Chapter 3

Characterization of the microbial community in the anaerobic/oxic/anoxic process with no sludge production and phosphorus recovery

SUMMARY

In this study, organisms playing an important role for nutrient removal in the anaerobic/oxic/anoxic process with no sludge production and phosphorus recovery were characterized by a PCR-cloning method and fluorescence in situ hybridization method. A total of 142 clones were amplified and yield 98 operation taxonomical units (OTUs). All OTUs comprised less than 6 clones, suggesting that the organisms in the process belonged to very different phylogenetic groups. Most clone sequences were affiliated with the Bacteroidetes, followed by the Betaproteobacteria and the Alphaproteobacteria. For Bacteroidetes, most OTUs were closely related to the sequences that had been obtained from activated sludge. For the sequences belonging to the Betaproteobacteria, the family Comamonadaceae, which have been reported as the extracellular poly-β-hydroxybutyrate (PHB) depolymerase producing denitrifying bacteria, was detected. Some intercellular PHB accumulating bacteria (the genus Amaricoccus Beijerinckia, Hyphomicrobium and Paracoccus) were detected in the process. These bacteria might play an important role for denitrification without extracellular organic carbon sources in the anoxic tank. The sequence related to Candidatus 'Accumulibacter phosphatis' and Actinobacterial PAOs, which are the major candidates of polyphosphate-accumulating organisms (PAOs), was not obtained in this study and it indicated that unidentified PAOs/DNPAOs (denitrifying PAOs) accumulate phosphorus in the oxic/anoxic tank of the process. FISH analysis demonstrated that the Betaproteobacteria and Alphaproteobacteria were prominent in the process.

3.1 INTRODUCTION

Activated sludge processes have been widely used to effectively remove nutrients from municipal wastewater. In wastewater treatment plants (WWTPs), treatment and disposal of excess sludge have been serious problems, and the treatment of excess sludge may account for 25% to 65% of total plant operating costs (Liu, 2003; Zhao and Kugel, 1997). Furthermore, the regulations regarding disposal have become increasingly strict in most countries as the amount of excess sludge increased with the expansion of populations and industries (Liu, 2003; Ødegaard, 2004).

In Chapter 2, to reduce both excess sludge and sludge production potential, a bench-scale continuous A/O/A system with no sludge production and phosphorus recovery was operated. In this process, excess sludge was solubilized by ozonation. The supernatant containing high amount of phosphorus was then passed a phosphorus adsorption column packed with zirconium-ferrite ($ZrFe_2(OH)_8$) adsorbent. To inhibit the phosphorus uptake in the oxic tank by polyphosphate-accumulating organisms (PAOs) and to induce the phosphorus uptake in the subsequent anoxic tank by DNPAOs, which has lower sludge production potential than PAOs, a part of the supernatant from the phosphorus adsorption column containing biodegradable organic carbons was added to the oxic tank. As a result, the oxic phosphorus uptake by PAOs was effectively inhibited and efficient nutrient removal was achieved with no excess sludge withdrawn.

The ratio of the anoxic phosphorus uptake rate (PUR) to the oxic PUR has been used as the indicator of the proportion of DNPAOs to total PAOs (Kuba *et al.*, 1997; Soejima *et al.*, 2006; Tsuneda *et al.*, 2006; Wachtmeister *et al*., 1997). The anoxic/oxic PUR ratio demonstrated that DNPAOs took a large part in phosphorus removal the same as previous studies whereas the population of total PAOs was lower than the previous studies (Kuba *et al.*, 1997; Soejima *et al.*, 2006; Tsuneda *et al.*, 2006). Additionally, the ratio of the anoxic PUR rate to the denitrification

rate was quite lower than previous studies, suggesting that denitrification was performed by other endogenous denitrifying organisms which were able to reduce nitrate/nitrite without extracellular organic carbon sources in the anoxic tank. Some of these endogenous denitrifying organisms have been called as denitrifying glycogen-accumulating organisms (DNGAOs) which have been reported the competitor of PAOs/DNPAOs because their ecophysiology is the same as PAOs/DNPAOs except for phosphorus uptake ability (Mino *et al.*, 1998; Seviour *et al.*, 2003). When the population of DNGAOs increased, the nutrient removal efficiency, especially for phosphorus removal efficiency, should be deteriorated (Mino *et al.*, 1998; Seviour *et al.*, 2003). Therefore, the characterization of the important organisms for nutrient removal in the A/O/A process with no production and phosphorus recovery was necessary.

In this study, PCR-Cloning and fluorescence *in situ* hybridization (FISH) were performed to characterize and to quantify the organisms playing important roles for nutrient removal in the A/O/A process with no sludge production and phosphorus recovery.

