İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHARACTERIZATION OF MICROBIAL COMMUNITY IN LAB SCALE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL REACTORS WITH QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

M.Sc. THESIS

İpek ERGAL

Department: Advanced Technologies
Programme: Molecular Biology-Genetics and Biotechnology

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521101123

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

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ANOKSİK ORTAMDA BİYOLOJİK FOSFOR GİDERİMİ YAPAN BAKTERİ ÇEŞİTLİLİĞİNİN KANTİTATİF GERÇEK ZAMANLI ZİNCİR TEPKİMESİ TEKNİĞİ İLE İNCELENMESİ

YÜKSEK LİSANS TEZİ

İpek ERGAL
521101123

İleri Teknolojiler Anabilim Dalı
Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Yrd. Doç. Dr. Alper Tunga AKARSUBAŞI

HAZİRAN 2013

İpek Ergal, a **M.Sc.** student of ITU **Institute of Science And Technology** student ID 521101123, successfully defended the **thesis** entitled "**Characterization Of Microbial Community In Lab Scale Enhanced Biological Phosphorus Removal Reactors With Quantitative Real-Time Polymerase Chain Reaction**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Assist. Prof. Dr. Alper Tunga AKARSUBAŞI**
İstanbul Technical University

Jury Members : **Assist. Prof. Dr. Alper Tunga AKARSUBAŞI**
İstanbul Technical University

Assoc. Prof. Dr. Ayten YAZGAN KARATAŞ
İstanbul Technical University

Assist. Prof. Dr. Mahmut ALTINBAŞ
İstanbul Technical University

Date of Submission : 03 May 2013
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To my grandfather,

FOREWORD

Firstly, I want to thank my advisor Assistant Professor Dr. Alper Tunga AKARSUBAŞI (Istanbul Technical University) for the opportunity to perform my Master's Thesis in his laboratory, his endless support, guidance and patience.
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İpek ERGAL
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ABBREVIATIONS

A : Amperes
APS : *Ammonium* peroxosulphate
BAFG : Biyolojik Aşırı Fosfor Giderimi
BLAST : *Basic Local Alignment Search Tool*
BOD : Biochemical oxygen demand
BPR : Biological Phosphate Removal
COD : Chemical Oxygen Demand
Ct : Cycle threshold
DGGE : Denaturant Gradient Gel Electrophoresis
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide triphosphate
EBPR : Enhanced Biological Phosphate Removal
FISH : Fluorescence in situ hybridization
OTU : Operational taxonomic unit
PCR : Polymerase Chain Reaction
PHA : Polyhydroxyalkanoate
PHB : Polyhydroxybutirate
QPCR : Quantitative Real-Time Polymerase Chain Reaction
RDP : Ribosomal Database Project
RNA : ribonucleic acid
rRNA : Ribosomal Ribonucleic acid
TAE : Tris-Acetate-EDTA
TEMED : Tetramethylethylenediamine
Tm : Melting Temperature
UPGMA : Unweighted Pair Group Method with Arithmetic Mean
V : Volts
WWTP : Waste Water Treatment Plants

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CHARACTERIZATION OF MICROBIAL COMMUNITY IN LAB SCALE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL REACTORS WITH QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

SUMMARY

Enhanced biological phosphorus removal (EBPR) is one of the most advanced and complicated, costeffective and environmentally friendly wastewater treatment processes. It is becoming more and more popular worldwide as a sustainable way to protect the receiving water bodies against eutrophication, and to remove P. The EBPR process is a well-studied system which is carried out by complex microbial communities. Because of the clearly defined boundaries, The EBPR process is very suitable as a model ecosystem in microbial ecology.

The principle of the EBPR processes is to enrich microorganisms that can accumulate excessive amounts of intracellular polyphosphate (polyP) by phosphate-accumulating organisms (PAOs) using sequential anaerobic-aerobic and/or anaerobic-denitrifying conditions.

PAOs take up anaerobically and store Carbon sources, particularly volatile fatty acids (VFA) as poly-β-hydroxyalkanoates (PHA) through the release of phosphorus (P) and degradation of glycogen. Higher amounts of phosphorus (P) are taken up when an electron acceptor is supplied (aerobic conditions) through PHA oxidation, which is accompanied by biomass growth and the regeneration of glycogen.

When compared to conventional EBPR, simultaneous denitrification and P removal can save on aeration, minimize sludge disposal and reduce the demand for the often-limiting carbon sources. In recent years, the studies about biological nutrient removal processes explored the existence of phosphate accumulating organisms which capable of using nitrate (NO_3-N) and/or nitrite (NO_2-N) as electron acceptor (denitrifying phosphate accumulating organisms (DPAO)) in anoxic environment. Investigation of the factors that affect the phosphorus removal in anoxic conditions is very important since it causes 50% decrease in excess sludge production and 30% decrease in oxygen consumption and provides efficient use of carbon source.

Literature studies are suggested that *Accumulibacter spp.* is one of the effective microorganism in both aerobic and anoxic EBPR systems.Therefore, to determine the effects of those microorganisms on the EBPR process became a great scientific interest.

In this study investigation the link between the performance of two successfully operated denitrifying phosphorus (P) removal systems and their microbial community structure was aimed. For this purpose, two sequencing batch reactors (SBRs) were operated in oxic and anoxic conditions in post and predenitrification configurations at Marmara University by our project partners. Samples from both post and predenitrification configurations performed to determine the putative existence of *Candidatus Accumulibacter phosphatis*, with Quantitative Real Time Polymerase Chain Reaction method (Q-RTPCR).

Conventional data showed that predenitrification system is more appropriate for wastewaters with low carbon/nitrogen ratio. While a rising quantity of *Candidatus Accumulibacter phosphatis* was obtained in samples of predenitrification system, samples of postdenitrification system exhibited a fluctuated quantity. Thus, it might be suggested that *Candidatus Accumulibacter phosphatis* found to be the dominant group in pre-denitrification configurations; however in post-denitrification configurations Actinobacter-related phosphate-accumulating organisms or other yet undefined groups of microorganisms may have important roles.

ANOKSİK ORTAMDA BİYOLOJİK FOSFOR GİDERİMİ YAPAN BAKTERİ ÇEŞİTLİLİĞİNİN KANTİTATİF GERÇEK ZAMANLI ZİNCİR TEPKİMESİ TEKNİĞİ İLE İNCELENMESİ

ÖZET

Günümüzde uygulanan biyolojik aşırı fosfor giderimi (BAFG) karışık bir proses olduğu kadar oldukça gelişmiş; maliyeti düşük, çevreye dost atıksu arıtma proseslerinden biridir. BAFG Fosforun giderimi ve potansiyel yeniden fosfor kazanımında, su kaynaklarını ötrofikasyona karşı korumada sürdürülebilir bir yol olduğundan, gün geçtikçe Dünya çapında daha da popüler hale geliyor.

