Chapter 5

Evaluation of polyphosphate-accumulating organisms indicators in the start-up phase in enhanced biological phosphorus removal process using conventional methods and microautoradiography-FISH

SUMMARY

Polyphosphate-accumulating organisms (PAOs) indicators in enhanced biological phosphorus removal (EBPR) process evaluated by conventional analyses and that by microautoradiography- fluorescence in situ hybridization (MAR-FISH) analysis were compared and discussed. Two types of activated sludge, EBPR sludge and non-EBPR sludge, were mixed in the different ratios and operated in the acetate-fed sequencing batch reactors (SBRs). During the start-up phase, three conventional indicators, the phosphorus content in biomass, the population density of Rhodocyclus-related PAO (RPAO) and the anaerobic phosphorus release, were analyzed. The increase of the population density of RPAO and the phosphorus content exhibited different behavior. This difference might be derived form the change in physiology of RPAO. The anaerobic phosphorus release also behaved different from the other two indicators. It might be resulted from the low acetate assimilation activity of PAOs and from the acetate assimilation by other bacteria. Both the acetate assimilation by α-Proteobacterial G-bacteria and no acetate assimilation by some RPAO were visualized by MAR-FISH analysis whereas the acetate assimilation by most of RPAO was also visualized. Conventional indicators evaluated in this study gave the different results because 1) each indicator is based on the each activity of PAOs, such as acetate assimilation, phosphorus uptake and growth activity, and 2) the activities of other organisms should contribute each indicator. MAR-FISH analysis is more appropriate for the determination of the actual substrate uptake activities, but the further identification of their phylogenetic affiliation and the determination of the biochemistry of PAOs and GAOs/G-bacteria are also still needed.

5.1 INTRODUCTION

Enhanced biological phosphorus removal (EBPR) processes are widely used to remove phosphorus from municipal and industrial wastewater. EBPR process essentially contains anaerobic and aerobic periods. In the initial anaerobic period, polyphosphate-accumulating organisms (PAOs) take up volatile fatty acids (VFAs) and polymerize them as polyhydroxyalkanoates (PHA) while releasing intercellular polyphosphate (PolyP) as orthophosphate (P_i) (Mino *et al*., 1998; Seviour *et al*., 2003). In the subsequent aerobic period, PAOs accumulate large amount of P_i in excess of released P_i as PolyP (Mino *et al*., 1998; Seviour *et al*., 2003).

Culture-independent molecular techniques have identified a member of the phylogenetically defined *Rhodocyclus*-relatives in the β-*Proteobacteria* as the dominant group in acetate-fed laboratory sequencing batch reactors (SBRs). These bacteria have been named '*Candidatus* Accumulibacter phosphatis' or *Rhodocyclus*-related PAOs, RPAO (Crocetti *et al*., 2000; Hesselmann *et al*., 1999; Kong *et al*., 2004; Liu *et al*., 2001). However, several studies reported that other PAOs candidates in the α-*Proteobacteria*, the γ-*Proteobacteria* and *Actinobacteria* existed in EBPR processes (Liu *et al*. 2001; Kawaharasaki *et al*., 1999; Kong *et al*., 2005).

Although the anaerobic-aerobic cyclic operations provide the selective advantage for growth of PAOs, other organisms are also able to grow in these operational conditions. These organisms, named glycogen-accumulating organisms (GAOs) or G-bacteria, which have a morphotype of cocci usually arranged in tetrads and/or clusters, were assumed to compete with PAOs for organic carbon uptake and to cause the deterioration of EBPR (Beer *et al*., 2004; Cech and Hartman, 1993; Crocetti *et al*., 2002; Kong *et al*., 2001, 2002a, 2002b, 2006; Lee *et al*., 2003; Liu *et al*., 1996, 1997; Maszenan *et al*., 1997, 2000, 2005; Meyer *et al*., 2006; Mino *et al*., 1998; Nielsen *et al*., 1999; Oehmen *et al*., 2004; Seviour *et al*., 2000, 2003; Wong *et al*., 2004).

. Culture-independent molecular techniques defined that GAOs belong to members of the lineage GB or '*Candidatus* Competibacter phosphatis' in the *γ-Proteobacteria* (Crocetti *et al*., 2002; Kong *et al*., 2002b, 2006; Nielsen *et al*., 1999). Members of *Amaricoccus* spp. and *Defluvicoccus vanus* belonging to α-*Proteobacteria* are also isolated or identified as G-bacteria (Beer *et al*., 2004; Maszenan *et al*., 1997, 2000, 2005; Meyer *et al*., 2006; Wong *et al*., 2004).

Therefore, the competition for carbon source uptake between PAOs and GAOs in the anaerobic period gives a significant effect on the phosphorus removal capacity. Conventionally, the phosphorus removal capacity is determined by the phosphorus content in biomass, the rates of phosphorus release, VFAs uptake, PHA synthesis in the anaerobic stage, and the rates of phosphorus uptake and glycogen synthesis in the aerobic stage. These indicators are based on the biochemical model of EBPR (Mino *et al*., 1998; Seviour *et al*., 2003). High phosphorus content and high phosphorus release exhibit high-phosphorus uptake potential and effective organic carbon assimilation by PAOs, respectively. High phosphorus uptake rate exhibited high growth activity of PAOs. On the other hand, not only PAOs but other organisms such as GAOs and denitrifying bacteria contribute the other indicators, VFAs uptake, PHA synthesis and glycogen. Therefore, these indicators do not always exhibit each activity of only PAOs and more powerful tools, with which the organic carbon uptake competition between PAOs and the others can be analyzed, are necessary to find an optimum operational condition for the stable and effective phosphorus removal.

Recently microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) technique has been widely applied for simultaneous evaluation of microbial community and mass transfer (Lee *et al*., 1999). MAR-FISH technique is a powerful tool to visualize target bacteria based on phylogenetic information and their *in situ* activity (substrate uptake activity) in single cell level. MAR-FISH analysis was applied for EBPR process in previous studies and the identification and characterization of ecophysiology of PAOs and GAOs were performed (Kong *et al*., 2001, 2002a, 2004, 2005, 2006; Lee *et al*., 2003). However, the differences of the results between the conventional parameters and MAR-FISH analysis were not discussed.