3.2 MATERIALS AND METHODS

3.2.1 Diversity and phylogenetic analysis by PCR-Cloning method

A sludge sample was collected from the bench-scale anaerobic/oxic/anoxic process with no sludge production and phosphorus recovery, which was operated in the previous study (Phase 4 in Chapter 2). Total DNA was extracted from the sample using Isoplant (Nippon Gene, Japan) according to the manufacturer's instructions. The 16S rRNA genes were amplified by PCR amplification by using universal primers, 341f and 907r (Muyzer *et al*., 1998). The PCR mixture contained 0.5 mM of each primer, 200 mM of dNTP, 1.0 mM $MgCl_2$, 1.25 U of Takara Ex Taq DNA polymerase (TAKARA BIO, Japan), 5 μL of 10× PCR Ex Taq buffer, and sterile water added to a final volume of 50 μL. The PCR amplifications were conducted in a model 9700 thermal cycler (Applied Biosystems,, USA) using the following protocol: 1 min at 94ºC, 25 cycles (60 s at 94ºC, 60 s at 55ºC, 60 s at 72ºC), 1 min at 72ºC. The presence of PCR product was confirmed by 2% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide. PCR products purification, cloning, plasmid DNA preparation and sequeincing with an ABI PRISM 3100-Avant DNA Sequencing system (Applied Biosystems, Japan) were performed as described previously (Osaka *et al*., 2006). A database search was conducted using BLAST from the DDBJ. The sequences determined in this study and those retrieved from the database were aligned using Clustal W (Thompson *et al*. 1994). Phylogenetic trees were constructed using Clustal W and Tree View (Page 1996) by the neighbor-joining method (Saitou and Nei 1987).

3.2.2 Fluorescence *in situ* hybridization (FISH) analysis

Sludge samples were collected from the A/O/A reactor in Phase 4 and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) solution (137 mM NaCl, 8.10 mM Na2HPO4, 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 1. 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 (Amann, 1995). Then, the samples were mounted in VECTASHIELD Mounting Medium (Vector, U.K.), and observed under a confocal laser scanning microscope (TCS4D, Leica Lasertechnik, Germany). Images of each probe binding-cells were collected and superimposed with the software Adobe Photoshop 6.0 (Adobe, USA). For direct counting of cells hybridized with each probe, fixed sludge samples were soaked in the same volume of sterile tripolyphosphorus buffer solution (400mg/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.

Table1 Oligonucleotide probes used in this study.

Probe name	Specificity	Probe sequence (5'-3')	Formamide (%)	Reference
EUB338	Most Bacteria	GCTGCCTCCCGTAGGAGT	20	Amann et al., 1990
EUB338II	*Planctomycetales*	GCAGCCACCCGTAGGTGT	20	Daims et al., 1999
EUB338III	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	20	Daims et al., 1999
ALF968	α-*Proteobacteria*	CGTTCGYTCTGAGCCAG	35	Neef, 1997
BET42a	β-*Proteobacteria*	GCCTTCCCACTTCGTTT	35	Manz et al., 1996
GAM42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	35	Manz et al., 1996
CF319a	*Cytophaga-Flavobacterium branch*	TGGTCCGTGTCTCAGTAC	35	Manz et al., 1996
PAO462	*Candidatus "Accumulibacter phosphatis*	CCGTCATCTACWCAGGGTATTAAC	35	Crocetti et al., 2000
PAO651	*Candidatus "Accumulibacter phosphatis*	CCCTCTGCCAAACTCCAG	35	Crocetti et al., 2000
PAO846	*Candidatus "Accumulibacter phosphatis*	GTTAGCTACGGCACTAAAAGG	35	Crocetti et al., 2000
GAOQ431	*Candidatus "Competibacter phosphatis"*	TCCCCGCCTAAAGGGCTT	35	Crocetti et al., 2002
GAOQ989	*Candidatus "Competibacter phosphatis"*	TTCCCCGGATGTCAAGGC	35	Crocetti et al., 2002

3.3 RESULTS AND DISCUSSION

3.3.1 Diversity and phylogenetic analysis by PCR-Cloning method

A total of 142 clones were amplified and classified as 98 operation taxonomical units (OTUs). All OTUs comprised less than 6 clones, suggesting that the organisms in the A/O/A process with no sludge production and phosphorus recovery belonged to very different phylogenetic groups and there was no prominent species. The quantitative contribution of clones affiliated with different divisions and subdivisions to total number of clones is shown in Fig.1. Most clone sequences were affiliated with the *Bacteroidetes* (39 clones belonging 26 OTUs), followed by the *Betaproteobacteria* (30 clones belonging to 25 OTUs) and the *Alphaproteobacteria* (22 clones belonging to 16 OTUs). Most sequences were closely related to the sequences obtained in activated sludges (Hoshino *et al.*, 2006; Osaka *et al.*, 2006).

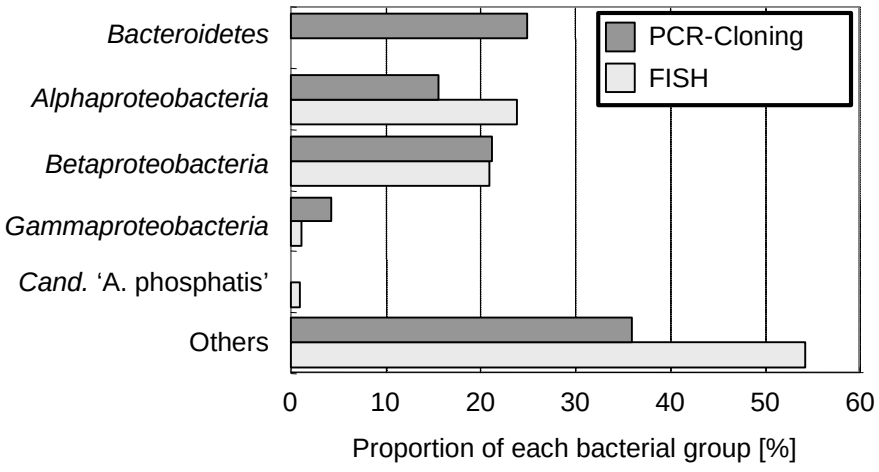

Fig.1 Quantitative contribution of each bacterial group determined by PCR-cloning method and FISH.

