Microbial community analysis involved in the aerobic/extended-idle process performing biological phosphorus removal

Tian-jing Zeng, Guo-jing Yang, Dong-bo Wang, Xiao-ming Li, Wei Zheng, Qi Yang and Guang-ming Zeng

ABSTRACT

Recently, it has been found that biological phosphorus removal can be achieved in an aerobic/extended-idle (AEI) process using both glucose and acetate as the sole substrate. However, the microbial consortiums involved in glucose-fed and acetate-fed systems have not yet been characterized. Thus the aims of this paper were to investigate the diversities and dynamics of bacterial communities during the acclimation period, and to quantify polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) in the systems. The phylogenetic analysis showed that the microbial communities were mainly composed of phylum *Proteobacteria*, *Bacteroidetes*, *Chlorobi* and another six kinds of unclassified bacteria. Fluorescence *in-situ* hybridization (FISH) analysis revealed that PAOs and GAOs accounted for 43 ± 7 and $16 \pm 3\%$ of all bacteria in the glucose-fed system, and 19 ± 4 and $35 \pm 5\%$ of total bacteria in the acetate-fed system, respectively. The results showed that the conventional PAOs could thrive in the AEI process, and a defined anaerobic zone was not necessarily required for putative PAOs growth.

Key words | biological phosphorus removal, fluorescence *in-situ* hybridization, polyphosphate accumulating organisms

Tian-jing Zeng
Guo-jing Yang
Xiao-ming Li
Wei Zheng
Qi Yang
Guang-ming Zeng
College of Environmental Science and Engineering,
Hunan University, Changsha 410082,
China;
Key Laboratory of Environmental Biology and Pollution Control (Hunan University),
Ministry of Education, Changsha 410082,
China

Dong-bo Wang (corresponding author)
State Key Laboratory of Pollution Control and Resources Reuse,
School of Environmental Science and Engineering,
Tongji University, 1239 Siping Road,
Shanghai 200092,
China
and
Jiangsu Tongyan Environmental Production Science and Technology Co. Ltd.,
Yancheng 224000,
China
E-mail: *wwyy44339745@yahoo.com.cn*

Xiao-ming Li
School of Environment Studies,
Guangxi University, Nanning 530004,
China

INTRODUCTION

Inorganic phosphate (Pi) is a principal limiting nutrient in freshwater ecosystems, and excessive Pi supply to freshwater negatively affects water quality and ecosystem balance through a process known as eutrophication. To reduce Pi levels in freshwater ecosystems, efficient and reliable Pi removal technologies are required. Because of the massive quantity of wastewater treated daily, any improvements in existing methods should have tangible economic and ecological consequences.

Aquatic Pi levels can be reduced through a process called enhanced biological phosphorus removal (EBPR), where Pi is removed from the wasted biomass as the biopolymer polyphosphate (poly-P) (Martín *et al.* 2006). Such a process is based on the usual consideration that the biomass needs to be recycled repeatedly through alternating anaerobic and aerobic (A/O) stages (Mino *et al.* 1998). So far, EBPR installations have been extensively used throughout the world since its fortuitous discovery in the 1950s (Srinath *et al.* 1959). When operated successfully, the A/O process is a relatively inexpensive and environmentally sustainable option for Pi removal. However, even operated under seemingly favorable operational conditions, deteriorations and even failures in Pi removal performances have been widely reported (Blackall *et al.* 2002). Especially in the temperate regions where the concentrations of volatile fatty acids (VFAs) in influent are low, the EBPR system is more prone to failure due to the excessive growth of glycogen-accumulating

doi: 10.2166/wst.2012.578

organisms (GAOs) (Nielsen *et al.* 1999; Wong *et al.* 2004). In these cases, periodic organic matter supplementation and/or chemical assistance may be required to attain compliance; as a result, the operational costs increase.

Our recent studies on possible alternatives to the conventional EBPR process have proved that biological phosphorus removal can be achieved in activated sludge with an aerobic/extended-idle (AEI) regime using both glucose and acetate as the sole carbon source (Wang *et al.* 2008, 2009). In contrast to the A/O process, a strict anaerobic phase was not conducted whereas an extended idle zone (e.g. 210 min) was operated between the decanting phase and the next aerobic phase. Although the extended-idle zone was also not aerated, mixture stirring was not required during this phase, showing that the AEI process is simpler than the A/O process. The AEI process might be an alternative supplement to the current EBPR and have a promising future in application due to simple operation control and tolerance of high nitrate levels (Wang *et al.* 2012a).