BAFG, karmaşık mikrobiyal komünite ile uygulanan iyi bilinen, belirlenmiş sınırları nedeniyle mikrobiyal ekolojide model ekosistem olmak için oldukça uygun bir prosestir. BAFG prosesinin prensibi ardışık anaerobik-aerobik ve/veya anaerobik-denitrifiye koşullarda hücre içinde aşırı miktarda polifosfatı depolayan mikroorganizmaların (PAO) zenginleşmesine dayanır. Yüksek fosfor içeriğine sahip bu mikroorganizmaların fazla çamurla sistemden atılması ile biyolojik aşırı fosfor giderimi gerçekleşmiş olur.

Ham atıksuyun içindeki kompleks yapıdaki organik madde anaerobik ortamda fermantasyon sonucu daha basit yapıda uçucu yağ asitlerine (UYA) (örn. asetik asit (HAc)) dönüşürler.

Fosfat depolayan mikroorganizmalar anaerobik ortamda oluşan uçucu yağ asitlerini hücre içinde poli- -hidroksialkanoatlar (PHA) (örn. poly-β-hidroksibütirat (PHB)) olarak sentezlerler. Sentezleme için gerekli olan enerji hücre içindeki polifosfat (ATP) ve glikojenin parçalanması ile elde edilir ve bunun sonucunda yüksek miktarlarda fosfor salınımı gerçekleşir. Daha sonra aerobik faza gelen PAO lar aerobik ortamda büyürler. Bünyelerindeki polifosfatı ve glikojeni geri kazanmak için gerekli olan karbon ve enerji kaynağı olarak daha önce anaerobik ortamda depoladıkları PHB'ları kullanırlar. Polifosfatın hücre içinde tekrar depolanması için gerekli olan enerji aerobik ortamda PHB oksidasyonu ile sağlanır.

Aerobik ortamda hücreye geri alınan fosfat miktarı anaerobik ortamda salınan fosfattan daha fazla olduğu için biyolojk aşırı fosfor giderimi gerçekleşmiş olur.

Son yıllarda biyolojik besi giderimi (BBG) prosesleri ile ilgili yapılan çalışmalar anoksik ortamda elektron alıcısı olarak nitratı (NO3-N) ve/veya nitriti (NO2-N) kullanarak fosfat depolama kabiliyetine sahip organizmaların (denitrifikasyon yapabilen fosfat depolayan organizmalar (DPAO)) varlığını ortaya çıkarmıştır. Klasik biyolojik fosfor giderimi (anaerobik-aerobik) ile kıyaslandığında çamur üretiminde %50, oksijen tüketiminde %30 azalma sağlayan ve karbon kaynağının verimli bir şekilde kullanıldığı anoksik ortamda biyolojik fosfor giderimini etkileyen faktörlerin incelenmesi önem teşkil etmektedir.

Literatür incelendiğinde *Accumulibacter* türlerinin aerobik ve anoksik BAFG giderim sistemlerinde etkin türlerden biri olduğu görülür. Bu nedenle bu mikroorganizmaların BAFG prosesi üzerine etkilerini belirlemek ilginç bir bilimsel araştırma konusu haline gelmiştir.

Bu çalışmada, başarıyla işletilmiş iki farklı denitrifiye fosfor giderim prosesi ve onların mikrobiyal komünite yapılarının arasındaki bağlantıyı araştırmak hedeflenmiştir. Bu amaçla Marmara Üniversitesindeki proje ortaklarımız; iki farklı ardışık kesikli reaktör, oksik-anoksik koşullarda ve sonda denitrifikasyon-önde denitrifikasyon konfigürasyonlarında incelenmiştir.

Konvansiyonel datalara göre, karbon/azot oranlarına sahip atıksular için önde denitrifikasyon proses konfigürasyonu daha uygundur.

Sonda denitrifikasyon-önde denitrifikasyon konfigürasyonlarından belirli zamanlarda alınan örneklerle, ortamda varsayılan *Candidatus Accumulibacter phosphatis* 'in varlığını kantitatif gerçek zamanlı polimeraz zincir tepkimesi metoduyla belirlenmesi için çalışılmıştır.

Önde denitrifikasyon proses örneklerinde *Candidatus Accumulibacter phosphatis* sayısal olarak yükselen bir profil sergilerken, sonda denitrifikasyon proses örnekleri dalgalanan bir profil sergilemiştir. Önde denitrifikasyon konfigürasyonunda *Candidatus Accumulibacter phosphatis* miktarsal olarak fosfat giderimiyle doğru orantılı olacak şekilde artmıştır. Önde denitrifikasyon konfigürasyonlarında *Candidatus Accumulibacter phosphatis'* in baskınlığını öne sürerken, sonda denitrifikasyon konfigürasyonlarında; Actinobacter türü fosfat depolama kabiliyetine sahip organizmalar ya da henüz belirlenememiş mikroorganizma grupları baskın olabileceğini işaret etmektedir.

Tüm bu sonuçlar ışığında; her iki proses konfigürasyonu için ortak bir problem olan nitrit ve/veya nitrat iyonunun anaerobik faza taşınmasını engellemek amacı ile anaerobik faz öncesinde çamurun anoksik koşullar altında gideriminin avantajlı sistem olarak önerilebilir.

1. INTRODUCTION

Following the industrial revolution, the surface waters became extremely polluted with discharging of domestic and industrial wastewaters. The discharged organic compounds created a nutrient rich environment for bacterial growth. Thus, the aquatic life was affected by loss of dissolved oxygen, and lead to severe environmental issues like (increasing of pathogens which generate health problems). As a result, development of wastewater treatment technologies have been accelerated by specially industrialized countries.

Biological treatment systems are composed of microbiological processes and they are based on the certain biomass concentration in the systems. Therefore types and concentration of microorganisms are very important factors that need to be tended the desired concentration. But the wastewaters do not contain enough concentration or desired types of bacteria for to remove inorganic and/or inorganic compounds. For waste removal is effectuated by some of the essential slow growing organisms. With the retention of biomass, needed concentrations can obtain. During time, stressing factors, which are developed by different environments or biochemical stages, create metabolic selection on required bacteria.