For the *Bacteroidetes*, except for the relative sequences (AOA-clone 37 and 106) to the genus *Runella*, no isolation was closely related to the sequences obtained in this study (Fig.2a). Moreover, most of sequences belonging to the *Bacteroidetes* were not closely related to any sequences in the database (below 97% similarity). The genus *Runella* isolated from diverse environmental habitats and *Runella limosa*, which has no ability to accumulate phosphorus and to reduce nitrate to nitrite, was recently isolated from acetated-fed sequencing batch reactor (SBR) with efficient phosphorus removal (Ryu *et al.*, 2006).

For the *Betaproteobacteria* (Fig.2b), 8 OTUs (11 clones) were affiliated with the family *Comamonadaceae*. Recently, the family *Comamonadaceae* has been reported as primary poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)-degrading denitrifying bacteria (Khan *et al.*, 2002). These bacteria could utilize the solid PHBV as electron donor under denitrifying conditions. The genus *Dechloromonas*, one of major denitrifying bacteria in wastewater treatment, was identified in this study. Ahn *et al.* (2002) reported genus *Dechloromonas* commonly existed in acetate-fed SBRs which operated with different electron accepters by using PCR-DGGE (denaturing gradient gel electrophoresis) analysis. No clone was closely related to *Candidatus* 'Accumulibacter phosphatis' which has been reported as one of major PAOs (DNPAOs). This result indicated that the other unidentified PAOs/DNPAOs played an important role for phosphorus removal in the A/O/A process.

For the *Alphaproteobacteria* (Fig.2c), most of sequences were affiliated with the isolated bacteria, the genus *Amaricoccus*, *Aminobacter*, *Beijerinckia*, *Deyosia*, *Hyphomicrobium*, *Nordella*, *Paracoccus* and *Roseomonas*. Maszenan *et al.* (1997) reported that the genus *Amaricoccus* isolated from activated sludge has been capable of both the poly-β-hydroxybutyrate (PHB) accumulation in an oxic condition and the nitrate reduction to nitrate. The genus *Aminobacter* is not denitrifying bacteria but PHB accumulating bacteria. The genus *Beijerinckia*, *Hyphomicrobium*, *Paracoccus* have both ability to accumulate PHB and to reduce nitrate to nitrite.

Two sequences ((AOA-clone 158 and 277) were closely related to *Candidatus*

‘Competibacter phosphatis’ belonging to the *Gammaproteobacteria*. These two sequences have no mismatch to the GAOQ431 probe specific for *Candidatus* ‘Competibacter phosphatis’. One OTU (AOA-clone 271) comprised 5 clones and was closely related to *Byssovorax cruenta*, a cellulose-degrading myxobacterium (Reichenbach *et al.*, 2006). One clone (AOA-clone 125) was closely related to an obligatory anaerobic bacterium, *Desulfobulbus elongates*. Actinobacterial PAOs (Kong *et al.*, 2005) were not detected in this study.

