ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

CHARACTERIZATION OF MICROBIAL COMMUNITY IN LAB-SCALE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL REACTORS WITH FLUORESCENCE IN SITU HYBRIDIZATION

M.Sc. THESIS

Mehmet Sefa ULUTAŞ

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Thesis Advisor: Asist. Prof. Dr. Alper Tunga AKARSUBAŞI

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ANOKSİK ORTAMDA BİYOLOJİK FOSFOR GİDERİMİ YAPAN BAKTERİ ÇEŞİTLİLİĞİNİN FLORESAN YERİNDE HİBRİTLEŞME (FISH) TEKNİĞİ İLE İNCELENMESİ

YÜKSEK LİSANS TEZİ

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HAZİRAN 2013

Mehmet Sefa Ulutaş, a **M.Sc.** student of ITU **Institute of Graduate School of Molecular Biology Genetics and Biotechnology Programe,** student ID **521111113**, successfully defended the **thesis** entitled "**CHARACTERIZATION OF MICROBIAL COMMUNITY IN LAB SCALE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL REACTORS WITH FLUORESCENCE IN SITU HYBRIDIZATION**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 03 May 2013
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To my grandma,

FOREWORD

Firstly, I would like to thank my advisor Assist Prof. Alper Tunga AKARSUBAŞI for his precious guidance and infinite support. It is a pride to be under his supervision during this thesis.
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June 2013

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ABBREVIATIONS

EBPR : Enhanced Biological Phosphate Removal
BAFG : Biyolojik Aşırı Fosfor Giderimi
BPR : Biological Phosphate Removal
FISH : Fluorescence in situ hybridization
DNA : Deoxyribonucleic acid
rRNA : Ribosomal Ribonucleic acid
ATP : Adenosine triphosphate
PHA : Polyhydroxyalkanoate
PHB : Polyhydroxybutirate
RFLP : Restriction Fragment Length Polymorphism.
Pi :Orthophosphate (Inorganic Phosphate)
COD : Chemical Oxygen Demand
DGGE : Denaturant Gradient Gel Electrophoresis
DNA : Deoxyribonucleic acid
TAE : Tris-Acetate-EDTA
PCR : Polymerase Chain Reaction
dNTP : Deoxyribonucleotide triphosphate
APS : Ammonium peroxosulphate
TEMED : Tetramethylethylenediamine
UPGMA : Unweighted Pair Group Method with Arithmetic Mean
BLAST : Basic Local Alignment Search Tool
RDP : Ribosomal Database Project
OTU : Operational taxonomic unit
CSLM : Confocal scanning laser microscopy
BSA : Bovine serum albumin
DAPI : 4', 6 diamidino-2-phenylindole
FA : Formamide
PBS : Phosphate buffered saline
PFA : Paraformaldehyde

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CHARACTERIZATION OF MICROBIAL COMMUNITY IN LAB-SCALE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL REACTORS WITH FLUORESCENCE IN SITU HYBRIDIZATION

ABSTRACT

Enhanced biological phosphate removal (EBPR) is a process that removes excessive phosphate from wastewater by phosphate accumulating organisms (PAOs) to prevent pollution of water source and increase the quality of water. Characterization of microbial community of EBPR reactor is one of the most important parameters for evaluating EBPR performance and biochemical pathways.

To understand the profile of denitrifying EBPR process; sequencing batch reactors of pre-denitrification and post-denitrification in oxic and anoxic conditions have been investigated where the reactors has been run by our project partners at Environmental Engineering Dept. Marmara University. Conventional parameters change such as COD, pH, Ammonia levels etc were regularly controlled to be able to check the correlation with microbial community. Microbial community, which is one of the most important parameters of the EBPR sludge that affects the performance of the system, has been investigated with molecular techniques, such as using fluorescent in situ hybridization (FISH), denaturant gradient gel electrophoresis (DGGE), clone library construction and sequencing.

In the EBPR reactors, generally phosphorus accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) were co-found. Actually, those two microorganisms are competitive in behavior.

The main aim of this study is identifying microbial community changes in terms of PAO/GAO competition and its relation with performance data in lab scale denitrifying EBPR reactor samples. Five pre-denitrification samples and 10 post-denitrification samples were studied. 16S rRNA genes of the 15 samples were amplified using Bacterial PCR and DGGE profiles of these PCR products were analyzed. Pre-denitrifying EBPR system has been found to be advantageous rather than post-denitrifying system in terms treatment efficiency. Thus, clone library of the first and the last sample of pre-denitrification samples were constructed.

Microbial community analysis of total population with DGGE, clone library, sequencing data and FISH analysis demonstrate that microbial diversity of pre-denitrification EBPR system show more taxonomic units than post-denitrification EBPR samples.

In the project, phosphate-accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) were studied with group specific probes using fluorescence in situ hybridization (FISH). So the ratios of PAOs and GAOs in EBPR samples were calculated. FISH analysis of EBPR samples show that phosphorus accumulating organisms (PAOs), bound with the FISH probe PAOmix were dominant throughout the almostly whole samples. In addition DGGE and clone library results insight that both the first and the last pre-denitrification sample have diverse microbial community.

ANOKSİK ORTAMDA BİYOLOJİK FOSFOR GİDERİMİ YAPAN BAKTERİ ÇEŞİTLİLİĞİNİN FLORESAN YERİNDE HİBRİTLEŞME (FISH) TEKNİĞİ İLE İNCELENMESİ

ÖZET

Nüfus yoğunluğu ile beraber artan su ihtiyacı; var olan su kaynaklarının korunmasını ve kirletilmiş sularında geri dönüşümünü gereklilik haline getirmiştir. Atık suların içeriğinin belirlenmesi ve atıksuyun doğaya dengeli bir şekilde salınması önemli hale gelmiştir. karbon kaynağı, azot ve fosfor gibi ekolojik sınırlayıcı besinlerin suda aşırı miktarda bulunması su ekosistemine zarar verir. Normal şartlarda sınırlayıcı etkiye sahip azot, Karbon ve fosforun sularda fazla bulunması ötrifikasyona ve mikrobiyal aktivitenin değişmesine neden olmaktadır. Avrupa birliği atıksu arıtma kriterlerine göre (91/271/EEC); nüfusu 100.000 den fazla olan şehirlerde sudaki azot ve fosfat miktarının sırasıyla 10 mg/L ve 1 mg/L den az olması gerekmektedir.

EBPR (Enhanced Biological Phosphate Removal) biyolojik aşırı fosfor giderimi olarak tanımlanan proses, fosfatın atık sulardan uzaklaştırılması işlemidir. İçme ve kullanma sularının kirlenmesini önlemek ve kalitesini arttırmak için atık sularda ötrifikasyona neden olacak aşırı fosforun sulardan uzaklaştırılması hayati bir önem taşımaktadır.

Biyolojik aşırı fosfor giderimi (BAFG) , fosfat giderimi yapan bakterileri kullanarak atıksularda bulunan fosforun giderilmesidir. Biyoloji fosfor giderimi reaktöründe zenginleştirilmiş durumda bulunan fosfat depolayan organizmalar ortamdaki fosfatı aerobik koşulda hücre içinde poly-fosfat (poly-pi) polimeri şeklinde depolar. Genel olarak iki fazdan oluşan biyolojik aşırı fosfor giderim (BAFG) reaktörünün ilk fazı anaerobik, ikinci fazı ise oksiktir. Anaerobik fazda, fosfat depolayan organizmalar (PAO'lar) ortamda bulunan karbon kaynağını kullanarak hücre içinde poly hidroksi alkanat'a (PHA) olarak depolar. Anaerobik koşulda bu dönüşüm gerçekleşmesi için; hücre içindeki glikojenin ve mevcut **Adenozin 3'-trifosfat** (ATP) parçalanır, böylece serbest kalan ortofosfat (Pi) hücre dışına salınır. Oksik fazda ise; depolanan PHA kullanılarak, fosfat depolayan organizmalar (PAO'lar) hücre içinde glikojen ve poly-fosfat polimeri şeklinde depolanmasını sağlanır.

Biyolojik aşırı fosfor giderimi (BAFG) reaktörünün ikinci aşaması olan oksik faz oksik/anoksik şeklinde iki koşulu sırayla taşıyorsa sonda-denitrifikasyon BAFG reaktörü olarak isimlendirilirken, oksik faz anoksik/oksik şeklinde sıralanmış ise önde-denitrifikasyon BAFG reaktörü olarak isimlendirilir. Çalışmada her iki reaktörden aylık numuneler alınarak mikrobiyal çeşitlilik/fosfor giderim performansları yönüyle değerlendirilmiştir.

Fosfor gideriminin yapıldığı BAFG reaktörlerindeki mikrobiyal toplulukların ne olduğunu ve belirli aralıklarla nasıl değiştiğini öğrenmek; hem BAFG prosesinin performansı hem de reaktördeki biyokimyasal yolağın nasıl işlediği ile ilgili soruların yanıtlanmasına yardımcı olacaktır.

Marmara Üniversitesi, Çevre Mühendisliği Bölümüde, Biyolojik aşırı fosfor giderimi (BAFG) oksik ve anoksik ortamda sonda denitrifikasyon ve önde denitrifikasyon

profillerinin anlaşılabilmesi için birçok farklı parametre üzerinde çalışmalar yapılmış. Oksik ve anoksik bekletme süreleri, çamur yaşları, oksik-anoksik tank hacim oranları, farklı giriş atıksu karekteristikleri çalışılmış, bunun yanı sıra sistemin biyokimyasal ve performansını etkileyen diğer önemli parametre olan mikrobiyolojik faktörlerin anlaşılabilir olması içinde örneklerde bulunan mikrobiyal toplulukların; floresan yerinde hibridizasyon (FISH), denature edici gradient jel elektoforezi (DGGE), klon kütüphane oluşturma ve sekanslama gibi moleküler teknikler kullanılarak incelenmesi yapılmıştır.

Genel itibariyle EBPR sistemlerinde fosfor depolayan organizmalar (PAO) ve glikojen depolayan organizmalar (GAO) bulunmaktadır. EBPR sistemlerinde bu iki bakteri gurubu rekabet halindedirler. Bu iki organizma anaerobik koşulda ortamdaki karbon kaynağı için rekabet durumundadırlar. Hem PAO'lar hem de GAO'lar anaerobik koşulda karbon kaynağını poly-hidroksi alkanat (PHA) ya da poly-hidroksi butirata (PHB) çevirerek hücre içinde depolarlar.

Bu çalışmada oksik ve anosik ortamda biyolojik aşırı fosfor giderimi (BAFG) yapan önde ve sonda denitrifikasyon örneklerin mikrobiyal toplulukların içerisindeki değişimini karakterize edilmesi, mikrobiyal dinamik/fosfor giderim performans değerlendirilmesi ve önde-denitrifikasyon sonda-denitrifikasyon reaktörlerinin karşılaştırılmasının yapılması hedeflenmektedir. Önde ve sonda denitrifikasyon örneklerin mikrobiyal topluluklarından oluşan 5 tane sonda denitrifikasyon 10 tane önde denitrifikasyon olmak üzere aylık olarak alınmış 15 tane örnek 16S rDNA geninin polimeraz zincir reaksiyonu (PZR) ile çoğaltılıp DGGE analizi yapılmış ve ilk ve sonuncu önde denitrifikasyon örneklerinin klon kütüphanesi oluşturulmuştur. Biyolojik aşırı fosfor giderim (BAFG) reaktöründen farklı zamanlarda alınan 15 örneğin analizinde post denitrifikasyon örneklerinin mikrobiyal topluluklarının zaman içerisinde değiştiği, pre-denitrifikasyon örneklerinin ise kısmen mikrobiyal biyo-çeşitliliğini koruduğu sonucuna varılmıştır. Tercih edilen bir BAFG sistemi olması açısından, önde denitrifikasyon prosesinin ilk ve son örneğinin klon kütüphanesi oluşturulmuş ve seçilen klonların sekanslaması yapılmıştır.

Önde ve sonda denitrifikasyon biyolojik aşırı fosfor giderim (BAFG) örneklerinin mikrobiyal çeşitliliğinin anlaşılabilmesi için ayrıca floresan yerinde hibritleşme (FISH) tekniği kullanılıştır. Fosfat depolayan organizmalara (PAOs) ve glikojen depolayan organizmalara (GAOs) ait ve 16S rRNA genini hedef alan spesifik problar kullanılarak önde ve sonda denitrifikasyon örneklerindeki PAO ve GAO yüzdelikleri DAPİ bayanmış toplam mikroorganizma sayısına göre hesaplanmıştır. Aynı teknik ile Actino-221 ve Actino-658 probları kullanılarak sırasıyla yuvarlak Aktinobakterilerin ve kısa-çubuk şeklindeki Actinobakterilerin örneklerdeki yüzdelikleri de belirlenmiştir.

Denaturant gradient jel elektroforez (DGGE) analiz sonuçları önde-denitrifikasyon örneklerinin mikrobiyal çeşitliliği, sonda-denitrifikasyon örneklerininkinden daha fazla olduğu görülmektedir. Önde-denitrifikasyon örneklerinden ilk ve sonuncusu ile yapılan klon kütüphanesi ve dizileme çalışması sonrasında bu BAFG örneklerinde farklı bakteri türlerinin olduğu belirlendi. İlk önde denitrifikasyon örneğinde; *Pseudomonas, Stenotrophomonas, Rhizobium, Acidovorax* ve *Clostridium* türlerine rastlanırken, sonuncu önde-denitrifikasyon örneğinde; *Stenotrophomonas, Acinetobacter, Achromobacter, Chryseobacterium* ve *Clostridium* türlerine rastlanmıştır.

Önde-denitrifikasyon ve sonda-denitrifikasyon EBPR örneklerinin PAOmix probu (fosfat depolayan organizmaları hedefleyen prob karışımı) ile yapılan FISH sonuçlarına göre tüm örneklerde fosfat depolayan bakteriler (PAO) baskın olarak gözükmektedir. Bütün örneklerde glikojen depolayan organizmaların (GAO) varlığı tespit edilmiştir.

DGGE analizleri, klon kütüphanesi, sekans verileri ve FISH teknikleri kullanılarak yapılan mikrobiyal kominite analizleri önde denitrifikasyon prosesindeki örneklerin mikrobiyal çeşitliliğinin daha fazla olduğu belirlenmiştir. Önde-denitriffikasyon sistemini, fosfat gideriminde sonda-denitrifikasyon sisteminden daha uygun bir BAFG olduğunu söylemek mümkündür.

1. INTRODUCTION

In the developed world, how a product is produced, and how it shall be consumed is an important issue. It is an important environmental concern that how domestic and industrial wastes are discharged to nature and what is the effect of these wastes to it. Because of that, it is a considerable issue, what are the compounds of these wastes and how they can be remediated. Hence, cleaning up the recreational waters, and conserving the quality of water source and remediation and controlling the reason of eutrophication conditions are significantly important. Thus, in wastewater, the limits for the biological removal application of carbon, nitrogen and phosphor have been increased. According to Europe unit urban wastewater cleaning instructions (91/271/EEC), in town more than 100.000 persons, nitrogen and phosphor amount must be less than 10 mg/L and 1 mg/L sequentially.

Carbon, nitrogen and phosphate are the major causes of eutrophication. As a result of eutrophication on the top of the water in summer, algeal communities are increasing. Due to enriched nutrient content of wastewater, dissolved oxygen gets lower and this causes fish death, water turbidity and decreasing of flora and fauna. Moreover, excessive nutrient content may trigger microbial activity which causes a change in microbial community such as a human harmful microorganism *Pfisteria.* (U.S E.P.A, 2001).

1.1 Aim of the Study

The purpose of this study is to characterize the microbial community and population dynamics of both lab-scale pre and post-denitrification processes followed by Enhanced biological phosphorus removal (EBPR) reactors and to be able to correlate such data with conventional wastewater treatment parameters like COD removal, pH change, Ammonia removal etc.

Therefore, changes in microbial communities of both EBPR reactors have been investigated with molecular methods. In terms of phosphate accumulating organisms

(PAOs) and glycogen accumulating organisms (GAOs) were investigated by using fluorescence in situ hybridization technique.

Besides FISH; PCR-DGGE based molecular approaches has been used to check microbial population dynamics.

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2. ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL (EBPR)

2.1 Phosphorus Cycle

Phosphorus cycle is a biogeochemical cycle that defines the path of the phosphate through the lithosphere, hydrosphere and biosphere. As can be seen phosphorus pathway does not include the atmosphere such as oxygen, nitrogen and carbon cycle, due to it is a non-metal cycle that does not has a gas cycle.

Phosphorus has a key role in circulation of energy through living organism, lithosphere and hydrosphere. The primary biological importance of phosphorus is being within the structure of Adenine three Phosphate (ATP). It also takes roles in driving enzymatic reaction or cellular transportation. Moreover, phosphorus holds the DNA and RNA molecules together like glue.

Generally, required phosphate is taken from plant which accumulate phosphorus from water and soil into their tissues, using it for organic molecules. Other organism can take up their required phosphate by consuming plants. During the decomposition of death animal, plant and other single cell organisms, phosphorus release to the soil and water again. By streams and rivers phosphorus can move to the water source and ocean. So the water cycle takes a key role within the overall phosphorus cycle. In figure 2.1 it was shown overview of phosphorus cycle.

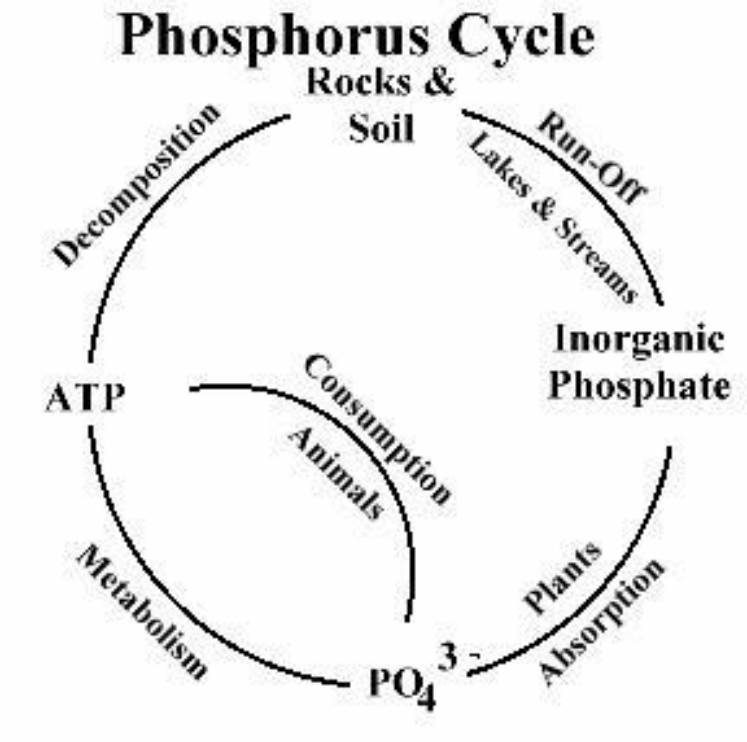


Figure 2.1 : An overview of phosphorus cycle.

2.2 Enhanced Biological Phosphorus Removal

Phosphor is the most important nutrient for microbial activity, In many clear water spring phosphor is limiting factor for biological activity. So, the removal of phosphor from wastewater prevents eutrophication substantially in water resource. Biological phosphor removal is performed by the way taken phosphor by bacteria for energy production or cell growth. Lots of Biological phosphor removal (BPR) mechanism has been designed until now. According to Meganck et al (1988) an EBPR system should be include the following conditions.

- An anaerobic reactor contains readily biodegradable energy and carbon source such as PHA, glycogen and acetate as being the first receiving of influent.
- The nitrate concentration in the anaerobic reactor should not be much more, Because of denitrifying bacteria were capable of respiration and depletion of organic source in the in the EBPR. Therefore, the presences of substrate cannot enough for the next oxic/anoxic zone for PAOs.
- Strict condition was need for anaerobic zone.

There are lots of different engineered BPR reactors. Generally these reactors based on two types. Te first is full biological process and the second is chemical/biological combination process. In figure2.2 there is a general view of a BPR reactor.