Further, the interesting finding might make a contribution to the current biological Pi removal theory if the mechanism of Pi removal behind the AEI regime can be explained clearly based on the microbial consortiums analysis. Though the most accepted polyphosphate-accumulating organism (PAO) *Candidatus Accumulibacter phosphatis* and GAOs have already been found to be dominant in an acetate-fed AEI reactor (Wang *et al.* 2012b), the microbial communities involved in the glucose-fed and acetate-fed systems have not yet been identified in detail, and these systems still remain 'black boxes' with limited understanding of them, leading to several questions. What are the structures of the communities in both systems? Are the microorganisms of the two systems the same? What levels do the main cells such as *Accumulibacter*, *Competibacter* and *Defluviicoccus*-related cells and other dominant bacteria, respectively, account for in the two communities?

To answer these questions, extensive molecular analysis of polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) combined with sequential DNA sequencing was conducted to detect the diversities and dynamics of bacterial communities during the acclimation period. Meanwhile, fluorescence *in-situ* hybridization (FISH) was applied to further quantify *Accumulibacter* (PAOs), *Competibacter* and *Defluviicoccus*-related cells (GAOs) and other dominant bacteria in these systems.

MATERIALS AND METHODS

Experimental device and synthetic media

Experiments were carried out in two reproductive sequencing batch reactors (SBRs) with a working volume of 12 L, which were made of lucite.

The G-SBR and A-SBR were respectively fed with glucose and acetate, which were mostly considered as detrimental and favorable substrates for EBPR (Mino *et al.* 1998; Oehmen *et al.* 2007), but they had almost the same influent amount of carbon element (0.36 g $C_6H_{12}O_6$ L^{-1} and 0.50 g CH_3COONa L^{-1}, respectively, implying about 11.97 and 11.96 mmol of C L^{-1}) and Pi concentration (15 mg L^{-1}). The concentrations of the other nutrients in the synthetic media fed to the two SBRs were the same (per liter): 0.12 g NH_4Cl, 0.01 g $MgSO_4{\cdot}7H_2O$, 0.005 g $CaCl_2$, and 0.5 mL of a trace metals solution. The trace metals solution containing the metal chelator of ethylenediaminetetraacetic acid has been described in a previous publication (Wang *et al.* 2008).

Operational methods

Activated sludge, taken from the first municipal wastewater treatment plant of Changsha, China, was inoculated to G-SBR and A-SBR simultaneously, and was acclimated according to the way described below. The 720 min cycles of both SBRs consisted of 240 min aerobic period, 28 min settling, 2 min decanting and 450 min idle periods, approximately. Synthetic wastewater was fed to the reactor during the first 2 min of the aerobic period. A volume of 7.8 L supernatant was discharged after the settling period for each SBR cycle, resulting in a hydraulic retention time of 18 h approximately. During aerobic phase, dissolved oxygen (DO) concentration was controlled at 3 ± 0.2 mg L^{-1} using an on/off control valve that was connected with a compressed air supply. The pH was controlled at 7–8 during aerobic period through addition of 0.5 M HCl and 0.5 M NaOH. The approximate 20 days of sludge retention time (SRT) was maintained in two SBRs by withdrawing 0.6 L of the sludge mixtures (once per day) from the reactors at the end of the aerobic period but before settling.

Chemical analytical methods

Sludge samples from the reactors were immediately filtered through a Whatmann GF/C glass microfiber filter (1.2 μm).

The filtrate was analyzed for total phosphorus (TP) and total organic carbon (TOC), and the filter was assayed for mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), glycogen and poly-hydroxyalkanoates (PHAs). For analysis of cell glycogen and PHAs, 0.5 mL of 1 N hydrochloric acid was immediately added to the samples to stop the bacterial activity. Glycogen was measured by the phenol–sulfuric method with glucose as standard (Wang *et al*. 2009). The analysis of poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV), and poly-3-hydroxy-2-methylvalerate (PH2MV) was described in our previous paper (Wang *et al*. 2009). The total PHAs was calculated as the sum of measured PHB, PHV, and PH2MV.

TP, MLVSS and MLSS were measured according to Standard Methods (APHA 1995), and DO and TOC were determined using a DO analyzer (Oxi-3210, Germany) and a TOC analyzer (ShimadzuTOC-500, Japan), respectively. Element analysis of sludge sample was conducted by an analyzer of scanning electron microscope with X-ray energy dispersive microanalysis (JSM-5910, Japan) after lyophilization pretreatment.

Microbial characterization

(1) Sludge sampling, DNA extraction and PCR amplification
10 mL of sludge sample from reactors was centrifuged at a speed of 3,500 rpm for 10 min, and then samples were frozen at −20 °C for further analysis. DNA was extracted with protease K and cetrimonium bromide (CTAB) according to the method of Yang *et al*. (2007). Cycle conditions for the amplification were as follows: 5 min at 94 °C, 35 cycles with each cycle consisting of 40 s at 94 °C, 40 s at 55 °C and 45 s at 72 °C, followed by a final 7 min extension at 72 °C.