(a) *Bacteroidetes*

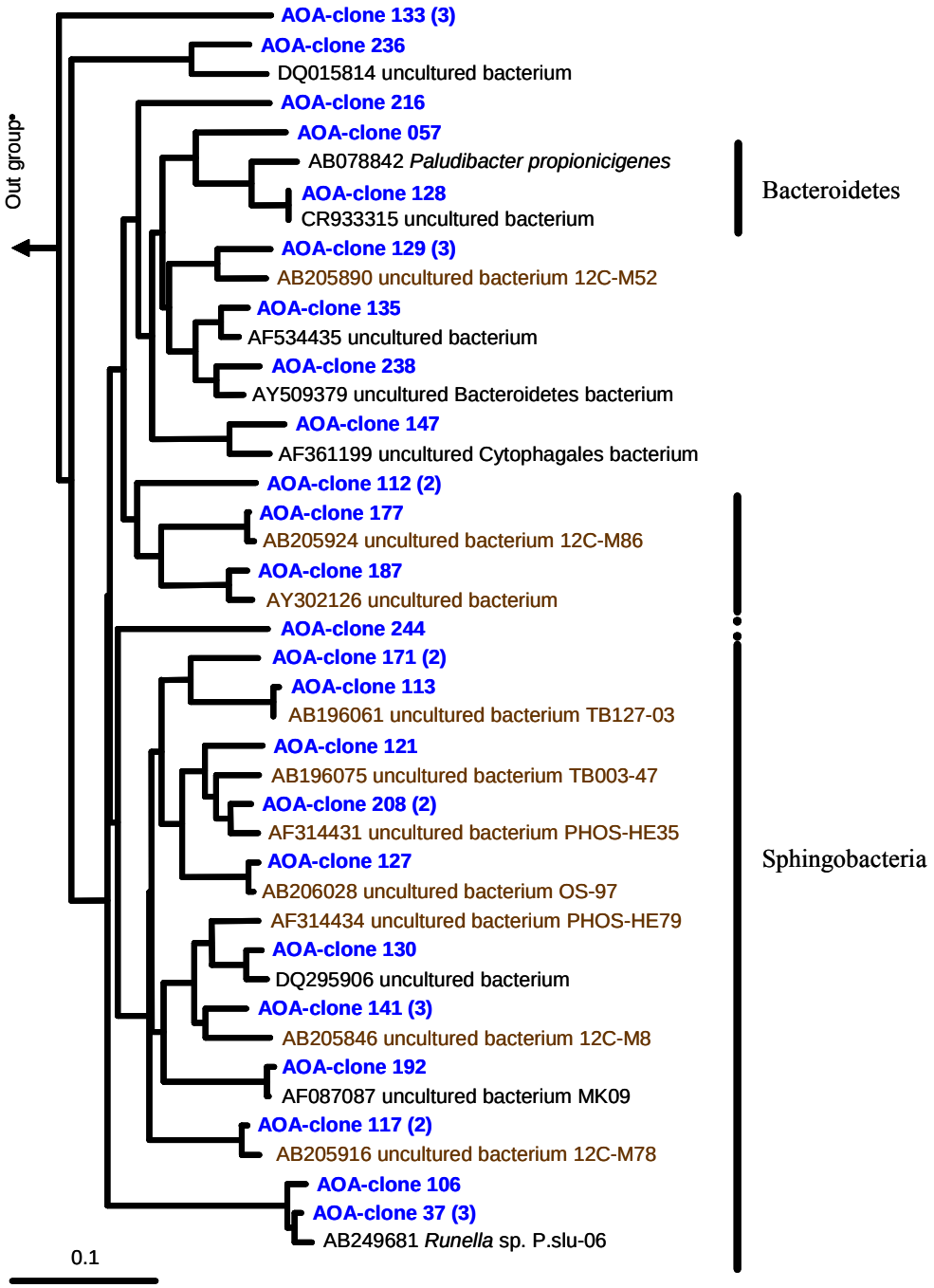

Fig. 2 Phylogenetic dendrogram based on 16S rRNA gene sequences in relation to members of the *Bacteroidetes* (a), *Betaproteobacteria* (b), *Alpha-, Delta-* and *Gammaproteobacteria* (c) and other bacterial groups (d). The trees were constructed using the neighbor-joining method. Genetic distances were calculated using Kimura's two-parameter method. The root of the tree was determined by using the 16S rRNA gene of *Methanosarcina mazei* (AB065295) as an outgroup. The theoretical length of terminal restriction fragments in *in silico* analysis is shown on the gray box. Scale bar indicates the 10% estimated difference in nucleotide sequence position. Clones obtained in this study are in blue boldface. The number of clones obtained is shown in parentheses.

