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Determination and Characterization of Microbial Community Structure of Activated Sludge

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Abstract

All data suggest that microbial community structures or samples of sludge with a content of phosphate between 8 and 12.3% were very similar but distinct from those containing phosphate at 1.8%. In all samples analyzed, ubiquinones, menaquinone and fatty acids were the main components. Dominance and E5 suggested that a large number of organisms belonging to the b and subclasses Proteobacteria and *Actinobacteria* from higher GMC Gram-positive bacteria, respectively, were present. Denaturing gradient gel electrophoresis analysis revealed at least 6-10 predominant DNA bands and numerous other fragments in each sample. Five major denaturing gradient gel electrophoresis fragments from each of 1.8% and 11.8% phosphate containing sludge samples, respectively, were successfully isolated and sequenced. Phylogenetic analysis of the sequences revealed that both 3% and 15% phosphate -containing sludge samples shared three common phylotypes which are separately associated with new bacterial groups of subclass C Proteobacteria, two E5 containing *Actinobacteria*, and *Caulobacter* spp. The subclass Proteobacteria. Phylogenetic analysis revealed useful phylotypes unique for both samples sludge. Therefore, changes in the phosphate content did not affect the composition and quantity prevailing microbial population, although specific phylotypes could not be unambiguously associated with EBPR.

Keywords: Activated sludge; Biomarker; DGGE; 16S rDNA; *Caulobacter*

Introduction

Nowadays, biological wastewater treatment plants are the most common biotechnological application in the world [1]. More than 15,000 WWTPs operate in the United States alone, 75% of which include a secondary biological treatment, processing billions of liters of sewage per day [2]. From the various alternatives of biological treatment systems that exist, conventional activated sludge (CAS) bioreactors are by far the most commonly used secondary treatment technology [3]. Despite of periodic improvements to the technology since its invention almost a century ago [3] and its ubiquitous global application, little is known about the underlying factors controlling the complex dynamics of the microbial populations interacting in the bioreactors and how those dynamic interactions affect the system's functional stability [4]. Methods based on the analysis of various biomarkers and 16S rRNA genes (rDNA) have been used to characterize and monitor microbial communities in various ecological systems. The biomarkers, cellular fatty acids and respiratory quinones, have been used routinely in taxonomy to characterize, differentiate and identify genera, species, and strains of bacteria [5]. Some studies have further shown that the biomarker `signature' of environmental samples can be statistically analysed and applied to differentiate community profiles [6-8]. 16S rDNA-based methods can provide more information on the phylogenetic structure of microbial communities than the biomarker method [9,10]. The number and intensity of resolved fragments gives an approximate estimate of the diversity of the predominant species, and further purification of fragments and sequence analysis provides an insight into the phylogenetic affiliatio of individual populations [11,12]. Combining the biomarkers and 16S rDNA-based approaches should greatly enhance the characterization of microbial communities found in various systems. At a 11.8% phosphate content the community was predominated by rod-shaped organisms that accumulated both polyphosphate and PHA, whereas at a 1.8% P content the community was dominated by PHA-accumulating cocci that neither accumulated polyphosphate nor aerobically took up phosphate when provided [13]. It was suspected that the loss of polyphosphate accumulating bacteria upon shifting the feed composition was due to loss of their energy pool (polyphosphate) which they used to transport and store carbon (e.g., PHA) under anaerobic conditions so that it could be subsequently used for growth under aerobic conditions. Our initial assumption was that polyphosphate accumulating bacteria would constitute a predominant fraction of the bacterial population in a 11.8% phosphate-containing sludge, and on altering sludge phosphate content to 1.8%, could be easily differentiated from non-polyphosphate accumulating bacteria using the biomarker and denaturing gradient gel electrophoresis approaches. Furthermore, the phylogenetic affiliatio of the predominant populations in 1.8% and 11.8%-phosphate containing sludge could be easily identifiable from sequence analysis of the predominant 16S rDNA denaturing gradient gel electrophoresis fragments. This study was aimed at testing the above assumption.