In this study, two types of activated sludge, EBPR sludge and non-EBPR sludge were mixed in the different ratios and operated in acetate-fed SBRs. During the start-up phase in each run, PAOs activity was determined as the phosphorus content in biomass (phosphorus uptake potential), the population density of RPAO (amount of PAOs) and the dissolved phosphorus concentration at the end of anaerobic period (effectiveness of organic carbon assimilation by PAOs). Then, the acetate assimilation activity was visualized by MAR-FISH analysis when all SBRs were judged to exhibit high activity in those conventional indicators. Finally, the results of the conventional indicators and those of MAR-FISH analysis were compared and discussed.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of EBPR and non-EBPR sludge and sequencing batch reactor operation

Two types of activated sludge were prepared. One type of the sludge (EBPR sludge) was originated from a municipal wastewater treatment process (WWTP) at Tokyo (Japan), which attains efficient EBPR. EBPR sludge had been cultivated in the anaerobic-aerobic acetate-fed sequencing batch reactor (SBR). Operational conditions were the same as below. After one month, this sludge exhibited high phosphorus removal capability and the phosphorus content in biomass became 6 % mg-P/mg-MLSS. The other type of the sludge (non-EBPR sludge) originating from the same WWTP had been cultivated in a conventional aerated reactor. As a result, non-EBPR sludge exhibited no phosphorus removal capacity and the phosphorus content in biomass was 3% mg-P/mg-MLSS.

These two types of activated sludge were inoculated to four cylindrical anaerobic-aerobic SBRs with different mixed ratios (Table 1). Each SBR has 4.7 L working volume and was operated with a cycle of 8 h, consisting of a filling period (15 min), an anaerobic period (90 min), an aerobic period (300 min), a settling period (60 min), and withdrawing period (15 min). In the withdrawing period, 2,350 ml of the supernatant was withdrawn and 2,350 ml fresh synthetic wastewater was added to each reactor in the next filling period. The hydraulic retention time (HRT) was adjusted to 16 h. Synthetic wastewater contained CH_3COONa as a sole organic carbon source, 384 g/m^3; KH_2PO_4, 65.9 g/m^3; $(NH_4)_2SO_4$, 142 g/m^3; $MgSO_4 \cdot 7H_2O$, 101 g/m^3; $CaCl_2 \cdot 2H_2O$, 14.7 g/m^3 and mineral salt solution (Smolders *et al.*, 1994), 1.5 ml/L. The sludge retention time (SRT) was adjusted to 20 days by withdrawing 235 ml of mixed liquor at the last 10 min in the aerobic stage once in a day. MLSS was maintained to 2,600 mg/L and temperature was adjusted at 20±1˚C.

Table 1 Mixed ratio of EBPR sludge and non-EBPR sludge

Run	EBPR sludge (%)	non-EBPR sludge (%)
1	100	0
2	50	50
3	10	90
4	0	100

5.2.2 Water quality analysis

Water quality analysis was performed according to standard methods (APHA, 1992). Dissolved ammonium, orthophosphate, nitrite and nitrate were analyzed by Traacs2000 (Bran+Luebbe K. K., Japan) after filtration with 0.2 μm filters (Millipore, United States). MLSS and the phosphorus content in biomass at the end of the aerobic period were also determined using standard methods (APHA, 1992).

5.2.3 Microbial community analysis by FISH

Sludge samples were collected from SBRs at the end of the aerobic stage and were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) solution (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, and 1.47 mM KH_2PO_4; pH 7.4) for 2 h at 4˚C. Fixed sludge samples were then washed with PBS solution. These sludge samples were stored in a 1:1 mixture of PBS and 98% ethanol at -20˚C.

Oligonucleotide probes used in this study are listed in Table 2. These oligonucleotide probes were commercially synthesized and labeled 5’ end with the fluorescein isothiocyanate (FITC) or indocarbocyanin 3 (Cy3) (TAKARA BIO, Japan). Fixed sludge samples were immobilized on gelatin-coated glass slides and dehydrated by successive passages through 50, 80 and 98% ethanol. The hybridization was performed according to the standard hybridization protocol[31]. Then, the samples were counterstained by 4’, 6’-diamidino-2-phenylindole (DAPI) solution (1μg/ ml), mounted in VECTASHIELD Mounting Medium (Vector, U.K.), and observed under an inverse epifluorescence microscopy (TE2000U, Nikon, Japan) or a confocal laser scanning

microscope (TCS4D, Leica Lasertechnik, Germany). Images of each probe binding-cells were collected and superimposed with the software AQUA-Lite (Hamamatsu Photonics K. K., Japan). For direct counting of cells hybridized with EUB338 or PAOMIX probe (PAO462, PAO651 and PAO846), fixed sludge samples were soaked in the same volume of sterile tripolyphosphprus buffer solution (400 mg/L) and sonicated for 60s with an ultrasonic disrupter (UR-20P, TOMY SEIKO, Japan) to disperse microbial flocs before immobilization on slides. At least 10 microscopic fields or 1,000 cells were counted for each sample.