(b) *Betaproteobacteria*

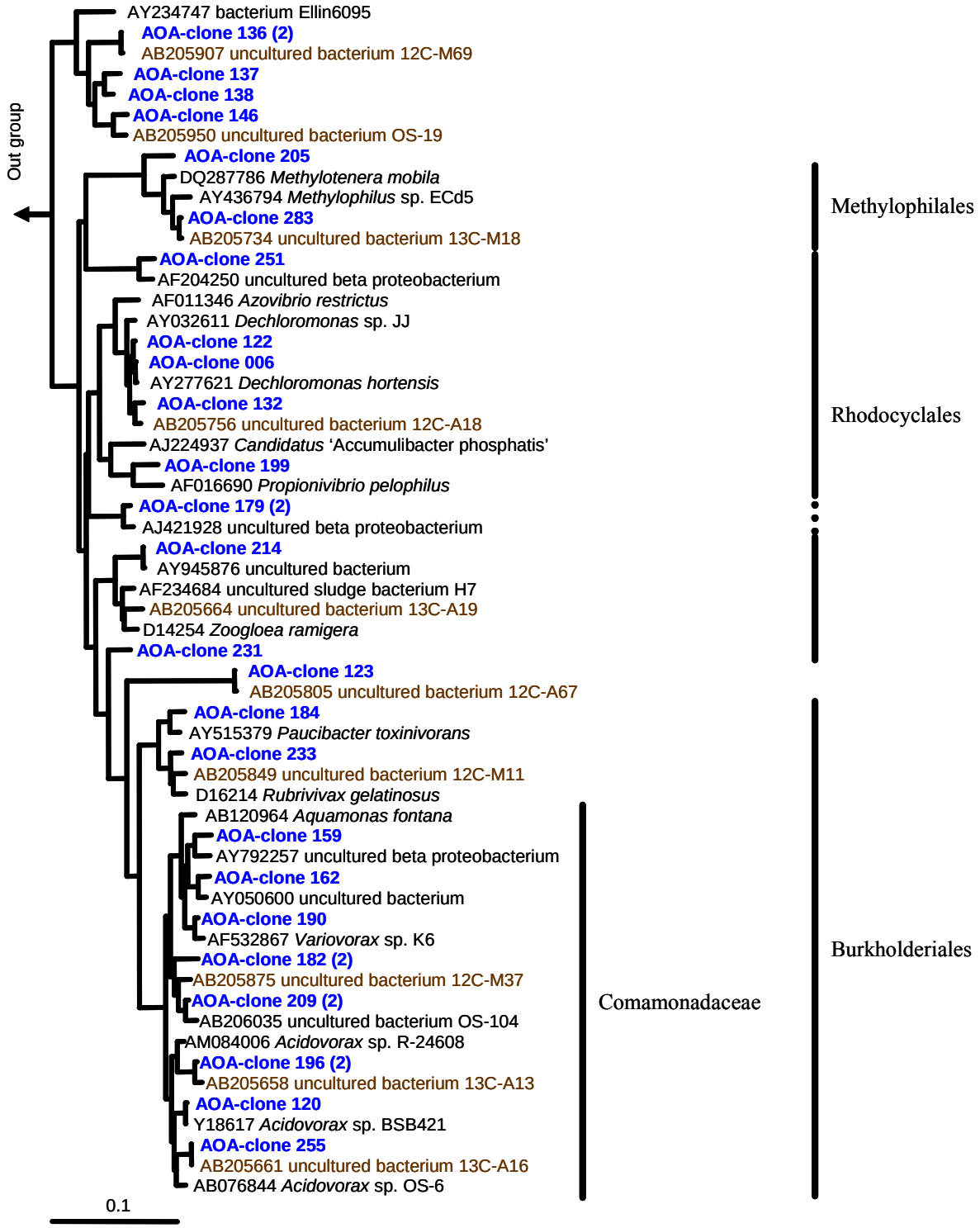

Fig. 2 continued.

(c) *Alpha-, Gamma-* and *Deltaproteobacteria*

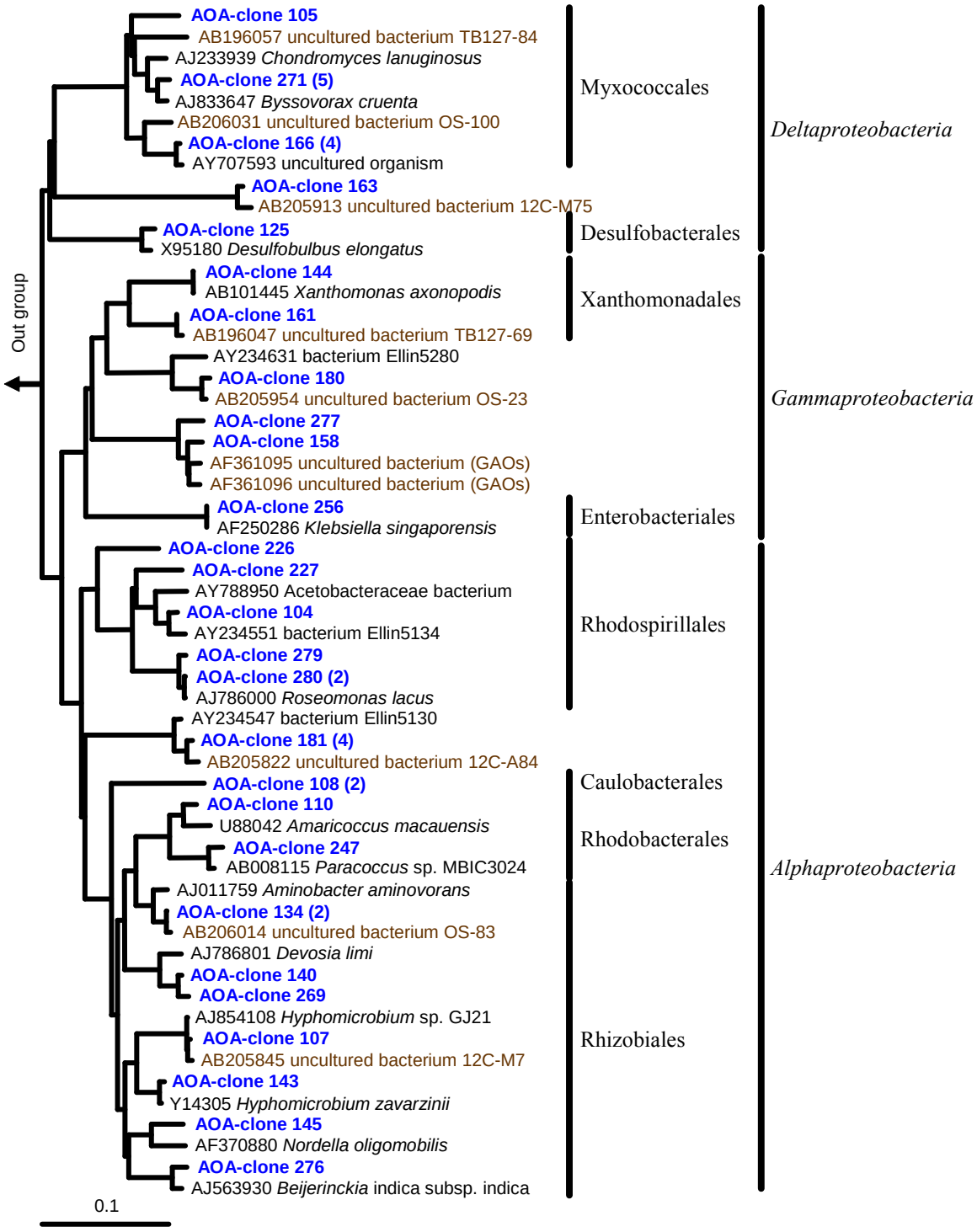

Fig. 2 continued.