The terms of phosphate removal comes from encouraging the accumulation of P in bacterial cells as in the form of polyphosphate (Poly-P). The excess level of this accumulation is called ‘’enhanced biological phosphate removal’’ (EBPR). Biological phosphor removal is performed by using microorganism sequentially in anaerobic-aerobic phase (polyphosphate accumulating organism, PAO) or anaerobic-anoxic phase (denitrifying phosphate accumulating organism, DPAO) (Fush and Chen, 1975)

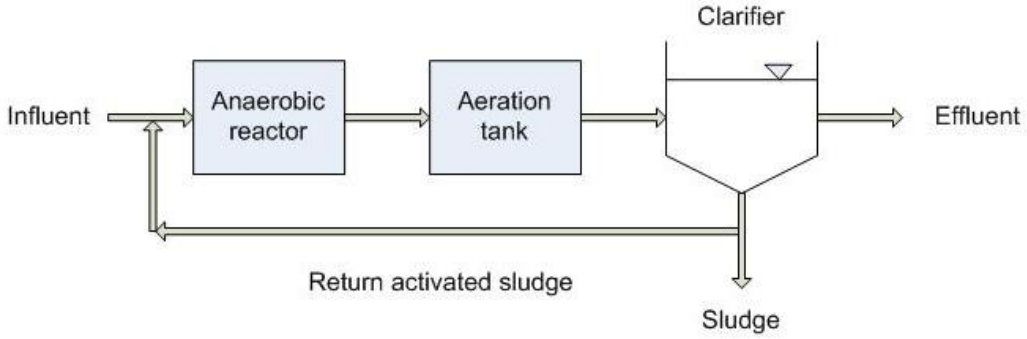


Figure 2.2 : A general view of enhanced biological phosphate removal reactor.

2.2.1 EBPR process with pre-denitrification

The commonly used biological nutrients removal (BNR) systems where pre-denitrification is implemented nitrogen, phosphorus removal from wastewater. Processes such as the A2O (Anaerobic-Anoxic-Aerobic) are based on pre-denitrification. The anoxic phase is located prior to the nitrifying aerobic stage. High internal mixed liquor recirculation is, therefore, required from the aerobic into the anoxic section in order to provide the PHA-rich PAO sludge (from the anaerobic stage) with the necessary NO_2 and NO_3 for denitrifying P removal. In the A2O process, Anoxic condition start with a rich level of carbon source because of intracellular storaged PHA. So beside the Phosphate removal, N removal from the system can also be achieved (figure 2.3).

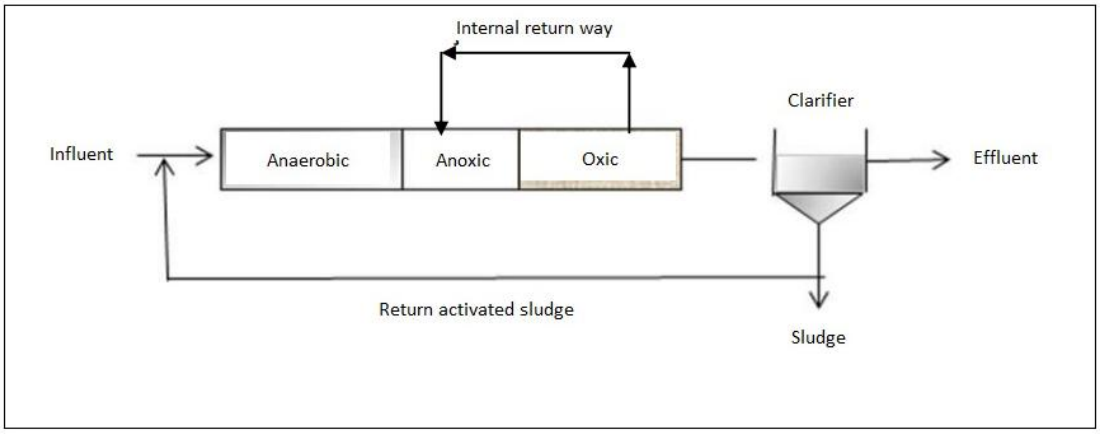


Figure 2.3 : Pre-denitrification EBPR reactor model.

2.2.2 EBPR process with post-denitrification

The second BNR process is based on post-denitrification. In this process the recurcilation of mixed liquor reduces. Post-denitrification process include Anaerobic-oxic and anoxic condition sequentially. So the process is called AOA process also. On the contrary pre-denitrification process, intracellular poly-β-hydroxyalkanoate (PHA) is low level, due to consuming the former oxic condition. So denitrification process remained with low carbon source (figure 2.4).

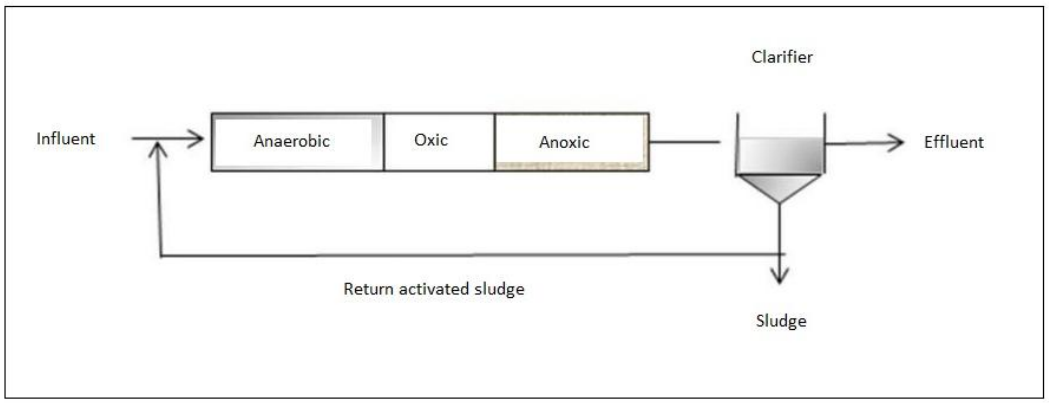


Figure 2.4 : Post-denitrification EBPR reactor model.

2.3 Chemical Characterization of Enhanced Biological Phosphate Removal

Many studies have been revealed to understand the biochemical structure of enhanced biological phosphor removal. The general acceptance is that, during the anaerobic phase, particular bacteria assimilate volatile fatty acid (VFA) and this converts leads to accumulation of PHA instead of bacterial growth. Figure 2.5 shows the chemical cycle that performed in the EBPR reactors in both anaerobic and aerobic conditions.

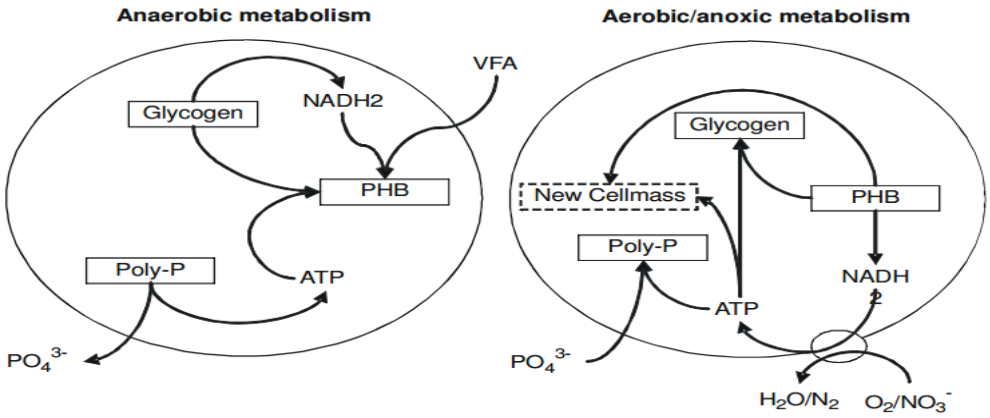


Figure 2.5 : Overview of biochemical pathways of EBPR systems both in anaerobic and aerobic conditions.

This conversion depends on substrate, when acetate is used, poly-β-hydroxybutyrate (PHB) formation is observed (Lemos et al, 1998). During the anaerobic stage, the biomass levels of PHA and PHB increase with parallel of decreasing level of acetate. Intracellular poly-P amount falls and the phosphate level in extracellular bulk increases (Mino et al, 1984). Then during the following aerobic/anoxic stage, PHA level in biomass decreases whereas glycogen level increasing. Moreover, phosphate amount decreases in extracellular liquid Poly-P is stored intracellular by phosphate accumulating organisms (PAOs) which can store phosphate up to 15 % level of their dry weight. In Figure 2.6 it could be seen that polyphosphate level is changing during anaerobic and aerobic conditions (Mino et al, 1998).

Phosphate removal is an inexpensive and environment-friendly when applied with enhanced biological phosphate removal (EBPR) treatment plants. The application of this treatment process has been increasing recently. Enhanced biological phosphate removal process is modified as enriched and increasing phosphate accumulating organism (PAO) which store and remove phosphate from the wastewater. Glycogen accumulating organisms (GAOs) are another major group of bacteria, which are present in EBPR plants. However, these bacterial groups are generally undesirable organisms due to their competition with PAOs for nutrient. Thus, PAOs and GAOs were focused microorganisms in the EBPR reactors (Mino et al, 1998).

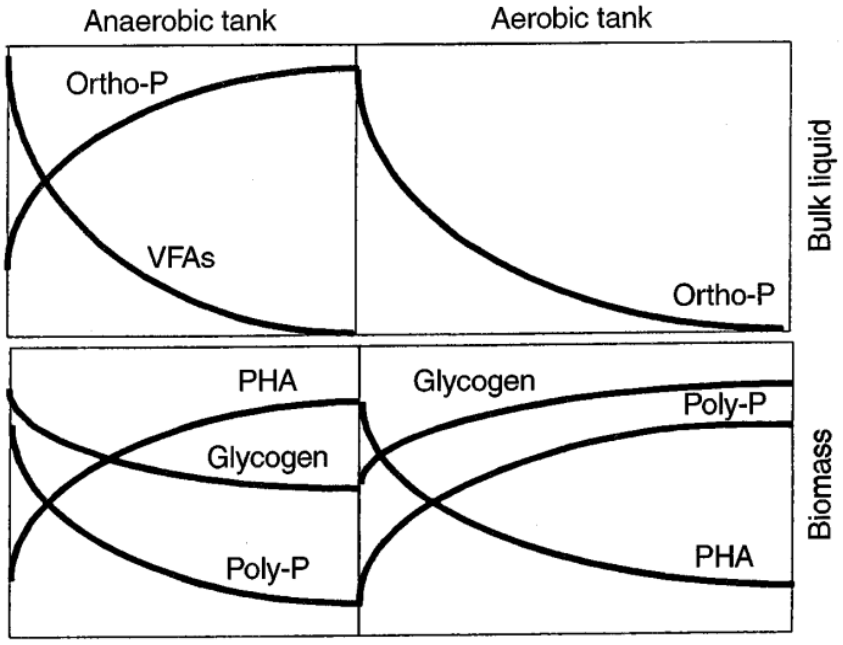


Figure 2.6 : The polyphosphate changes in anaerobic and aerobic tanks.

3. MICROBIOLOGY OF ENHANCED BIOLOGICAL PHOSPHATE REMOVAL

3.1 Identification of PAOs

There are many attempts to isolate phosphate accumulating organism from EBPR systems. Classical culture dependent techniques have been used to isolate and characterize pure bacterial cultures for a long time over 30 years. Until now, *Pseudomonas, Aeromonas, Acinetobacter, Micrococcus* genera members were found (Jenkins and Tandoi, 1991; Van Loosdrecht et al., 1997; Mino et al., 1998). *Acinetobacter* was first proposed to be the primary organism responsible for P removal in EBPR by Fush and Chen (1975) by staining techniques and microscopic observation. According to this study; these microorganism are non-motile rod shaped, coccus shaped and PHB staining gram positive bacteria. *Acinetobacter* was long assumed to be the sole PAO present in EBPR plants.

However, the use of culture independent techniques such as fluorescence in situ hybridization (FISH), 16S rRNA-based clone libraries, restriction fragment length polymorphism (RFLP) or denaturing gradient gel electrophoresis (DGGE) demonstrated that a high diversity of phylogenetic groups are present in lab- and full-scale EBPR sludges (Seviour et al., 2003). Wagner et al. (1994) have showed that the culture dependent methods especially agar plate growth, overestimate the *Acinetobacter* spp. It was with the use of specific FISH probes that *Acinetobacter* was shown to have little significance in full-scale plants when compared to members of other phylogenetic groups, such as the *Betaproteobacteria* and *Actinobacteria* Then it has been understood that this *Acinetobacter* was not highly responsible for phosphate removal in EBPR systems (Kampfer et al, 1996).

Nakamura et al (1995) isolate a high GC gram-positive microorganism called *Microlunatus phosphovorus, which* is assumed another EBPR responsible organism strain NM-1 from the EBPR systems. However, *Microlunatus phosphovorus* species,

which is found as PAO in EBPR systems, not generally representative of the EBPR systems and also they don't have typical characteristics of PAOs. The organism can accumulate poly-P under aerobic condition and use this poly-P under anaerobic condition but neither glycogen nor PHA were cycled within their cells (Seviour et al., 2003). Stante et al (1997) isolate a gram negative *Lampropedia* bacteria from the EBPR system. Similarly, Maszenan et al (2000) isolate *Tetrasphaera spp* from the EBPR sytems. But these are also not represent the characteristics of PAOs. Mino et al (1998) indicated that instead one dominant organism, a mixture of organisms are more affective for representing of EBPR systems.

3.1.1 Actinobacteria a new type of PAO

Many studies have previously investigated the presence of *Actinobacteria* in EBPR systems. Actinobacteria is a gram positive bacteria that have been suggested to be potential PAO. Some isolates of *Actinobacteria* have the ability of aerobic Pi accumulating after taking up organic substartes under anaerobic conditions. Kawaharasaki et al (1999) obtained pure culture of gram negative *Actinobacteria* which are thought as PAO. Crocetti et al (2000) was studied laboratory scale sequential batch reactors (SBRs) as model of biological phosphorus removal batch reactors. In the EBPR sludge, β-2 *Proteobacteria* that is considered as responsible in EBPR, was found dominantly. In this study, *Actinobacteria* was found as the second dominant microorganism. Two distinctive group, *Rhodocyclus spp.* related and *Propionibacter pelophilus* were found in same sludges with clone library of PCR-amplified.

Actinobacteria include Microlunatus phosphovorus and *Tetrasphaera elongate* (strain ASP12) However their Pi accumuating depend on anaerobic uptake of glucose (for *Microlunatus phosphovorus*) and amino acid mixture (for *Tetrasphaera elongate*) instead of short fatty acids. In addition, PHA is not detected as intracellular polymer (Kong et al, 2005). Clone library analysis and MAR-FISH analysis using two new oligonucleotide probes (Actino-221 and Actino-658) revealed that there are both morphotypes, cocci in clusters of tetrads and short rods in some EBPR systems (Kong et al, 2005). These organisms were particularly abundant in industrial wastewater plants, Although, they have also been found in domestic plants.

3.1.2 Candidatus accumulibacter phosphatis

Instead of the classic method, using molecular method to investigate the poly-phosphate accumulating organisms (PAOs) led to characterization of bacteria phylogenetically closed with the *Rhodocyclus* group of the *Betaproteobacteria* are numerically dominant in the EBPR systems. The member of those bacteria have been called ‘’ *Candidatus Accumulibacter phosphatis’’* (referred to as the *Accumulibacter*) (Hesselmann et al., 1999; Crocetti et al., 2000). Generally used FISH probes for *’Accumulibacter’* are the mix of PAO462, PAO651 and PAO846. Those probes are covering almost whole phosphate accumulating organisms (Table: 3.1).

3.1.3 Denitrifying phosphate accumulating bacteria

Large amount of phosphorus is taken up by PAO during an electron acceptor is supplied which is oxygen in aerobic condition. This led to oxidation of PHA through the biomass and glycogen formation. Alternatively, nitrate (NO_3) or nitrite (NO_2) can be used as electron acceptors in anoxic conditions instead of oxygen. The using of nitrate and nitrite as electron acceptor also lead to dentirification. So this process is advantageous because both N and P are removed simultaneously. (Kuba et al., 1993).

In a recent study by Carvalho and his colleagues (2006) have been operated using two SBRs under anaerobic–aerobic conditions and gradually acclimatized to anaerobic-anoxic conditions. The used carbon source fed to the SBRs was acetate and propionate, respectively. The anaerobic–anoxic–aerobic batch tests are suggesting the presence of two different groups of PAOs (DPAOs and non-DPAOs). (Carvalho et al., 2006).

Table 3.1 : Most common 16S rRNA-targeted probes used for FISH detection of organisms of relevance in EBPR systems (Oehmen et al., 2007).

Probe	Sequence 5′-3′	Specificity	Reference
Probes designed for (potential) PAOs			
PAO462	CCGTCATCTACWCAGGGTATTA AC	Most *Accumulibacter*	Crocetti et al. (2000)
PAO651	CCCTCTGCCAAACTCCAG	Most *Accumulibacter*	Crocetti et al. (2000)
PAO846	GTTAGCTACGGCACTAAAAGG	Most *Accumulibacter*	Crocetti et al. (2000)
RHC439	CNATTTCTTCCCCGCCGA	*Rhodocyclus/ Accumulibacter*	Hesselmann et al. (1999)
RHC175	TGCTCACAGAATATGCGG	Most *Rhodocyclaceae*	Hesselmann et al. (1999)
PAO462b	CCGTCATCTRCWCAGGGTATTA AC	Most *Accumulibacter*	Zilles et al. (2002a)

Table 3.1 (Continued): Most common 16S rRNA-targeted probes used for FISH detection of organisms of relevance in EBPR systems (Oehmen et al., 2007).

Probe	Sequence 5′-3′	Specificity	Reference
Probes designed for (potential) PAOs			
PAO846b	GTTAGCTACGGYACTAAAAGG	Most *Accumulibacter*	Zilles et al. (2002a)
Actino-221a	CGCAGGTCCATCCCAGAC	*Actinobacteria*—potential PAOs	Kong et al. (2005)
Actino-658a	TCCGGTCTCCCCTACCAT	*Actinobacteria*—potential PAOs	Kong et al. (2005)
Probes designed for (potential) GAOs			
Gam1019	GGTTCCTTGCGGCACCTC	Some *Gammaproteobacteria*	Nielsen et al. (1999)
Gam1278	ACGAGCGGCTTTTTGGGA	Some *Gammaproteobacteria*	Nielsen et al. (1999)
GAOQ431	TCCCCGCCTAAAGGGCTT	Some *Competibacter*	Crocetti et al. (2002)
GAOQ989	TTCCCCGGATGTCAAGGC	Some *Competibacter*	Crocetti et al. (2002)
GB	CGATCCTCTAGCCCACT	*Competibacter* (GB group)	Kong et al. (2002b)
GB_G1 (GAOQ989)	TTCCCCGGATGTCAAGGC	Some *Competibacter*	Kong et al. (2002b)
GB_G2	TTCCCCAGATGTCAAGGC	Some *Competibacter*	Kong et al. (2002b)
GB_1 and 2	GGCTGACTGACCCATCC	Some *Competibacter*	Kong et al. (2002b)
GB_2	GGCATCGCTGCCCTCGTT	Some *Competibacter*	Kong et al. (2002b)
GB_3	CCACTCAAGTCCAGCCGT	Some *Competibacter*	Kong et al. (2002b)
GB_4	GGCTCCTTGCGGCACCGT	Some *Competibacter*	Kong et al. (2002b)
GB_5	CTAGGCGCCGAAGCGCCC	Some *Competibacter*	Kong et al. (2002b)
GB_6 (Gam1019)	GGTTCCTTGCGGCACCTC	Some *Competibacter*	Kong et al. (2002b)
GB_7	CATCTCTGGACATTCCCC	Some *Competibacter*	Kong et al. (2002b)
SBR9-1a	AAGCGCAAGTTCCCAGGTTG	*Sphingomonas*-related organisms—potential GAOs	Beer et al. (2004)
SBR9-1b	TGTTAGGGGCTTAGACCT	*Sphingomonas*-related organisms—potential GAOs	Beer et al. (2004)
SBR8-4	CACCGAAGCACTAAGTGCCC	*Sphingomonas*-related organisms—potential GAOs	Beer et al. (2004)
TFO_DF218	GAAGCCTTTGCCCCTCAG	*Defluviicoccus*-related organisms (cluster 1)	Wong et al. (2004)
TFO_DF618	GCCTCACTTGTCTAACCG	*Defluviicoccus*-related organisms (cluster 1)	Wong et al. (2004)
DF988	GATACGACGCCCATGTCAAGGG	*Defluviicoccus*-related organisms (cluster 2)	Meyer et al. (2006)
DF1020	CCGGCCGAACCGACTCCC	*Defluviicoccus*-related organisms (cluster 2)	Meyer et al. (2006)

3.2 Identification of GAOs

Glycogen accumulating organisms (GAOs) was as known ‘’G bacteria’’ storage the glycogen aerobically and utilize it anaerobically. GAOs accumulate the corbon source and storing this as PHA.