(2) DGGE screening
The DGGE profile of the PCR-amplified DNA was obtained following the method of Muyzer *et al*. (1993) using a DCode™ Universal Mutation Detection System (Bio-Rad). The acrylamide concentration in the gel was 6% and the denaturing gradient was 30–60%. The 100% denaturant solution contains 7 mol L^{-1} urea, 40% (v/v) formamide, 8% acrylamide/bis-acrylamide (37.5: 1) and 0.5× TAE buffer (pH 8) in Milli-Q water. The 0% denaturant solution contains 8% acrylamide/bis-acrylamide (37.5:1) and 0.5× TAE buffer (pH 8) in ultrapure water. Thirty microliters of purified PCR products mixed with 6 μL loading buffer were transferred to the bottom of the sample holes in the gel. Gels were run in 0.5× TAE buffer at 55 °C for 15 h at 140 V. Gels were stained with SYBR™ Green I and were digitized in UV light with the Gel Doc 2000 System.

(3) DNA sequencing and phylogenetic analysis
Bands were excised from DGGE gel under UV light, and a bead beating method was sequentially applied to extract DNA. The purified DNA was re-amplified using the primer pair GC341f/907r, and the products were ligated into the Target Clone (Toyobo, Japan).

Phylogenetic identity was determined by comparing the cloned partial 16S rRNA gene fragment sequences with those of the reference microorganisms available in the GenBank by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

(4) Nucleotide sequence accession numbers
The nucleotide sequence data reported in this paper have been submitted to the GenBank using the program Sequin win32 and have been assigned the following accession numbers: GU123142-GU123162 and GU191145.

(5) FISH analyses
Sludge samples (taken from the reactors at the end of aerobic phase during steady operation period) were fixed in 4% formaldehyde for 20 h at 4 °C, and then subjected to freeze–thaw treatment to improve the penetration of oligonucleotide probes. Cells were attached to poly-L-lysine-coated slides and dehydrated by sequential washes in 50, 80, and 100% ethanol (3 min each). The hybridization and washing procedures were the same as those described by Nielsen *et al*. (1999). The following oligonucleotide probes, EUBmix labeled with 5′FITC (containing EUB338-I, EUB338-II and EUB338-III, specific for most bacteria), PAOmix labeled with 5′AMCA (containing PAO462, PAO651 and PAO846, specific for *Accumulibacter*) and GAOmix labeled with 5′Cy3 (containing GAOQ431, GAOQ989 and GB_G2, specific for *Candidatus Competibacter phosphates*; TFO-DF218, TFO-DF618, DF988, DF1020, specific for *Defluviicoccus*-related organisms) were used for hybridization and are listed in Supporting information Table S1 (available online at http://www.iwaponline.com/wst/067/578.pdf). Additionally, the Zra (5′- CTGCCGTACTCTAGTTAT-3′) probe (Rosselló-mora *et al*. 1995) labeled with 5′Cy3 was used to target *Zoogloea* sp. For quantitative FISH analysis, at least 20 microscopic fields were analyzed for the hybridization of individual probes using a confocal scanning laser microscope (FV 500) with image database software (VideoTesT Album3.0). Each

was expressed as a percentage of the total area fluorescing with the EUBmix probes.

RESULTS AND DISCUSSION

SBR performance during steady-state operation

As shown in Table 1, low levels of TOC and NH_4^+-N in effluent were determined in both glucose-fed (G-SBR) and acetate-fed (A-SBR) reactors, and the significant increases of effluent NO_3^--N concentrations indicated that nitrification occurred in both reactors. By comparing the influent NH_4^+-N with the effluent NH_4^+-N, NO_2^--N, and NO_3^--N, it can be concluded that denitrification occurred in both parent reactors. This point can be supported by the presence of some denitrifiers in the microbial communities (Figure 1 and Table 2). The reason for this denitrification presence might be due to some small granules forming in the systems, which resulted in some DO gradient. In addition, since each reactor was aerated by an air diffuser, DO concentration in some regions may be below 3 ± 0.2 mg L^{-1}, which also caused denitrification. Effluent TP concentrations in G-SBR and A-SBR demonstrated that excess TP removal was achieved in both systems in excess of metabolic requirement. Moreover, elemental analysis of sludge samples from two reactors after 93 days of operation also showed that there were substantial accumulations of phosphorus in activated sludge in both systems (Supporting information Figure S1, http://www.iwaponline.com/wst/067/578.pdf). The facts explained that biological phosphorus removal was obtained in both SBRs.