(d) Other bacterial groups

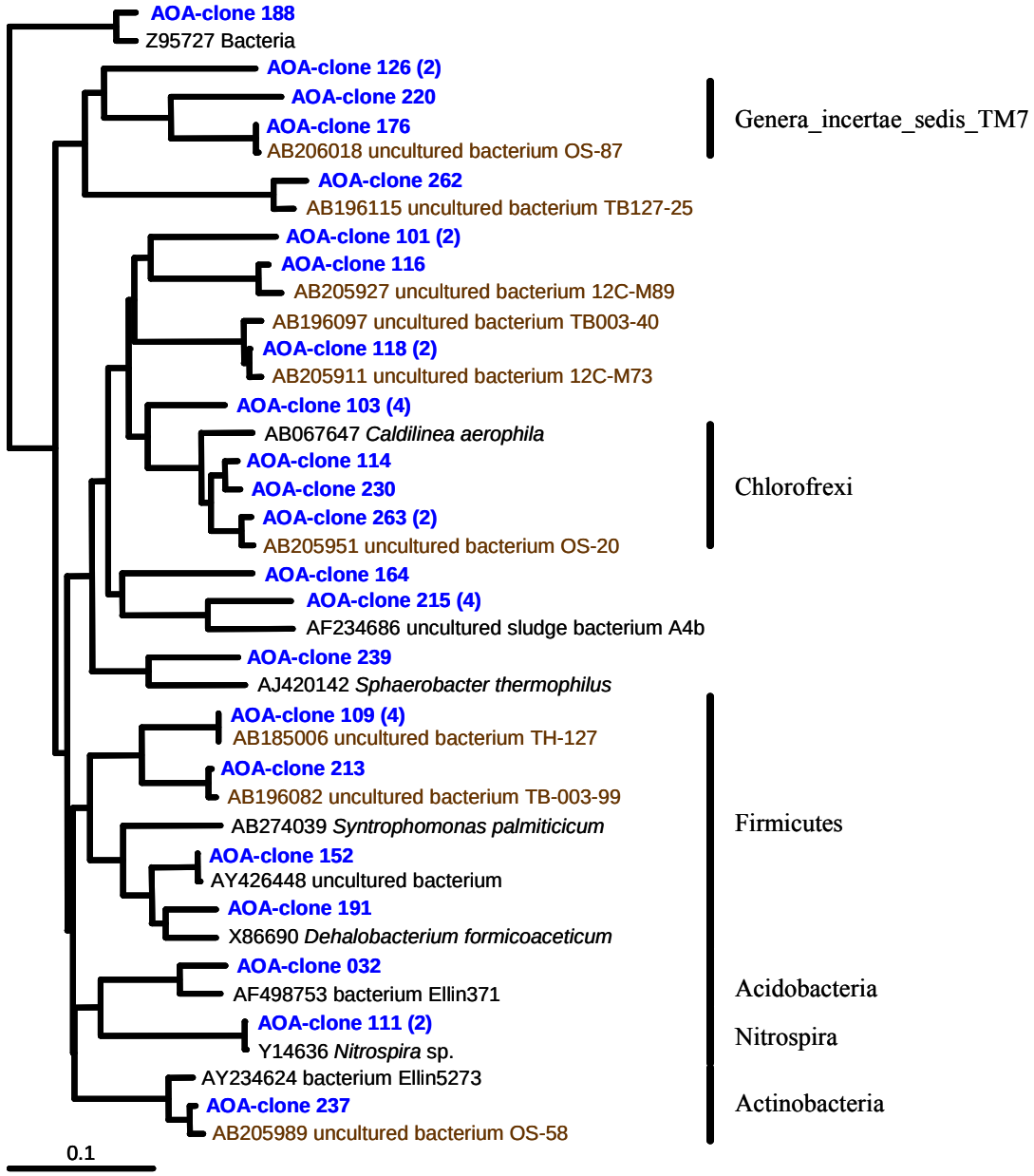

Fig. 2 continued.

3.3.2 FISH analysis

The population of each bacterial group (the *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*), *Candidatus* 'Accumulibacter phosphatis' and *Candidatus* 'Competibacter phosphatis' was evaluated by FISH analysis. The proportion of each bacterial group is shown in Fig.1. The *Alphaproteobacteria* and the *Betaproteobacteria* were the prominent in the process (24% and 21% of EUB probes-binding cells, respectively). These proportions were almost the same as the PCR-cloning analysis. However, the phylum *Bacteroidetes* was not detected in this study. The CF319a probe was commonly used to detect the *Bacteroidetes* in enhanced phosphorus removal processes (Crocetti *et al.*, 2000, 2002; Liu *et al.*, 2001; Wong *et al.*, 2005). However, according to probeBase (Loy *et al.*, 2003), only 38% of the phylum *Bacteroidetes* sequences in database has no mismatch to the CF319a probe. Therefore, the population density of the *Bacteroidetes* should be underestimated. O'Shllivan *et al.* (2002) designed new degenerate probe, CF560, specific for the phylum *Bacteroidetes*. This probe has no mismatch to all sequence belonging to the phylum *Bacteroidetes* obtained in this study and should be used in the future study.

