Monitoring microbial community structure and succession of an A/O SBR during start-up period using PCR-DGGE

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Abstract

Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) protocol was employed for revealing microbial community structure and succession in a sequential anaerobic and aerobic reactor performing enhanced biological phosphorus removal (EBPR) during start-up period. High phosphorus removal was achieved after 15 d. On day 30, phosphorus removal efficiency reached to 83.2% and the start-up was finished. DGGE profiles of periodical sludge samples showed that dominant microbial species were 19 OTUs (operational taxonomy units). Unweighted pair-group method using arithmetic averages (UPGMA) clustering analysis revealed that rapid community succession correlated to low phosphorus removal rate and high phosphorus removal efficiency reflected on steady community structure. Sequencing results indicated that determined sequences (12 OTUs) belonged to Proteobacterium, Actinobacteria, Gemmatimonadales and unaffiliate group. *Proteobacterium*, *Tetrasphaera elongate* and *Gemmatimonas aurantiaca* may act important roles in phosphorus removal. With little amount as known glycogen accumulating organisms, *Candidatus* Competibacter phosphatis still at accumulating-phase had limited effect on microbial community structure. When climax community was obtained, dominant microbes were 14 OTUs. Microbes in a large amount were uncultured bacterium *Thauera* sp., uncultured γ-*Proteobacterium* and *Tetrasphaera elongata*.

Key words: biological phosphorus removal; polyphosphate accumulating organisms; glycogen accumulating organisms

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Introduction

Because of an increased eutrophication of lakes and reservoirs, the enhanced biological phosphorus removal (EBPR) process is widely implemented in wastewater treatment plants to remove phosphorus, a limiting nutrient in algal blooms. In activated sludge polyphosphate accumulating organisms (PAOs) are capable of storing orthophosphate as intracellular polyphosphate, leading to phosphorus removal via biomass wasting. However, in deteriorated EBPR systems, glycogen accumulating organisms (GAOs) are often observed in a large amount (Thomas *et al.*, 2003). The biochemistry pathway of GAOs is similar to PAOs except for performing anaerobic phosphorus release or aerobic phosphorus uptake. Thus GAOs do not contribute to phosphorus removal but increase the anaerobic VFA (volatile fatty acid) requirements (Saunders *et al.*, 2003). Studies have found that a high COD/P ratio (e.g., >50 mg/mg) in the wastewater feed tends to favor the growth of GAOs instead of PAOs (Mino *et al.*, 1998). A low COD/P ratio (e.g., 10–20 mg/mg) should be more favorable for the growth of PAOs. The amount of volatile fatty acids (VFAs), the carbon source, pH and the

temperature also effect the microbial competition (Oehmen *et al.*, 2007). GAOs have indeed been found in numerous full-scale EBPR plants (Crocetti *et al.*, 2002; Burow *et al.*, 2007). Minimizing the growth of GAOs in the EBPR systems has been a hot topic recently.

Since Fuhs and Chen firstly isolated *Acinetobacter* of γ-*Protebacteria* from high P removal capacity biomass based on culture-dependent techniques, isolation of PAOs was carried out for decades (Mino *et al.*, 1998). *Microlunatus phosphovorus*, *Lampropedia* spp. etc., were ever considered as functional PAOs and then denied because of their disagreement with biochemistry pathway of PAOs (Nakamura *et al.*, 1995; Santos *et al.*, 1999; Stante *et al.*, 1997; Seviour *et al.*, 2003).

Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) was firstly applied to molecular ecology by Muyzer *et al.* (1993). It is particularly effective in analyzing the microbial community structure of non-culturable environmental samples such as microorganisms responsible for EBPR. It showed that a high diversity of phylogenetic groups presented in lab- and full-scale EBPR sludges (Seviour *et al.*, 2003) which verified EBPR system is a complex microbial community. γ-*Proteobacteria*, β-*Protebacteria* and *Actinomyces* sp. etc.

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were reported to be responsible for EBPR (Onuki *et al.*, 2002; Schuler *et al.*, 2002; Nielsen *et al.*, 1999; Yu *et al.*, 2005).