Table 1 | Summary of performance parameters in two SBRs during steady-state operation

	G-SBR	A-SBR
TOC in effluent (mmol L^{-1})	[a]0.13 ± 0.03	0.09 ± 0.02
TOC removal efficiency (%)	98.9 ± 0.2	99.2 ± 0.2
TP in effluent (mg L^{-1})	0.15 ± 0.08	3.53 ± 0.85
TP removal efficiency (%)	99.0 ± 0.5	78.0 ± 5.9
NH_4^+-N in effluent (mg L^{-1})	0.61 ± 0.49	0.72 ± 0.34
NH_4^+-N removal efficiency (%)	98.1 ± 1.9	97.7 ± 1.3
NO_2^--N in effluent (mg L^{-1})	0.22 ± 0.13	0.18 ± 0.09
NO_3^--N in effluent (mg L^{-1})	7.2 ± 3.5	10.1 ± 2.9
MLSS (mg L^{-1})	6,757 ± 633	4,136 ± 607
MLVSS (mg L^{-1})	4,428 ± 571	3,243 ± 426

[a]Results are mean values ± standard deviation (SD) obtained over a two week period after stabilization for 60 days (more than three times the SRT).

Sequencing results and phylogenetic analysis

Twenty-two distinct bands were excised for further PCR re-amplification.

Figure 1 illustrates the DGGE image of the PCR-amplified 16S rDNA genes from the acclimation phase. There

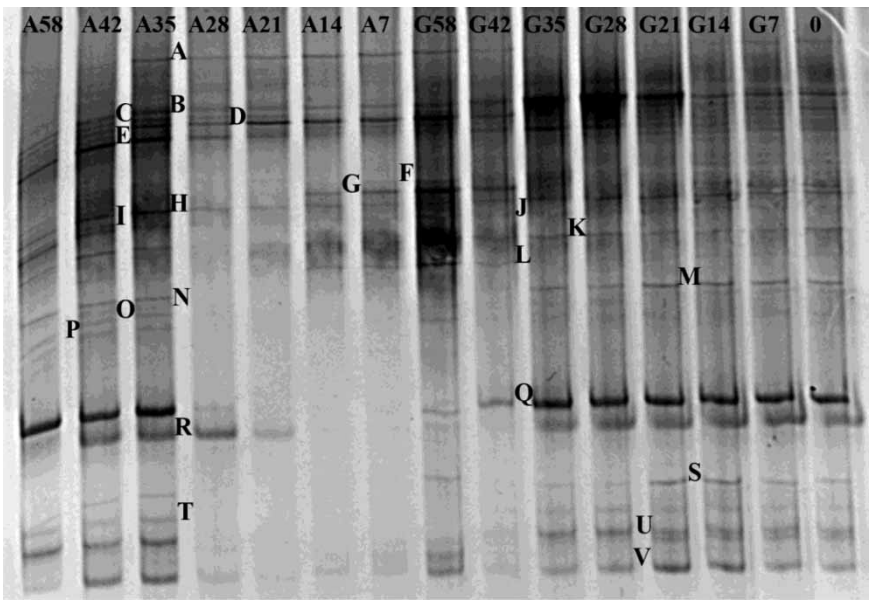

Figure 1 | DGGE profiles of 16S rDNA fragments obtained from the acclimation phase of two SBRs. 0: seed sludge; Gi: sludge sample obtained from G-SBR on day *i*; Ai: sludge sample obtained from A-SBR on day *i*. The letters represent different bands in the figure.

Table 2 | Phylogenetic analysis of the dominant DGGE bands from the glucose-fed and acetate-fed systems in comparison to the clone library