Methods

Activated sludge enrichment

Three sequential batch reactors (A1, A2 and A3) were used with a mixture of acetate and peptone as carbon sources in alternating anaerobic and aerobic conditions as previously described [14]. All reactors were subjected to eight rounds of 3 hours per day, which consisted of a 50 min period followed by anaerobic aerobic phase of 80 min and 50 min and the sediment phase. Early in the anaerobic phase, the substrate was introduced to maintain an organic load of 0.38-0.88 kg/cm^2, third residence time of the sludge was maintained between

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7 and 8 d, and pH was controlled between 7 and 8. The phosphate content in each loop reactor was controlled between about 1.8 and 14% using total organic carbon reports from different phosphate [14]

Chemotaxonomic biomarker analyses

Hiraishi et al. procedures for the analysis of respiratory tract-quinone were described by Ref. [11]. In short, respiratory quinones were extracted with a chloroform:methanol mixture, which was purified by thin layer chromatography and qualitatively analyzed by reverse phase HPLC equipped with an array detector photodiodes. Determination of other quinones, including demethyl menaquinone and rhodoquinone, has not been attempted because these quinones are previously found in the methods of enhanced biological phosphate removal [11,12]. Ubiquinones and menaquinones n isoprene units are abbreviated n-Q and E-n, respectively. E-n (Hx) represents a partially hydrogenated menaquinone x with hydrogen atoms on the side chain containing n isoprene units (Rajendran et al.). Total cellular fatty acids were analyzed using a protocol described by other researchers. Cluster analysis (statistical) was used to statistically distinguish models in the respiratory quinone and total cellular fatty acid data. Dendrograms were constructed using the unique link and euclidean distance rules in the statistical program. The analysis of variance was also used to test the changes in individual quinone components from mud samples from the reactors containing different phosphate contents sludge

PCR amplification and isolation of DNA

Deoxyribo nucleic acid from activated sludge was obtained after cell lysis, extraction with chloroform, phenol and precipitation with ethanol using a protocol described previously [13,14]. This DNA preparation was used as a template in PCR mixtures - a reaction which contained 1x PCR buffer, 200 microM each dNTP, 1.5 mM $MgCl_2$, 0.1 microM each loop primer, 5% DMSO and 2.5 U Taq DNA polymerase in a final volume of 100 ml. For the amplification of 16S rDNA for denaturing gradient gel electrophoresis analysis, 968FGC forward primer with a GC-clamp [15] and reverse primer 1392R [16] were used. The polymerase chain rection was performed in a Perkin Elmer 9600 thermocycler using a thermal program described previously [17]. Amplification of DNA was verified by electrophoresis of 2 ml of the PCR product on a 1% gel agarose in TAE 1X buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM EDTA).

Denaturing gradient gel electrophoresis

Denaturing Gradient Gel Electrophoresis was carried out using a D-Gene system according to manufacturer's instructions. PCR products were loaded onto an acrylamide gel at 6% in 1X TAE buffer. The denaturing gradient in the gel was formed by mixing two stock solutions of 6% acrylamide containing 40% denaturing formamide and 60% denaturing formamide deionized with a denaturing AG501 -X8 mixed bed resin before being used (Riesner et al.). DNA fragments were visualized by silver staining as described by other scholars.

Isolation, cloning and sequencing

Deoxyribo nucleic acid sequences of specific fragments in the denaturing gradient gel electrophoresis gels were determined. Fragment was excised from the gel with a razor blade and the DNA was eluted overnight in 100 l of buffer of TAE. Each DNA fragments amplified by PCR were DGGE primers described above, ligated into the pCRII vector and transformed into competent *E. coli* cells. Clones having fragments of target DNA were chosen randomly gain acceptance ten clones using the denaturing gradient gel electrophoresis gel primers and comparing the electrophoretic mobility of the amplicon with the fragments in the original sample. The selected DNA fragments were sequenced using 968FGC and terminal 1392R primers and Taq Dye Deoxy Terminator Cycle Sequencing Kit.