Since process performance is likely to be strongly effected by the microbial composition, it is recommended that further studies be devoted in linking operational factors and phosphorus removal performance with microbial community structure predicting microbial population dynamics in EBPR system. The function of various microbes in forming a steady microbial community for better phosphorus removal is not clear. Furthermore, the succession of microbial community has not been well described, from which the information about adaptability of PAOs switched into high phosphorus content environment will be obtained. For this, an anaerobic-aerobic sequencing batch reactor (A/O SBR) to achieve better phosphorus removal with acetate as the carbon source was used. PCR-DGGE protocol was employed for revealing microbial community structure and succession during start-up period. A medium COD/P ratio (30 mg/mg) was investigated to determine the microbial competition of PAOs with GAOs.

1 Materials and methods

1.1 Reactor operation

A laboratory-scale A/O SBR with 2.5 L working volume was operated at (22 ± 1)°C to achieve EBPR. Seed sludge was taken from aerobic tank of Wenchang WWTP (waste water treatment plant) in Harbin, China. The running cycle of the reactor was 6 h consisting of a 90-min anaerobic period (8-min filling period included), a 240-min aerobic period, a 15-min sludge settling period and another 15-min for replacing supernatant. The components of synthetic wastewater (pH 7.0 ± 0.2) were as follows (per 50 L): NaAc·3H$_2$O 43.81 g, NH$_4$Cl 3.7 g, KH$_2$PO$_4$ 1.389 g, K$_2$HPO$_4$ 2.076 g, MgSO$_4$·7H$_2$O 2.5 g, CaCl$_2$·2H$_2$O 1.2 g, Peptone 1.25 g, trace elements liquid 15 mL. COD and PO$_4^{3-}$-P were 300 and 10 mg/L, respectively.

1.2 Chemical analysis

COD (chemical oxygen demand), MLSS (mixed liquor suspended solids), MLVSS (mixed liquor volatile suspended solids) were analyzed by standard methods (APHA, 1995). The PO$_4^{3-}$, NO$_3^-$ and NO$_2^-$ concentrations of the supernatant were measured by ion chromatography (DIONEX ICS-3000, USA) with an ion-pac AS11-HC column. TOC was measured by a TOC analyzer (TOC-VCPN, Shimadzu, Japan).

1.3 Microbial community analysis

1.3.1 Sampling, sample preparation and DNA extraction

The sludge samples of 0 (seed sludge), 6, 11, 15, 19, 26, and 30 d during start-up period were taken from the aerobic end, and stored in –20°C immediately. As all the sampling were finished, thawed the samples in 37°C water bath, then centrifuged at 9000 ×*g* (Beckman 64R, J-25, USA) in 1.5 mL eppendorf tube for 5 min. The supernatant

was removed and the sediment was cleaned up twice with PBS (phosphate buffered saline) solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na$_2$HPO$_4$, 2 mmol/L KH$_2$PO$_4$, pH 7.4). The extraction of total genomic DNA was carried out by DNA Extraction Mini Kit (Watson, China) and detected by 0.8% (*W/V*) agarose gel electrophoresis.

1.3.2 PCR amplification

The region corresponding to positions 968 and 1401 in the 16S rDNA of *Escherichia coli* was PCR-amplified using the forward primer BSF968GC (5'-CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3') and reverse primer BSR1401 (5'-CCCCGTCAATTC-CTTTGAGTTT-3') (Nuebel *et al.*, 1996). The PCR amplification was conducted in an automated thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, USA). In order to improve the amplification specificity, Touchdown PCR was performed to provide sufficient DNA samples for DGGE, using the following protocol: initial denaturation of DNA for 5 min at 94°C; 30 cycles of 40 s at 94°C, 40 s at 55°C, and 30 s at 72°C; decreasing 0.1°C per cycle to 51.5°C, followed by a final extension for 8 min at 72°C. The PCR mixtures had a final volume of 30 μL which contained 3 μL of 10× Ex Taq buffer (containing Mg^{2+}), 0.6 μL of each primer (20 pmol/L), 1.5 μL dNTP (2.5 mmol/L), 100 ng template and 0.2 μL Ex Taq (Takara, Japan). After PCR amplification, PCR products were electrophoresed in 0.8% (*W/V*) agarose gel for determining amplification effort.