			Phylogenetic relationship			
Band	**Sequence length (bp)**	**Accession**	**Closest relatives**	**Accession**	**Similarity (%)**	**Phylogenetic division**
A	565	GU123142	Uncultured unclassified bacterium 16S rRNA gene from clone QEEB2BC05	CU917960	90	
B	579	GU123144	Uncultured *Saprospiraceae* bacterium clone Epr129 16S ribosomal	EF523446	98	*Bacteroidetes*
C	579	GU123147	Uncultured *Saprospiraceae* bacterium clone Epr129 16S ribosomal	EF523446	98	*Bacteroidetes*
D	588	GU123152	*Zoogloea ramigera* gene for 16S ribosomal RNA	D14257	99	*Betaproteobacteria*
E	588	GU123158	*Zoogloea* sp. EMB 357 16S ribosomal RNA gene, partial sequence	DQ413172	99	*Betaproteobacteria*
F	564	GU123149	Environmental 16s rDNA sequence from Evry wastewater treatment plant anoxic basin	CU466712	97	
G	565	GU191145	Environmental 16s rDNA sequence from Evry wastewater treatment plant anoxic basin	CU466712	97	
H	579	GU123162	Environmental 16s rDNA sequence from Evry wastewater treatment plant anoxic basin	CU466728	99	
I	579	GU123150	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence	AB231424	99	
J	586	GU123159	Uncultured *Betaproteobacteria* bacterium 16S rRNA gene from clone	CU926136	95	*Betaproteobacteria*
K	577	GU123155	Uncultured *Chlorobi* bacterium 16S rRNA gene from clone QEDN2DF11	CU927353	99	*Chlorobi*
L	586	GU123156	*Zoogloea resiniphila* partial 16S rRNA gene, strain PIV-3C2y	AJ505854	93	*Betaproteobacteria*
M	582	GU123153	Uncultured *Bacteroidetes* bacterium 16S rRNA gene	CU927760	94	*Bacteroidetes*
N	587	GU123161	*Azoarcus* sp. NSC3 16S ribosomal RNA gene, partial sequence	GQ389714	97	*Betaproteobacteria*
O	588	GU123154	*Dokdonella* sp. CC-YHH031 16S ribosomal RNA gene, partial sequence	GQ281768	93	*Gammaproteobacteria*
P	587	GU123160	Uncultured betaproteobacterium clone REV_R1PII_9A 16S	FJ933475	97	*Betaproteobacteria*
Q	586	GU123148	Uncultured *Nitrosomonadaceae* bacterium partial 16S rRNA gene, clone	AM934971	92	*Betaproteobacteria*
R	588	GU123151	Uncultured *Rhodocyclaceae* bacterium partial 16S rRNA gene, clone Z7	AM268345	97	*Betaproteobacteria*
S	587	GU123157	*Thauera* sp. TS4 16S ribosomal RNA gene, partial sequence	EU073070	99	*Betaproteobacteria*
T	578	GU123143	*Runella* sp. HMD1032 16S ribosomal RNA gene, partial sequence	GQ144416	97	*Sphingobacteria*
U	586	GU123146	Uncultured *Burkholderiaceae* bacterium partial 16S rRNA gene, clone	AM936570	92	*Betaproteobacteria*
V	565	GU123145	Uncultured bacterium clone N1903_10 16S ribosomal RNA gene, partial	EU104270	90	

existed many visible bands showing complexity and diversity of microbial ecology in the sludge samples, and the bacterial community structure in G-SBR was different from that in A-SBR. Twenty-two distinct bands were excised for further PCR re-amplification. Table 2 summarizes the sequence length, the closest species found in the GenBank and the degree of similarity by Blast analysis. Among these sequences, 12 bacteria clones belonged to phylum *Proteobacteria* (10 to *Betaproteobacteria*, one to *Gammaproteobacteria* and one to *Sphingobacteria*), three bacteria clones belonged to phylum *Bacteroidetes* and one sequence belonged to phylum *Chlorobi*. In addition, there were another six bacteria clones that could not affiliate to a putative group.

Sequencing results shown in Table 2 and DGGE profiles displayed in Figure 1 revealed that members of *Betaproteobacteria* were extensively represented in both communities. The *Betaproteobacteria* class plays important roles in organic degradation, nitrogen and phosphorus removal as well as floccule formation, and a substantive domination of *Betaproteobacteria* has been observed in many natural environments (drinking water), engineered systems (activated sludge) and anaerobic digestion of sludge (Rivière *et al*. 2009). In seed sludge, bands Q and R showed intense fluorescent signals. Band R showed 97% of sequence similarity to family *Rhodocyclaceae*, which is generally considered as PAOs widely existing in the A/O process (Ahn *et al*. 2007). Band Q was most closely related to family *Nitrosomonadaceae* with 92% similarity. The group *Nitrosomonadaceae* are ammonia-oxidizing bacteria and are commonly present in natural and engineered environments. Although it was uncertain whether band Q was a member of *Nitrosomonadaceae* because of its 92% similarity, a high level of nitrate concentration in effluent demonstrated its occurrence in both mature communities (Table 1). Moreover, two organisms affiliated with *Betaproteobacteria* were determined with low fluorescent signals in both communities (bands N and S were closely related to genus *Azoarcus* sp. and *Thauera* sp. with 97 and 99% similarities, respectively). Preliminary results showed that *Betaproteobacteria* related to *Azoarcus–Thauera* complex were probably abundant denitrifiers in activated sludge, and their presence in two communities indicated that denitrification might occur to a certain extent. Notably, one species of *Zoogloea* sp. (band E) and two strains of this species, *Zoogloea ramigera* (band D) and *Zoogloea*