Phylogenetic analyses

Partial DNA sequences obtained in this study were compared with 16S rRNA sequences available in Gen Bank using the NCBI BLAST program. The bridge closely related NCBI blast search sequences and 16S rRNA important sequences of environmental clones and bacterial isolates obtained from EBPR process were retrieved and aligned with these denaturing gradient gel electrophoresis bands sequenced using the Clustal W program [18]. A phylogenetic tree was constructed from the evolutionary distance matrix based on the algorithm of two parameters using the method of Kimura neighbor-joining [19]. The analysis was performed with the mega program [20], and the deviation in alignment sites were excluded from the pair wise comparison.

Results and Discussion

Community composition as revealed using chemotaxonomic biomarkers

Table 1 shows the respiratory quinone profiles of activated sludge samples with different phosphate contents. Both ubiquinone and menaquinones were detected in all sludge samples. S8 and S10 were the most dominant ubiquinones present in all samples, Followed by S7 and S9. The high content of S8 and S10 could reflect the predominance of members of the b and has Subclasses, respectivement, of the Proteobacteria [21]. E5 was the dominant menaquinone detected (70%), and is found in mainly members of the *Actinobacteria* of the high G+C Gram-positive bacteria [22]. Fatty-acid profiles revealed that two major monounsaturated fatty acids, C16: 1 and C18 and a saturated fatty acid, C16: 0, accounted for more than 60% of total fatty acids present in all sludge communities. In addition, lower water equivalent of C16: 0i were detected. The profiles of both respiratory quinones and fatty acids for different samples taken from reactors at different phosphate analyzed statistically. Similar results of ANOVA have shown that the difference in the mean percentage of Q8, S10 and E5 was observed between a mud phosphate 1.8% from R1 and phosphate sludge from the reactor R3 11.8%. Since Q - 8, S10 and E5 (H%) were the main components of ubiquinone and menaquinone, the difference observed on changing the sludge content phosphate 12.3 to 1.8% suggested trips population occurring in the chamber of a Proteobacteria subclass and *actinobacteria* respectively. However, significant differences in -8 Q, Q-10 and E in R1 with 1.8% phosphate, and R3 with 1.8% phosphate, it is difficul to draw generalized conclusions about their role in eliminating phosphates. As a result of respiratory quinone profiling fatty acid data (Figure 1b) also revealed marked differences in the

Phosphate content		A1	A2	A3		
Phosphate/carbon		1.8	7.2	1.8	7.4	11.8
Ratio		2:100	10:100	2:100	10:100	20:100
Ubiquinones	S7	0.12	1.8	0	1.74	0.58
	S8	4.8	6.8	1.2	1.28	1.28
	S9	0.28	0.48	1.4	0.74	0.74
	S10	4.9	0.92	6.5	0.91	1.3
Menaquinones	E6	0	1.5	0.5	0	1.21
	E7	0	0	1.28	0	1.81
	E8	1.3	1.2	0.6	0.25	0
	E8 (H4)	1.7	2.8	1.4	1.4	0.91
	E9	0.8	3	0.52	1.12	1.31
	E9 (H4)	0.28	1.4	1.2	0.81	0.74

Table 1: Respiratory quinone profile.