Similarly to phosphate accumulating organism, difficulties have been encountered to isolate GAOs (Mino et al., 1998). However, applying molecular technique such as DGGE, clone library of 16S rRNA gene and FISH led to identification of this organis in EBPR systems (Nielsen et al., 1999, Crocetti et al., 2002). The microorganisms, which found in EBPR systems, are belonged to Gammaprpteobacteria typically showed GAO phenotype. Coretti et al.,(2002) was named this organism as ‘’Candidatus Competibacter Phosphatis’’ generally abbreviated as ‘’Competibacter’’.

Kong et al., (2002) designed additional FISH probes (GAOQ431, GAOQ989 and GB) to target a new GAO group which was found in full-scale and lab-scale EBPR systems (Table3.2). Those probes provides more robust molecular tool for investigation of GAOs.

3.3 Molecular Techniques Used for Characterization of Microbial Community

Molecular techniques give a new sight as a strong alternative to the classical technique, to define the characterization of both microbial community and biochemical structure of EBPR systems. Wagner et al (1994) and Kampfer et all (1996) were found sludges from EBPR process contained high G+C content gram positive bacteria by using fluorescence in situ hybridization (FISH) that is a molecular technique using oligonucleotide probes. But, Bond et all (1995) used clone library approaches and indicated that only few high G+C content gram positive bacteria were found in EBPR. Bond also showed that the critical differences between EBPR and non-EBPR processes by existing of beta subclasses of *proteobacteria* proposing that this group have an important role in EBPR processes. Ahn et al (2001) also was found Rhodocyclus spp. And Dechlorimonas spp. that are belonged to the beta subclass of *proteobactaria* by using polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). This study showed that denitrifying phosphate-accumulating organisms (DPAO) plays an important role in EBPR. DPAOs use nitrogen as electron acceptor under anoxic condition and perform release

phosphorus and nitrogen simultaneously. The fluorescence in situ hybridization and other molecular techniques indicate that EBPR sludges phylogenetically classes several distinguishable microbial community. Brdjanovic et all (1997) and Liu et all (1997) demonstrated that EBPR sludges have several different DNA fragments showing that the sludges are not dominated by a single microorganism. However, several dominant bacteria by using Denaturing Gradient Gel Electrophoresis (DGGE) and Restriction Fragment Length Polymorphism (RFLP) were reported.

4. MATERIALS AND METHODS

4.1 Description of EBPR System

In this study, the basic aim is the characterization of bacterial communities in Marmara University, Environmental Engineering Department lab scale EBPR samples. Additionally, changes in the microbial community during phosphate removal . The characterization of bacterial community was done with phylogenetic analysis of samples by using 16S rRNA gene and DGGE (Denaturing Gradient Gel Electrophoresis) profile of bacterial community. Firstly DNA extraction was performed, 16S rRNA gene was amplified by using universal primers for Bacteria domain. Then, DGGE profile was detected. After that, the group specific probes were used for fluorescence in situ hybridization of EBPR samples.

4.2 Material and Equipment

The equipment, chemicals and solution and FISH images were listed in Appendix A, Appendix B and Appendix C and Appendix D respectively.

4.3 Methods

4.3.1 Sample collection and storage

Duplicate samples were taken from EBPR every month during phosphor removal processes from the Marmara University Environmental Engineering Department. There was five post-denitrification and 10 pre-denitrification EBPR samples as shown in Table 4.1. Samples were stored at -20 ^{o}C for further analysis.

Table 4.1 : Samples and sampling dates.

Sample	Samples Feature	Sampling date	FISH Samples
S1	Post-denitrification samples	29.06.2009	X
S2	Post-denitrification samples	04.08.2009	X
S3	Post-denitrification samples	15.10.2009	X
S4	Post-denitrification samples	02.12.2009	X
S5	Post-denitrification samples	01.03.2010	X
O1	Pre-denitrification samples	10.03.2010	X
O2	Pre-denitrification samples	07.05.2010	X
O3	Pre-denitrification samples	10.06.2010	X
O4	Pre-denitrification samples	05.08.2010	
O5	Pre-denitrification samples	22.09.2010	
O6	Pre-denitrification samples	03.01.2011	
O7	Pre-denitrification samples	26.05.2011	X
O8	Pre-denitrification samples	27.06.2011	X
O9	Pre-denitrification samples	28.07.2011	X
O10	Pre-denitrification samples	12.08.2011	X

4.3.2 DNA extraction

Total genomic DNA from post-denitrification and pre-denitrification samples were extracted using FastDNA Spin Kit for soil (MP, Biomedical, USA). Before DNA extraction, 500 µl distilled water was added to all samples and vortexed. Then, samples were centrifuged at 14.000 rpm for 2 minute and supernatants of all samples were removed, and pellets were used for further analysis.

After DNA extraction the amount of DNA was measured using Qubit fluorometer (Invitrogen, USA) and analyzed on agarose gel that was prepared using 1% (w/v) agarose in 1XTAE buffer. Electrophoresis was done at 120 V for 20 minute. Gel was visualized under Gel Doc (BIORAD, USA) imaging system. Lastly, extracted DNA samples were diluted to 10 ng/mL for further analysis.

4.3.3 Polymerase chain reaction (PCR)

4.3.3.1 PCR overview

In this study; archeal and bacterial 16S rRNA gene fragments were amplified with PCR method.

4.3.3.2 Bacterial PCR

Universal 16S rRNA gene primers were used to identification and study of bacterial community. Amplification of 16S rDNA was done using Vf-GC and Vr primers (Table 4.2). GC clamped forward primer was used for DGGE analysis. The

ingredient of PCR is listed at Table 1 (Appendix C). The total volume is 25 µl per samples. PCR condition for Vf-GC/Vr primer set was given at Table 4.2. For wider region of 16S rRNA gene, a second primer set which name is 63f and 1387r were used (Table 4.2). 63f and 1387r primer set were used to obtain PCR products to prepare clone library. PCR condition for 63f/1387r primer set was given at table 4.2. PCR amplicons were visualized by agarose gel electrophoresis with 1% w/v concentration of agar following the staining with ethidium bromide in 1XTAE buffer. Visualization of agarose gel was done using GelDoc^{TM} (BIORAD, USA). All PCR products were stored at 4 ºC until DGGE analysis.

4.3.3.3 Archeal PCR

For identification of archea, universal 16S rDNA gene primers were used. Amplification of 16S rDNA of archeal samples were done by using nested PCR technique. Fistly, arch-7f and arch-1384r primers were used for amplification of big region. Than, the arch344f-gc and univ522r priers were used. The ingredient of PCR is listed at table1 (Appandix C). The total volume is 25 µl per samples. PCR amplicons were visualized by agarose gel electrophoresis with 1% w/v concentration of agar following the staining with ethidium bromide in 1XTAE buffer. Visualization agrose gel was done using GelDoc^{TM} (BIORAD, USA). All PCR products were stored at 4 ºC until DGGE analysis.

Table 4.2 : Vf-GC and Vr primer set for 16S rRNA gene.

Primer name	Primer sequence	Reference
GC – Vf	5'-GC***GGCCTACGGGAGGCAGCA-3'	Muyzer et al., 1993
Vr	5'-ATTACCGCGGCTGCTGG-3'	Muyzer et al., 1993
***GC-	5'-CGCCCGCCGCGCGGCGGCGGG CGGGGCGGGGGCACGGGGG-3'	Muyzer et al., 1993
Eu1387r	GGGCGGWGTGTACAAGGC	
Eub63f	CAGGCCTAACACATGCAAGTC	
Arch7f	TTCYGGTTGATCCYGCC	Lueders et al., 2004
Arch1384r	CGGTGTGTGCAAGGAGCA	Lueders et al., 2004
Arch 344fGC	GC**-GAC GGG GHG CAG CAG GCG CGA	Raskin et. al, 1994
Univ 522r	GWA TTA CCG CGG GKG CTG	Raskin et. al, 1994

Table 4.3 : PCR condition for GC-Vf/Vr and 63f/1387r.

GC - Vf – Vr			**63f - 1387r**		
Temperature (°C)	**Time**	**Cycles**	**Temperature (°C)**	**Time**	**Cycles**
95	5 min.	1	94	3 min.	1
95	30 sec.	35	94	30 sec.	35
55	30 sec.		49	30 sec.	
72	1 min.		72	45 sec.	
72	10 min.	1	72	10 min.	1
12	∞	1	4	∞	1

4.3.4 Denaturing gradient gel electrophoresis (DGGE)

4.3.4.1 DGGE overview

Denaturing gradient gel electrophoresis separate DNA molecule based on melting behavior of double strand DNA molecule. Even same length DNA with different sequence is separated by DGGE due to sequence specific melting temperature. The lower melting DNA domain opens primarily whereas the higher melting DNA domain opens later. The partial melting of DNA molecule suddenly reduces its mobility in acrylamide gel (Molecular Cell Physiology, 2001). In traditional agarose gel electrophoresis similar size of DNA results in same band, this limitation can be overcome by using DGGE. This method firstly, developed by Fisher and Lerman (1983).

In DGGE, the denaturing condition is created by uniform running temperature between 50 and 65 ^{o}C and a linear gradient is formed with urea and formamide. Gel gradient is initiated from 0% percentage through 100% percentage denaturing agent including (Figure4.1).

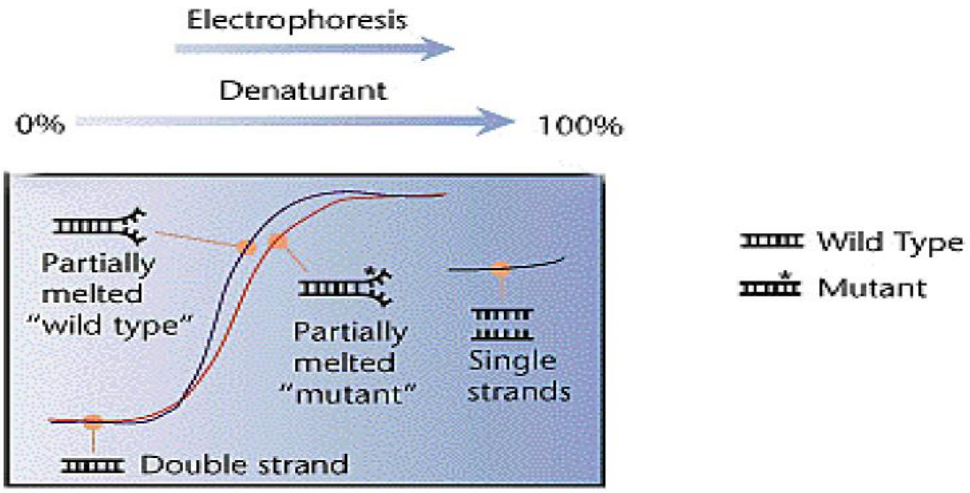


Figure 4.1 : Polyacrylamide gel gradient from 0% to 100%.

4.3.4.2 DGGE gel preparing

Two kind of 10% of polyacrylamide gel [acrylamide:bisacrylamide (37.5:1)] solutions which contain denaturing gradient 100% and 0% were prepared for double strand DNA separation (Table2.5).

Firstly glass plate, spacer and comp were cleaned carefully. Gel sandwich was assembled according to spacers. High and low solutions were prepared from stock solution of denaturant and acrylamide. Both two solutions were 16 ml. Each gel was 32 ml totally. 160 µl 10% APS (Ammonium Per sulfate) and 16 µl TEMED were added to each solution and mixed. The solutions were poured to gradient maker tubes. Current pump was opened. The combs were inserted at a specific angle and gels polymerize at 45-60 minutes. Combs were removed from gels and wells were washed immediately with TAE buffer to clean the wells.

Table 4.4 : DGGE conditions.

Bacterial 16S rDNA (GC-VF, VR)	
Gel percentage	%10
Denaturing Gradient	%35 - %65
Running Voltage	180 volt
Running Temperature	60^{o}C
Running Time	960 min.
Staining	15 min.
De-staining	20 min.

4.3.4.3 DGGE fingerprinting analysis

Interpretation of DGGE profile is difficult because of diverse microbial communities such as sediments, soil, wastewater and biofilm samples. For this reason, to analyze DGGE profiles in a right inference, computer based programs were used.

Each band and their intensity in DGGE gels responses to different 16S rRNA gene based operational taxonomic unit. Computer programs which are based on mathematical equations, define these band according their location and intensity.

Most used algorithm programs for phylogenetic tree former are Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) and Neighbor Joining. The main principle of this programs are that each peak and distance among them are separated as taxa (Fitch, 1967).

4.3.5 Cloning for PCR product

4.3.5.1 Cloning overview

Cloning is transferring an isolated DNA fragment from its original organism to a new organism by using a plasmid or a virus which is called vector. Three basic stage is needed for cloning. At first, the target DNA is isolated from original organism and DNA is cut by enzymes. This first step is performed by the activity of restriction enzymes likely EcoRI, BamHI and TaqI endonuclease enzymes. Secondly, target DNA is inserted to a vector which is artificially designed plasmid or virus. At the end, this vector is transformed to a host cell which is generally Escherichia coli (Glick et al, 2005).

4.3.5.2 The TOPO Kit for sequencing procedure

TOPO TA cloning® kit for sequencing (Invitrogen, USA) was used in this study. TOPO TA cloning® kit has high efficiency of insertion of *Taq* polymerase-amplified PCR product into a plasmid vector for sequencing. TOPO TA cloning® kit does not require ligase, post-PCR procedures or PCR primers containing specific sequences. Taq polymerase has terminal transferase activity which adds single deoxyadenosine (A) to the 3’ end of PCR product. TOPO TA cloning® kit supply a single, overhanging 3’ deoxythymidine (T) base. This performs the insertion of PCR product to linearized vector.

TOPO TA cloning® kit allow positive selection via lethal *E. coli ccdB* gene (Bernard and Couturier, 1992). The *ccdB* gene contained vectors fuse to *lacZα* fragment. While insertion of PCR product was performed, *lacZα* gene expression is prevented. Cells which contain non-recombinant vector are killed. So blue/white screening was not required.

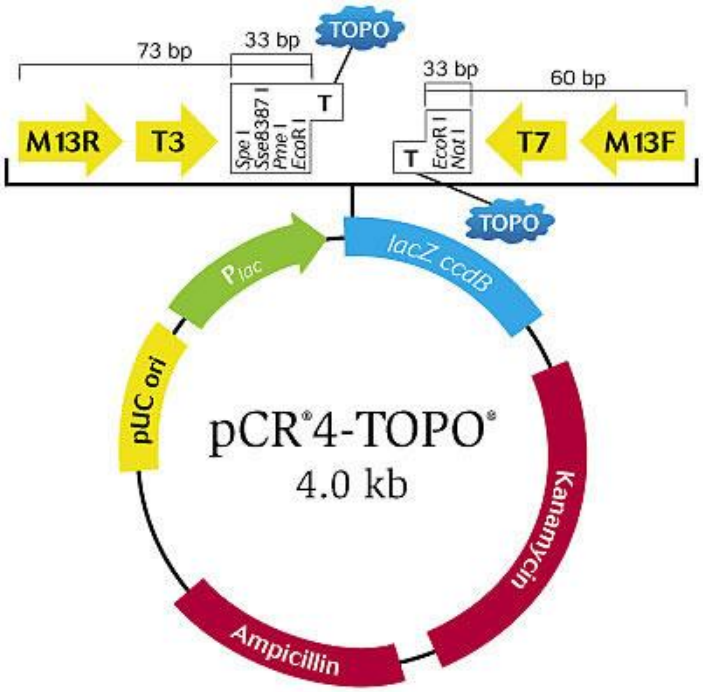


Figure 4.2 : Plasmid vector with PCR product insertion (Invitrogen, USA).

4.3.5.3 Performing the TOPO Cloning® Reaction

Firstly TOPO Cloning® reaction was prepared. Table4.5 show ingredient and amount of them.

Table 4.5 : TOPO Cloning® reactions components and amounts.

Reagent	Chemically Competent *E. coli*
Fresh PCR product	0.5 to 4 µl
Salt Solution	1 µl
Dilute Salt Solution	--
Sterile Water	Add to a final volume of 5 µl
TOPO vector	1 µl

2 µl of the TOPO Cloning® reaction (prepared before) were added to chemically competent *Escherichia coli* cells. İncubated on ice for 30 minutes. Cells were applied

heat-shockng for 30 second at 42 ^{0}C without shaking. Then, tubes transferred to ice immediately. After that, 250 µl of room temperature S.O.C medium. Tubes were incubated with horizantely shaker at ^{o}C for 1 hour. Spreading of 50-75 µl was performed from each transformation on 50 µg/ml kanamycin slective plate and incubated at 16 hours at 37 ^{o}C. Then, formed colonies were chosed randomly for kanamycin included LB broth inocultaion for overnigth. Lastly, Plasmid isolation of overnigth incubated cells were perfromed by plasmid isolation kit (Invitrogen, Usa).

4.3.6 Phylogenetic analysis

Sequencing data were analysed by using Basic Local Alignment Search Tool (BLAST) server of the National Centre for Biotechnology Information using the BLAST algorithm. The sequenced clones nucleotide sequences were queried against nucleotide sequence server database.

Firstly BLAST finds similar sequences with query sequence, BLAST algorithm applies statistical theory to compute bit score and expect value (E-value) for each alignment pair. Bit score demonstrates the quality of the alignment. Higher bit scores mean better alignment. E-value shows the statistical significant of the alignment. During the study, sequences were retrieved from two different clone libraries representing first pre-denitrification sample (O1) and last pre-denitrification sample (O10) collected after EBPR process. Overall, 20 sequences were retrieved from two different clone libraries.

After finding the closest GENBANK matches, molecular evolutionary and phylogenetic analyses were operated using *MEGA* version 5.10 (Tamura et al, 2007). A phylogenetic tree was constructed with Neighbor-Joining analysis available on Ribosomal Database Project. RDP classifier was used to obtain cultured isolates that were affiliated with sequences available. (Wang et al.,2007, Cole et al., 2009).

4.4 Fluorescence In Situ Hybridization

Fluorescence in situ hybridization is a molecular technique that target to rRNA molecule by specific probes. First of all; this technique is a staining method that allows identification of samples such as bacteria or archea without cultivation. The stained samples were visualized by epifluorescence and confocal laser microscopy or flow cytometry (Giovannoni et all., 1988; Amann et all., 1990). Two kind of probes

were described by Trebesus et all (1994). These are polynucleotide DNA probes and oligonucleotide probes. However, Fluorescence in situ hybridization generally used with oligonucleotide probes which are fluorescently labeled. FISH technique were used exclusively for dedection and characterization of bacteria, analyze structure composition of bacteria and determination of numerical aspect of bacteria (Alfreider et all., 1996; Simon et all., 1999). Generally, bacterial ribosomes contain one copy of each of 5S, 16S and 23S subunit. In bacterial phylogeny, 16S rRNA was focused on (Woese et all., 1990).

FISH technique can be identified as follows; sampling of the part which will be analyzed, sample fixation with ethanol and paraformaldehyde, hybridization of fluorescent labeled probes, washing of non-hybridized probes and lastly microscopic vizualation either with fluorescent or confocal scanning laser microscopes.