Table 2 Oligonucleotide probes used in this study

Probe name	Specificity	Probe sequence (5'-3')	Formamide (%)
EUB338	Most Bacteria	GCTGCCTCCCGTAGGAGT	20
PAO462	*Rhodocyclus* -related PAO	CCGTCATCTACWCAGGGTATTAAC	35
PAO651	*Rhodocyclus* -related PAO	CCCTCTGCCAAACTCCAG	35
PAO846	*Rhodocyclus* -related PAO	GTTAGCTACGGCACTAAAAGG	35
ALF1b	α-*Proteobacteria*	CGTTCGYTCTGAGCCAG	20
BET42a	β-*Proteobacteria*	GCCTTCCCACTTCGTTT	35
GAM42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	35
CF319a	*Cytophaga-Flavobacterium branch*	TGGTCCGTGTCTCAGTAC	35
AMAR839	*Amaricoccus* spp.	CTGCGACACCGAACGGCAAGCC	20
DF988*	*Defluvicoccus vanus* -relative organisms	GATACGACGCCCATGTCAAGGG	35
DF1020*	*Defluvicoccus vanus* -relative organisms	CCGGCCGAACCGACTCCC	35
H966*	-	CTGGTAAGGTTCTGCGCGTTGC	-
H1038*	-	AGCAGCCATGCAGCACCTGTGTGGCGT	-

5.2.4 Functional analysis by MAR-FISH

At the end of the aerobic period in 89 days operation, sludge samples were collected from Runs 1 and 4 and moved into tubes. The sludge samples were diluted to 1,000 mg-MLSS/L by ddH_2O. After 60 min settling, half of supernatant was removed from the tubes in the same manner as SBR operation. Then synthetic wastewater with [1,2-^{14}C] acetate (Amersham Bioscience) was added to the tubes (the final concentration 10 μCi/mg-MLSS). The synthetic wastewater was adjusted to maintain the same acetate loading per MLSS as applied for SBR operation. Then sludge samples were incubated for 90 min anaerobically. As a negative control for MAR, sludge samples pasteurized at 70°C for 10 min and unpasteurized sludge samples were

incubated with the radioactive acetate and nonradioactive substrates, respectively. After incubation, the sludge samples were collected and MAR-FISH was carried out according to the procedure described by Lee *et al*. (1999).

5.3 RESULTS

5.3.1 Performance of SBRs

Two types of sludge, EBPR sludge and non-EBPR sludge, were inoculated to four SBRs with different mixed ratios (Table1). After 54 cycles operation, all SBRs exhibited typical EBPR profile: rapid phosphorus release at the anaerobic period and luxury phosphorus accumulation in biomass in the subsequent aerobic period (Fig.1). Only in Run 4, a small amount of phosphorus was assimilated in the last of anaerobic period. This phenomenon has been seen in other studies but the clear explanation has not been done (Crocetti *et al*., 2000; Hesselmann *et al*., 1999; Kong *et al*., 2002a). The time course of the phosphorus content in biomass, the population density of RPAO and the dissolved phosphorus concentration at the end of anaerobic period in each runs are shown in Fig. 2. In Run 1, where only EBPR sludge was inoculated, the biomass maintained high phosphorus content (around 8%) and RPAO were predominant (2.0×10^8 cell/ml) from the beginning of the operation. High amount of phosphorus release by PAOs was also confirmed at the end of anaerobic period. Therefore, the operational condition of SBR should be adequate for phosphorus removal and RPAO played an important role for phosphorus removal in the reactor.

In Run 2, the profiles of the phosphorus content, the population density of RPAO and the anaerobic phosphorus release became the same as Run 1 after only 2 days operation despite the equal volume of non-EBPR sludge were mixed with EBPR sludge (Fig. 2A, B).

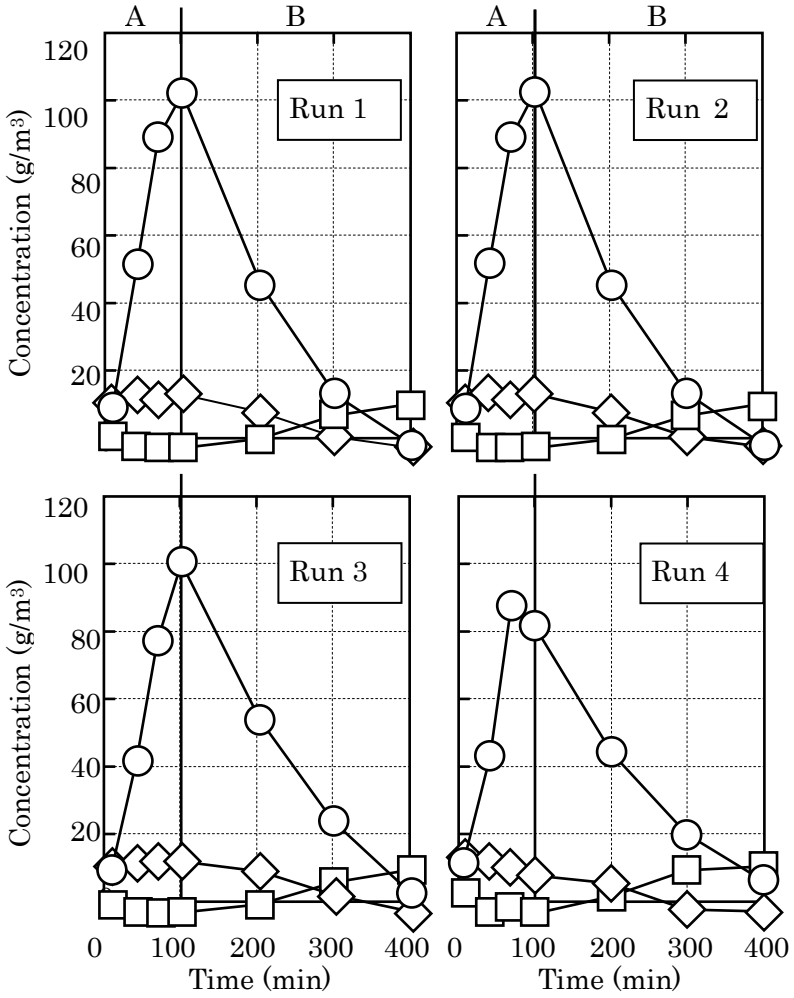


Fig. 1 Chemical changes during 54th cycle of each run. Each run exhibited typical EBPR profile. Nitrogen removal was also achieved by nitrifying bacteria and denitrifying bacteria. A, anaerobic period; B, aerobic period. Symbols: ○, PO_4-P; □, NO_{2+3}-N;◇, NH_4-N.