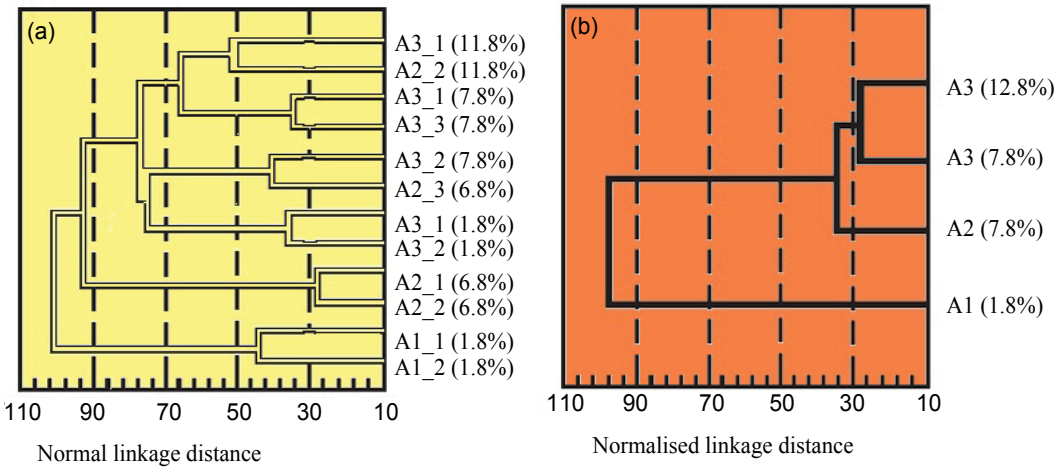

Figure 1: Dendrogram of the cluster analysis on the profiles of respiratory quinones (a) and cellular fatty acids (b) for samples containing different sludge P contents.

structure of microbial communities in reactor enhanced biological phosphate removal containing different levels of phosphate

Community structure as revealed using DGGE

The fragments of 16S rDNA genes amplified from sludge samples containing 11.8 (A3), 7.8 (A2 and A3), 1.8% (A3) phosphate content were solved using denaturing gradient gel electrophoresis (Figure 2). On the basis of the intensity of the fragment of about 8.2 predominant fragments and many others have been observed in each sample. The electrophoretic mobility and intensity of major bands were similar in the sludge samples A3 with phosphate content ranging from 7.8 to 11.8%, suggesting the predominant proposal populations of microbial communities are not detectable different. Furthermore, the grounds of 16S rDNA fragments from sludge samples having a phosphate content of about 8% were very similar to each other, indicating that the enrichment of microbial populations was reproducible to phosphate content. A further reduction of the phosphate content of the sludge from 7.8% to 2 Detectable caused a change in fingerprints of denaturing gradient gel electrophoresis: at least three of the seven bands dominate the phosphate sludge 8% have disappeared and six new groups appeared predominant in the phosphate sludge of 1.8%. Obviously, the energy depletion of the pool for carbon uptake excluded some of the dominant microbial populations that were perhaps involved in an enhanced biological phosphate removal activity, leading to the selection of other populations microbial in the process. Consequently, as the results of the biomarker, it can be concluded that the variation of ratio phosphate of 20: 100-2 100 causes not only the decrease in sludge phosphate content, but also the change in the types and plenty of predominantly microbial populations in enhanced biological phosphate removal systems. Referring to the observation of denaturing gradient gel electrophoresis bands predominant common in both high and low phosphate samples sludge percentage further suggested that the microbial ecology occurring in the system of enhanced biological phosphate removal was more complicated than our initial assumption. This polyphosphate accumulation and non-polyphosphate accumulating bacteria were represented by bands of denaturing gradient gel electrophoresis. Identification of 16S rRNA sequences predominant DGGE bands allow such explanations postulated. However, we must recognize that rRNA sequence information rarely allows a physiological function to be assigned to each population. More features, polyphosphate and PHA training are not phylogenetically conserved [22-24].

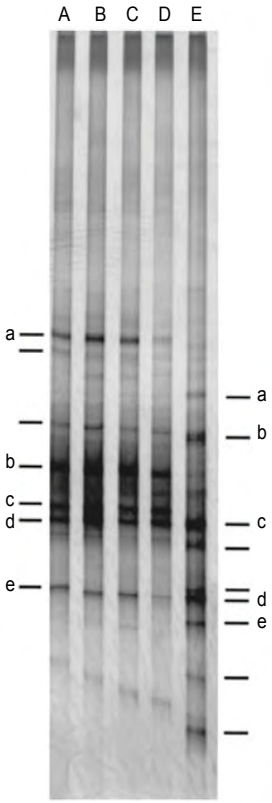

Figure 2: Polymerase chain reaction-Denaturing gradient gel electrophoresis analysis of activated sludge sample.