Fixation step usually includes fixation but also permeabilization of the cells as well. Cells to be analyzed are fixed to retain their original shape integrities and this process makes them permeable to short oligonucleotides. Common fixation solutions are alcohols and aldehydes. While alcohols precipitate protein, aldehydes make cross-link the cell walls. Generally, in most studies 4% paraformaldehyde is used to fix and permeabilize the cells.

Hybridization is the term of binding of two complementary oligonucleotide polymer. In FISH, hybridization is the term given to the binding fluorescently labeled probes to the ribosolamal oligonucleotides in fixed cells. Hybridization is generally depends on three condition; the presence of target nucleotides, the complementarity between fluorescently labeled probes and target nucleotides and stringency of hybridization condition. The stringency of the hybridization is the optimal condition for specific binding of labeled probes to the targets oligonucleotides. This condition can be counted as temperature, salt concentration and denaturation concentration. In addition, washing steps affect the stringency conditions. Sequentially, washing step is performed. Washing is the removing of excessive and non-hybridizing probes. Washing condition also effects the overall hybridization. Washing temperature, salt concentration and washing incubation time are important parameters for a better washing and hybridization.

4.4.1 Fluorescence in situ hybridization procedure

Fixation, hybridization and washing steps were performed according to folloving methods (Amann et al, 1990). The probes which were used in this study was given in table4.5.

4.4.1.1 Fixation and permeabilization

An appropriate amount of bacterial sample (typically 1 ml) is taken and centrifuged at 13,000 × *g* for 3 minutes. Sample is washed once with phosphate buffered saline (PBS) and added 1 ml PBS and mixed by using the vortex. Solution is centrifuged at 13,000 × *g* for 3 minutes and supernatant is removed. Pellet is resuspended in 0.25 ml of PBS and then, 0,75 ml PFA to the solutin and solution is vortexed. Then immedietly, sample is centrifuged at 13,000 × *g* for 3 minutes and supernatant is removed. After fixation, cells are washed; centrifuged at 13,000 × *g* for 3 minutes, and superntat is removed and 1 ml PBS is added to the mix. After all, Sample is centrifuged at 13,000 × *g* for 3 minutes, supernatant is removed and PBS and absolute ethanol (1:1, v/v) are added to the mix. This suspension can be stored for a long-term at -20°C.

4.4.1.2 Hybridization and washing

10 µl of cell suspension is added (approx. 10^6 cells) to the wells of a gelatine-coated teflon slide, and allowed to air dry at 37°C for 2 hours. Dehydration is performedion by immersing the slide for 3 minutes each in a series of universal tubes, containing decreasing concentrations of ethanol; 50, 80 and 96% ethanol respectively. Slide is air-dried. 10 µl of hybridisation buffer **(Appendix C)** is added (with the appropriate formamide concentration) containing 1 µl of the appropriate probe(s) (concentration 50 ng μl^{-1}), e.g. 9 µl HB plus 1 µl probe. Slide is hybridised at the appropriate temperature for the probe (usually 46°C) for 1.5 hours in an isotonic moisture chamber. The slide is flused with 2 ml of the appropriate wash solution **(Appendix C)** (for the probe being used) and the slide is immersed immediately in enough volume of the same wash solution as to cover the wells of interest at 48°C for 15 minutes. Again washing step is repeated. Finally, the slide is rinsed thoroughly with distilled water (pour over the slide) and air dried. Slides may be stored in the dark at -20°C, or viewed immediately.

Table 4.6 : The probes that used in this study and optimal hybridization condition for each probe.

Probe	Specificity	Formamid Con. (%)	NaCl Conc. mM	Hib. Temp.	Washing Temp.	References
PAO462	Most *Accumulibacter*	35	84	46 ^{o}C	48 ^{o}C	Carvalho et al, 2007
PAO651	Most *Accumulibacter*	35	84	46 ^{o}C	48 ^{o}C	Carvalho et al, 2007
PAO846	Most *Accumulibacter*	35	84	46 ^{o}C	48 ^{o}C	Carvalho et al, 2007
Actino-221	*Actinobacteria*—potential PAOs	30	112	46 ^{o}C	48 ^{o}C	Kong et al, 2005
Actino-658	*Actinobacteria*—potential PAOs	40	56	46 ^{o}C	48 ^{o}C	Kong et al, 2005
GAOQ431	Some *Competibacter*	35	84	46 ^{o}C	48 ^{o}C	Carvalho et al, 2007
GAOQ989	Some *Competibacter*	35	84	46 ^{o}C	48 ^{o}C	Carvalho et al, 2007
GB_G2	Some *Competibacter*	35	84	46 ^{o}C	48 ^{o}C	Carvalho et al, 2007
EUB338	most Bacteria	0-50	900-28	46 ^{o}C	48 ^{o}C	Amann et al, (1990)
EUB338-II	Planctomycetales	0-50	900-28	46 ^{o}C	48 ^{o}C	Amann et al, (1990)
EUB338-III	Verrucomicrobiales	0-50	900-28	46 ^{o}C	48 ^{o}C	Amann et al, (1990)

It was aimed to indentify the distribution of phosphorus accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) in the post-denitrification (S1, S2, S3, S4 and S5) and pre-denitrification (O1, O2, O3, O7, O8, O9 and O10) samples that were sampled from Marmara University, Environmental Engeenering Department EBPR system. FISH technique was used to assess the dynamics of these considerably important organisms in EBPR systems. Specific probes which are PAOmix (PAO-462, PAO-651 and PAO846) and GAOmix (GAO431, GAO989 and GB_G2) were used respectively to determine the ratio of PAO and GAO group in the EBPR samples. Also, DAPI staining was applied to samples for entire DNA staining.

In addition, flourescence in situ hybridization of post-denitrification (S1, S2, S3, S4 and S5) and pre-denitrification (O1, O2, O3, O7, O8, O9 and O10) samples were performed with *Actinobacteria* specific Actino-221 preobe (short rods *Actinobacteria*) and Actino-658 probe (cocci shaped *Actinobacteria*).

Positive and negative controls were performed simultaneously with fluorescence in situ hybridization. Positive controls were applied with the probes EUBmix (EUB338-I, EUB338-II, EUB338-III). In addition for negative control, NonEUB-338 probe was used. FISH slides were visualised with confocal scaning laser microscope.

Slides were analysed with a Confocal Scaning Laser microscope, Lecia confocal software program.. For each sampe, 3 random fields of view were obtained. In order

to determine the relative abundance total microorganisms in each field of view dapi stained samples images were taken. The hybridized microorganisms within total microorganisms determined with dapi staining were viewed from fluorescence images. The FISH images analysed using image J (Image processing and analysis in java, counts for 3 random fields of view were obtained for each samples, and the avarage cell count was calculated.

5. RESULTS

5.1 Performance and Sampling

Sampling was performed during both pre-denitrification and post-denitrification EBPR systems operation. Conventional data, such as oxic phosphate removal, anoxic phosphate removal, anaerobic phosphate removal amounts during EBPR process post-denitrification EBPR and pre-denitrification were recorded by our colleagues at Marmara University, Environmental Engineering Department. Figure5.1 and figure5.2 show the activity of microbial community especially PAOs.

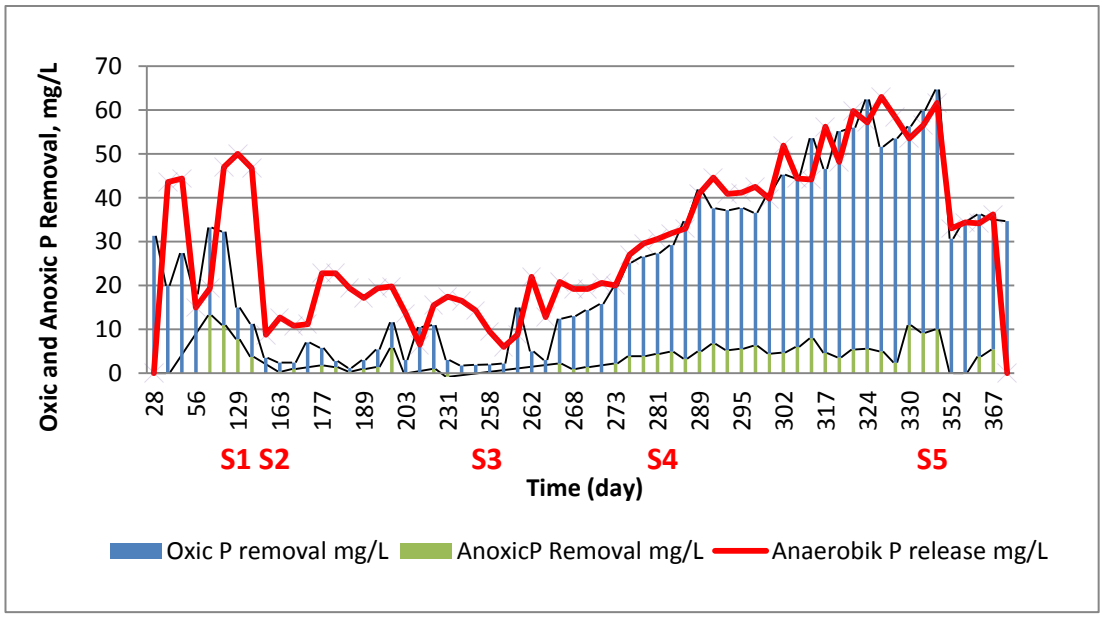


Figure 5.1 : Phosphate release and removal under post-denitrification condition.

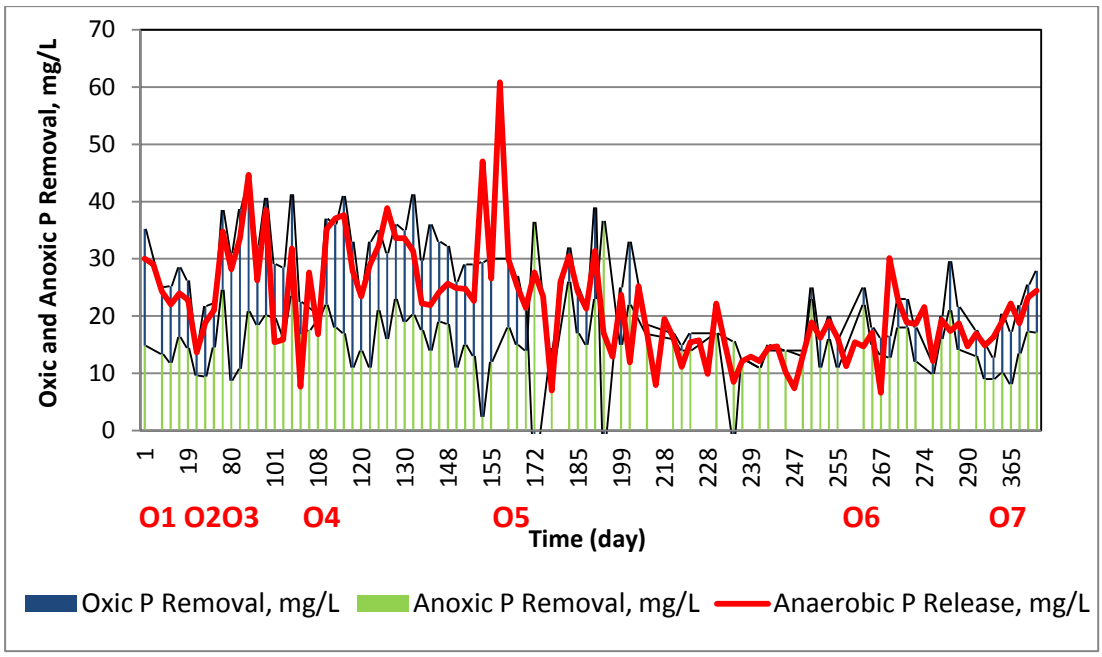


Figure 5.2 : Phosphate release and removal under pre-denitrification condition.

Figure 5.3 and figure 5.4 show the PO_4-P influent and effluent amount in post-denitrification and pre-denitrification respectively. The studied EBPR samples were marked acording to sampling date in figure5.3 and figure5.4 as can be seen form those figure, phosphate removal reach the 65 mg/L in some point.

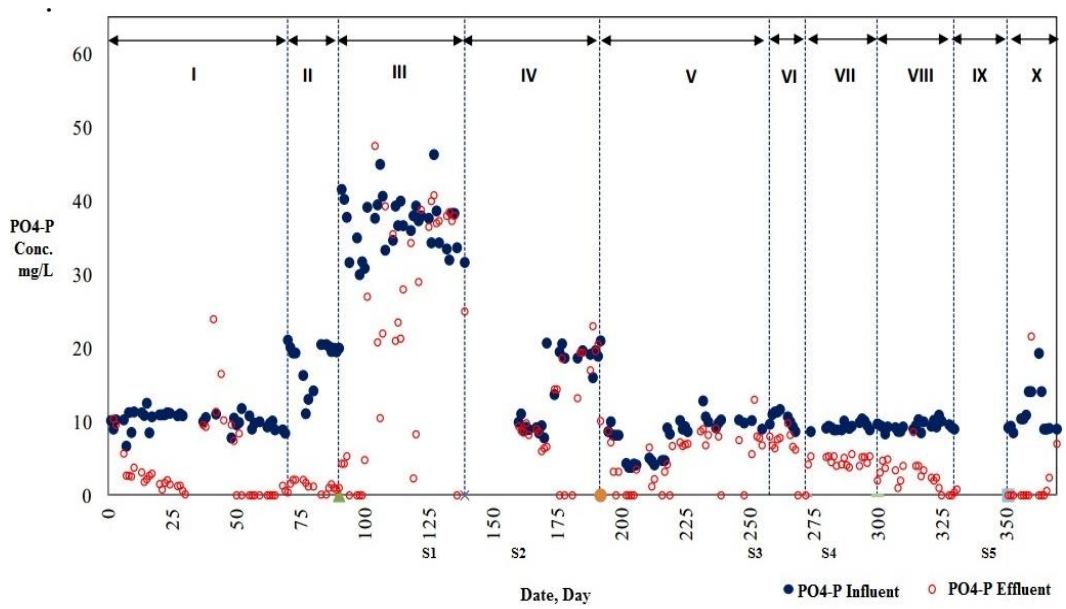


Figure 5.3 : Influent and effluent PO4-P in post-denitrification.

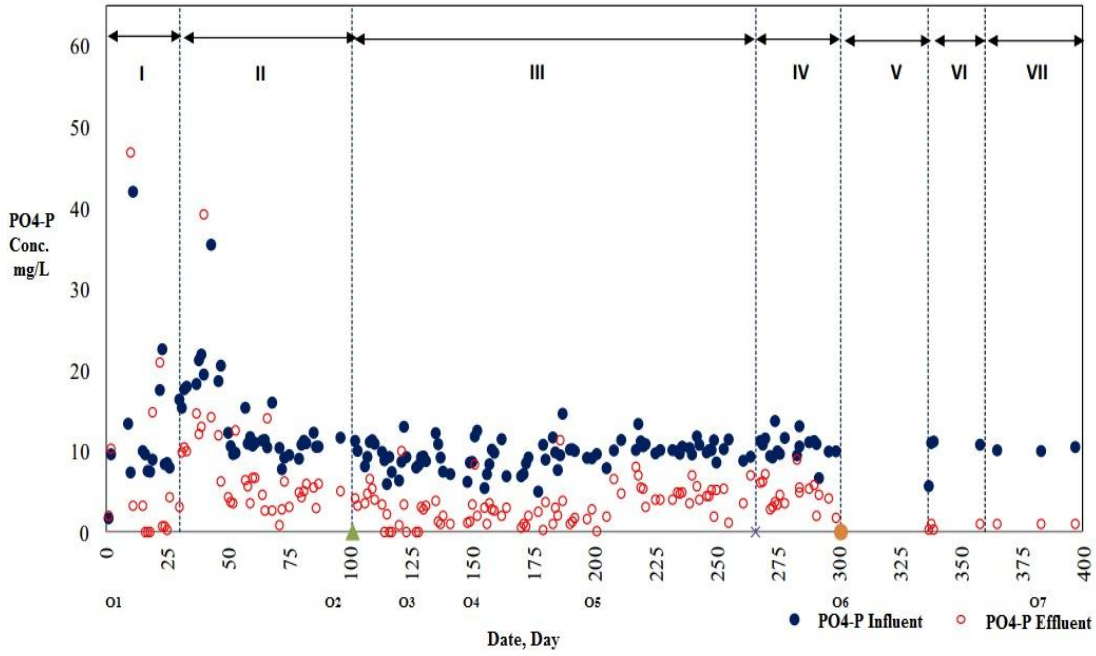


Figure 5.4 : Influent and effluent PO4-P in p**re**-denitrification.

5.2 DNA Extraction Results

The genomic DNA from a total of 15 samples; 5 samples from pre-denitrification and 10 samples from post-denitrification were extracted and the amounts of DNA were measured using Qubit fluorometer (Table5.1). Extraction result was also analyzed using agarose gel electrophoresis visualized by GelDoc™ (BIORAD, USA) (Figure5.5). As it can be seen from Qubit DNA measurement, DNA amount of Pre-denitrification samples are higher than post-denitrification samples. As can be seen from Table5.1 the DNA amount of post-denitrification (S series) samples are very low while DNA amount of pre-denitrification (O series) samples were high which could be correlated with wash-outs and reactor running conditions.

Table 5.1 : DNA amounts measured fluorometrically using Qubit fluorometer.

Sample	Samples Description	Sampling date	Qubit Fluorometer Result (µg/mL)
S1	Post-denitrification samples	29.06.2009	10,6
S2	Post-denitrification samples	04.08.2009	49
S3	Post-denitrification samples	15.10.2009	16,5
S4	Post-denitrification samples	02.12.2009	29,5
S5	Post-denitrification samples	01.03.2010	13
O1	Pre-denitrification samples	10.03.2010	26,9
O2	Pre-denitrification samples	10.06.2010	12,2
O3	Pre-denitrification samples	09.07.2010	25,3
O4	Pre-denitrification samples	05.08.2010	34,1
O5	Pre-denitrification samples	22.09.2010	27
O6	Pre-denitrification samples	03.01.2010	23
O7	Pre-denitrification samples	23.03.2011	150
O8	Pre-denitrification samples	27.06.2011	64,5
O9	Pre-denitrification samples	28.07.2011	90,4
O10	Pre-denitrification samples	12.08.2011	122

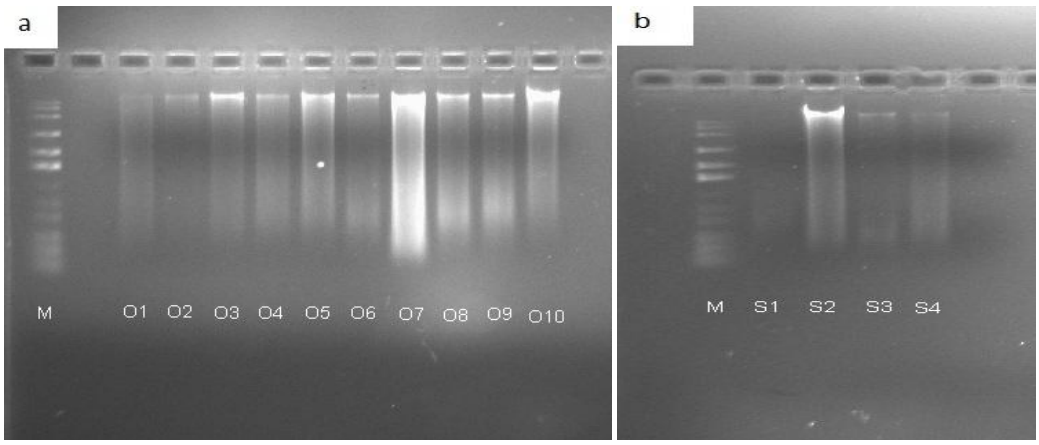


Figure 5.5 : Gel electrophoresis view of extracted samples from from pre-denitrification and post-denitrification. (a) pre-denitrification samples, (b) post-denitrification samples.