1.3.3 Denaturing Gradient Gel Electrophoresis

DGGE was performed using the Dcode^{TM} Universal Mutation Detection System (BioRad, USA). The PCR products were electrophoresed directly to 6% polyacrylamide gel in 1× TAE buffer containing a linear gradient ranging from 30% to 60% denaturant (denaturation of 100% corresponding to 7 mol/L urea and 40% (*V/V*) formamide). The gradient gel was cast with a gradient delivery system (Model 475, Bio-Rad, USA). Electrophoresis was run at 60°C with a constant voltage of 120 V. After 5 h of electrophoresis, the gel was stained with AgNO$_3$, and then visualized by a scanner (Power-Look1000, Umax, Taiwan, China).

1.3.4 Sequencing and cluster analysis

Prominent DGGE bands were excised from DGGE polyacrylamide gel for 16S rDNA fragment sequencing. The reamplified DNA fragments were purified by Gel Recovery Purification Kit (Watson, China) and cloned by the pMD19-T plasmid vector system (TaKaRa, Japan). The DNA sequences were determined using the chain-termination method in an ABI 3730 stretch sequencing system by a commercial service (Sangon, China). Vector sequence was cut off and the rest was compared in GenBank using BLASTN program to obtain the most similar 16S rDNA fragments. Cluster analysis of DGGE patterns was performed with unweighted pair-group method using arithmetic averages (UPGMA) clustering algorithm.

2 Results and discussion

2.1 Reactor performance

The start-up period of reactor lasted for a month. The MLSS of the reactor increased from 1902 to 2315 mg/L as the microorganism growth. The COD removal efficiency was about 85% without large fluctuation and outlet COD concentration was less than 100 mg/L for all the time. Phosphorus removal and anaerobic phosphorus release performance in the start-up period can be divided into two stages, as shown in Fig. 1. In the former 15 d, good phosphorus removal efficiency was not achieved (only from 38.3% to 49.0%) and anaerobic phosphorus release was not notable. At the following 15 d, phosphorus removal performance and phosphorus release increased rapidly. On day 30, phosphorus removal efficiency and anaerobic outlet phosphorus concentration reached to 83.2% and 19.22 mg/L, respectively.

2.2 DGGE profiles and UPGMA clustering analysis

The DGGE profiles of sludge samples in start-up period are shown in Fig. 2. Since each band can be considered as an operational taxonomy unit (OTU), the DGGE profiles
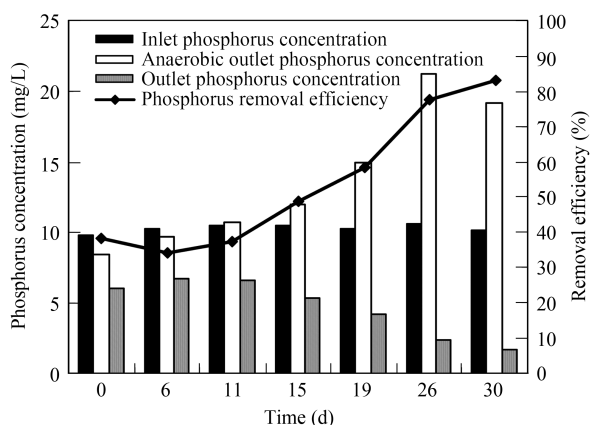

Fig. 1 Concentration and removal efficiency of PO_4^{3-}-P during start-up period.
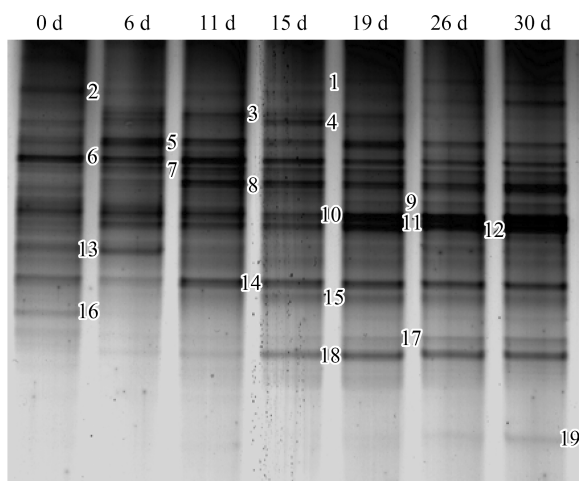

Fig. 2 DGGE profiles of sludge samples in start-up period.

showed that dominant microbial species were 19 OTUs, which indicated a diverse microbial community. Microbial community structure was much complex on day 0 while most microbes existed in few quantities.