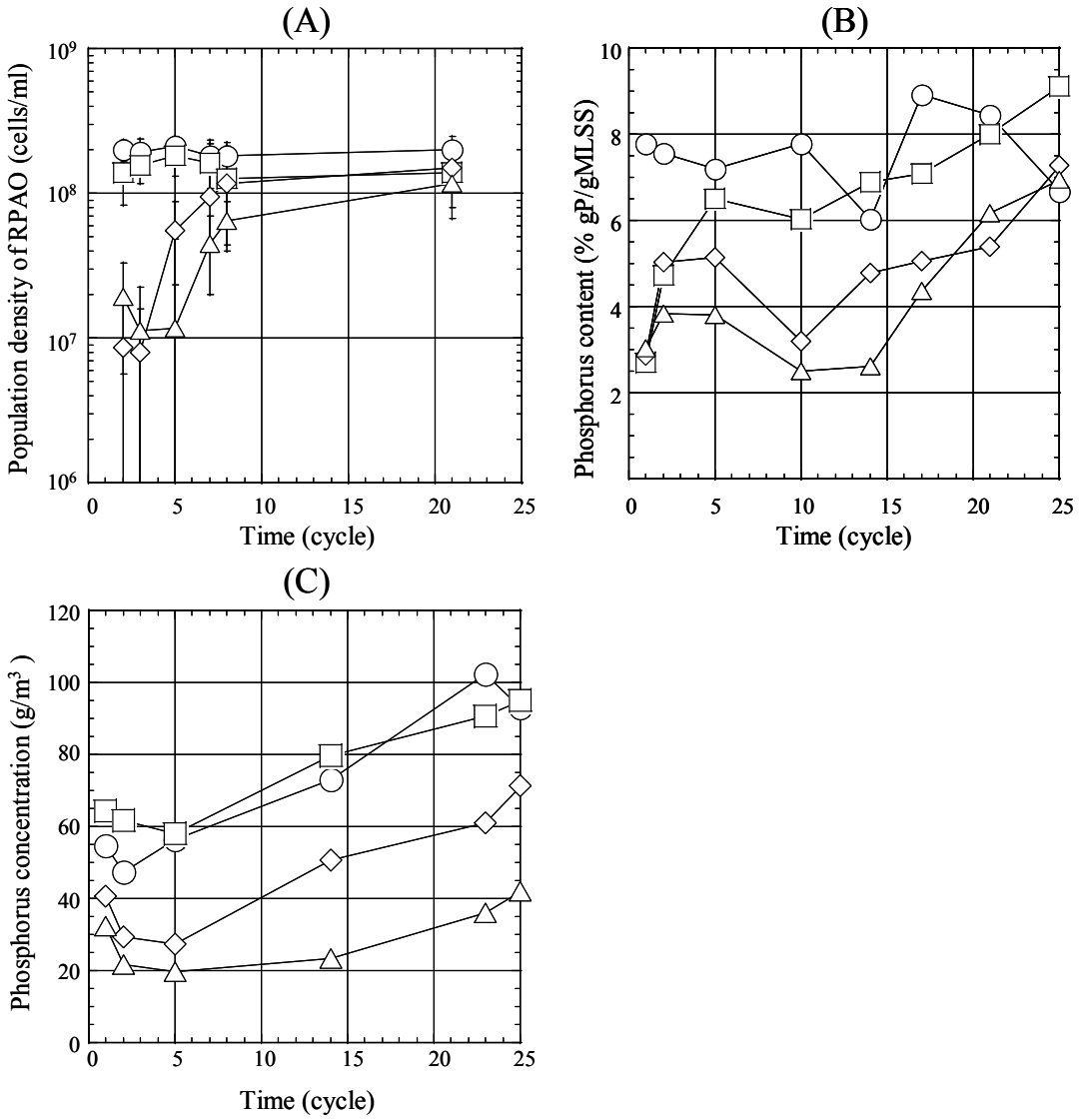


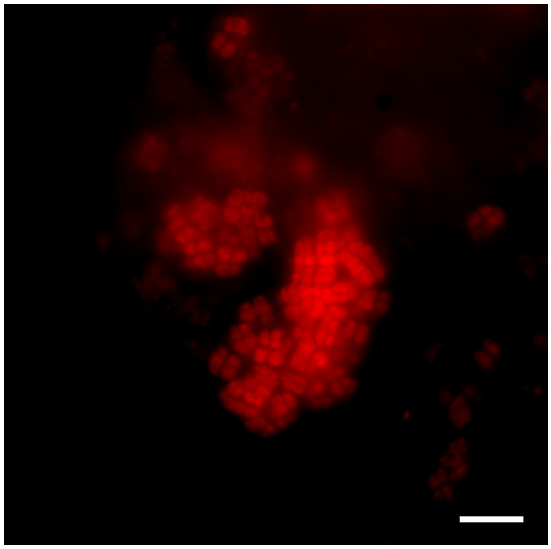
Fig. 2 Profiles of the population density of RPAO (A), the phosphorus content in biomass (B) and the phosphorus concentration at the end of the anaerobic period (C) during 25 cycle operation. Symbols: ○, Run1; □, Run2; ◇, Run3; △, Run4.

In Runs 3 and 4, composed by mainly non-EBPR sludge (90% and 100%, respectively), the phosphorus content, the population density of RPAO and the anaerobic phosphorus release were increased. However, the start-up points, which are the times when the increases of each indicator were confirmed, were later than Run 2. The population density of RPAO in Runs 3 and 4 increased rapidly after 3 and 5 cycles operation, respectively (Fig. 2A). The significant increases in the phosphorus contents in Runs 3 and 4 were also confirmed in 10 and 14 cycles operation, respectively (Fig. 2B). Then, both the population density of RPAO and the phosphorus content became the same as Run 1 within 25 cycles operation. On the other hand, the anaerobic phosphorus release in Runs 3 and 4 increased slowly (Fig. 2C).