5.3 PCR Results

Presence of archaeal and bacterial communities was detected using domain specific 16S rRNA gene primers. DNA products obtained in PCR reactions served as a template for subsequent DGGE methodology to determine microbial diversity of each sample. Briefly, 10 pre-denitrification and 5 post-denitrification EBPR samples were screened for both bacterial and archeal diversity, and 15 EBPR samples were

screened for bacterial diversity only. After the amplification, all of the PCR products were of the expected length.

5.3.1 Bacterial PCR Results

Extracted DNA samples from pre-denitrification and post-denitrification samples were successfully amplified by 16S rRNA gene complementary Vf-GC/Vr primer sets. PCR products were used for further DGGE analysis. Figure5.6 shows the PCR results of samples. PCR products of samples are approximately 190 bp length.

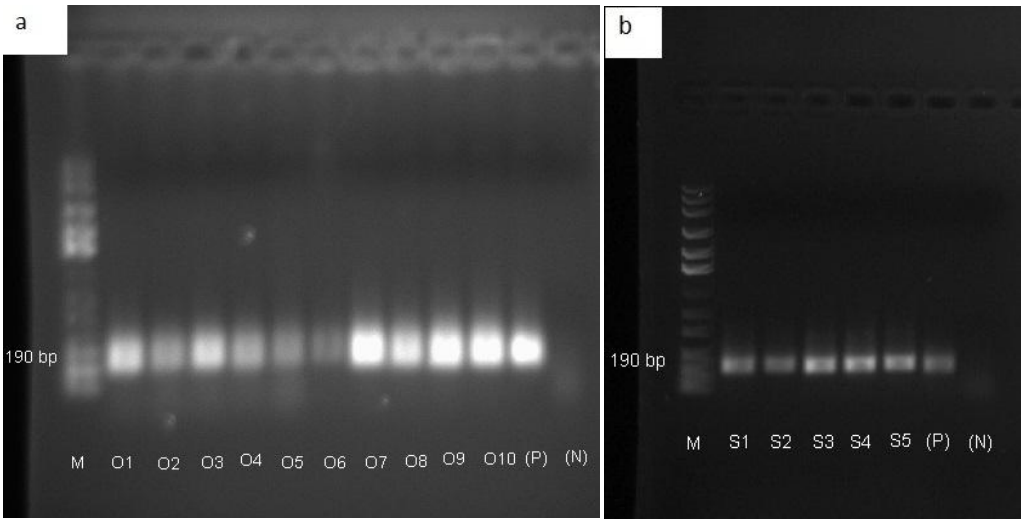


Figure 5.6 : Bacterial amplification results of pre-denitrification (a) and post-denitrification (b) by Vf-GC/Vr PCR primer set.

5.3.2 Archeal PCR results

For identification of Archeal communities, domain specific universal 16S rDNA gene primers were used. Amplification of 16S rDNA of archeal samples were done using nested PCR technique with former arch-7f and arch-1384r primers. Archeal community were detected for neither pre-denitrification nor post-denitrification samples. Figure5.7 demonstrates the PCR results of the arch-7f and arch-1384r primer sets.

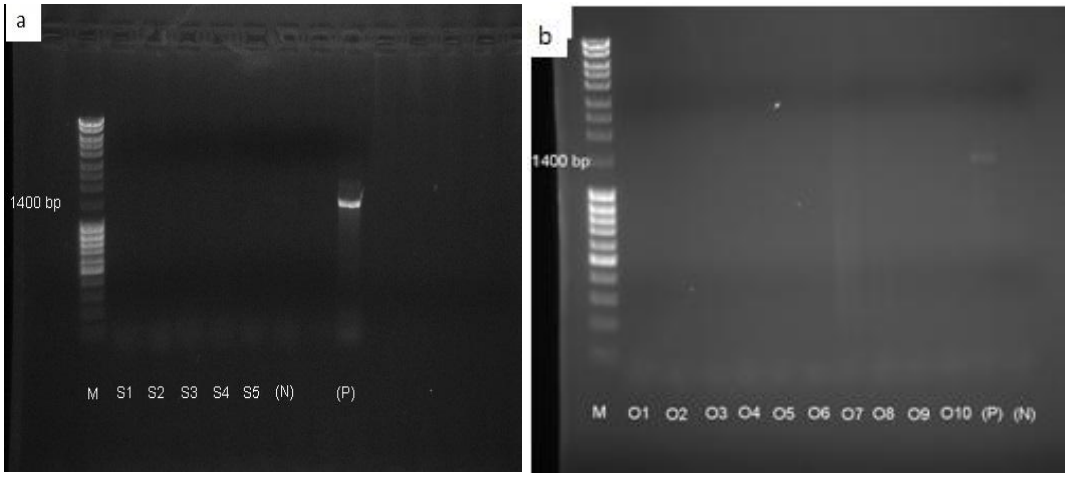


Figure 5.7 : Archeal PCR results of pre-denitrification (a) and post-denitrification (b) by arch-7f and arch-1384r primer set.

5.4 DGGE Results

After PCR amplification of 10 pre-denitrification samples (O series) and 5 post-denitrification samples (S series) form EBPR by using 16S rRNA gene specific VfGC and Vr primers, 190 bp length amplicons were obtained. Then, these PCR amplicons were run using DGGE analysis. Obtained DGGE profiles indicate different profiles between pre-denitrification samples and post-denitrification. Besides DGGE band patterns display diversity change through samples. Figure 5.8 and 5.9 show DGGE profiles of pre-denitrification and post-denitrification EBPR samples.

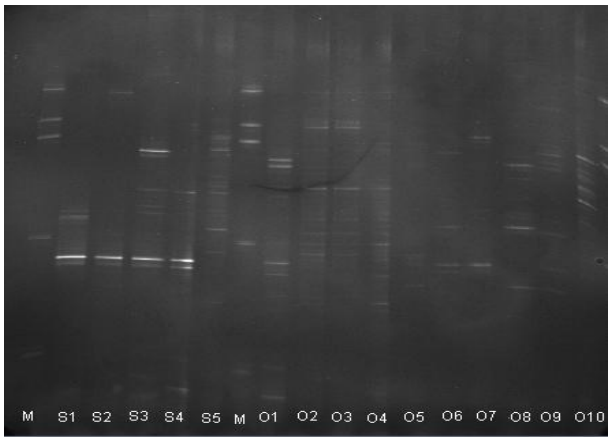


Figure 5.8 : DGGE profile of both pre-denitrification (O series) and post-denitrification (S series) samples, M is marker.

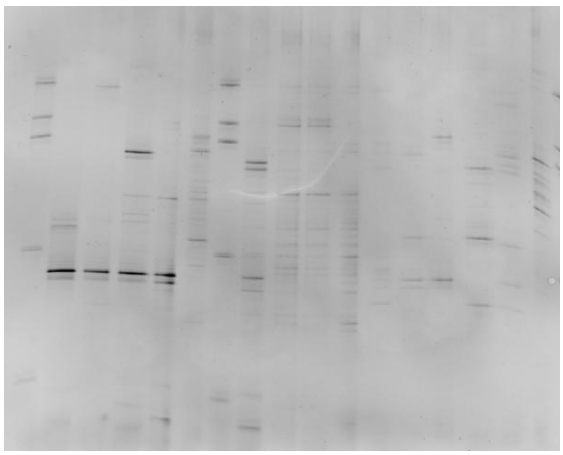


Figure 5.9 : Inverted image of DGGE profile of both pre-denitrification (O series) and post-denitrification (S series) samples, M is marker.

5.4.1 Community analysis of DGGE profiles

DGGE profile patterns of pre-denitrification and post-denitrification EBPR samples were clustered according to their bacterial 16S rRNA gene products. Figure 5.10 shows the clustering analysis (Pearson correlation coefficient-UPGAMA) of 15 samples and they were shown to cluster closely together. Pre-denitrification and post-denitrification samples clustered in two separate groups except for two samples. First exception is S5 sample which is post-denitrification sample that clustered together with pre-denitrification samples. Second exception was O1 sample which is pre-denitrification sample, clustered with post-denitrification samples according to DGGE profile.

Pearson Correlation [0,0%-100%]

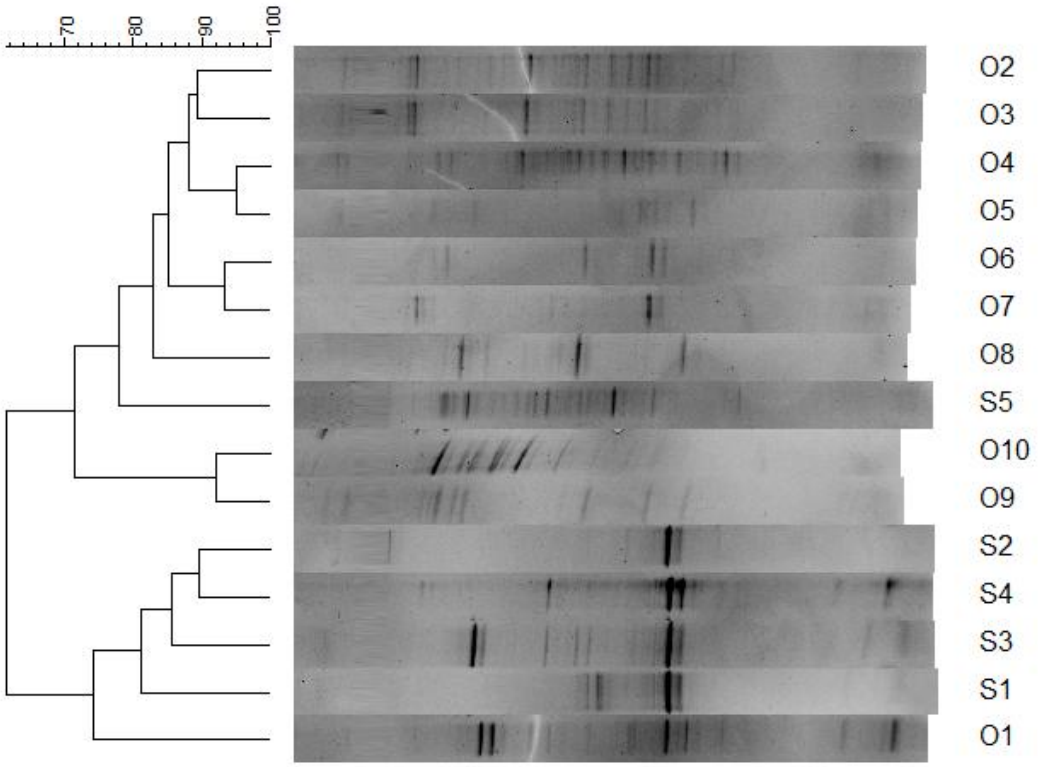


Figure 5.10 : Similarity (Pearson correlation coefficient- UPGAMA) of the DGGE banding patterns of 15 EBPR samples.

Similarity percentages of pre-denitrification samples (O series) and post-denitrification samples (S series) were given in Table 5.2. Similarity percentage between binary samples shows the similarity of all EBPR samples community among each other .

Table 5.2 : Similarity percentages (%) of pre-denitrification samples (O series) and post-denitrification samples (S seies).

	Similarity percentage														
Samples	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	S1	S2	S3	S4	S5
O1	100	83.43	68.91	71.28	76.15	76.79	72.59	64.97	51.81	47.99	67.51	75.23	75.49	77.43	71.23
O2	83.43	100	89.14	86.26	89.28	88.47	86.02	80.97	72.27	64.05	67.33	75.02	70.29	75.63	82.75
O3	68.91	89.14	100	89.22	87.25	80.41	74.71	80.76	80.23	73.02	56.27	65.52	60.41	65.75	79.60
O4	71.28	86.26	89.22	100	94.87	89.89	81.67	83.02	75.53	76.36	56.98	60.79	59.14	62.79	81.97
O5	76.15	89.28	87.25	94.87	100	93.38	86.28	84.25	78.16	77.37	60.77	63.03	63.12	63.34	80.76
O6	76.79	88.47	80.41	89.89	93.38	100	93.24	84.11	67.45	65.32	65.92	68.99	64.26	69.62	78.33
O7	72.59	86.02	74.71	81.67	86.28	93.24	100	83.31	66.39	60.23	59.36	65.82	59.99	67.61	69.65
O8	64.97	80.97	80.76	83.02	84.25	84.11	83.31	100	82.07	74.25	52.25	59.35	57.36	61.43	71.61
O9	51.81	72.27	80.23	75.53	78.16	67.45	66.39	82.07	100	92.03	42.13	46.04	42.41	41.20	64.13
O10	47.99	64.05	73.02	76.36	77.37	65.32	60.23	74.25	92.03	100	40.40	39.16	39.14	32.21	62.71
S1	67.51	67.33	56.27	56.98	60.77	65.92	59.36	52.25	42.13	40.40	100	89.09	78.21	75.49	65.21
S2	75.23	75.02	65.52	60.79	63.03	68.99	65.82	59.35	46.04	39.16	89.09	100	86.89	89.39	71.09
S3	75.49	70.29	60.41	59.14	63.12	64.26	59.99	57.36	42.41	39.14	78.21	86.89	100	84.26	69.83
S4	77.43	75.63	65.75	62.79	63.34	69.62	67.61	61.43	41.20	32.21	75.49	89.39	84.26	100	73.33
S5	71.23	82.75	79.60	81.97	80.76	78.33	69.65	71.61	64.13	62.71	65.21	71.09	69.83	73.33	100

Community similaritiy percentages between the pre-denitrification and the post-denitrification EBPR samples among each other were demonstrated in Table 5.3. As it can be seen from Figure 5.11, population dynamic of both pre-denitrification (O series) samples and post-denitrification samples (S series) remain almost stable. Especially, pre-denitrification samples demonstrate more conserved stracture.

Table 5.3 : Similarity percentages between pre-denitrification EBPR (O series) and post-denitrification (S series).

Binary samples	Percntage (%)	Binary samples	Percntage (%)
O1-O2	83.43	S1-S2	89.09
O2-O3	89.14	S2-S3	86.89
O3-O4	89.22	S3-S4	84.26
O4-O5	94.87	S4-S5	73.33
O5-O6	93.38		
O6-O7	93.24		
O7-O8	83.31		
O8-O9	82.07		
O9-O10	92.03		

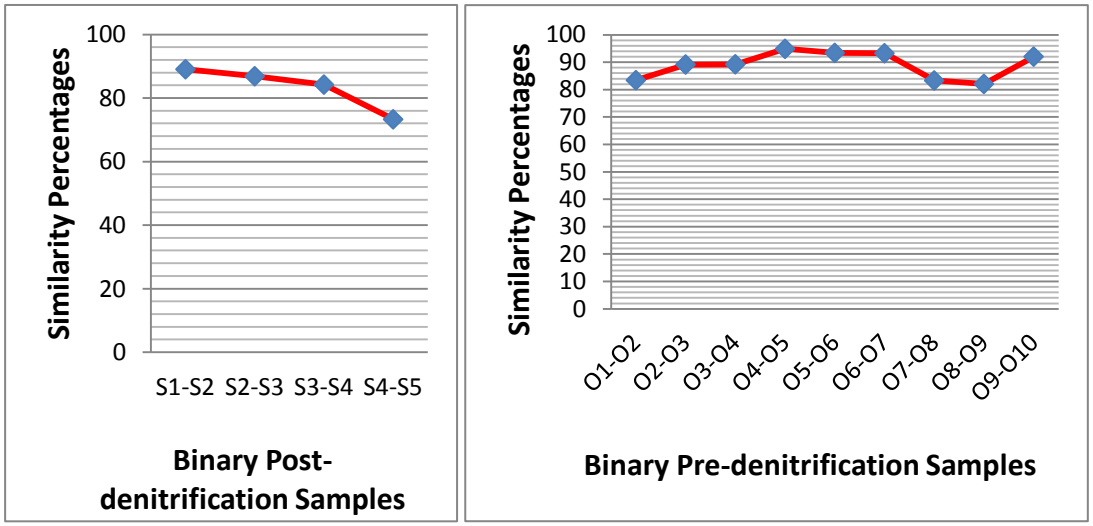


Figure 5.11 : Similarity percentage of post-denitrification (S series) and pre-denitrification (O series) samples among each other.

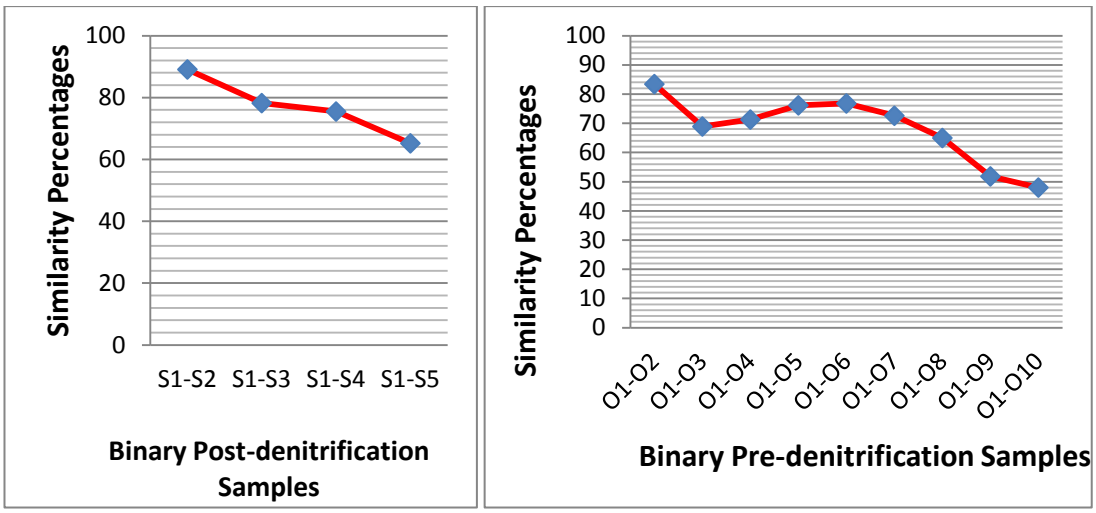


Figure 5.12 : Communities similarity between first samples of both post-denitrification and pre-denitrification among other post-denitrification and pre-denitrification samples.

5.5 Fluorescence In Situ hybridization (FISH) Results

In the second part of the study, it was aimed to indentify the distribution of phosphorus accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) in the EBPR samples that were sampled from Marmara University, Environmental Engeenering Department EBPR system. FISH technique was used to assess the dynamics of these considerably important organisms in EBPR systems. Specific probes which are PAOmix (PAO-462, PAO-651 and PAO846) and GAOmix (GAO431, GAO989 and GB_G2) were used respectively to determine the ratio of PAO and GAO group in the EBPR samples. Also, DAPI staining was applied to samples for entire DNA staining.

Positive and negative controls were performed simultaneously with fluorescence in situ hybridization. Positive controls were applied with the probes EUBmix (EUB338-I, EUB338-II, EUB338-III). In addition for negative control, NonEUB-338 probe was used. FISH slides were visualised with confocal scaning laser microscope.

5.5.1 PAOs and GAOs FISH results

The Flourescence in situ hybridization of post-denitrification (S1, S2, S3, S4 and S5) and pre-denitrification (O1, O2, O3, O7, O8, O9 and O10) samples were performed with group specific PAOmix and GAOmix probe. Moreover, the Flourescence in situ hybridization of same samples were performed with *Actinobacteria* specific Actino-221 and Actino-658 probe to visualize viable *Actinobacteria* population.. The FISH images were analysed with Image J (image processing and analysis in java) and the

percentages of PAOs, GAOs, rod saheped Actinobacteria and coccus shaped Actinobacteria in total bacterial community were obtained. The FISH analysis of all samples were given in Table 5.4. The FISH result show that phosphate accumulating organisms are more dominant than glycogen accumulating organisms in all sample. The FISH analysis show that the short rods *Actinobacteria* (targeted by probe Actino-658) were more aboundant than the cocci shaped *Actinobacteria* (targeted by Actino-221).