Since DGGE profiles can make a half-quantitative analysis of microbes, relative quantities of various microbes during start-up period are shown in the profiles. The quantities of some microbes increased (band 8, 10, 12, 15, 18 etc.), whereas others decreased (band 3, 13, etc.) or even clear away (band 4, 16, etc.). There are still some microbes keeping their quantities, such as band 5, 6, and 14. Community structure changed a lot in the former 15 d while kept almost the same from then on. With the enrichment of some functional microbes, community structure was simple and steady at the end which led to better phosphorus removal.

The UPGMA clustering analysis was used to analysis community similarity among different time (Fig. 3). It showed that microbial community structure in seed sludge had the lowest similarity. The rapid change of community structure at the first 6 d resulted from phosphorus shock load. Synthetic wastewater (10 mg P/L) was unfit for existence of some microbes original from municipal wastewater (about 3.2 mg P/L). PAOs responsible for phosphorus removal tended to be dominant in reactor with high phosphorus. In the following time, community succession rate declined. Community structure of 6 and 11 d, 19 and 26 d was almost the same. The community structure of former 15 d was obviously different from latter 15 d. When compared with phosphorus removal effect (Fig. 1), a correlation between community structure and phosphorus removal effect can be detected. In the former 15 d, rapid community succession correlated to lower phosphorus removal rate, whereas higher phosphorus removal efficiency reflected on steady community structure after then.
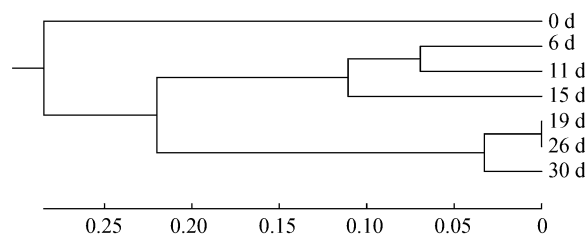

Fig. 3 UPGMA cluster analysis of DGGE profiles.

2.3 Sequencing results and functional analysis of microbes

16S rDNA fragments extracted from prominent DGGE bands were re-amplified, purified, cloned to PMD19-T vector, and sequenced. The closest relative strains available in the GenBank database were obtained using Blastn Program (Table 1). Among the 19 OTUs, the similarity between 12 OTUs and the closest sequences were more than 94%, which indicated that they may belong to the same genus. In these sequences, 6 bacteria clones belonged to *Proteobacterium* (1 to α-*Proteobacterium*; 2 to β-*Proteobacterium*; 3 to γ-*Proteobacterium*). Two bacteria clones were most similar to Actinobacteria and