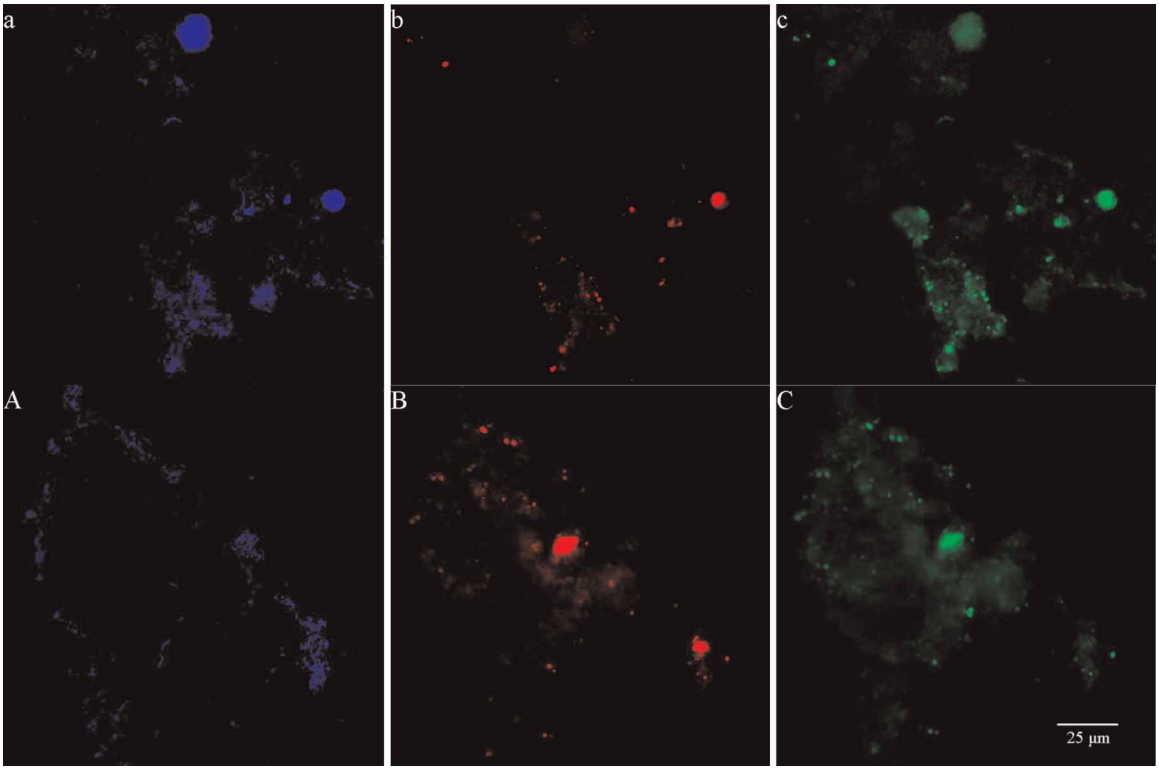

Figure 2 | FISH micrographs of bacteria communities from glucose-fed system (a–c) and acetate-fed system (A–C) hybridizing with PAOmix (a and A), GAOmix (b and B) and EUBmix (c and C) probes specific for *Accumulibacter* (PAOs), *Competibacter* and *Defluviicoccus*-related organisms (GAOs) and the dominant bacteria, respectively. Samples were obtained after steady operation (on day 118).

resiniphila (band L), were detected with intensified fluorescent signals in lanes A58 and G58, respectively. *Zoogloea* are floc-forming bacteria commonly found in the activated sludge and can grow under aerobic and/or anaerobic conditions (Tsai *et al*. 2005).

Band O showed 93% of similarity to *Dokdonella* sp., which belonged to class *Gammaproteobacteria*. The genus *Dokdonella* is usually present in industrial wastewater treatment plant. Band T was most closely related to genus *Runella* of class *Sphingobacteria* with 97% similarity, and this genus has been observed in industrial wastewater treatment process and aerobic granulation system.

Band B was affiliated with family *Saprospiraceae* of phylum *Bacteroidetes* with 98% similarity, and band M showed 94% of similarity to phylum *Bacteroidetes*. The phylum *Bacteroidetes* extensively exists in waste activated sludge (Zhou *et al*. 2010). Band K was most closely related to phylum *Chlorobi* and it has been previously detected in some Pi removal ecosystems. Furthermore, there were another six unclassified bacteria and their characters are unclear so far.

FISH analysis

The other concern was the quantifications of *Accumulibacter* (PAOs) and *Competibacter* and *Defluviicoccus*-related organisms (GAOs) in the AEI systems. Through applying a set of probes targeting the PAO and GAO groups, it was demonstrated that *Accumulibacter* (PAOs) ($43 \pm 7\%$ of total bacteria) were present in relatively higher abundance in the glucose-fed reactor than the *Competibacter* and *Defluviicoccus*-related organisms (GAOs) ($16 \pm 3\%$ of total bacteria, Figure 2a–c). Nevertheless, *Accumulibacter* (PAOs) only accounted for $19 \pm 4\%$ of the bacterial