Research in the field of wastewater handling mainly during the past tewenty years has been directed toward the development of effective and dependable treatment methods for phosphorus removal. Although phosphorus recovery from wastewater streams an interesting option because of the depletation of rocks of P, the main focus of the current research is on averting water bodies from being enriched with nutrients (N, P).

Even though phosphorus removal is considered as very complex processes in wastewater treatment, it has turn out one of the most reliable and effective method for nutrient removal and it is applicated in numerous WWTPs.

Biotechnological methods used in phosphorus removal are important scientific interest. The Enhanced Biological Phosphorus Removal (EBPR) is based on the supplying of raw wastewater under anaerobic conditions and circulation of activated sludge between anaerobic and aerobic phases. Specially the denitrifying (anoxic)

phosphorus removal is highlighted as really promissing process in by the side of the conventional aerobic system, proceeding with certain energy savings, high efficiency, and stable EBPR performance. With this type of configuration, phosphorus accumulating organisms (PAOs) are enriched and become dominant in activated sludge. These high phosphorus content microorganisms are removed by sludge discharging and so enhanced biological phosphorus removal can be achieved.

1.1 Aim of the Study

In this study investigation the link between the performance of two successfully operated denitrifying phosphorus (P) removal systems and their microbial community structure was aimed. For this purpose, two separate sequencing batch reactors (SBRs) were operated in oxic and anoxic conditions in post and predenitrification configurations at Marmara University. Samples from both post and predenitrification configurations performed to determine the putative existence of *Candidatus Accumulibacter phosphatis*, with Quantitative Real Time Polymerase Chain Reaction method.

DNA from both postdenitrification reactor samples and predenitrification reactor samples were extracted, and polymerase chain reaction (PCR) is used to amplify *Candidatus Accumulibacter phosphatis* 16S rDNA genes fragments. In addition, Quantification of *Candidatus Accumulibacter phosphatis* 16S rDNA genes copy numbers were screened by quantitative real time polymerase chain reaction (QPCR).

2. PHOSPHORUS (P)

Virtually, phosphorus means “bearer of light”. in the presence of oxygen, elemental P (P^o) glows and is easily flamable. P is discovered isolating P^o from urine by the German alchemist Henning Brandt, in 1669. Until the 1800s, P^o was used to make weapons. Many studies have been performed about the abundance of P in tissues of animals and plants and extraction of P from these tissues, specificaly P-rich bone. By the late 1800s, the extraction of P moved from tissues to rock P. Rocks of P are still the primary source of P for human use especially apatite (a variety of tri-calcium phosphate minerals). The P extracted is mainly used in the oxidized form (phosphates) infertilizer, detergent and feed additives. These human uses of P have adveresly affected P cycling on the planet.

2.1 Phosphorus Cycle

The P cycle is a very slow biogeochemical cycle where movement of P from rock through soils to the oceans occurs very slow (500 million years; Figure 2.1). On the other hand, P cycles relatively fast through animals and plants. Usually, P-based compounds are solids which exist on Earth, there is relatively low contribution via atmosphere in the movement of P, in comparison with many other biogeochemical cycles.

The global phosphorus cycle is elucidated under four main components: (i) Phosphorus containing rocks being effected by weather and tectonic uplift; (ii) enrichment of rivers with phosphorus by soil, which is produced by chemical weathering and physical erosion of rocks. (iii) transport of this phosphorus to lakes and the ocean via rivers; and (iv) sedimentation of phosphorus related with mineral and organic substances and residence in sediments.

The major source of P is the weathering of primary minerals to terrestrial ecosystems. biological and geochemical processes contol weathering of primary minerals. Mineral type, topography, climate, andbiota affect the rate of P release (Cross et al., 1995). Weathering rates are increased by rising temperature,

precipitation, and slope. Through chemical weathering, the dissolution of apatite is increased by inorganic acids (produced by plants and microbes) and acids (from reactions of rain water and soil CO2). P releases from the rock irreversibly. P can be adsorbed to soil or sediment particles and, also dissolved and moves with surface water to the ocean. In terrestrial and aquatic ecosystems, plants and algae assimilate dissolved P from soils and water. And it reach to animals through herbivores to carnivores. By the excretion of urine and feces, and death and decomposition of fungi, P is released back into soils.