As an example the FISH results of O8 EBPR sample (pre-denitrification sample) is given in figure5.13 Analysis of O8 samples with rRNA targeted oligonucleotide probes shows that 45,53 ± 2,3 % (mean and standart deviation) of total population belongs to phosphorus accumulating organisms (PAOs) and 6,91 ± 1,5 % of total microbial community belongs to glycogen accumulating organisms (GAOs). All FISH images were given in **AppendixD** (FISH Images).

Table 5.4 : Relative abundance of microbial group.

	Microbial Group (Probe name)			
Samples	Pao (%)	Gao (%)	Actino-221(%)	Actino-658 (%)
S1	62,9± 3,6	9,60±1,4	11,15±1,3	20,82±1,9
S2	76,6± 3,8	4,67±1,3	16,43±1,6	36,37±2,6
S3	49,1± 3,24	23,40±2,3	4,71±1,0	31,13±2,2
S4	47,4± 2,4	22,47±1,9	3,781±1,2	22,81±1,9
S5	44,6± 2,5	19,28±1,7	9,25±1,5	18,95±1,6
O1	57,0± 2,25	36,00±3,0	13,08±1,9	39,82±3,0
O2	53,3± 3,7	25,25±2,5	13,85±1,7	26,24±2,5
O3	47,9± 3,09	12,15±1,3	2,37±0,7	20,06±1,8
O7	27,1 ± 2,1	6,24±1,2	9,65±1,1	3,52±0,9
O8	45,5± 2,3	6,91±1,4	26,04±2,4	9,98±1,2
O9	62,5± 3,5	25,20±2,2	33,54±2,7	4,12±0,9
O10	35,9± 2,13	46,25±3,1	18,34±2,5	0,44±0,2

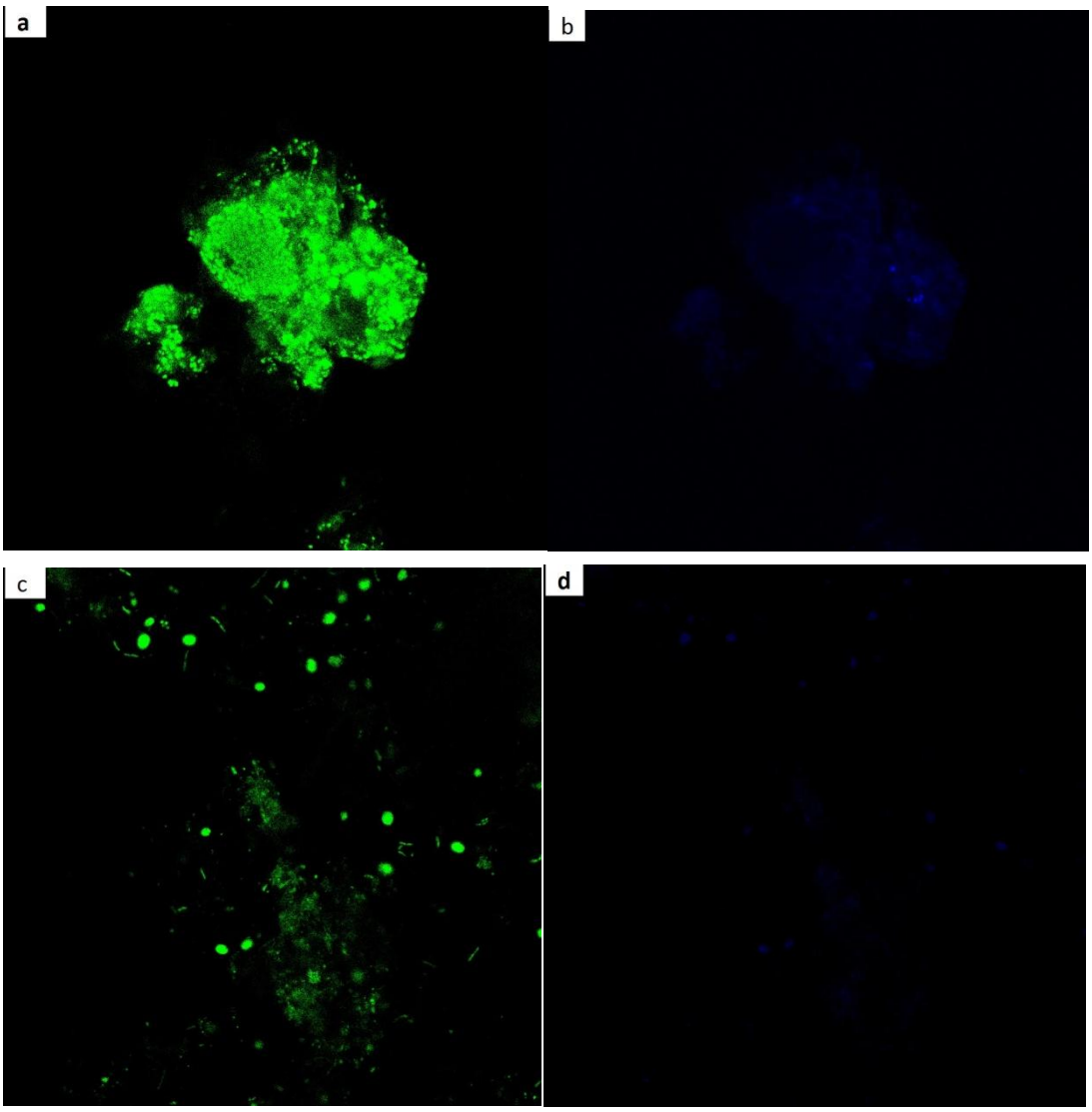


Figure 5.13 : Image examples of microbial groups in the O8 (pre-denitrification) sample. a and c) Dapi staining of O8 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, d) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

FISH analysis of PAO, GAO and *Actinobacteria* diverse significantly among samples. Generally in all samples, except sample O10, phosphate accumulating organisms were dominant. It was shown from table5.4 that in the last sample (pre-denitrification sample) the glycogen accumulating organism sare more than phosphate accumulating organisms. Moreover, FISH analysis with the Actino-221 and Actino-658 probes demonstarte that the aboundance of short rods Actinobacteria (targeted by probe Actino-658) are 19,52 % of DAPI stained total microorganisms while the aboundance cocci shaped Actinobacteria are 13,52 % of DAPI stained total microorganisms. Hovewer, as it can be seen from the figure 5.14 trough the last

samples the aboundance of short rod Actinobacteria are decreasing, contraversary, the aboundance of cocci shaped Actinobacteria are increasing.

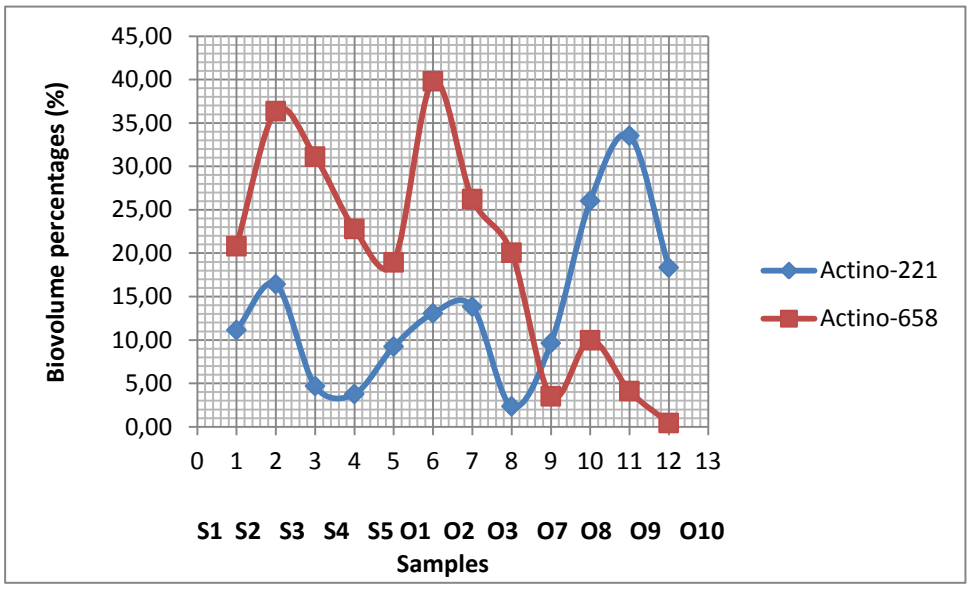


Figure 5.14 : The percentages of of short rods *Actinobacteria* (targeted by probe Actino-658) and cocci shaped *Actinobacteria* throughout DAPI stained total microorganisms.

5.6 Cloning and Phylogenetic Analysis Results

Major bands of DGGE profiles were also subject to phylogenetic analysis. 12 clone from first pre-denitrification sample (O1) 16 clone from and last pre-denitrification sample (O10) were formed. O1 and O10 samples were found to be affiliated with several different bacterial classes including Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria from the diverse Proteobacteria phylum and also from Firmicutes group affiliated OTUs (Table 5.5). In addition Figure 5.15 and figure 5.16 show the abundance of clone library results of first and last pre-denitrification samples respectively.

Table 5.5 : Phylogenetic affiliations, closest matches and number of clones from pre-denitrification samples (O1 and O10).

Sample Group	Phylum	Order	Clone	Accession Number/ Closest GenBank Affiliate	
O1	GAMMA PROTEOBACTERIA	Pseudomonadales	O1-2	EU652469.1	Pseudomonas Sp.
			O1-5	AB685628.1	Pseudomonas Sp.
			O1-7	HQ665008.1	Pseudomonas Sp.
			O1-10	NR028986.1	Pseudomonas Sp.
		Xanthomonadales	O1-8	EF111221.1	Stenotrophomonas sp.
	ALPHA PROTEOBACTERIA	Rhizobiales	O1-4	JX292365.1	Rhizobium
	BETA PROTEOBACTERIA	Burkholderiales	O1-11	AJ012071.1	Acidovorax Sp.
	FIRMICUTES	Clostridiales	O1-9	AJ229234.1	Clostridium Sp.
	Unclassified		O1-3	DQ168649.1	Unclassified Porphyromonadaceae
	Uncultured		O1-6	FQ659844.1	Uncultured
O10	GAMMA PROTEOBACTERIA	Xanthomonadales	O10-4	JX949966.1	Stenotrophomonas sp.
			O10-10	KC503914.1	Stenotrophomonas maltophilia
			O10-15	KC884682.1	Stenotrophomonas sp.
		Pseudomonadales	O10-9	GQ202270.1	Acinetobacter Sp.
			O10-14	AJ633634.1	Acinetobacter Sp.
	BETA PROTEOBACTERIA	Burkholderiales	O10-5	KC252767.1	Achromobacter xylosoxidans
	FLAVOBACTERIIA	Flavobacteriales	O10-6	JX827629.1	Chryseobacterium Sp.
	FIRMICUTES	Clostridiales	O10-7	AY548785.1	Clostridium Sp.
			O10-12	AY548785.1	Clostridium Sp.
	Unclassified		O1-3	DQ168649.1	Unclassified Porphyromonadaceae

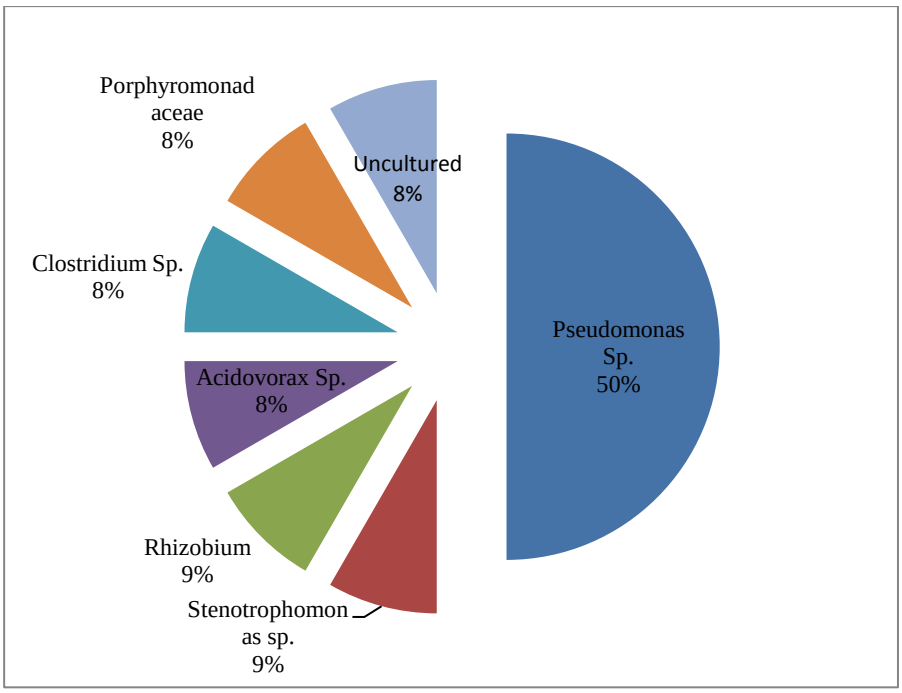


Figure 5.15 : The percentages of microbial strains found in clone library of first pre-denitrification sample.

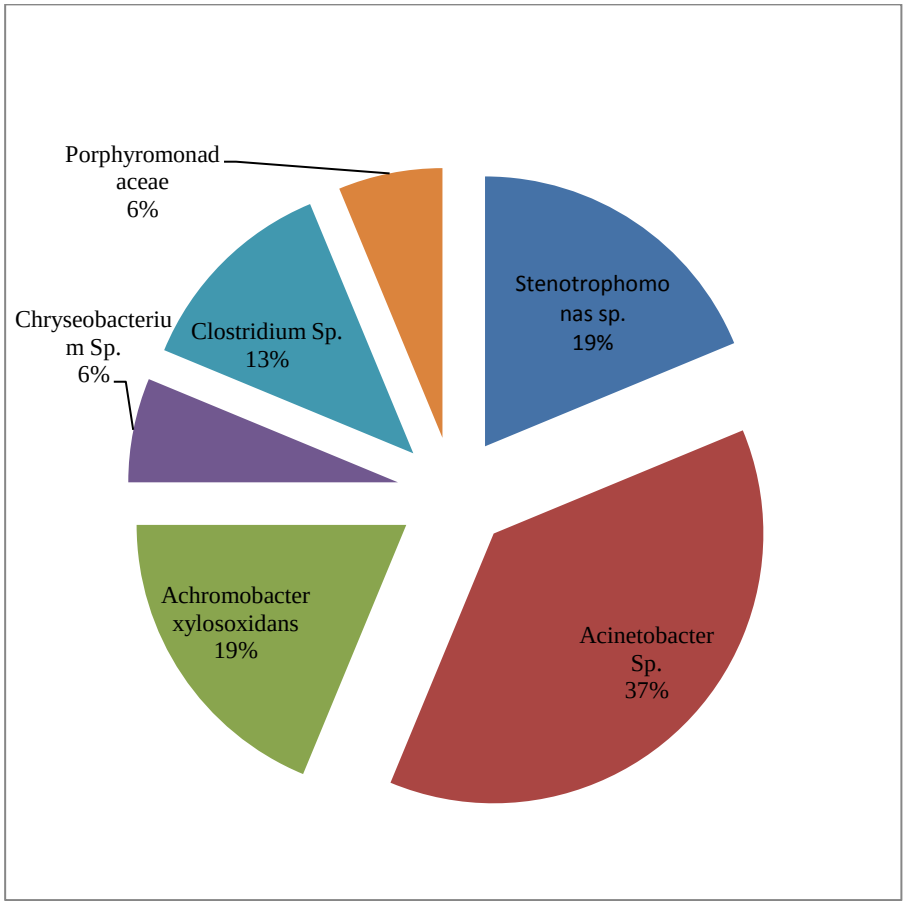


Figure 5.16 : The percentages of microbial strains found in clone library of last pre-denitrification sample.

Majority of the clones analyzed in the first pre-denitrification sample (O1) closely related to (more than 95%) to *Pseudomonas* sp. of Gammaproteobacterium group. This genus is an aerobic gram-negative proteobacterium. *Pseudomonas* sp. have been found in some EBPR reactors (Lötter, 1984; Li et al.,2003). Some Pseudomonas species is a well know for degradation of polycyclic aromatic hydrocarbons (O'Mahony, 2006. As can be seen from table 5.5 , figure 5.15 and figure5.17. Pseudomonas sp. was only found in O1 clones. (first pre-denitrification sample). Six of O1 clones (clone-2, clone3, clone-5, clone-7, clone-10 and clone-12) belong to *Pseudomonas* sp. *Acidovorax* is also a bacterial genus order of Betaproteobacteria commonly found in EBPR sludge (Melasniemi et al., 1998).

In addition, *Stenotrophomonas* sp. *Rhizobium*, and *Clostridium* sp. were found in O1 clones.

EBPR inoculum was obtained from Paşaköy biological wastewater treatment plant (WWTP).Thus in many industrial treatment systems; they are important role players for degradation of organic material.

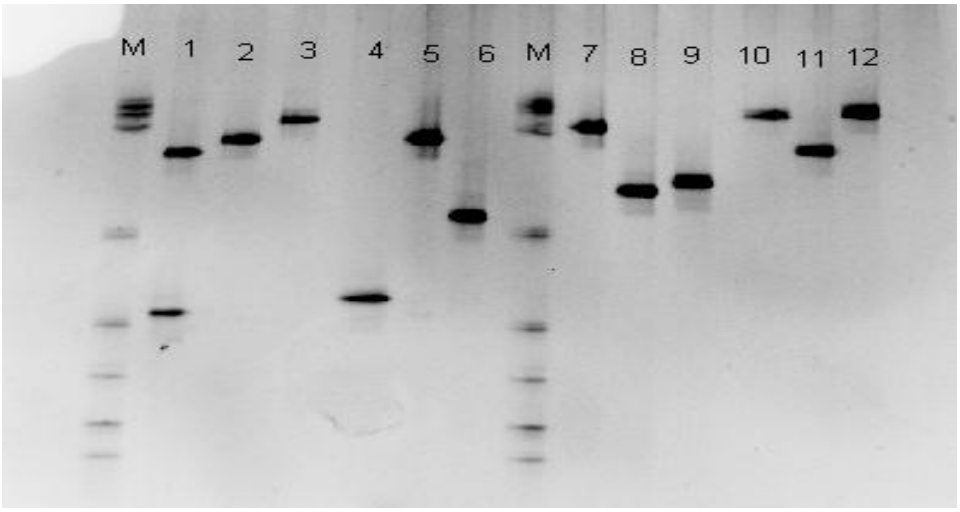


Figure 5.17 : DGGE view of first pre-denitrification sample's clones (O1).

During the EBPR process; initial microbial populations in pre-denitrification samples changed significantly. While analysed clones were affiliated with *Pseudomonas* sp. at the first pre-denitrification sample (O1), after approximately 400 day EBPR process, microbial community shifted towards Acinetobacter genus and *Stenotrophomonas* genus order of Gammaproteobacteria and the majority (56,25 %) of the analysed clones belonged to this two genus. Totaly 6 clones (Clone-1, Clone-

2, clone-3, clone-9, clone13 and clone-14) belonged to *Acinetobacter* genus, which was almostly found in all EBPR samplesin different ratio (Fuhs and Chen, 1975; Oehmen, 2007). Hovewer it is belived that *Acinetobacter* is not significiantly responsible for EBPR systems (Oehmen, 2007). Clone-5 from the last pre-denitrification sample (O10) closely related to (more than 97%) *Achromobacter xylosoxidans* (KC252767.1) and similarly clone-8 and clone-16 are belonged to *Achromobacter* sp. based on DGGE profile (figure5.18).