Table 1 Partial 16S rDNA sequence similarity of bands 1–19 on DGGE profiles

Band	The closest sequences (GenBank number)	Similarity	Putative division
1	Uncultured α-*Proteobacterium* clone Ebpr9 (AF255630.1)	94%	α-*Proteobacterium*
2	Uncultured bacterium clone SBRQ157 (AF361092.1)	96%	–
3	Type 0803 filamentous bacterium strain Ben05B (X86070.1)	90%	–
4	*Brevundimonas* sp. dcm7A (AF430126.1)	90%	α-*Proteobacterium*
5	*Frigovirgula patagoniensis* (AF450134.1)	90%	Clostridia
6	*Thauera* sp. Al7 (AY570693.1)	90%	β-*Proteobacterium*
7	Uncultured *Verrucomicrobium* DEV055 (AJ401121)	96%	–
8	Uncultured bacterium FukuN108 (AJ289984.1)	94%	β-*Proteobacterium*
9	*Dyella ginsengisoli* strain LA-4 (EF191354.1)	97%	γ-*Proteobacterium*
10	*Thauera* sp. R-24450 (AM231040.1)	99%	β-*Proteobacterium*
11	*Nitrospira* sp. strain RC25 (Y14639.1)	91%	*Nitrospirales*
12	Uncultured γ-*Proteobacterium* clone AKYG1642 (AY921834.1)	99%	γ-*Proteobacterium*
13	Uncultured α-*Proteobacterium* strain C11-18 (AJ294357.1)	92%	α-*Proteobacterium*
14	*Tetrasphaera elongata* strain ASP12 (AB051430.1)	98%	*Actinobacteria*
15	Uncultured *Mycobacteriaceae* bacterium clone Elev_16S_1104 (EF019585.1)	91%	*Actinobacteria*
16	Uncultured bacterium clone YSK16S-27 (EF612990.1)	94%	–
17	*Gemmatimonas aurantiaca* (AB072735.1)	100%	Gemmatimonadales
18	Uncultured bacterium clone FG-EBPR-61 (AY521685.1)	98%	–
19	*Candidatus* Competibacter phosphatis clone SBRQ22 (AY172162.1)	99%	γ-*Proteobacterium*

–: unaffiliated group.

Gemmatimonadales. There are other 4 bacteria clones that can not affiliate to putative group.

Recently, *Rhodocyclus* genus from β-*Protebacteria* (related to *Candidatus* Accumulibacter phosphates) has gained increasing attention as the most possible PAOs candidates and a group of Actinobacteria is probably a new type of PAOs (Ahn *et al.*, 2002; Lee *et al.*, 2004). The previous publications showed that GAOs may belong to γ-*Protebacteria*, such as *Candidatus Accumulibacter* phosphates, and α-*Protebacteria* (Nielsen *et al.*, 1999; Wong *et al.*, 2004; Meyer *et al.*, 2006).

In this study, bacteria clones of *Proteobacterium* can not be found in seed sludge and then accounted for larger proportion at the end of start-up period. *Thauera* sp. (band 10) and uncultured γ-*Proteobacterium* (band 12), performed an increasing trend especially from 19 d, and uncultured bacterium of β-*Proteobacterium* (band 8) from 11 d. Most of them were uncultured bacteria clone without fully understanding on their function for phosphorus metabolism. As quantities of *Proteobacterium* increased along with obvious improvement of phosphorus removal on day 11 and 19, they may have a positive effect in this reactor. At the end, *Proteobacterium* formed important part in microbial community structure with better phosphorus removal.

Tetrasphaera elongata from Actinobacteria represented by band 14 existed in large amount all over the start-up period, even in seed sludge. Hanada *et al.* (2002) isolated *T. elongata* by traditional method from an A/O SBR, exhibiting positive Neisser-staining and implying a role in the biological phosphorus-removal process. As the seed sludge performed phosphorus removal to some extend, the effect and perfect adaptation for both low and high phosphorus of *T. elongate*, which the only strain in seed sludge was proposed as PAOs, should not be neglected and require further study.

Gemmatimonas aurantiaca from Gemmatimonadales was firstly isolated by Zhang *et al.* (2003) and was proved of the ability to accumulate polyphosphate. *Gemmatimonas aurantiaca* (band 17) was not detected in seed sludge. It appeared on day 15 and grew better in the following time. Although *G. aurantiaca* always lived in little amount, its appearance corresponded to rapid increasing of phosphorus removal effect obviously. Therefore, it plays substantial role in the EBPR system but has a long adapting phase when converted to high phosphorus. The present results suggest that *G. aurantiaca* had stronger ability of phosphorus removal even at low quantities.