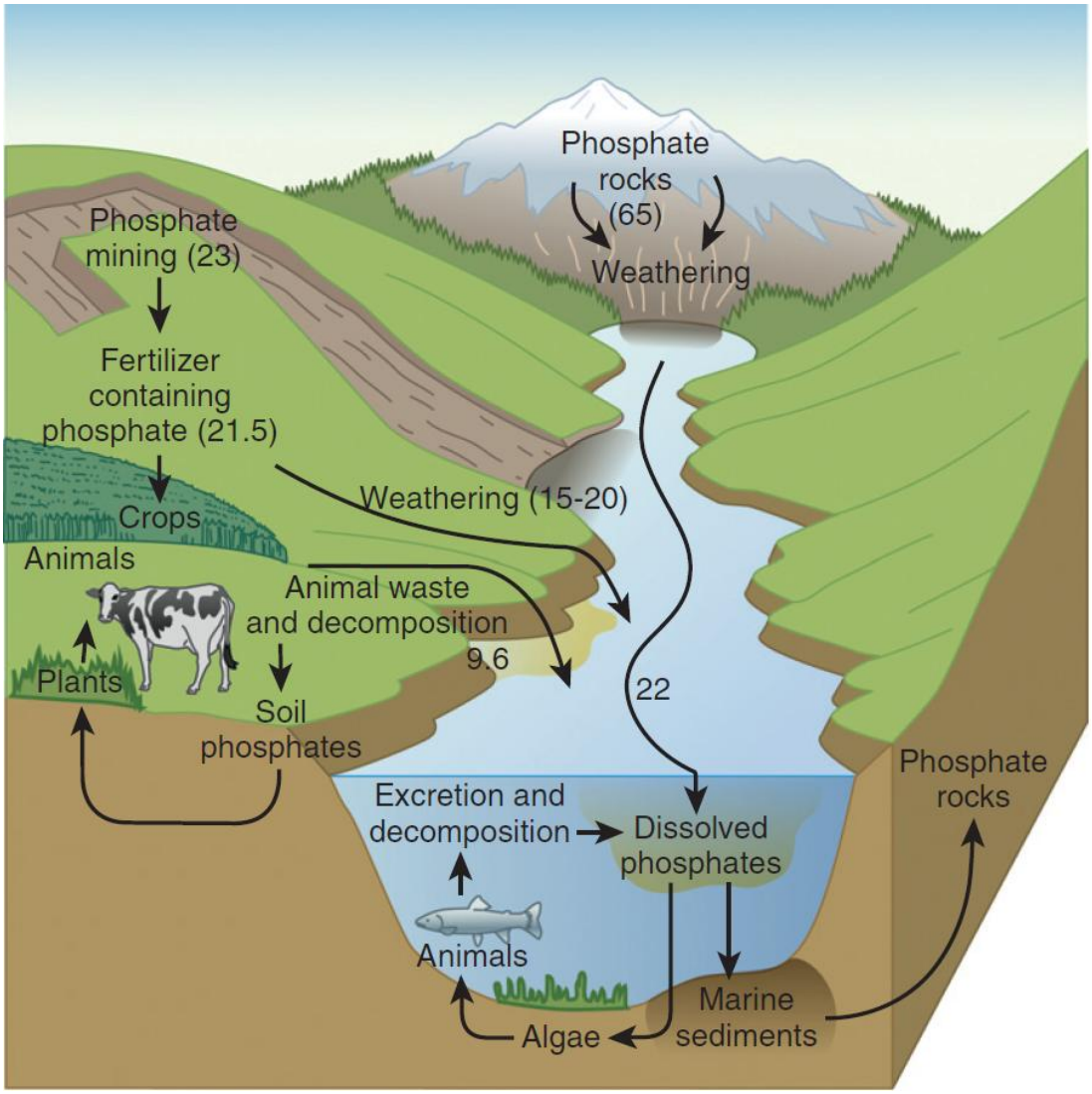

Figure 2.1 : Summary of the phosphorus cycle. Standing stocks in rocks given in petagrams; other fluxes and stocks given in teragrams. (Cordell et al. 2009, MacDonald et al. 2011).

Phosphorus is a necessity for every vibrant. It has major roles in primary biochemical reactions such as energy transfer (adenosine triphosphate, ATP), important in genetic

materials (DNA, RNA) and structure strengthening obtained with phospholipid mebranes and bones.

Phosphorus have three sources in terrestrial systems: living organisims (biomass), soil and bedrock (Benitez-Nelson,2000). Continental vegetation is assisted by the soil and the basic way of soil to get phosphorus is by the weathering of continental bedrock, where atmospheric residues are comparatively insignificant. Dissolution of phosphorus containing minerals, one of which is apetite ($Ca_{10}(PO_4)_6(OH, F, CL)_2$) defined as the most phosphorus enriched mineral in crustal rocks, from bedrock results in the weathering. Microbial activity produces natural acids that drives reactions responsible of mineral wheathering. This so mentioned weathering solubilizes phosphate in soil, ready to be absorbed by terrestrial plants. Phosphate is returned to the soil after the decomposition of the plant (Figure 2.2).

Some soil components, mainly aluminum oxyhydroxides and ferric iron, pull phosphorus from soil, thus result in low phosphate concentrations in it. It is assumed that terrestrial phosphorus bioavailability is most critically controled by sorption. Even though, plants have various physiological methods to obtain phosphorus from soil regardless of low concentrations.

Exemplarily, numerous plants are able to increase root surface area and volume in the need of absorbtion optimization. In another example, chelating compounds, acids and/or enzymes produced from plant roots and/or related fungi can solubilize phosphorus bound by calcium around the root environment. Plants also absorb most of the phosphorus before litterfall, and recycle the litter most efficiently, this way minimize the phosphorus loss. In excessive unfertile soil conditions (e.g., tropical rain forests) all the phosphorus is accumulated in biomass, due to highly efficient phosphorus recycling, leaving the soil with no phosphorus at all.

