, respectively). Although only one clone (1.1%) affiliated with *Nitrosomonas* was found in the AN clone library from the conventional A²/O system due to the relatively low abundance (about 2% [5,31]), the impact of sludge bulking on ammonia oxidation was also observed. The ammonia removal for the conventional and inverted A²/O systems decreased from 94.9 ± 7.5% and 98.0 ± 0.7% in the non-bulking phase to 93.5 ± 3.1% and 90.8 ± 7.2%, respectively, in the serious bulking phase.

As shown in Table 1, only nitrite and ammonia oxidations were affected by sludge bulking, while the functions of phosphorus removal and denitrification as well as COD removal were not decreased. It is known that *M. parvicella* could utilize diverse types of organic compounds (long-chain fatty acids, oleic and palmitic acids, trioleic acid and etc. [22]), and may also be able to function as a PAO [9]. Thus the dominance of *M. parvicella* did not lead to the deterioration of the phosphorus and COD removal functions. *M. parvicella* was believed not to reduce nitrite to nitrogen gas [22]. However, many other bacteria could reduce nitrite, such as the members of *Rhodobacteraceae* (accounting for 8.1 and 5.2% respectively for the ASB and ISB libraries) and *Hyphomicrobium* (accounting for 2.0 and 2.1% respectively for the ASB and ISB libraries) [32]. Thus the denitrification performance was not adversely affected, either, as indicated by the equivalent TN removals (68.5–83.6% during sludge bulking period vs. 66.5–75.5% during non-bulking period). No filamentous bacteria have been reported to function as AOB and NOB, which could explain the accumulation of nitrite and ammonia when serious sludge bulking occurred.

Table 3

Distribution of major functional bacteria groups among bacterial 16S rRNA gene clone libraries for conventional and inverted A²/O systems.

	AN	AB	ASB	AR	IN	IB	ISB	IR
PAO (<i>Candidatus Accumulibacter phosphatis</i>) (%)	3.3	4.1	0	0	5.5	6.6	0	1.2
PAO (<i>Tetrasphaera</i>) (%)	0	2.0	2.0	1.1	0	2.2	1.0	2.4
AOB (<i>Nitrosomonas</i>) (%)	1.1	0	0	0	0	0	0	0
NOB (<i>Nitrospira</i>) (%)	6.7	6.1	0	0	5.5	6.6	0	0
Denitrifying bacteria (<i>Thauera</i>) (%)	13.3	9.2	0	1.1	16.5	5.4	0	0

4. Conclusions

Sludge bulking caused by the overgrowth of *M. parvicella* resulted in significant shift in bacterial compositions from *Proteobacteria* dominance to *Actinobacteria* dominance, accompanied with the losses of important functional bacteria including PAO (*Candidatus Accumulibacter phosphatis*), AOB (*Nitrosomonas*), NOB (*Nitrospira*) and denitrifying bacteria (*Thauera*). Accumulation of nitrite and ammonia was observed during serious bulking, while the phosphorus removal performance was not decreased because *M. parvicella* could behave as a PAO. Our results demonstrated that sludge bulking is not just a solid–liquid separation issue, it may also affect process performance by changing relevant bacterial populations. So more efforts should be made in order to secure the stability of activated sludge processes.

Author contributions

Qian Li performed part of experiments; Juan Wang performed part of experiments, and was responsible for data analysis and manuscript preparation. Rong Qi, Valter Tandoi and Min Yang participated in the discussions for the research, and provided suggestions for the revision of the manuscript.

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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.procbio.2014.08.005.

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