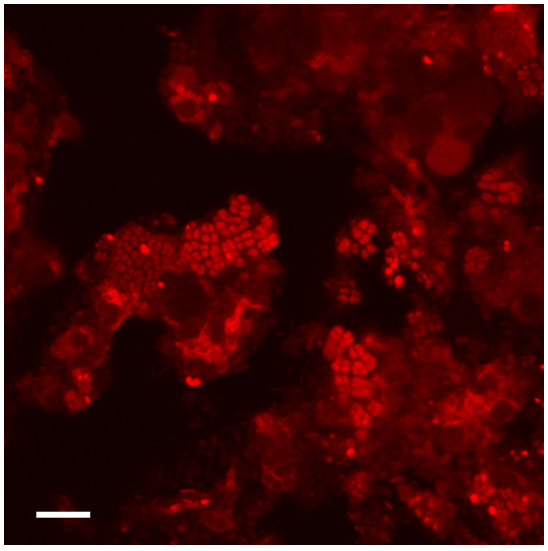
5.3.2 Observation of microbial community by FISH

In 25 cycles operation, large cocci, packaged in tetrad cluster, also became dominant (Fig. 4). PolyP staining by DAPI revealed that these organisms did not accumulate polyP in the aerobic period, and thus these organisms were not PAOs. These organisms were named tetrad-forming organisms (TFOs) and/or G-bacteria (Seviour *et al*., 2000). To identify the phylogenetic affiliation of G-bacteria in this study, *in situ* hybridization with the group-specific probes ALF1b, BET42a, GAM42a and CF319a were performed. As a result, G-bacteria was affiliated with the α-*Proteobacteria*, which responded to ALF1b probe (Fig. 3A). Members of *Amaricoccus* spp., *Defluvicoccus vanus*-relative G-bacteria and *Sphingomonas*-relative G-bacteria belonging to α-*Proteobacteria* are isolated or identified as G-bacteria (Beer *et al*., 2004; Maszenan *et al*., 1997, 2000, 2005; Meyer *et al*., 2006; Wong *et al*., 2004). Although AMAR839 specific for *Amaricoccus* spp. could not hybridized with cells, DF988 and DF1020 bound to those, which were identified as *D. vanus*-relative G-bacteria (Fig.3B, C).

(A)



(B)



(C)

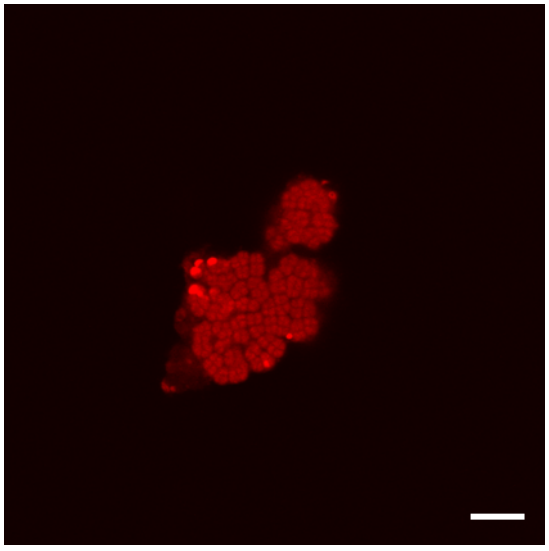


Fig. 3 FISH images from SBR sludge (Run 4).α-*Proteobacterial* G-bacteria, responding to ALF1b probe (A), *D. vanus*-relative G-bacteria, responding to DF988 probe (B) and *D. vanus*-relative G-bacteria, responding to DF1020.

5.3.3 Functional analysis by MAR-FISH

After 89 days operation, sludge samples collected from Runs 1 and 4 were subjected to MAR-FISH analysis. In the negative control samples, no silver grain was observed and thus chemographic and non-specific adsorption did not occur. In the batch experiments for MAR-FISH analysis, phosphorus release was confirmed and this showed that the conditions in the SBRs were imitated in the batch experiment. Most of all PAOMIX probe-binding cells in Run 1 were covered with thick silver grains (Fig.4 A, B). This micrograph revealed that RPAO actively assimilated [^{14}C]-acetate in the anaerobic period. However, some RPAO in Run 4 had less activity for acetate assimilation and non-RPAO exhibited high activity (Fig.4 C, D).

Functional analysis for α-*Proteobacterial* G-bacteria was also conducted. As a result, α-*Proteobacterial* G-bacteria were also covered with thick silver grains and it indicated G-bacteria assimilated [^{14}C]-acetate, and then the competition for acetate assimilation between G-bacteria and PAOs was confirmed (Fig.4E, F). Acetate assimilation by Alf1b-negative cells was also confirmed (Fig.4E, F).