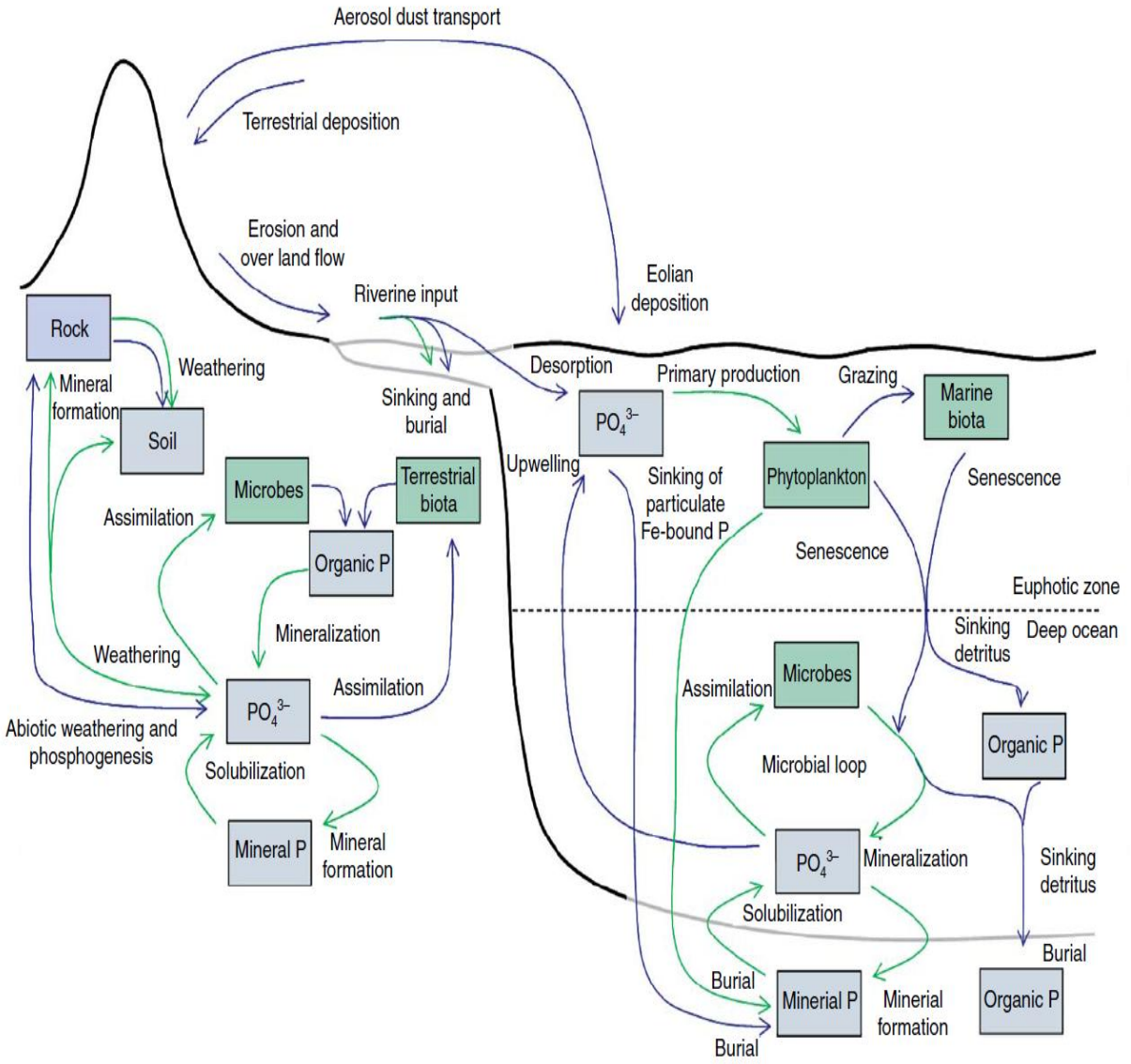

Figure 2.2 : Schematic diagram of the phosphorus cycle. Showing phosphorus reservoirs(living in green boxes; nonliving in gray boxes), physical transport pathways (blue arrows), and microbially mediated transformations (green arrows).

Total amount of various chemical forms of phosphorus is exposed to paticular changes during soil development. In the first stages, phosphorus sources are in the form of primary minerals like apetite. In midstage soil, organic phosphorus along with less soluble secondary minerals increase in percentage while primary minerals lessen. In late stage; soil organic phosphorus and refractory minerals together include the main phophorus partition (Figure 2.3).

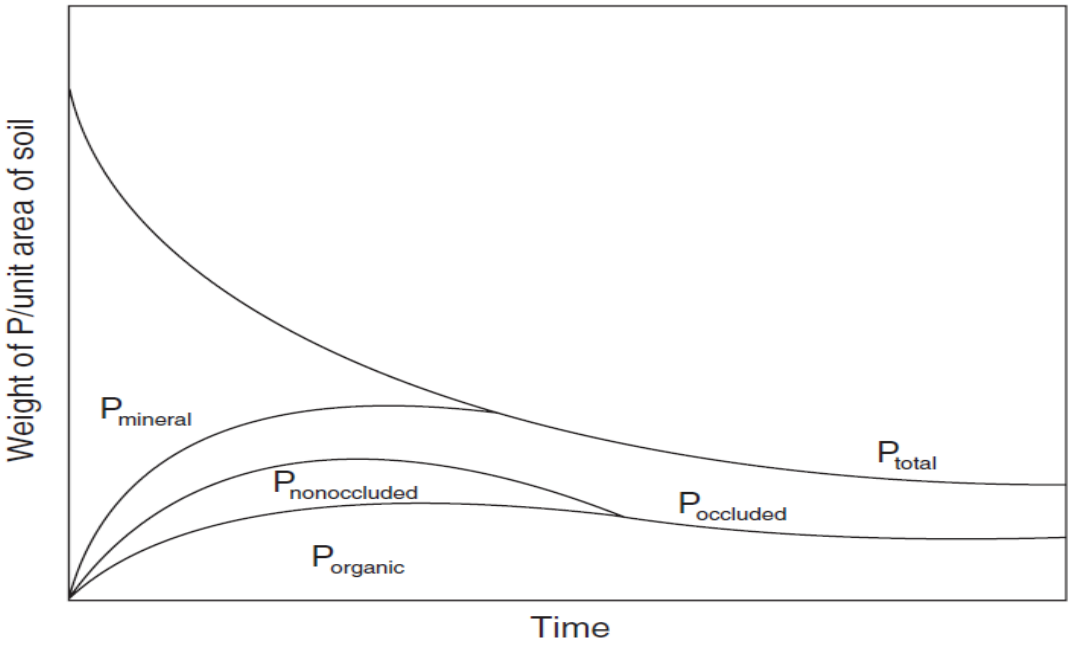

Figure 2.3 : The fate of phosphorus during pedogenesis.
Some of primary mineral P could lost from system leaching (decrease in P_{total}) and also could reincorporated into nonoccluded, occluded and organic fractions within the soil.
Nonoccluded P: phosphate sorbed to surface of hydrous oxides of iron and aluminum, and calcium carbonate. Occluded P: phosphate present within the mineral matrix of discrete mineral phases. Organic P: organic matter returned to soil from vegetation supported by the soil (Ruttenberg, 2001).

2.2 Microbial Phosphorus Metabolism

Phosphorus is a macro-nutrient and it is taken up as inorganic phosphate by almost all microorganisms for use in the synthesis of nucleic acids, nucleotides (ATP), polyphosphates, phospholipids, phosphosugars, and other cell components. Some microorganisms do not use phosphorus immediately. When they are temporarily limited by other nutrients, excess phosphate could be stored intracellulary. When the standart conditions returned the polyphosphates are degraded. Phosphate is mobilized for synthesis of ATP and other cellular constituents (Figure 2.4).

Phosphorus bound in organic form is usually not directly reachable to living organisms (Cotner and Wetzel, 1992). Before the assimilation of organic phosphorus, it need to be released from the organic moiety by mineralization (Jansson et al., 1988; Shan et al., 1994).

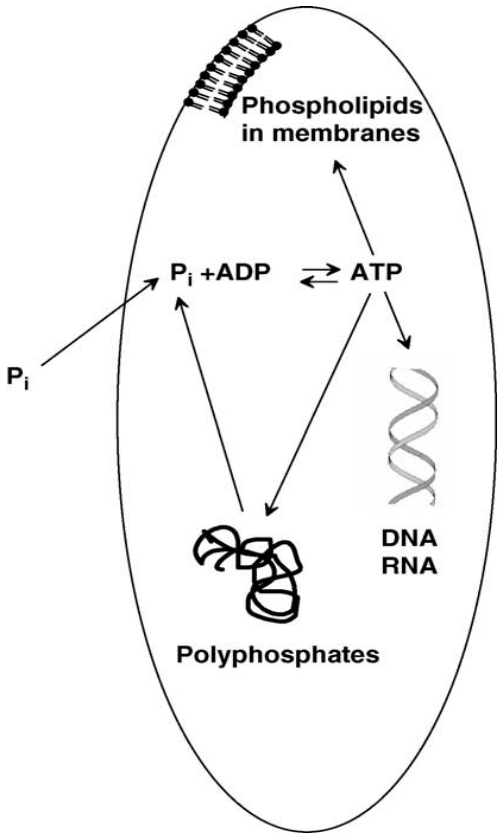

Figure 2.4 : Schematic presentation of t phosphorus transformations in prokaryotes.
P_i; for inorganic phosphorus.