Phylogeny of predominant populations

Seven and nine major fragments from mud samples containing 11.8 and 1.8% phosphate content, respectively, were excised from the gel and purified. The purified DNA was amplified using the same primers and subjected to denaturing gradient gel electrophoresis in a gel together with the initial sample. Perhaps due to the quality of the recovered DNA, only five large fragments of each sample gave PCR products. Since re-amplification of one fragment often results in the formation of several amplicons, direct sequencing of the fragment

could not be re-amplified (data not shown). Instead, the fragment was reamplified cloned and clones with the correct insert were identified by comparing the electrophoretic mobility of the cloned fragments with that of the target fragment found in the original sample. The comparative analysis of these partial 16S rRNA sequences (Figure 3) revealed the phylogenetic affiliatio of ten sequences recovered. Among the ten sequences determined, two fragments DGGE found in sludge samples phosphate 1.8% and 11.8% were identified. The six remaining fragments were unique and have found that in either 1.8% or 11.8% sample phosphate sludge. A pair of common fragments (Figure 2) was

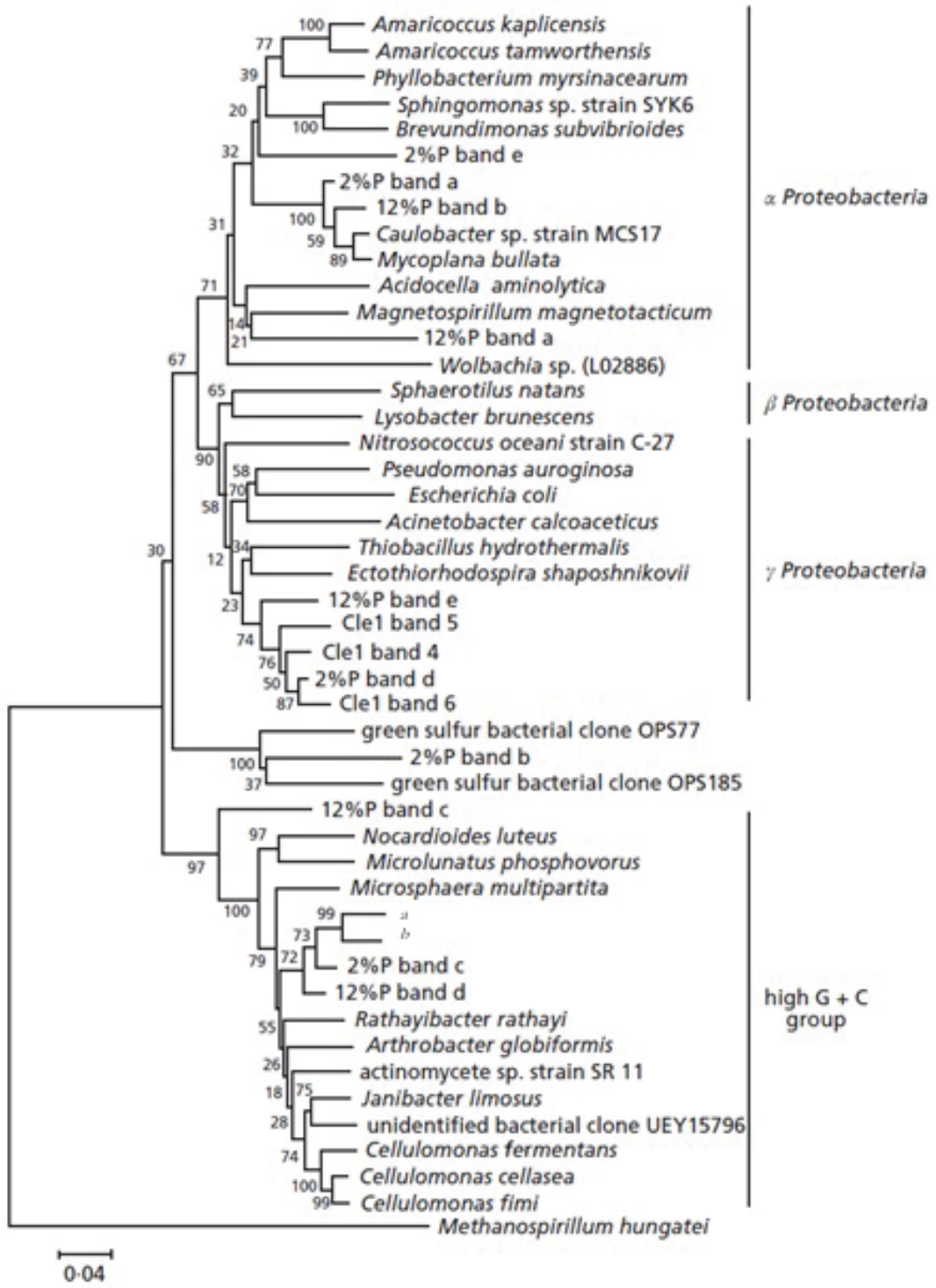

Figure 3: Phylogenetic distribution of numerically dominant populations in sludge samples containing either 2% or 12-3% P, based on the partial sequence of 16S rDNA genes resolved using DGGE. The distance-matrix consensus tree was calculated using the neighbour-joining method with bootstrapping. The 16S rDNA sequence of *Methanospirillum hungatei* (belonging to the domain Archaea) was used to root the tree.