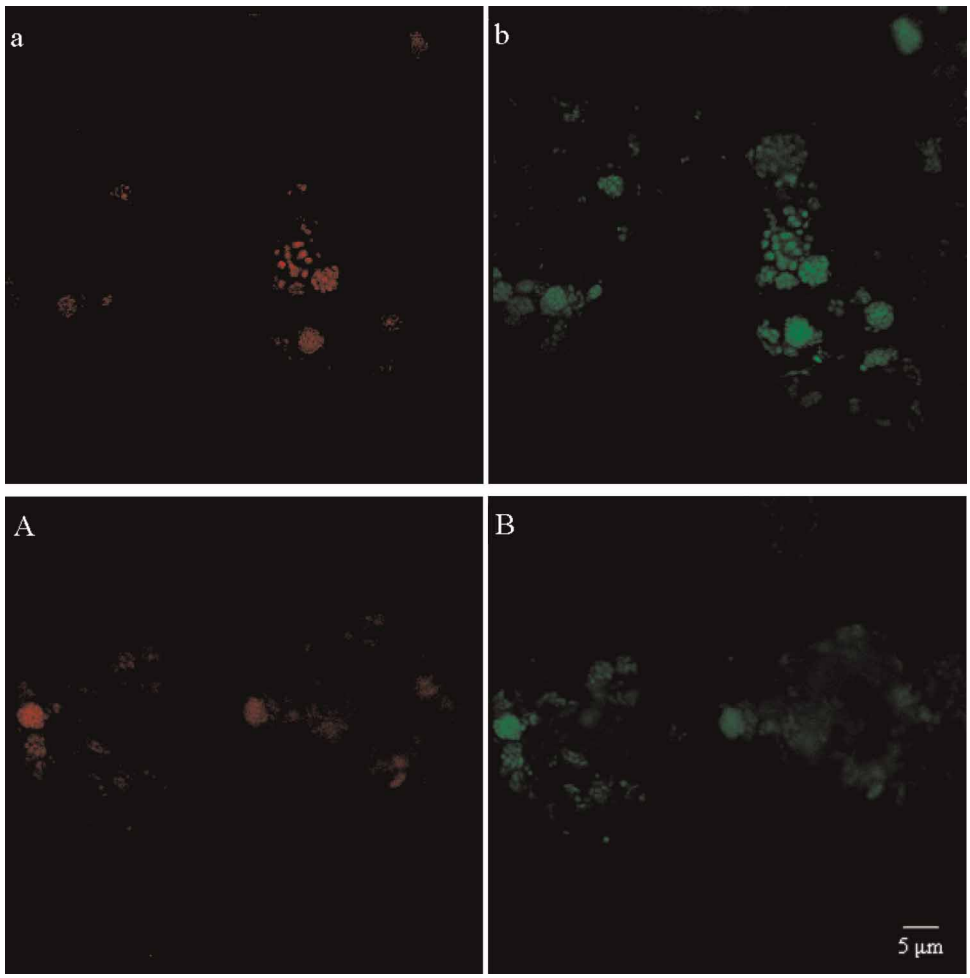

Figure 3 | FISH micrographs of activated sludge (On day 118) from glucose fed system (a, b) and acetate fed system (A, B) hybridizing with Zra (a and A) and EUBmix (b and B) probes specific for *Zoogloea* sp. and the dominant bacteria, respectively. Samples were obtained after steady operation.

population and GAOs were present in a high abundance in acetate-fed reactor (35 ± 5% of all bacteria, Figure 2 A–C). The results correlated well with the observed phosphorus removal performance of two systems, and showed that the conventional PAOs could thrive in both glucose-fed and acetate-fed AEI processes, and a defined anaerobic zone was not necessarily required for putative PAOs growth. Recently, other researchers have found the conventional PAOs can grow under a wide variety of environments (Ahn *et al*. 2007).

Furthermore, FISH quantification revealed that both reactors contained plenty of *Zoogloea* sp., which accounted for 27 ± 4 and 36 ± 6% of all bacteria in the sludge, respectively (Figure 3). *Zoogloea* sp., one species of aerobic Gram-negative bacteria, is found to exist extensively in organically enriched aqueous environments, and has long been considered the typical activated sludge bacteria responsible for the formation of activated sludge flocs. Roinestad & Yall (1970) reported that a strain of this species (*Zoogloea ramigera*) was capable of forming poly-P when excess Pi was added to a Pi-starved culture under pure cultured conditions; thus it might be a potential PAO in the AEI systems. However, as yet this capability has never been identified in activated sludge mixtures or processes; further investigations are required to confirm it.

CONCLUSIONS

This paper displayed a significant novel microbial consortium involved in the AEI process performing biological phosphorus removal. The microbial communities in these systems were mainly composed of phyla *Proteobacteria*, *Bacteroidetes*, *Chlorobi* and another six kinds of unclassified bacteria. FISH analysis showed that PAOs and GAOs accounted for 43 ± 7 and 16 ± 3% of all bacteria in the glucose-fed system, and 19 ± 4 and 35 ± 5% of total bacteria in the acetate-fed system, respectively.

ACKNOWLEDGEMENTS

This research was financially supported by the project of National Natural Science Foundation of China (Grant No. 51078128), Shanghai Postdoctoral Scientific Program (12R21415700), China Postdoctoral Science Foundation (2012M510888), National Science Foundation of Jiangsu Province (BK2012253) and Zhejiang Provincial Natural Science Foundation of China (LQ12E08001).