2.3 Eutrophication

P cycle is highly affected by postindustrial human activities, including agricultural practices, phosphorus mining and deforestation, which increase the mobility of phosphorus and cause accumulation of P in soils and aquatic environments.

Several factors make contribution to the mobilization of P by those activities.

It has been suggested that in terrestrial and freshwater habitats, the net storage of phosphorus has increased 75% over preindustrial levels. Eventually, the excessive growth of phytoplankton as a response to over- enrichment of a growth-limiting nutrient (eutrophication) has become a serious environmental problem in estauries and lakes throughout the world. It has been been observed in many ecosystems.

The accumulation of phophorus is increased in water bodies by agricultural applications.

Microbial community undergoes important chages when phosphorus of cultivation practices is washed off into water bodies and limits the production. Increase in the phytoplankton, some of which is toxic or inconvient species (such as *Pfiesteria* sp.) that mean harm to humans and aquatic organism. When phytoplankton started to die a way, accumulate at the bottom of the water body and dispersed by heterotropic microbial community. Anoxic environmental conditions are observed in bottom water and also, due to low levels of light, photosynthetic phytoplankton, can not grow. Anoxia brings harm to benthic invertabrate communities and causes fish kills. Sensitive aquatic ecosystems, when exposed to abundant phosphorus caused by humans, are possible to lose underwater aquatic vegetation, have coral reefs die, poison humans through shellfish and have a decrease in biodiversity (Mackey et al., 2009).

In wastewater total phosphorus concentration (inorganic and organic forms) is approximately 10–20 mg/L. Wastewater effluents specially phosphorus cause eutrophication which leads to significant changes in water quality and lowers the value of surface waters for industrial and recreational uses. Reducing P inputs to receiving waters contol eutrophication (Hammer, 1986).

3. PHOSPHORUS REMOVAL

3.1 Importance of Phosphorus Removal

One of the main aim of biological wastewater treatment is removal of biodegradable organic matter that measured in terms of chemical/biochemical oxygen demand (COD/BOD). But the accumulation of nutrients like nitrogen (N) and phosphorus (P) in estuaries leads to pollution. Untreated wastewater discharge, stimulates the growth of photosynthetic organisms specillay algae (e.g. cyanobacteria). This process, known as eutrophication and it is a global concern in pollution of surface waters. Eutrophication causes decrease in oxygen generating activity, production of toxic compounds, depletion of oxygen (DO) and desirable flora and fauna, thus loss of plant and animal species. (Gijzen and Mulder, 2001; Seviour *et al.*, 2003; EEA, 2005).

As an outcome, for discharge of excess nutrients (N, F) wastewater treatment technologies have been developed.

In natural waters, one of the important limiting factors for biological growth are phosphorus and nitrogen. Cyanobacteria fix the molecular nitrogen from the atmosphere and eliminate the requirement for ammonia (NH_3-N) or nitrate (NO_3^--N), because of that phosphorus is more critical (Seviour *et al*., 2003). According to studies with large water reservoirs while phosphorus concentration is 8-10 μg P/L eutrophication does not occur, even at nitrogen concentration is high (as 4-5 mg N/L) (Korstee *et al.*, 1994).

Wastewater treatment systems are major P sources, because nutrient releases from urban wastewater systems contains higher P than any other sources. Consuming P-rich sludge diminish soluble P concentrations in wastewater. Therefore, controlling P concentration in wastewater treatment systems prevent eutrophication and excessive algal bloom in surface water bodies.

Phosphorus is a key element in all known forms of life, almost every process obtains phosphorus in form of adenosine-5'-triphosphate (ATP) (Metcalf and Eddy, 2003). Also phospholipids (present in the membrane), deoxyribonucleic acid (DNA) and

ribonucleic acid (RNA) have P in their structure, accordingly phosphorus is an essential nutrient for the growth process of organisms.

3.2 Enhanced Biological Phosphorus Removal (EBPR)

Two main approaches are used for P removal in municipal wastewater treatment systems. The first process is chemical removal and the other is biological phosphate removal process (BPR), known as enhanced biological phosphorus removal (EBPR). Also hybrid technologies which combine chemical and biological processes, is utilised for P removal (Figure 2.5).

Because of environmental, economic and operational benefits of biological phosphorus removal (BPR) processes is used in the wastewater treatment systems. Controlling eutrophication in effluent obtaining water is the most important benefit as environmental concern.

P is removed very quickly from the wastewater with the chemical process which is a simple and reliable method. End of the 1959, Biological phosphate removal was discovered by accident in full-scale wastewater treatment plants, and the first full-scale processes were designed and introduced around 1970s.

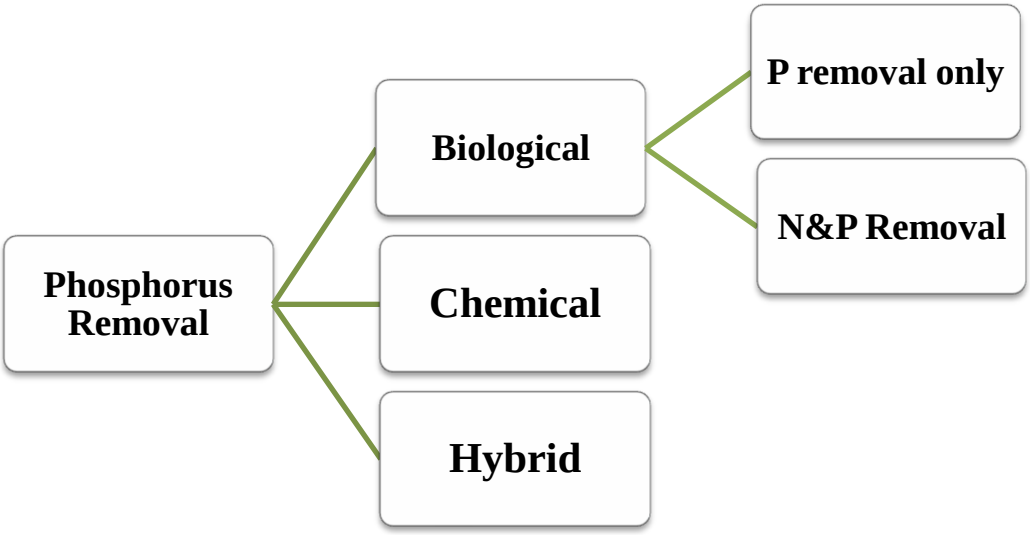

Figure 3.1 : Processes of phosphor removal.