closely related to a novel isolated but-not-yet-group bacterial c subclass of the Proteobacteria (Figure 3) found in anaerobic acetate medium fed by the aerobic activated sludge that has not accumulated phosphate (Nielsen et al.). This group of new bacteria could accumulate granular inclusions, and possibly PHA composed of two populations with identical morphology coccoid (3-4 cells were ~ l m diameter). Large cocci with morphological characteristics similar to those of the group according to the invention have also been observed as a predominant population at the beginning of our 1.8% phosphate mud sample. Though the ability of this novel group to accumulate phosphate remains unclear, it was also observed in a good EBPR process with PHA accumulation. Although the ability of this novel group of accumulating phosphate remains uncertain, it was also observed in a good process EBPR with PHA accumulation by the combination of fluorescent in situ hybridization staining and PHB in (data not presented). This is probably because the group can perform the new carbon metabolism in the process of EBPR, and has not completely overwhelmed by the phosphate batteries. Thus, a low content S9, a major component of the ubiquinone c subclass Proteobacteria, was observed in all samples of sludge (Table 1). Another common DGGE fragment at both 2 and 12 ± 3% Sludge phosphate (Figure 2) was closely linked to the *actinobacteria*. The cultured bacteria were most closely related two isolates, the strain and b, from the reactor containing 1.8% phosphate [25-28]. These two isolates accumulated PHA but not polyphosphate granular inclusions and contain the same major menaquinone component [E5 (% M)] [29] as seen in the sludge sample. It is likely that these two organizations are not particularly involved in phosphorus removal. A good example is between a phosphate-accumulating bacteria, Microlunatus phosphovorus [30] and non-phosphate accumulating bacteria *Micropruina glycogenica* [31], two phylogenetically related genera isolated from EBPR process. Since different *actinobacteria* may contain different proportions of nemaquinones [32], the observed change in E5 (H%) contained about changing the phosphate content may reflect a population shift between phosphate accumulation and *actinobacteria* phosphate accumulating with different contents nemaquinone. Nevertheless, these data confirmed that the members of this novel bacterial population of *actinobacteria* are widely distributed in the EBPR systems, and other reports have suggested [33-36] are partly responsible atoms and optionally phosphate metabolism observed in both reactors 1.8% and 11.8% containing phosphate. DGGE fragments (Figure 2) that were phylogenetically related to *Caulobacter* species of Proteobacteria of the subclass were observed in both the 1.8% systems and containing 11.8% phosphate -even though these fragments do not migrate to the same position on the gel, DGGE. It therefore appears, as demonstrated herein and by other studies [37], that of 16S rDNA fragments that are phylogenetically closely related may have different positions migration in a DGGE gel. *Caulobacter* spp. are often found in environments with low organic carbon content [38], but were detected in sewage treatment systems [32,34,36] and a process of EBPR full scale [39]. The presence of *Caulobacter* may be a reason for the significant amount S10 detected in all samples of sludge (Table 1). In more common groups, the phylogenetic analysis (Figure 3) revealed fragments or specific bacterial populations in both 1.8% and 11.8% of sludge containing