The *Alphaproteobacteria* and the *Betaproteobacteria* were prominent in the A/O/A process with no sludge production and phosphorus recovery. Considering the results of the PCR-cloning analysis, intercellular PHB accumulating *Alphaproteobacteria* was prominent in the reactor. Some of them have denitrification ability. Then, it was suggested that these organisms reduce nitrate in the oxic tank without extracellular organic carbon sources. The prominence of the *Betaproteobacteria* also suggested that the family *Comamonadaceae* also reduce nitrate with depolymerization of extracellular PHBV/PHB. Therefore, PHBV/PHB should be the key substrate in the A/O/A process with no sludge production and phosphorus recovery. One of major PAOs, *Candidatus* 'Accumulibacter phosphatis', was quite low abundance in the A/O/A process (1%). This result also suggested that unidentified PAOs/DNPAOs should play an important role for phosphorus removal in the process. On the other hand, *Candidatus* 'Competibacter phosphatis' detected by the PCR-Cloning method was not observed.

3.3.3 Microbial community in the A/O/A process with no sludge production and phosphorus recovery

In Chapter 2, to reduce both excess sludge and sludge production potential, a bench-scale continuous A/O/A system with no sludge production and phosphorus recovery was operated. The anoxic/oxic PUR ratio demonstrated that DNPAOs took a large part in phosphorus removal the same as previous studies whereas the population of total PAOs was lower than the previous studies (Kuba *et al.*, 1997; Soejima *et al.*, 2006; Tsuneda *et al.*, 2006). Additionally, the ratio of the anoxic PUR rate to the denitrification rate was quite lower than previous studies, suggesting that denitrification was performed by other endogenous denitrifying organisms which were able to reduce nitrate/nitrite without extracellular organic carbon sources in the anoxic tank.

A total of 142 clones were amplified and classified as 98 operation taxonomical units (OTUs). All OTUs comprised less than 6 clones, suggesting that the organisms in the A/O/A process with no sludge production and phosphorus recovery belonged to very different phylogenetic groups and there was no prominent species. Most clone sequences were affiliated with the *Bacteroidetes* (39 clones belonging 26 OTUs), followed by the *Betaproteobacteria* (30 clones belonging to 25 OTUs), the *Alphaproteobacteria* (22 clones belonging to 16 OTUs). Most sequences were closely related to the sequences obtained in activated sludges (Hoshino *et al*., 2006; Osaka *et al.*, 2006).

For the *Bacteroidetes*, except for the relative sequences (AOA-clone 37 and 106) to the genus *Runella*, no isolation was closely related to the sequences obtained in this study (Fig.2a). FISH analysis demonstrated that the CF319a probe, which is commonly used, was not suitable to detect all the member of the phylum *Bacteroidetes*.

The family *Comamonadaceae* in the *Betaproteobacteri*a, which has been reported as primary PHBV-degrading denitrifying bacteria, was detected in the reactor. These bacteria could utilize the solid PHBV as electron donor under denitrifying conditions (Khan *et al.*, 2002). On the other hand, the genus *Beijerinckia*, *Hyphomicrobium* and *Paracoccus,* which have both ability to accumulate PHB and to reduce nitrate to nitrite, were detected. These results suggested that these bacteria contributed to denitrification without extracellular organic carbon sources.

Khan *et al.* (2002) reported the cometabolism of PHBV-degrading non-denitrifying bacteria and denitrifying bacteria might also contribute in part to the overall denitrification activity of activated sludge. In this study, the genus *Dechloromonas* and *Zoogloea* major denitrifying bacteria in wastewater treatment, was identified and these denitrifying bacteria might utilize degraded PHBV as electron donor. It is difficult to estimate each activity for denitrification (intercellular PHB accumulating denitrifying bacteria, PHBV denitrifying bacteria, denitrifying bacteria); however, those different types of denitrification contributed the high diverse microbial community in the A/O/A process with no sludge production and phosphorus recovery.

The major PAOs/DNPAOs candidates, *Candidatus* 'Accumulibacter phosphatis' and *Actinobacterial* PAOs, were not detected in this study, suggesting that unidentified PAOs/DNPAOs should play an important role for phosphorus removal in the A/O/A process with no sludge production and phosphorus recovery. Identification of these unidentified PAOs/DNPAOs was difficult because of high-diverse microbial community and this high-diverse microbial community suggested that not only one prominent PAOs/DNPAOs but some phylogenetically different PAOs/DNPAOs contributed to phosphorus removal.

3.4 CONCLUSIONS

In this study, PCR-Cloning and fluorescence *in situ* hybridization (FISH) were performed to characterize and to quantify the organisms playing important roles for nutrient removal in the A/O/A process with no sludge production and phosphorus recovery. Main conclusions are as follows.