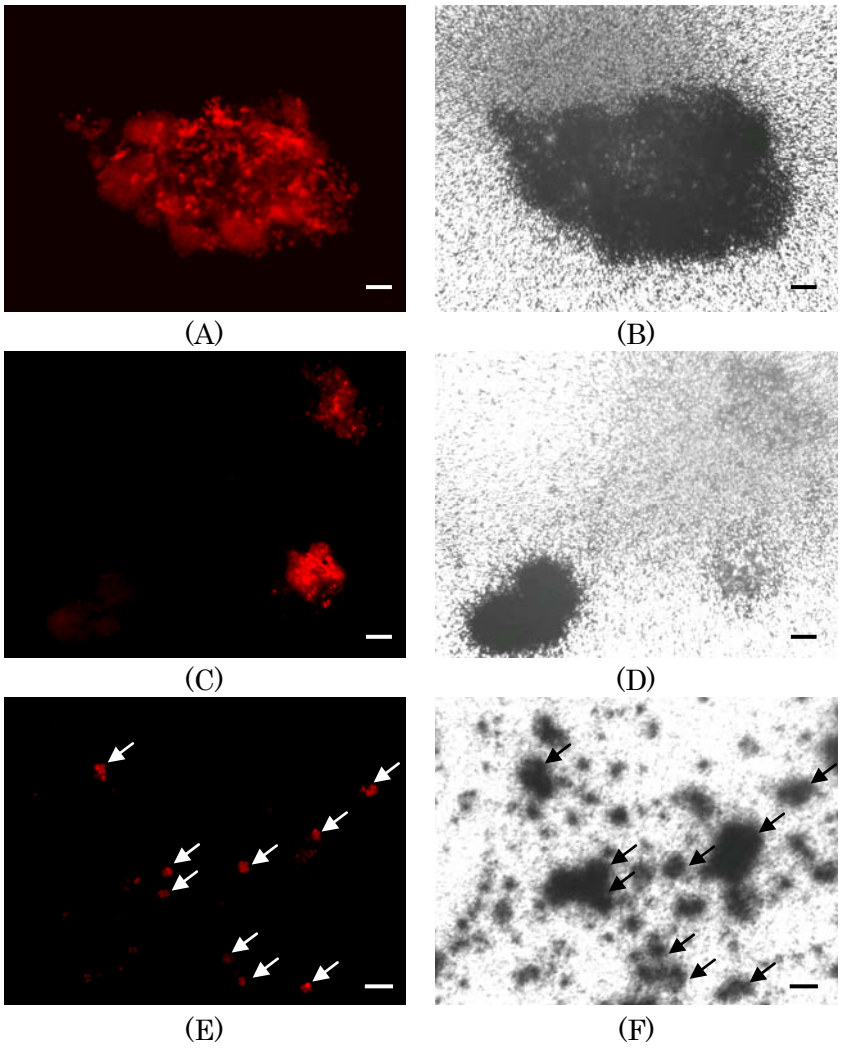


Fig. 4 MAR-FISH images from SBRs. Anaerobic [^{14}C]-acetate assimilation by RPAO (PAOMIX probes-binding cells) in Run 1(A, B). No anaerobic [^{14}C]-acetate assimilation was observed by any RPAO in Run 4(C, D). Anaerobic [^{14}C]-acetate assimilation by α-*Proteobacterial* G-bacteria (ALF1b probe-binding cells shown by the arrows) in Run 1 (E, F). (B), (D), (F) are microautoradiographs corresponding to the epifluorescence images shown in (A), (C), (E), respectively. Bars (in all images), 10 μm.

5.4 DISCUSSION

In EBPR process, PAOs play an important role for phosphorus removal. RPAO has been identified as major PAOs in laboratory SBR reactors and full-scale WWTPs (Crocetti *et al*., 2000; Hesselmann *et al*., 1999; Kong *et al*., 2004; Liu *et al*., 2001). RPAO assimilate acetate and polymerize them as PHA while releasing intercellular PolyP as Pi (Mino *et al*., 1998; Seviour *et al*., 2003). This intercellular storage is then used as energy for growth and accumulation of PolyP (Mino *et al*., 1998; Seviour *et al*., 2003). Therefore, the phosphorus content in biomass, population of RPAO and the amount of anaerobic phosphorus release have been recognized as indicators for PAO activities: the phosphorus content in biomass as phosphorus uptake potential; the population density of RPAO as the amount of PAOs: the amount of anaerobic phosphorus release as effectiveness of organic carbon assimilation by PAOs.

In this study, two types of sludge, EBPR sludge and non-EBPR sludge were inoculated to four SBRs with the different mixed ratios and these conventional analytical methods were applied for monitoring PAOs activity. The time courses of the phosphorus content, the population density of RPAO and the amount of anaerobic phosphorus release in Run 1 indicated that operational condition in this study was suitable for EBPR process and RPAO played an important role for phosphorus removal.

The significant increases in the phosphorus content and the population density were also observed in all Runs mixed with non-EBPR sludge. As the non-EBPR sludge ratio was high, the start-up point became later. However, the start-up points of the population density of RPAO and the phosphorus content were different. For example, in Run 4, the population density of RPAO increased exponentially from 5 cycles operation while the phosphorus content increased gradually from 14 cycles operation. According to this result, it was hypothesized that RPAO could grow without additional aerobic polyP accumulation. RPAO became prominent in the reactor utilized internal polyP, which accumulated before operation and a little additional polyP

after a couple of operations, and internal carbons sources such as PHA. After the dominance, RPAO accumulated polyP and this resulted in the increase of the phosphorus content. The clear explain for this hypothesis should be done in future but it has been impossible to use pure culture.

On the other hand, the increase in the phosphorus concentration at the end of the anaerobic period in Run4 was lower than that in other Runs after 25 cycles operation (Fig.2C). The population of RPAO and the phosphorus content were almost the same as other Runs and it indicates that the reactor had enough PAOs population and phosphorus uptake potential. Therefore, it was found that the low anaerobic phosphorus release resulted from the low availability of acetate in the anaerobic period according to the biochemical mode of EBPR (Mino *et al*., 1998; Seviour *et al*., 2003).

MAR-FISH analysis was performed to determine the acetate assimilation activity of RPAO and α-*Proteobacterial* G-bacteria. Most of RPAO assimilate [^{14}C]-acetate anaerobically in both Runs 1 and 4, to which only EBPR sludge and only non-EBPR sludge was inoculated, respectively (Fig. 4 A, B). These results emphasized that RPAO play an important role for phosphorus removal in this study. However, no silver grains covered some flocs mainly composed of RPAO in Run 4 (Fig. 4 C, D). Lee *et al*. (2003) reported the same phenomenon. They explained that this phenomenon was due to the differences in the acetate assimilating activity among RPAO and/or the metabolic differences between phylogenetically different, but closely related RPAO. These differences might be the suitable explanation for this phenomenon. The quantification of RPAO by FISH is useful for obtaining the amount of PAOs; however, these results revealed that sometimes this quantification would overestimate the amount of PAOs, which actually exhibited the biochemical model of EBPR, because of the presence of non-active PAOs for acetate assimilation.