Sufficiently low levels of P could produced in treated wastewater by chemical and biological processes, but EBPR has very convenient advantages.

a- Removal of P efficiency of EBPR systems is very high. Similar to chemical P removal, EBPR systems achieve P effluent concentrations of less than 1 mg/L, and depending on wastewater characteristics and operating conditions, chemical processes generally need high chemical dosages.
b- EBPR systems; reduce sludge production. Absence of chemical sludge, decreases production of total sludge, and prevent problems of effluent salinity in the chemical process.
c- It provide higher reusing of produced sludges. Through the implementation of side-stream processes, The P present in sewerage is recovered with EBPR process.
d- Because of there is no depositing harmful residual (like iron,aluminum) in the treated effluent, EBPR process is environmentally-friendly and has no ecological effects.
e- Besides, operating costs are very low, especially because of total sludge production is decreased. Moreover, dewatering chemical sludge is quite difficult and chemical ingredient increase the dewatering costs.

Therefore EBPR is one of the comment method to design new treatment plants. EBPR system has become more interesting in the way of microbial ecological research. In EBPR, Under repeated cycles of anaerobic and anoxic/aerobic conditions, large amounts of inorganic phosphate (Pi) are accumulated in intracellular polyphosphate (polyP) form by microorganisms. Those microorganisms have complex physiology and consume abundant amount of inorganic phosphate (Pi) more than any other metabolite. These organisms were called ‘polyphosphateaccumulating organisms (PAOs)’ (Mino *et al*., 1998).

A standard EBPR system consists of anaerobic and aerobic zone, and eventually wasting high polyP contents sludge remove P from the system (Figure 2.6 (a)). Figure 2.6 (b) shows the metabolic models in the liquid and biomass, to picture the process. [Oehmen et. al. (2007)].

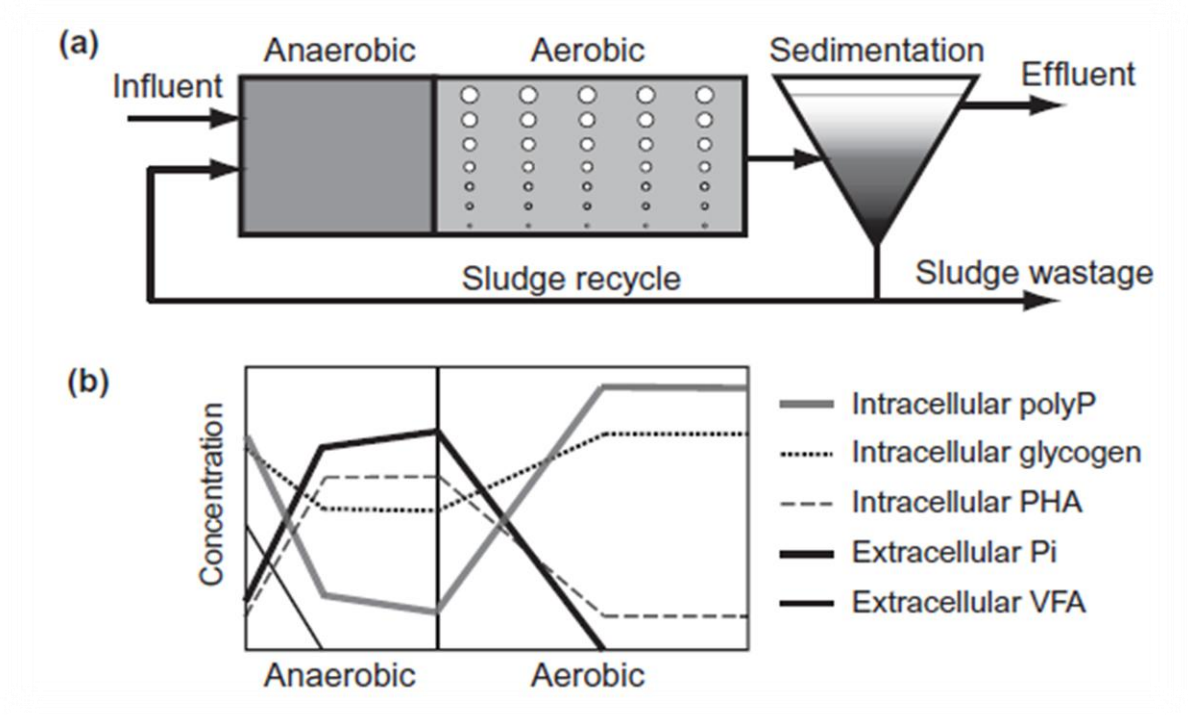

Figure 3.2 : Traditional EBPR process with anaerobic/aerobic configuration (a). During an EBPR cycle excibition characteristic chemical profile (b) (Shaomei He et. al 2011).

EBPR generally starts with an anaerobic stage where volatile fatty acids (VFA) such as acetate (HAc) and propionate (HPr) (Figure 2.6 (a)), and this cause the flourusment of polyphosphate-accumulating organisms (PAO) in the system.

In the anaerobic phase, VFA present in the influent is stored as poly- β-hydroxyalkanoates (PHA) by PAO. The stored PHA could be composed by poly-β-hydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHB) or poly-β-hydroxy-2-methylvalerate (PH_2MV) depend on the type of of VFA supplied (e.g. HAc or HPr) (Smolders *et al*., 1994a; Oehmen *et al*., 2005c). The hydrolysis of internally stored poly-phosphate (poly-P) provide most of the required energy for transportation of VFA to cell membrane and stored as PHA. (Mino *et al*., 1998). During the PHA storage, it is assumed that the essential reducing power is provided by glycogen. The required energy for to cover the anaerobic maintenance needs is also supplied by poly-P hydrolysis and under anaerobic conditions with poly-P hydrolysis cause releasing of orthophosphate (PO_4^{3-}-P) released into the bulk liquid (Figure 2.7) (Smolders *et al*., 1994a; Mino *et al*., 1998).