Candidatus Competibacter phosphatis (band 19) appeared at the end of the start-up period (on day 26) with little amount. The organism belonging to the γ-*Proteobacteria* was identified as GAO phenotype and named by Crocetti *et al.* (2002). It has frequently dominated lab-scale cultures fed with acetate (Crocetti *et al.*, 2002; Zeng *et al.*, 2003; Oehmen *et al.*, 2004). Better phosphorus removal here indicated that *Candidatus* Competibacter phosphatis occurrence had not caused serious negative effect as mentioned by other researchers. This is mainly because all the previous studies have been conducted at an already deteriorated EBPR system. Little information on the beginning of GAO competition process and succession is available. The results showed that an accumulating-phase was necessary for GAO mature and a lag-stage existed when taking the performance of the reactor into consideration. It was also demonstrated that it is possible for an EBPR system to operate with an insignificant *Candidatus* Competibacter phosphatis population.

For the bacteria clones which have the similarity more than 94% but represent unaffiliated group (4 OTUs), they all belonged to uncultured bacterium with less knowledge. Some of them are familiar in EBPR processes (band 2, 18), which may benefit phosphorus removing and stable community structure. Moreover, the similarity between some sequencing results and the closest sequences are less than 94% (7 OTUs), which indicated that their identities were uncertain.

2.4 Stable microbial community structure at the end of start-up period

Figure 2 shows that the structure of microbial community and the relative quantity of various microbes tended to be stable from day 19. Corresponding to better phosphorus removal efficiency, a stable climax community was achieved at the end of start-up period.

DGGE profiles showed that dominant microbes in climax community were 14 OTUs. Microbes in a large amount were uncultured bacterium (band 8), *Thauera* sp. (band 10), uncultured γ-*Proteobacterium* (band 12), *T. elongata* (band 14) and uncultured bacterium (band 18). Among these microbes, *T. elongata* had been isolated and proved to have the ability of accumulating polyphosphate. Since the function of microbes may not be in proportion with their quantity, the action of microbes which account for little proportion should not be neglected, such as *G. aurantiaca* mentioned before. The function of other microbes, such as uncultured bacterium (band 8, 18), need further study.

3 Conclusions

A SBR reactor was operated under sequential anaerobic and aerobic conditions for phosphorus removal fed with acetate. The 30 d start-up period can be divided into two stages when analyzing the phosphorus removal efficiency. The 15 d lag phase followed by a rapid increasing of phosphorus removal efficiency which reached to 83.2%.

DGGE profiles of sludge samples showed that dominant microbial species were 19 OTUs during start-up period. UPGMA clustering analysis revealed that lower phosphorus removal rate correlated to rapid community succession and higher phosphorus removal efficiency reflected on steady community structure.

Sequencing results indicated that determined sequences (12 OTUs) belonged to *Proteobacterium*, Actinobacteria, Gemmatimonadales and unaffiliate group. *Proteobacterium* may have a positive effect on a phosphorus removal microbial community structure. *Tetrasphaera elongate* and *G. aurantiaca* may act important roles in phosphorus removal in this study condition. With insignificant amount as known GAOs, *Candidatus* Competibacter phosphatis still at accumulating-phase had a limited influence on phosphorus removal and microbial community structure.

A stable climax community was achieved at the end of start-up period. Dominant microbes in climax community were 14 OTUs. Microbes in a large amount were uncultured bacteriums, *Thauera* sp., uncultured γ-*Proteobacterium* and *T. elongata*. The action of microbes which account for little proportion should not be neglected, such as *G. aurantiaca* and *Candidatus* Competibacter phosphatis.

References

Ahn J, Daidou T, Tsuneda S, Hirata A, 2002. Characterization of denitrifying phosphate-accumulating organisms cultivated under different electron acceptor conditions using polymerase chain reaction-denaturing gradient gel electrophoresis assay. *Water Research*, 36: 403–412.

APHA (American Public Health Association), 1995. Standard Methods for the Examination of Water and Wastewater (19th ed). Washington DC.

Burow L C, Kong Y H, Nielsen J L, Blackall L L, Nielsen P H, 2007. Abundance and ecophysiology of *Defluviicoccus* spp., glycogen accumulating organisms in full-scale wastewater treatment processes. *Microbiology*, 153: 178–185.

Crocetti G R, Banfield J, Keller J, Blackall L L, 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. *Microbiology*, 148: 3353–3364.

Hanada S, Liu W T, Shintani T, Kamagata Y, Nakamura K, 2002. *Tetrasphaera elongata* sp. nov., a polyphosphate-accumulating bacterium isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology*, 52: 883–887.