Large cocci, packaged in tetrad cluster named TFOs or ‘G-bacteria’, also became prominent in the SBRs. FISH analysis revealed that these G-bacteria were hybridized with both DF988 and DF1020 probes, designed for *Defluvicoccus*-relative G-bacteria (Fig.3). These

G-bacteria assimilated acetate in the anaerobic period (Fig.4E, F). Therefore, in Run 4, PAOs could not assimilate acetate effectively and acetate was utilized by those other organisms. Meyer *et al*. reported that these organisms became the dominant in their SBR with no phosphorus removal, which was operated for enrichment of GAOs by using low phosphorus concentration synthetic wastewater (Propionate, 132 g/m^3; PO_4-P 2 g/m^3) (Meyer *et al*., 2006; Oehmen *et al*., 2005). Kong *et al*. visualized the acetate assimilation by α-*Proteobacterial* G-bacteria, dominated in the glucose/acetate fed SBR with no phosphorus removal, by using MAR-FISH analysis (Kong *et al*., 2001). The presence of those α-*Proteobacterial* G-bacteria were reported in the SBRs with no phosphorus removal as above, however, efficient phosphorus removal capacity kept at least three months despite the α-*Proteobacterial* G-bacteria exhibited acetate assimilation activity in the acetate-fed SBR in this study (Fig.4E, F). This phenomenon, discrepancy of the efficient phosphorus removal and the presence of G-bacteria, was reported by Kong *et al*. (2002a) and Liu *et al*. (1997). Both studies reported the complete and stable phosphorus removal was achieved when the influent P/TOC ratio was more than 1/10 by using acetate-fed SBRs with the presence of G-bacteria. Kong *et al*. also reported that the population density of *α-Proteobacterial* G-bacteria, which assimilated acetate and polymerized PHA, was increased with the decrease of P/TOC ratio (Kong *et al*., 2002a). In this study, the influent P/TOC ratio was 1/7.5 (P/COD = 15/300) and efficient phosphorus removal was achieved same as these studies (Kong *et al*., 2002a; Liu *et al*., 1997). Therefore, although the precise phylogenetic affiliation of the *α-Proteobacterial* G-bacteria was not assessed in their study, G-bacteria can co-exist with PAOs the same as those studies.

Anaerobic acetate assimilation by G-bacteria and other non-PAOs in the efficient EBPR as shown in Fig.4 causes the underestimation of PAOs acetate assimilation activity because not only PAO but also GAO/G-bacteria contribute anaerobic phosphorus release/organic carbon uptake ratio, anaerobic phosphorus release/PHA synthesis ratio. MAR-FISH analysis could visualize the substrates uptake activity of target organisms but the quantification of those activities in a complex microbial community is impossible. Quantitative MAR-FISH

(QMAR-FISH) has been developed to determine the quantitative uptake of specific substrates of filamentous bacteria in an activated sludge sample (Nielsen *et al*., 2003). However, this analysis requires pre-quantification of substrate uptake rate in a pure-culture system. Therefore, the each indicator of PAOs and GAOs could not be determined individually.

In conclusion, during the start-up phase for phosphorus removal, the increase of the population density of RPAO and the phosphorus content exhibited different behavior despite these indicators are closely related to each other. This difference might be derived form the change in physiology of RPAO but it is impossible to explain this phenomenon. The anaerobic phosphorus release also behaved different from the other two indicators. This might be resulted from the low acetate assimilation activity of PAOs and from the acetate assimilation by other bacteria. This other bacterial acetate assimilation was visualized by MAR-FISH analysis. Both high and no acetate assimilation by RPAO were also visualized. These results indicated that conventional indicators gave the different results because each indicator is based on the different activity of PAOs, such as acetate assimilation, phosphorus uptake and growth activity, and those activities of other organisms should contribute those indicators. FISH analysis would overestimate PAOs activity because of the presence of RPAO cells which do not assimilate acetate and the apparent phosphorus release would underestimate PAOs contribution because of other bacterial acetate assimilation. MAR-FISH analysis is more appropriate for the determination of the actual substrates uptake activities of organisms such as organic carbon assimilation and phosphorus uptake using radioactive substrates, but the further identification of their phylogenetic affiliation and the determination of the biochemistry of PAOs and GAOs/G-bacteria are also still needed.

5.5 CONCLUSIONS

In this study, two types of activated sludge, EBPR sludge and non-EBPR sludge were mixed in the different ratios and operated in acetate-fed SBRs. During the start-up phase in each run, PAOs activity was determined as the phosphorus content in biomass (phosphorus uptake potential), the population density of RPAO (amount of PAOs) and the dissolved phosphorus concentration at the end of anaerobic period (effectiveness of organic carbon assimilation by PAOs). Then, the acetate assimilation activity was visualized by MAR-FISH analysis when all SBRs were judged to exhibit high activity in those conventional indicators. Finally, the results of the conventional indicators and those of MAR-FISH analysis were compared and discussed. Main conclusions are as follows.

(1) Both the acetate assimilation by *α-Proteobacterial* G-bacteria and no acetate assimilation by some RPAO were visualized by MAR-FISH analysis whereas the acetate assimilation by most of RPAO was also visualized.
(2) Conventional indicators evaluated in this study gave the different results because 1) each indicator is based on the each activity of PAOs, such as acetate assimilation, phosphorus uptake and growth activity, and 2) the activities of other organisms should contribute each indicator.
(3) MAR-FISH analysis is more appropriate for the determination of the actual substrate uptake activities, but the further identification of their phylogenetic affiliation and the determination of the biochemistry of PAOs and GAOs/G-bacteria are also still needed.

5.6 REFERENCES

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A. (1990) Combination of 16s ribosomal-RNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial-populations. Applied and Environmental Microbiology **56** (6), 1919-1925.

Amann, R.I. (1995) *In situ* identification of microorganism by whole cell hybridization with rRNA-targeted nucleic probes. In: Akkerman, ADC, van Elsas JD, de Bruijin FJ (eds) Molecular microbial ecology manual, Kluwer Academic Publishers, Dordrecht, pp1-15

APHA, AWWA, WEF (1992) Standards methods for the examination of water and wastewater 18th edn. American Public Health Association, Washington, DC.

Beer, M., Kong, Y.H., Seviour, R.J. (2004) Are some putative glycogen accumulating organisms (GAO) in anaerobic: aerobic activated sludge systems members of the alpha-Proteobacteria? Microbiology-SGM **150** (7), 2267-2275.