REFERENCES

Ahn, J., Schroeder, S., Beer, M., McIlroy, S., Bayly, R. C., May, J. W., Vasiliadis, G. & Seviour, R. J. 2007 Ecology of the microbial community removing phosphate from wastewater under continuously aerobic conditions in a sequencing batch reactor. *Appl. Environ. Microbiol.* **73**, 2257–2270.

APHA (American Public Health Association) 1995 *Standard Methods for the Examination of Water and Wastewater*, 19th edn. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.

Blackall, L. L., Crocetti, G., Saunders, A. M. & Bond, P. L. 2002 A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants. *Anton. Leeuw.* **81**, 681–691.

Martín, H. G., Ivanova, N., Kunin, V., Warnecke, F., Barry, K. W., McHardy, A. C., Yeates, C., He, S., Salamov, A. A., Szeto, E., Dalin, E., Putnam, N. H., Shapiro, H. J., Pangilinan, J. L., Rigoutsos, I., Kyrpides, N. C., Blackall, L. L., McMahon, K. D. & Hugenholtz, P. 2006 Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. *Nat. Biotechnol.* **24**, 1263–1269.

Mino, T., van Loosdrecht, M. C. M. & Heijnen, J. J. 1998 Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Res.* **32**, 3193–3207.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. 1993 Profiling of complex microbial population by DGGE analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. *Appl. Environ. Microbiol.* **62**, 2676–80.

Nielsen, A. T., Liu, W. T., Filipe, C., Grady, L., Molin, S. & Stahl, D. A. 1999 Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. *Appl. Environ. Microbiol.* **65**, 1251–1258.

Oehmen, A., Lemos, P. C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L. L. & Reis, M. A. M. 2007 Advances in enhanced biological phosphorus removal: from micro to macro scale. *Water Res.* **41**, 2271–2300.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P. & Sghir, A. 2009 Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *ISME J.* **3**, 1–15.

Roinestad, F. A. & Yall, I. 1970 Volutin granules in *Zoogloea ramigera*. *Appl. Microbiol.* **19**, 973–979.

Rosselló-mora, R. A., Wagner, M., Amann, R. & Schleifer, K. 1995 The abundance of *Zoogloea ramigera* in sewage treatment plants. *Appl. Environ. Microbiol.* **61**, 702–707.

Srinath, E. G., Sastry, C. A. & Pillai, S. C. 1959 Rapid removal of phosphorus from activated sludge. *Experimentia* **15**, 339–343.

Tsai, Y. P., You, S. J., Pai, T. Y. & Chen, K. W. 2005 Effect of cadmium on composition and diversity of bacterial communities in activated sludges. *Int. Biodeter. Biodegr.* **55**, 285–291.

Wang, D., Li, X., Yang, Q., Zeng, G., Liao, D. & Zhang, J. 2008 Biological phosphorus removal in sequencing batch

reactor with single-stage oxic process. *Bioresour. Technol.* **99**, 5466–5473.

Wang, D., Li, X., Yang, Q., Zheng, W., Liu, Z., Liu, Y., Cao, J., Yue, X., Shen, T., Zeng, G. & Deng, J. 2009 The probable metabolic relation between phosphate uptake and energy storages formations under single-stage oxic condition. *Bioresour. Technol.* **100**, 4005–4011.

Wang, D., Li, X., Yang, Q., Zheng, W., Wu, Y., Zeng, T. & Zeng, G. 2012a Improved biological phosphorus removal performance driven by the aerobic/extended-idle regime with propionate as the sole carbon source. *Water Res.* **46**, 3868–3878.

Wang, D., Yang, G., Li, X., Zheng, W., Wu, Y., Yang, Q. & Zeng, G. 2012b Inducing mechanism of biological phosphorus removal driven by the aerobic/extended-idle regime. *Biotechnol. Bioeng.* **109**, 2798–2807.

Wong, M. T., Tan, F. M., Ng, W. J. & Liu, W. T. 2004 Identification and occurrence of tetrad-forming *Alphaproteobacteria* in anaerobic–aerobic activated sludge processes. *Microbiology* **150**, 3741–3748.

Yang, Z. H., Xiao, Y., Zeng, G. M., Xu, Z. Y. & Liu, Y. S. 2007 Comparison of methods for total community DNA extraction and purification from compost. *Appl. Microbiol. Biotechnol.* **74**, 918–925.

Zhou, S., Zhang, X. & Feng, L. 2010 Effect of different types of electron acceptors on the anoxic phosphorus uptake activity of denitrifying phosphorus removing bacteria. *Bioresour. Technol.* **101**, 1603–1610.

First received 28 March 2012; accepted in revised form 29 August 2012