Lee Y S, Kim G H, Park B J, Park K Y, 2004. Denaturing gradient gel electrophoresis analysis of bacterial populations in 5-stage biological nutrient removal process with step feed system for wastewater treatment. *The Journal of Microbiology*, 42(1): 1–8.

Meyer R L, Zeng R J X, Giugliano V, Blackall L L, 2005. Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. *FEMS Microbiology Ecology*, 52(3): 329–338.

Meyer R L, Saunders A M, Blackall L L, 2006. Putative glycogenaccumulating organisms belonging to Alphaproteobacteria identified through rRNA-based stable isotope probing. *Microbiology*, 152: 419–429.

Mino T, Van Loosdrecht M C M, Heijnen J J, 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Research*, 32(11): 3193–3207.

Muyzer G, Waal E C, Uitterlinden A G, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. *Applied and Environment Microbiology*, 59: 695–700.

Nakamura K, Hiraishi A, Yoshimi Y, Kawaharasaki M, Masuda K, Kamagata Y, 1995. *Microlunatus phosphovorus* gen. nov., sp. nov., a new Gram-positive polyphosphate-accumulating bacterium isolated from activated-sludge. *International Journal of Systematic and Evolutionary Microbiology*, 45(1): 17–22.

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. *Applied and Environment Microbiology*, 65(3): 1251–1258.

Nuebel U, Engelen B, Felske A, 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology*, 178: 5636–5643.

Oehmen A, Yuan Z, Blackall L L, Keller J, 2004. Short-term effects of carbon source on the competition of polyphosphate accumulating organisms and glycogen accumulating

organisms. *Water Science and Technology*. 50(10): 139–144.

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

Onuki M, Satoh H, Mino T, 2002. Analysis of microbial community that performs enhanced biological phosphorus removal in activated sludge fed with acetate. *Water Science and Technology*, 46(1-2): 145–154.

Santos M M, Lemos P C, Reis M A M, Santos H, 1999. Glucose metabolism and kinetics of phosphorus removal by the fermentative bacterium *Microlunatus phosphovorus*. *Applied and Environment Microbiology*, 65(9): 3920–3928.

Saunders A M, Oehmen A, Blackall L L, Yuan Z, Keller J, 2003. The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. *Water Science and Technology*, 47(11): 37–43.

Schuler J A, Onuki M, Satoh H, Mino T, 2002. Density separation and molecular methods to characterize enhanced biological phosphorus removal system populations. *Water Science and Technology*, 46(1-2): 195–198.

Seviour R J, Mino T, Onuki M, 2003. The microbiology of biological phosphorus removal in activated sludge systems. *FEMS Microbiology Review*, 27(1): 99–127.

Stante L, Cellamare C M, Malaspina F, Bortone G, Tilche A,

1997. Biological phosphorus removal by pure culture of *Lampropedia* spp. *Water Research*, 31(6): 1317–1324.

Thomas M, Wright P, Blackall L, Urbain V, Keller J, 2003. Optimisation of Noosa BNR plant to improve performance and reduce operating costs. *Water Science and Technology*, 47(12): 141–148.

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

Yu S L, Liu Y N, Jing G L, Zhao B, Guo S, 2005. Analysis of phosphate-accumulating organisms cultivated under different carbon sources with polymerase chain reaction-denaturing gradient gel electrophoresis assay. *Journal of Environment Sciences*, 17(4): 611–614.

Zeng R J, Yuan Z, Keller J, 2003. Model-based analysis of anaerobic acetate uptake by a mixed culture of polyphosphate-accumulating and glycogen-accumulating organisms. *Biotechnology and Bioengineering*, 83(3): 293–302.

Zhang H, Sekiguchi Y, Hanada, Hugenholtz P, Kim H, Kamagata Y, Nakamura K, 2003. *Gemmatimonas aurantiaca* gen. nov., sp. nov., a Gram-negative, aerobic, polyphosphate accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. Nov. *International Journal of Systematic and Evolutionary Microbiology*, 53: 1155–1163.