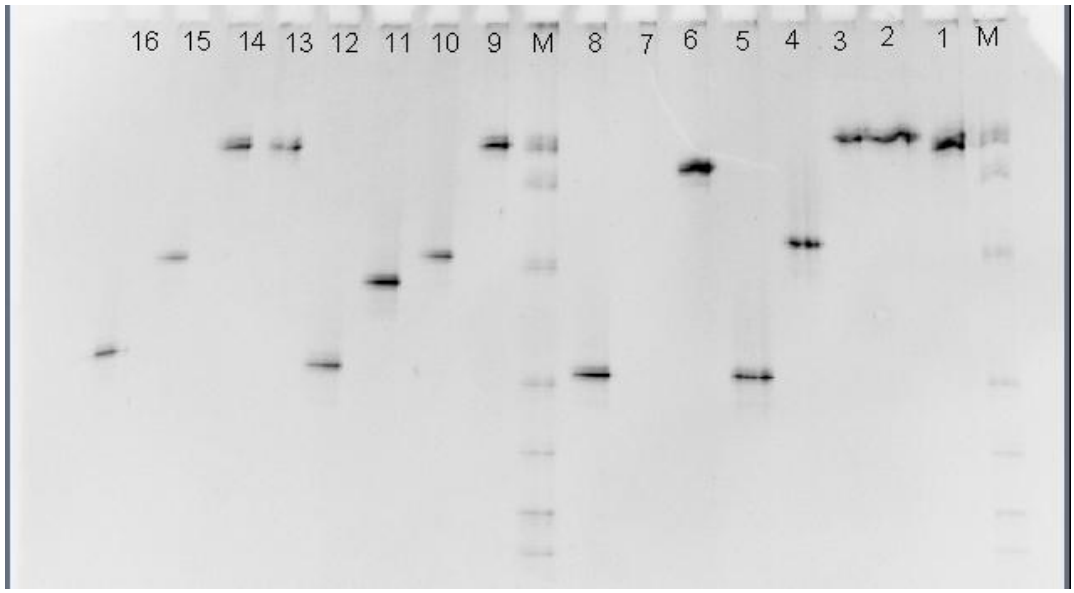
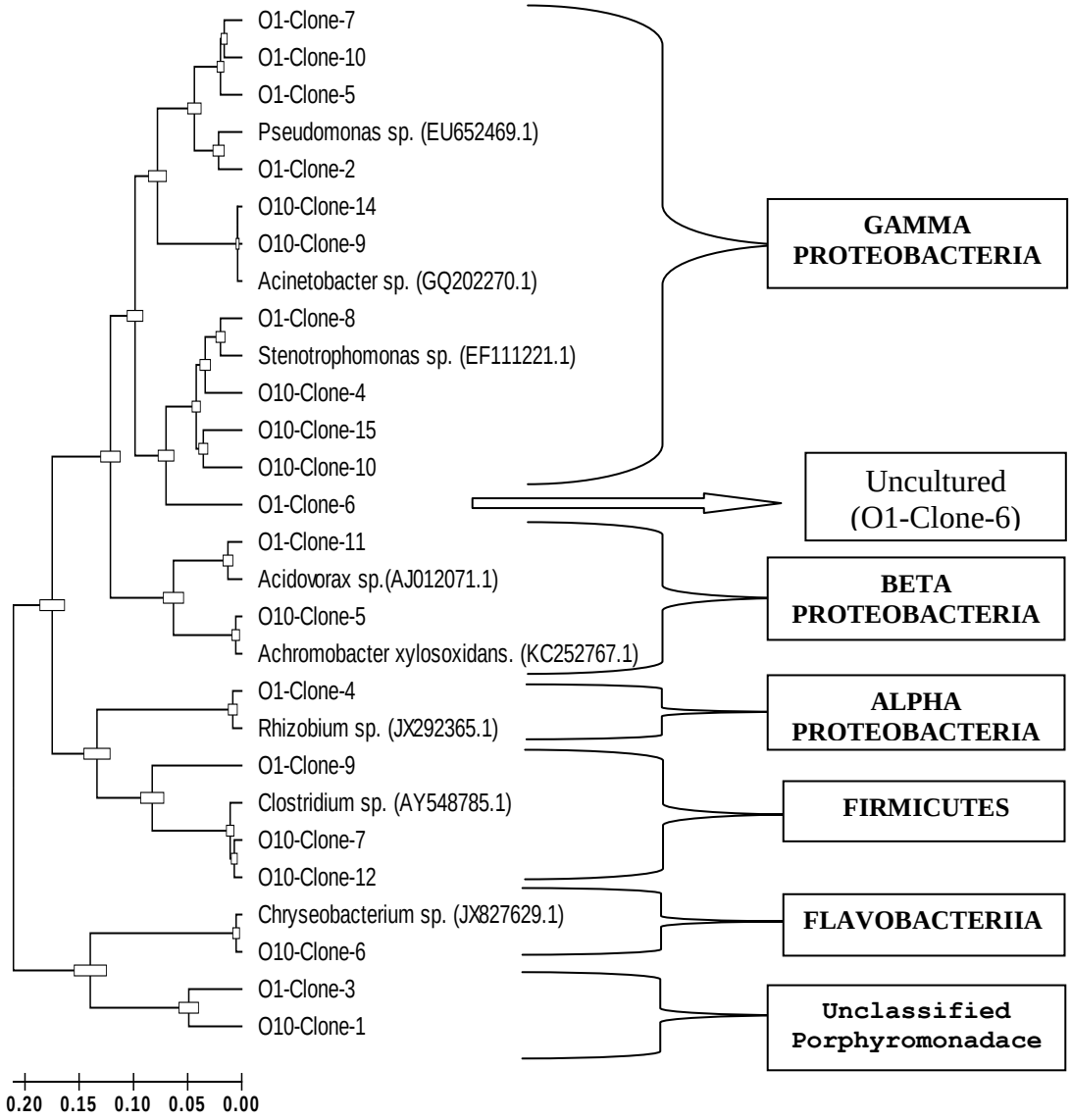


Figure 5.18 : DGGE view of last pre-denitrification sample's clones (O10).

Phylogenetic tree was constructed using Molecular Evolutionary genetics Analysis (MEGA). As can bee seen from the plygenetic tree (figure 5.19) the most aboundant group is Gammaproteobacteria. Totally 10 clone were closely related to Gammaproteobacteria which includes genus of *Pseudomonas*, *Acinetobacter* and *Stenotrophomona.* Those proteobacteria comed together in phylogenetic tree. There are two groups of Betaproteobacteria in which OTUs of PAO and GAO sludge were both found: *Acidovorax* and *Achromobacter*. Among them, *Acidovoraxis* a bacterial genus commonly found in EBPR sludge (Melasniemi et al., 1998). Betaproteobacteria is the mostly responsible and numerically dominant in the EBPR systems (Hesselmann et al., 1999; Crocetti et al., 2000) So it was significance to find this group in pre-denitrification clone library.



Fifure 5.19 : Phylogenetic tree illustrating the relationship between the closest relatives in the RDP and GenBank databases and bacterial 16S rRNA gene sequences retrieved from the the first (O1) and the last (O10) pre-denitrification samples. The tree was constructed by the Neighbor-joining matrix from weighted pairwise comparisons made by the Jukes and Cantor (1969) algorithm in RDP Tree Builder. The scale bar represents 2% difference in nucleotide sequence.

6. CONCLUSION

Microbial community analysis of both pre-denitrification and post-denitrification EBPR samples was performed during this study. Changes in EBPR community profile of 15 samples were investigated using 16S rRNA gene based DGGE analysis using bioinformatic tools like Bionumerics, Mega, Blast etc. PCR-DGGE analysis of EBPR samples demonstrated different diversity patterns between pre-denitrification and post-denitrification processes. Bionumerics analysis of DGGE patterns (Pearson correlation coefficient-UPGAMA) showed that pre-denitrification communities cluster closely together as in post-denitrification communities according to their community similarity. DGGE analysis demonstrated that pre-denitrification EBPR samples contains higher OTU numbers than post denitrification samples.

FISH analysis of EBPR samples showed that phosphorus accumulating organisms (PAOs), bound with the FISH probe PAO mix were dominant throughout the whole samples. Microbial community analysis of total population with DGGE and FISH demonstrate that microbial community of pre-denitrification EBPR samples is more diverse than post-denitrification EBPR samples.

In pre-denitrification EBR samples mainly there were two groups of Betaproteobacteria in which of PAOs and GAOs were both found: *Acidovorax* and *Achromobacter*. According to ,*Acidovorax* is a bacterial genus commonly found in EBPR sludge (Melasniemi et al., 1998). Betaproteobacteria is mostly responsible and numerically dominant in the EBPR systems (Hesselmann et al., 1999; Crocetti et al., 2000).

FISH analysis with the Actino-221 and Actino-658 probes demonstrate that the abundance of short rods Actinobacteria (targeted by probe Actino-658) are 19,52 % with respect to DAPI stained total microorganisms while the abundance of cocci shaped Actinobacteria are 13,52 %. Hovewer, there is a significant lack of consistency between the FISH and the clone library analyses on the presence of Gram-positive bacteria, especially Actinobacteria. FISH results showed that

Actinobacteria, mainly consisting of two targeted probes (Actino-221 and Actino-658) defined as actinobacterial PAO groups, constituted approximately 33 % of the DAPI stained total microorganisms, while no *Actinobacterial* clone was retrieved in the clone library analysis. This could be due to the difficulty of extracting DNA from Gram-positive bacteria (Beer et al., 2004), PCR bias and/or low cloning efficiency (Head et al., 1998; Kong et al., 2007).

In such comporision study, pre-denitrification process was found to be preferred EBPR system. Because of required low carbon source and carbon/nitrogen ratio better treatment effeciencies achieved.

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APPENDICES

APPENDIX A : Laboratory Equipment.
APPENDIX B : Chemicals.
APPENDIX C : Solutions and Buffers.
APPENDİX D : FISH Images.

APPENDIX A

Laboratory equipment

Autoclave	TOMY SX-700E
Balances	Precisa BJ610, OHAUS pioneer
Centrifuges	Sigma 1-14
DGGE system	BIORAD DCODE universal mutation system
DRY heating thermostat block	BIO TDB 100, BIOSAN
Electrophoresis system	BIORAD mini sub cell GT
Fluorometer	Invitrogen, Qubit
Gel documentation system	BIORAD GELDOC
Incubators	Nüve EN120
Labwater	
Laminar flow	Faster BH-EN 2003
Magnetic stirrer, heater	Heidolph MR hei-standard
Microwave oven	Vestel MD17
Multimeter with data acquisition	Keithley 2700
PCR Thermocycler	BIORAD C1000 thermal cycler
pH meter	Mettler Toledo MP220
Pipettes	Eppendorf 2.5 µl, 10 µl, 20 µl, 100 µl, 1000 µl
Power supply	BIORAD power pac 300
Pure water systems	USF Elga UHQ-PS-MK3, Elga
Refrigerators	Whirlpool +4^{o}C, -20^{o}C, Vestel -20^{o}C; Haier -80^{o}C
The FastPrep instrument	Q-BIOgene, FP220A
Vortex	Heidolph reax top
Water Bath	Memmert

APPENDIX B

Chemicals

Ammonium Peroxosulfate	Sigma-Aldrich
Cloning Kit For Sequencing	Invitrogen
EDTA molecular biology reagent	Sigma-Aldrich
Ethyl alcohol absolute	Sigma-Aldrich
Formamide deionized solution	Sigma-Aldrich
Hot start *taq* polymerase	Qiagen
LB base	Invitrogen
LB Broth powder	Sigma-Aldrich
NaOH	Reidel-de Haën
Pottassium Hydrogen phosphate	J.T. Baker
Primers	Iontek
SOC Broth BioChemika, for microbiology	Fluka
Sodium Acetate Anhydrous	Sigma-Aldrich
Sodium Phosphate, Monobasic	Sigma-Aldrich
taq polymerase	INTRON
TEMED	Biorad
TRIS-HCl	Sigma-Aldrich
UREA	Fluka
Polyvinyl pyrrolidone	Sigma-Aldrich
Phosphate buffered saline	Sigma-Aldrich
Denhardt's solution	Sigma-Aldrich
40%ACRYLAMIDE/BIS SOLUTION 37.5:1	BIORAD
Acetic acid extra pure %99.5	Sigma-Aldrich

APPENDIX C

Solutions and Buffers

Table 1 : Reagent used for PCR

Reagents	Volume (µl)
dH_2O	17,75
10X PCR Buffer	2,5
Forward primer	0,5
Reverse primer	0,5
dNTPs	2,5
Taq polymerase	0,25
DNA template	1

Table 2 : Hybridization buffer for FISH

	Examples:	**10% FA**	**20% FA**	**30% FA**
4.5 M NaCl	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Sterile water	**Y ml**	**0.3 ml**	**0.2 ml**	**0.1 ml**
250 mM NaH_2PO_4, pH (7.0)	0.1 ml	0.1 ml	0.1 ml	0.1 ml
Denhardt's solution (× 50)	**0.2 ml**	**0.2 ml**	**0.2 ml**	**0.2 ml**
Poly (A) (20 mg/ml)	0.05 ml	0.05 ml	0.05 ml	0.05 ml
0.5 M EDTA	10 µl	10 µl	10 µl	10 µl
10% SDS	10 µl	10 µl	10 µl	10 µl
Deionised formamide (FA)	**X ml**	**0.1 ml**	**0.2 ml**	**0.3 ml**
Total Volume (approx.)	1 ml	1 ml	1 ml	1 ml

Table 3 : Washer buffer for FISH

Equivalent % formamide	0	10	20	30	40	50	60	70	80	90
Equivalent conc. NaCl (mM)	900	450	225	112	56	28	14	7	3.5	1.75
4.5M NaCl (ml)	4	2	1	0.5	0.25	0.125	0.063	0.031	0.016	0.008
0.5 M EDTA, pH 8.0 (µl)	-	-	200	200	200	200	200	140	70	36
200 mM Tris-HCl, pH 7.2 (ml)	2	2	2	2	2	2	2	2	2	2
10% SDS (µl)	200	200	200	200	200	200	200	200	200	200
Make up to total approx. volume with Sterile water (ml):	20	20	20	20	20	20	20	20	20	20

APPENDIX D

FISH Images:

PAOs and GAOs FISH Images

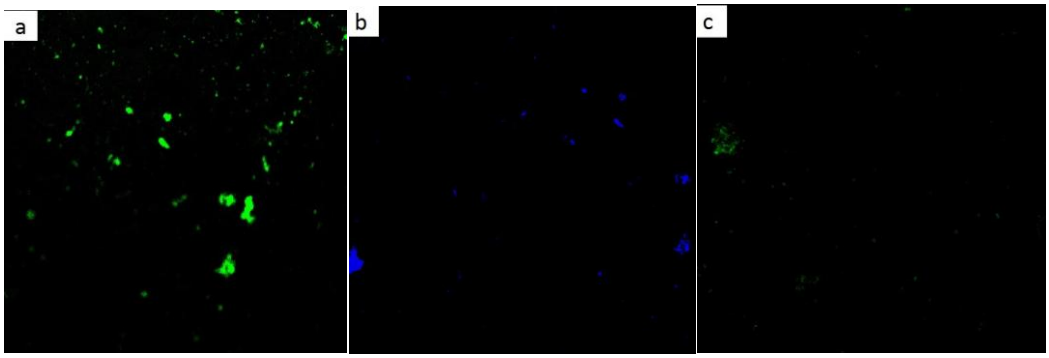

Figure 1: Images example of microbial groups in the S1 (first post-denitrification) sample. a) Dapi staining of S1 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, c) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

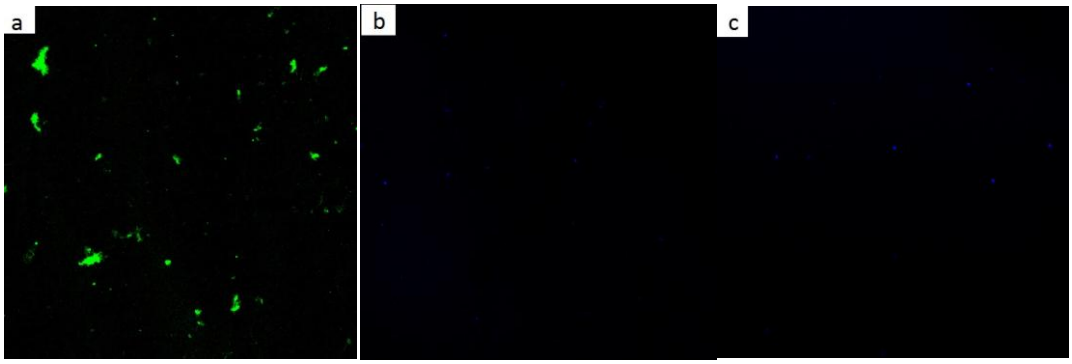

Figure 2: Image examples of microbial groups in the S2 (post-denitrification) sample. a) Dapi staining of S2 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, c) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

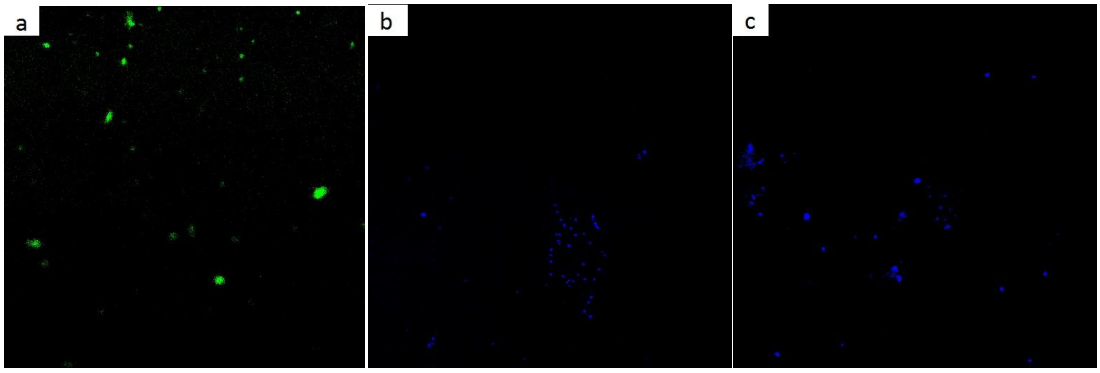

Figure 3 : Images example of microbial groups in the S3 (post-denitrification) sample. a) Dapi staining of S3 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, c) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

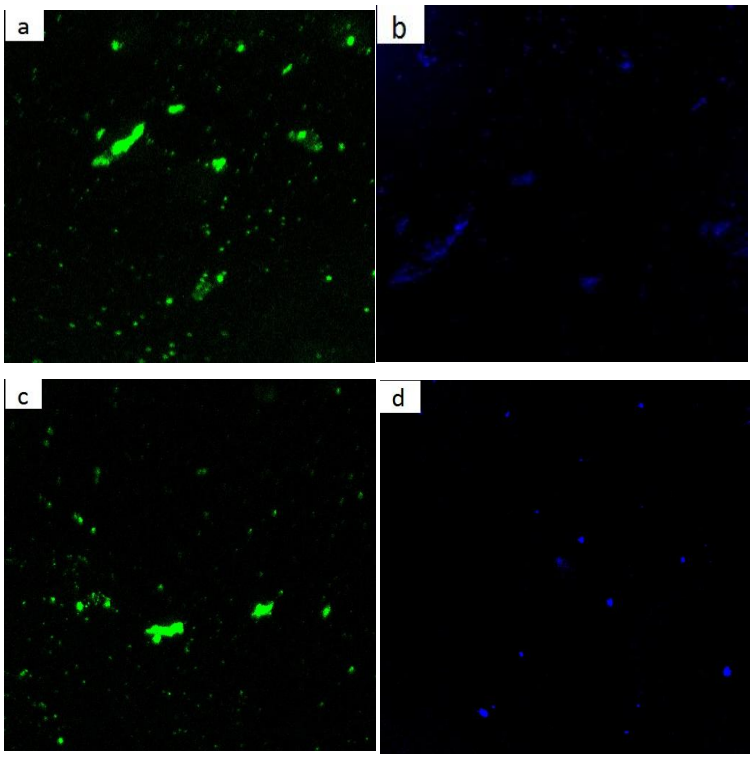

Figure 4 : Images example of microbial groups in the S4 (post-denitrification) sample. a and c) Dapi staining of S4 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, d) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