(1) The organisms in the A/O/A process with no sludge production and phosphorus recovery belonged to very different phylogenetic groups and there was no prominent species. Most clone sequences were affiliated with the *Bacteroidetes*, followed by the *Betaproteobacteria* and the *Alphaproteobacteria*.

(2) For the *Alphaproteobacteria* (24% of EUB-probes-binding cells), the genus *Amaricoccus* *Beijerinckia*, *Hyphomicrobium* and *Paracoccus,* which have both ability to accumulate PHB and to reduce nitrate to nitrite, contributed to denitrification in the anoxic tank.

(3) For the *Betaproteobacteria* (21% of EUB-probes-binding cells), the family *Comamonadaceae* in the *Betaproteobacteri*a, which has been reported as primary PHBV-degrading denitrifying bacteria, was detected and these bacteria contributed to denitrification.

(4) The major PAOs/DNPAOs candidates, *Candidatus* 'Accumulibacter phosphatis' and *Actinobacterial* PAOs, were not detected in this study, suggesting that unidentified PAOs/DNPAOs should play an important role for phosphorus removal in the A/O/A process with no sludge production and phosphorus recovery.

3.5 REFERENCES

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.

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.

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** (2), 403-412.

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.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology **22** (3), 434-444.

Hoshino, T., Terahara, T., Yamada, K., Okuda, H., Suzuki, I., Tsuneda, S., Hirata, A., Inamori, Y. (2006) Long-term monitoring of the succession of a microbial community in activated sludge from a circulation flush toilet as a closed system. FEMS Microbiology

Ecology **55** (3), 459-470.

Khan, S.T., Horiba, Y., Yamamoto, M., Hiraishi, A. (2002) Members of the family *Comamonadaceae* as primary poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifiers in activated sludge as revealed by a polyphasic approach. Applied and Environmental Microbiology **68** (7), 3206-3214.

Kong, Y., 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.

Kuba, T., van Loosdrecht, M.C.M., Brandse, F.A., Heijnen, J.J. (1997) Occurrence of denitrifying phosphorus removing bacteria in modified UCT-type wastewater treatment plants. Water Research **31** (4), 777-786.

Liu, Y. (2003) Chemically reduced excess sludge production in the activated sludge process. Chemosphere **50** (1), 1-7.

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.

Loy, A., Horn, M., Wagner, M. (2003) probeBase - an online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Research **31** (1), 514-516.

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 Bacteriology. **47** (3), 727-734.

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., Brinkhoff, T., Nubel, U., Santegoeds, C., Schafer, H., Wawer, C. (1998) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Akkermans, A.D.L., v. Elsas, J.D., de Bruijin, F.J. (Eds.) Molecular Microbial Ecology Manual, Kluwer Academic Publishers, Dordrecht, the Netherlands, 3.4.4., pp 1–27.

Neef, A. (1997) Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse von komplexen mikrobiellen Biozönosen. PhD thesis, Technische Universität München, Munich, Germany

Ødegaard, H. (2004) Sludge minimization technologies - an overview. Water Science and Technology **49** (10), 31-40.

Osaka, T., Yoshie, S., Tsuneda, S., Hirata, A., Iwami, N., Inamori, Y. (2006) Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. Microbial Ecology **52** (2), 253-266.

Page, R.D.M. (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences **12** (4)**,** 357–358.

Reichenbach, H., Lang, E., Schumann, P., Sproer, C. (2006) *Byssovorax cruenta* gen. nov., sp. nov., nom. rev., a cellulose-degrading myxobacterium: rediscovery of'*Myxococcus cruentus*' Thaxter 1897. International Journal of Systematic and Evolutionary Microbiology **56,** 2357–2363.

Ryu, S.H., Hien, T.T., Park, W., Kim, C.J. (2006) *Runella limosa* sp. nov., isolated from activated sludge. International Journal of Systematic and Evolutionary Microbiology **56**, 2757-2760.

Saitou N, Nei M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4** (4), 406–425.

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

Soejima. K., Oki, K., Terada, A., Tsuneda, S., Hirata, A. (2006) Effects of acetate and nitrite addition on fraction of denitrifying phosphate-accumulating organisms and nutrient removal efficiency in anaerobic/aerobic/anoxic process. Bioprocess and Biosystems Engineering **29** (5-6), 305-313.

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22** (22), 4673–4680.

Tsuneda, S., Ohno, T., Soejima, K., Hirata, A. (2006) Simultaneous nitrogen and phosphorus removal using denitrifying phosphate-accumulating organisms in a sequencing batch reactor. Biochemical Engineering Journal **27** (3), 191-196.

Wachtmeister, A., Kuba, T., van Loosdrecht, M.C.M.and Heijnen, J.J. (1997) A sludge characterization assay for aerobic and denitrifying phosphorus removing sludge. Water Research **31** (3), 471–478.

Wong, M.T., Mino, T., Seviour, R.J., Onuki, M., Liu, W.T. (2005) *In situ* identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. Water Research **39** (13), 2901-2914.

Zhao, Q.L., Kugel, G. (1997) Thermophilic/mesophilic digestion of sewage sludge and organic waste. Journal of Environmental Science and Health, A **31** (9), 2211–2231.