phosphate. Due to the sequence of 16S rRNA obtained short and the absence of closely related sequences based on 16S rRNA phylogenetic exact location of these populations was difficult Band E (1.8% phosphate) and the web (11.8% phosphate) were both associated with the subclass of Proteobacteria. Band v (11.8% phosphate) and the band b (1.8% phosphate) were associated with high GC group green sulfur bacteria unidentified, respectively. These results were consistent with the result that significant amounts of S10 and E5 were present in all sludge samples, and a change in their composition about changing the phosphate content was detected. Although these unique populations in the sludge 11.8% phosphate were perhaps the predominant populations performing metabolism EBPR, capacities regarding the polyphosphate metabolism and PHA is unknown and warrants further study. Furthermore, contrary to the respiratory quinone result, none of the dominant DGGE fragments extracted and sequenced have been associated with the subclass of β-Proteobacteria. This could be due to our inability to retrieve some sequences of DGGE bands predominant (Figure 2) that may have belonged to the subclass. The stigma associated with the mining community of DNA and PCR amplification in some cases [31,33,34-36,40] could also lead to misrepresentation of the true fingerprint of the community. It is also possible that the bacteria which produce large amounts of S8, but are not part Proteobacteria b- exist and may be present in the activated sludge systems. In summary, our current understanding of the diversity of microbial populations in the process of EBPR has mainly come from studies using fluorescent in situ hybridization [30-34,36,38,39] and analysis of environmental 16S rDNA clone libraries [38-40]. All studies suggest a high degree of phylogenetic diversity, at least 30 different phylotypes big branches domain bacteria. However, no study to date has identified a specific phylotype directly associated with the polyphosphate accumulation, PHA or both. We first suspected that inconclusive results were attributed to the use of sludge samples from EBPR full scale processes in which the diversity of the population is influenced by a variety of environmental factors (e.g., several substrates, electron acceptors and constant changes in the P} C power ratio) [30,32,34-36], and accumulation of bacteria phosphate are not necessarily the majority population. Our study showed that even in well-controlled EBPR systems and enriched detectable microbial populations were phylogenetically diverse. Although a change in the structure of the microbial community on changing the P sludge content was observed from the biomarker and fingerprinting DGGE, our results further found that microbial communities in 1.8% and 11.8% P containing mud not only included the predominant bacterial populations specific to each mud, but also populations phylogenetically closely related shared. It was also suspected that the specific phylogenetic groups could include both non-phosphate accumulation and accumulation phosphate populations. Thus, the combined use of biomarkers and methods DGGE was insufficien to identify organisms that accumulate phosphates. One promising approach is to combine micro autoradiography with fluorescent in situ hybridization to link functional traits in phylogenetic population in activated sludge process [38]. This approach with refined hierarchical set of probes [36-39,40] should allow a better understanding of the organizations responsible for EBPR.

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