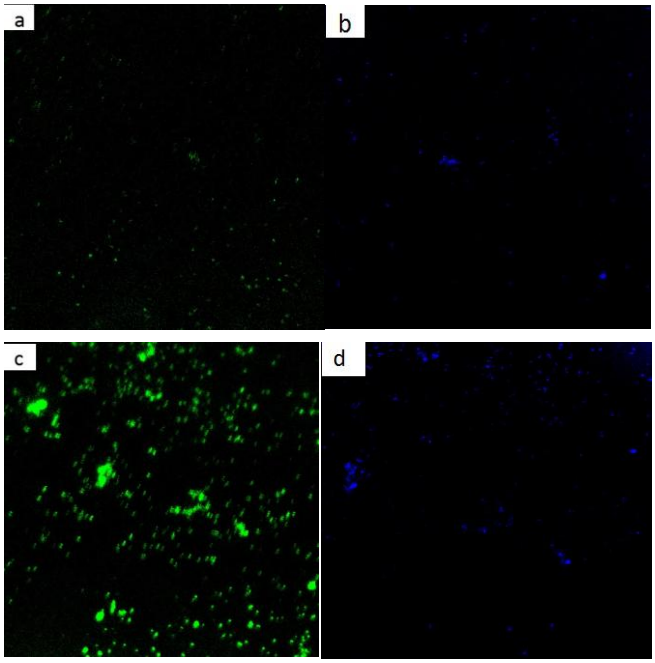

Figure 5 : Image examples of microbial groups in the S5 (post-denitrification) sample. a and c) Dapi staining of S5 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, d) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

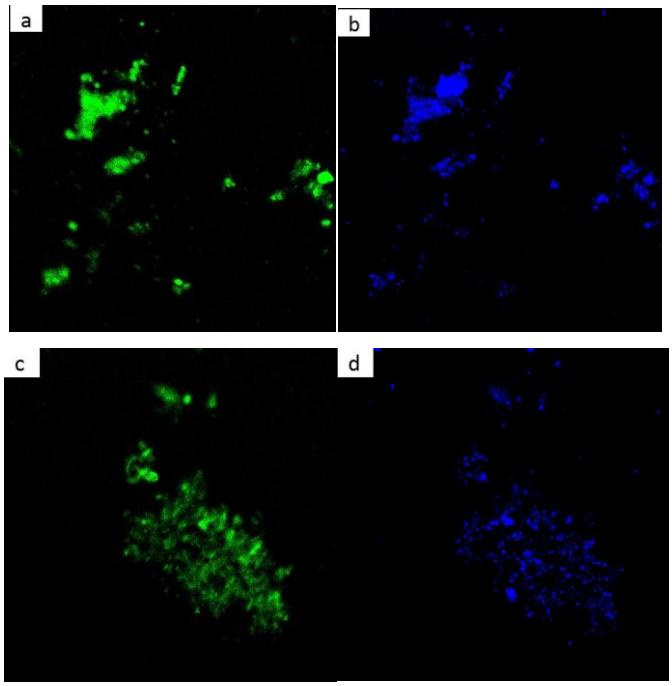

Figure 6 : Image examples of microbial groups in the O1 (pre-denitrification) sample. a and c) Dapi staining of O1 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, d) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

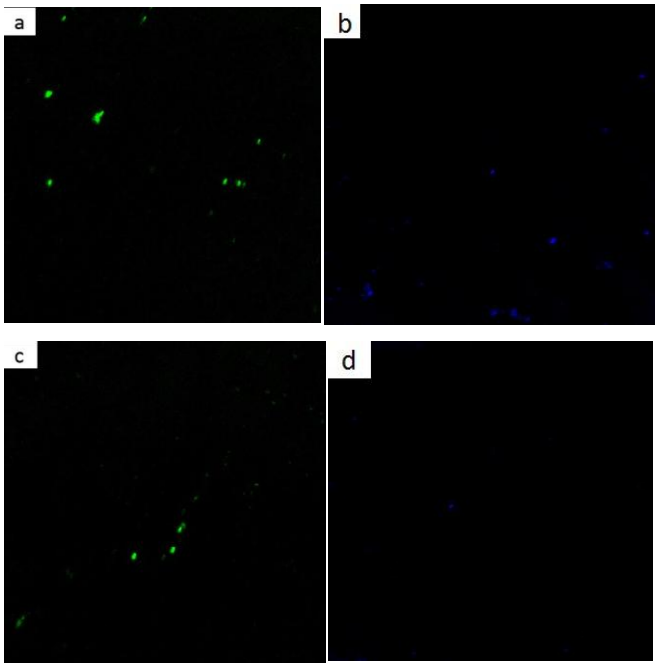

Figure 7 : Image examples of microbial groups in the O2 (pre-denitrification) sample. a and c) Dapi staining of O2 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, d) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

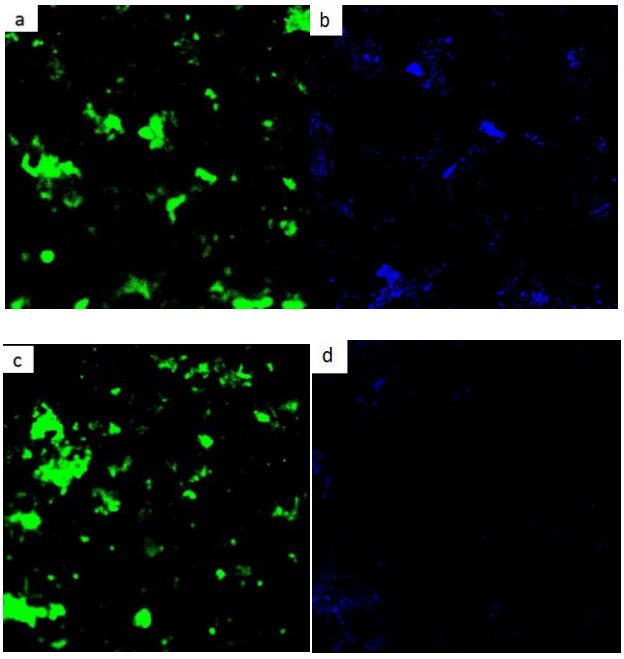

Figure 8 : Image examples of microbial groups in the O3 (pre-denitrification) sample. a and c) Dapi staining of O3 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, d) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

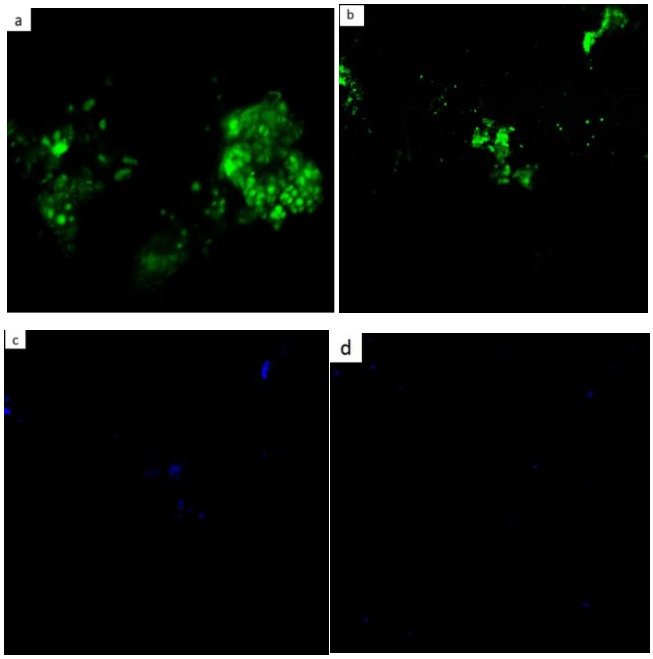

Figure 9 : Image examples of microbial groups in the O7 (pre-denitrification) sample. a and b) Dapi staining of O7 sample, c) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, d) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

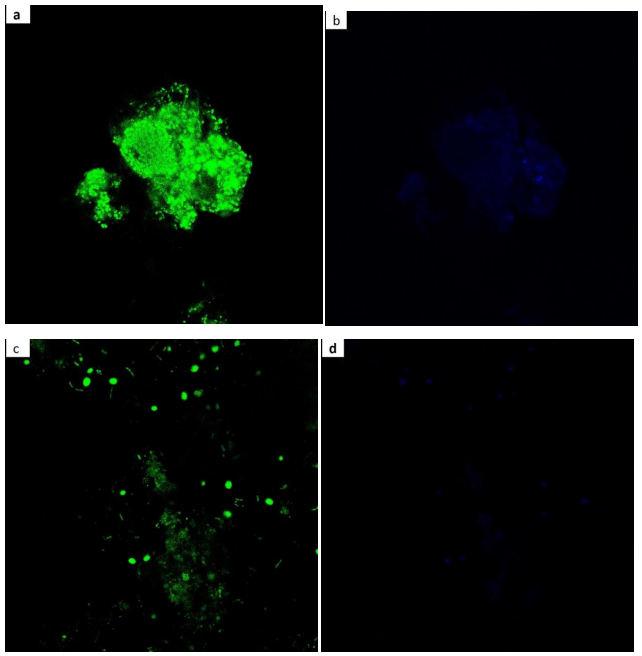

Figure 10: Image examples of microbial groups in the O8 (pre-denitrification) sample. a and c) Dapi staining of O8 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, d) FISHimage of confocal scaning laser microscope of GAOs specific porbe sample.

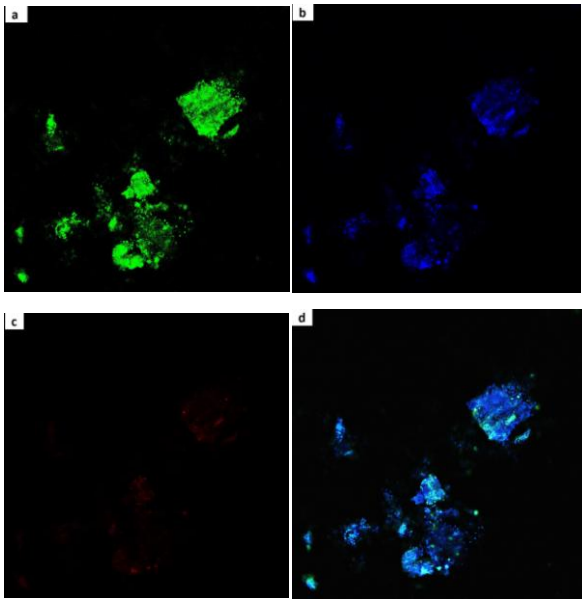

Figure 11 : Image examples of microbial groups in the O9 (pre-denitrification) sample. a) DAPİ staining of O9 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, c) FISH image of confocal scaning laser microscope of GAOs specific porbe sample, d) Overlay view of same samples with DAPİ, PAO and GAO probes.

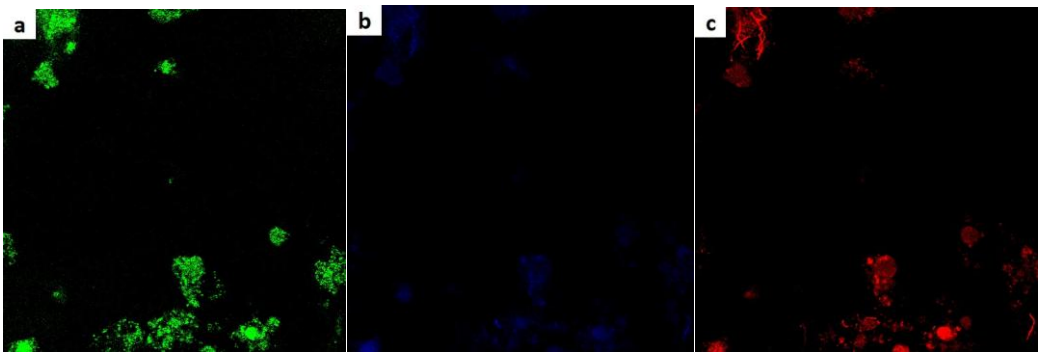

Figure 12 : Image examples of microbial groups in the O10 (pre-denitrification) sample. a) DAPİ staining of O10 sample, b) FISH image of confocal scaning laser microscope of PAOs specific porbe sample, c) FISH image of confocal scaning laser microscope of GAOs specific porbe sample.

Actinobacteria FISH results

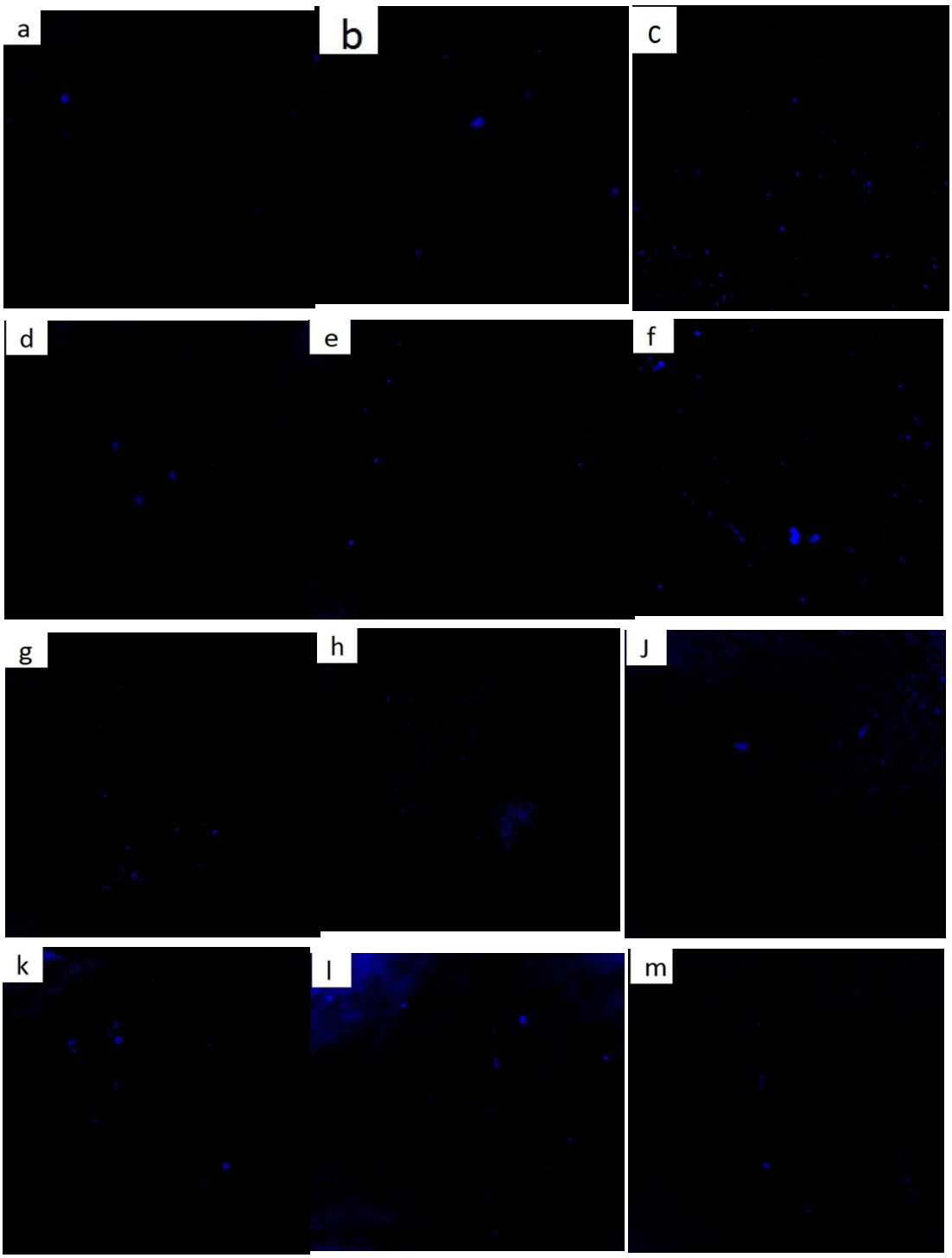

Figure 13 : Image examples of microbial groups in the post-denitrification (S1, S2, S3, S4 and S5) and pre-denitrification (O1, O2, O3, O7, O8, O9 and O10) samples respectively (a, b, c, d, e, f, g, h, j, k, l, m) with *Actinobacteria* specific Actino-221.

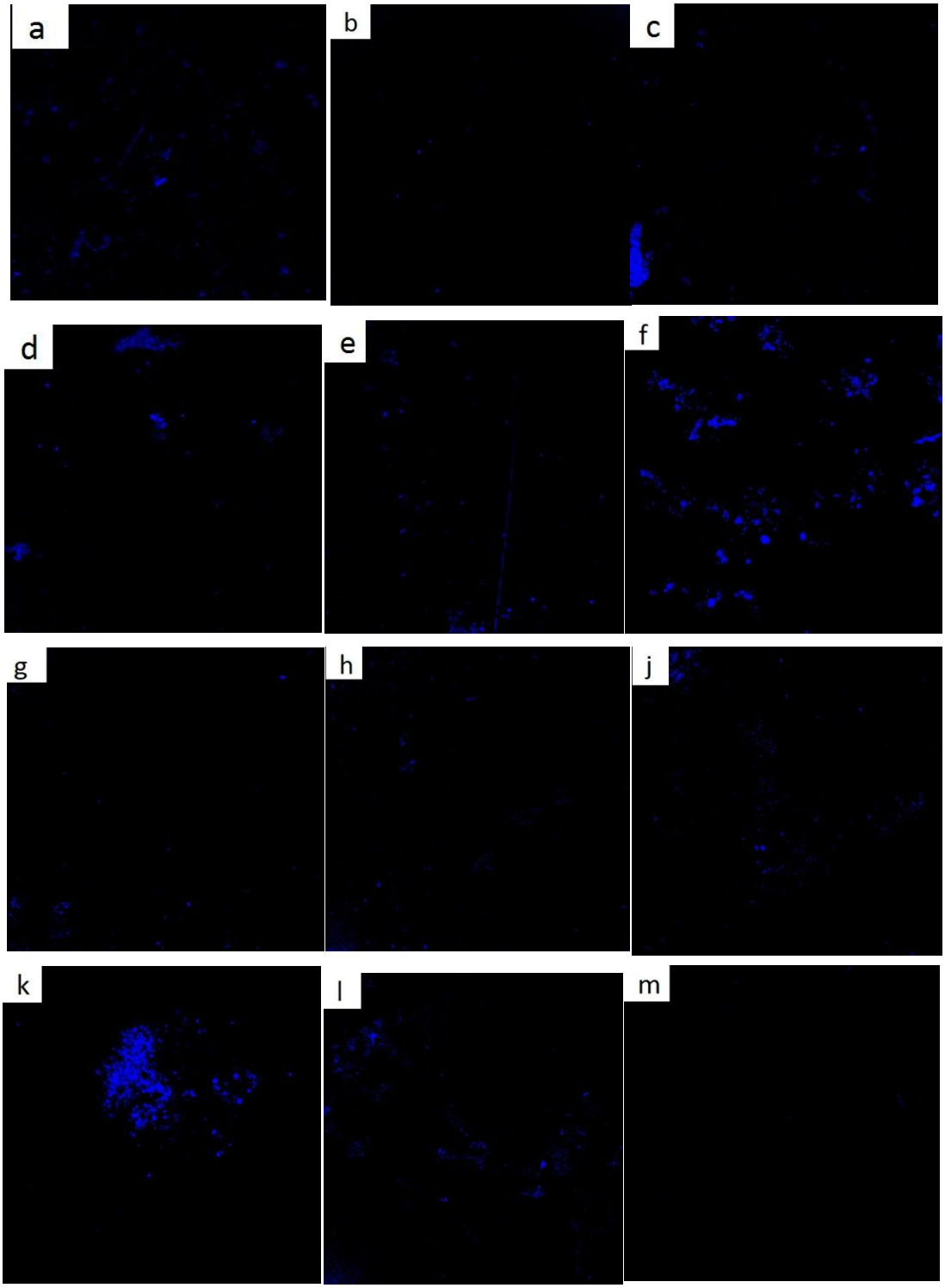

Figure 14 : Image examples of microbial groups in the post-denitrification (S1, S2, S3, S4 and S5) and pre-denitrification (O1, O2, O3, O7, O8, O9 and O10) samples respectively (a, b, c, d, e, f, g, h, j, k, l, m) with *Actinobacteria* specific Actino-658.

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