Cech, J.S., Hartman, P. (1993) Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphorus removal systems. Water Research **27** (7), 1219-1225.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L,L, (2000) Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantification. Applied and Environmental Microbiology **66** (3), 1175-1182.

Crocetti, G.R., Banfield, J.F., Keller, J., Bond, P.L., Blackall, L.L. (2002) Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology-SGM **148** (11), 3353-3364.

Hesselmann, R.P.X., Werlen, C., Hahn, D., van der Meer, J.R., Zehnder, A.J.B. (1999) Enrichment, phylogenetic analysis and detection of bacterium that performs enhanced

biological phosphate removal in activated sludge. Systematic and Applied Microbiology **22** (3), 454-465.

Kawaharasaki, M., Tanaka, H., Kanagawa, T., Nakamura, K. (1999) *In situ* identification of polyphosphate-accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4',6-diamidino-2-phenylindol (DAPI) at a polyphosphate-probing concentration. Water Research **33** (1), 257-265.

Kong, Y.H., Beer, M., Seviour, R.J., Lindrea, K.C., Rees, G.N. (2001) Structure and functional analysis of the microbial community in an aerobic: anaerobic sequencing batch reactor (SBR) with no phosphorus removal. Systematic and Applied Microbiology **24** (4), 597-609.

Kong, Y.H., Beer, M., Rees, G.N., Seviour, R.J. (2002a) Functional analysis of microbial communities in aerobic-anaerobic sequencing batch reactors fed with different phosphorus/carbon (P/C) ratios. Microbiology-SGM **148** (8), 2299-2307.

Kong, Y.H., Ong, S.L., Ng, W.J., Liu, W.T. (2002b) Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. Environmental Microbiology **4** (11), 753-757.

Kong, Y.H., Nielsen, J.L., Nielsen, P.H. (2004) Microautoradiographic study of *Rhodocyclus*-related polyphosphate accumulating bacteria in full-scale enhanced biological phosphorus removal plants. Applied and Environmental Microbiology **70** (9), 5383-5390.

Kong, Y.H., Nielsen, J.L., Nielsen, P.H. (2005) Identity and ecophysiology of uncultured *Actinobacterial* polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. Applied and Environmental Microbiology **71** (7), 4076-4085.

Kong, Y.H., Xia, Y., Nielsen, J.L., Nielsen, P.H. (2006) Ecophysiology of a group of uncultured *Gammaproteobacterial* glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants. Environmental Microbiology **8** (3), 479-489.

Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.H., Wagner, M. (1999) Combination of fluorescent *in situ* hybridization and

microautoradiography - a new tool for structure-function analyses in microbial ecology. Applied and Environmental Microbiology **65** (3), 1289-1297.

Lee, N., Nielsen, P.H., Aspegren, H., Henze, M., Schleifer, K.H., Jansen, J.L. (2003) Long-term population dynamics and *in situ* physiology in activated sludge systems with enhanced biological phosphorus removal operated with and without nitrogen removal. Systematic and Applied Microbiology **26** (2), 211-227.

Liu, W.T., Mino, T., Nakamura, K., Matsuo, T. (1996) Glycogen accumulating population and its anaerobic substrate uptake in anaerobic-aerobic activated sludge without biological phosphorus removal. Water Research **30** (1), 75-82.

Liu, W.T., Nakamura, K., Matsuo, T., Mino, T. (1997) Internal energy-based competition between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus removal reactors - Effect of P/C feeding ratio. Water Research **31** (6), 1430-1438.

Liu, W.T., Nielsen, A.T., Wu, J.H., Tsai, C.S., Matsuo, Y., Molin, S. (2001) *In situ* identification of polyphosphate- and polyhydroxyalkanoate-accumulating traits for microbial populations in a biological phosphorus removal process. Environmental Microbiology **3** (2), 110-122.

Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Systematic and Applied Microbiology **15** (4), 593-600.

Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Rees, G.N., McDougall, B.M. (1997) *Amaricoccus* gen. nov, a gram-negative coccus occurring in regular packages or tetrads, isolated from activated sludge biomass, and descriptions of *Amaricoccus veronensis* sp. nov, *Amaricoccus tamworthensis* sp. nov, *Amaricoccus macauensis* sp. nov, and *Amaricoccus kaplicensis* sp. nov. International Journal of Systematic and Bacteriology **47** (3), 727-734.

Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Wanner, J. (2000) A fluorescently-labelled r-RNA targeted oligonucleotide probe for the *in situ* detection of G-bacteria of the genus *Amaricoccus* in activated sludge. Journal of Applied Microbiology **88** (5), 826-835.

Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Janssen, P.H., Wanner, J. (2005) *Defluvicoccus vanus* gen. nov., sp nov., a novel Gram-negative coccus/coccobacillus in the '*Alphaproteobacteria*' from activated sludge. International Journal of Systematic and Evolutionary Microbiology **55** (5), 2105-2111.

Meyer, R.L., Saunders, A.M., Blackall, L.L. (2006) Putative glycogen-accumulating organisms belonging to the *Alphaproteobacteria* identified through rRNA-based stable isotope probing. Microbiology-SGM **152** (2), 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.

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 Environmental Microbiology **65** (3), 1251-1258.

Nielsen, J.L., Christensen, D., Kloppenborg, M., Nielsen, P.H. (2003) Quantification of cell-specific substrate uptake by probe-defined bacteria under in situ conditions by microautoradiography and fluorescence *in situ* hybridization. Environmental Microbiology **5** (3), 202-211.

Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J. (2005) Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnology and Bioengineering. **91** (2), 162-168.

Seviour, R.J., Maszenan, A.M., Soddell, J.A., Tandoi, V., Patel, B.K.C., Kong, Y.H., Schumann, P. (2000) Microbiology of the 'G-bacteria' in activated sludge. Environmental Microbiology **2**, 581-593.

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.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J. (1994) Model of the anaerobic metabolism of the biological phosphorus removal process - stoichiometry and pH influence. Biotechnology and Bioengineering **43** (6), 461-470.

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-SGM **150** (11), 3741-3748.