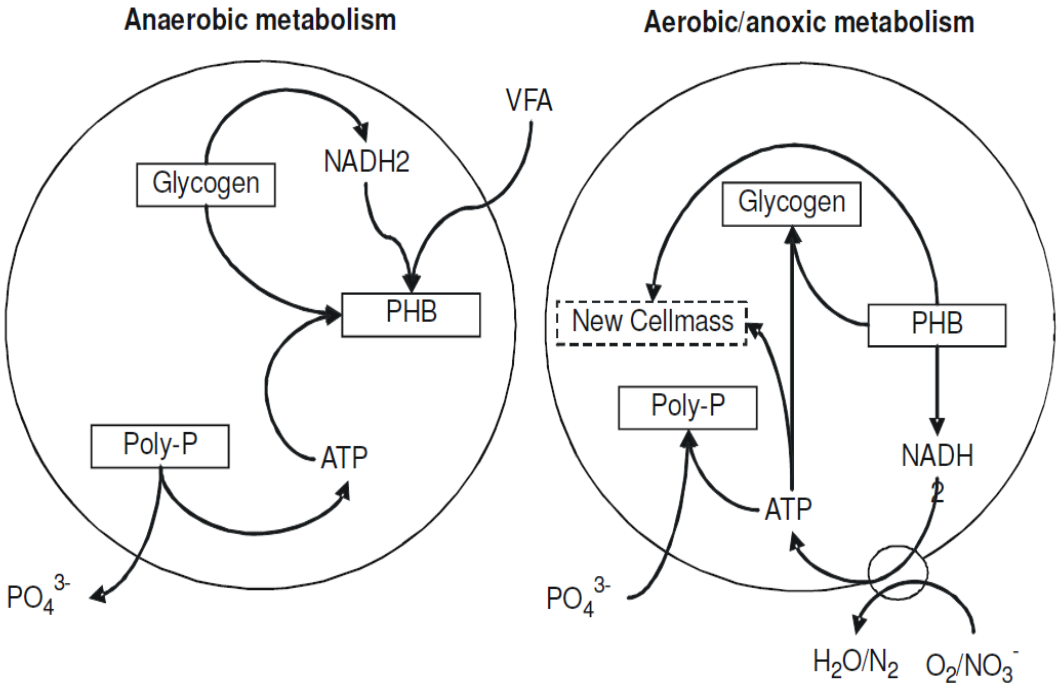

Figure 3.3 : Schematic presentation of metabolic reactions of PAO under anaerobic and aerobic/anoxic conditions (Smolders et al, 1994).

In the aerobic (or anoxic) stage, PHA, stored as carbon and energy source under anaerobic conditions, is utilized by PAO for taking up higher amounts of orthophosphate than those released in the anaerobic stage (Smolders *et al*., 1994b). The stored intracellular PHA is also used for the refilling of the intracellular glycogen pool, growth of biomass, and covering the aerobic maintenance requirements (Smolders *et al*., 1995) . When sludge contains high poly-P ingredient, total removal of P from wastewater is accomplised through removal of activated sludge.

3.2.1 Microbial Characteristics of PAOs

When activated sludge, mixed with the influent wastewater) is presented in an anaerobic phase, the microorganisms which are able to take anaerobically up carbon sources from the influent are advantaged. In the anaerobic±aerobic configuration, polyphosphate accumulating organisms (PAOs) similarly are became dominant.

PAOs hydrolyze stored poly-P for supplying energy. Therefore, PAOs store the carbon sources as the form of polyhydroxyalkanoates (PHA) which are accompained by poly-P degradation and orthophosphate is released in the anaerobic phase. In the

subsequent aerobic phase, PAOs aerobically grow and for to recover the poly-P level, orthophosphate is taken up by PAOs using PHA as carbon and energy source.

So far, any confirmed PAO, demonstrating all of the characteristics of EBPR, has been cultivated in isolation. Because of the major changes, observing single organism is difficult in EBPR process.

In the begining of 2000s, some of the major organisms were identified with help of the availability of molecular microbiological tools which proved the early descripted organisms were not correct. Fuhs and Chen (1975) made the first morphological description of PAOs based on microscopic observation of PAO-enriched sludge. They, usually exist in cluster, were described as non motile cocci or rods according to chemical stainnig methods (poly- β-hydroxybutyrate (PHB) staining). The predominance of *Acinetobacter spp*. has been reported by Fuhs and Chen with using culture dependent identification methods and Buchan,1983; Lotter,1985; Wentzel et al.,1986 also reported the predominance of *Acinetobacter spp*. using similar methods, in EBPR processes. But, *Acinetobacter spp*. which were isolated and identified cultuvation-dependent methods, have failed to possess PAO metabolism. With using molecular microbiological tecniques it has been shown that classical culture-dependent methods for bacterial counting are highly selective for *Acinetobacter spp*. (Wagner et al., (1994)). Any pure culture of *Acinetobacter spp* have shown high phosphorus removal characteristics (Jenkins and Tandoi, 1991; van Loosdrecht et al.,1997).

Studies on the identification of PAO related organisms were continued several years. A high G+C possitive coccus shaped bacterium, *Microlunatus phosphorus* is isolated and considered as a candidate dominant PAO in EBPR systems by Nakamura et al. (1995). But NMR studies showed that under anaerobic conditions, *M. phosphorus* could not able to take up acetate as carbon source and convert to PHA (Seviour et al.,2003). Also in continuous studies which are the dual staining with polyphosphate staining dye, DAPI for the in situ identification of bacteria, and oligonucleotide probes, it was observed that the number of *M. phosphorus* was low to be dominant in EBPR by Kawaharasaki et al., (1999).

A PHB storing strain *Lampropedia spp.* which has basic PAO metabolism, is isolated by Stanta et al., (1997). But according to predictions of EBPR models, acetate-uptake phosphate-release ratio of *Lampropedia spp.* was very low.

For isolation of PAOs, responsible from EBPR, many study have been done, but none of them have succeeded to represent all the characteristics of typical EBPR process (Mino et al.,1998; Oehmen et al.,2007). Using molecular techniques makes easier to identify microorganisms which show high EBPR performance and present predominantly in sludges. (Bond et al.,1999)

In the end of 1999, a new genus and species is proposed as *'Candidatus Accumulibacter phosphatis'* by Hesselmann et al., (1999). *Candidatus Accumulibacter phosphatis* is also suggested as *Rhodocyclus*-related bacterium (Crocetti et al., 2000). In full scale EBPR processes, *Candidatus Accumulibacter phosphatis* and *Rhodocyclus* related organisms are important in the phosphorus removal activity (Zilles et. Al., 2002).

It was confirmed with chemical staining that these organisms are able to store polyphosphate and PHA and show predomination in acetate fed lab-scale EBPR systems. Abundance of *Candidatus Accumulibacter phosphatis* which correlated to content of phosphorus in the sludge, is shown in many lab scale EBPR reactors and different full scale EBPR processes across the world. In some studies, it was found that 4-22 % of all bacteria was *Candidatus Accumulibacter phosphatis* (Oehmen et al. 2007). With excellent EBPR performance richness of this microorganism could be as high as 90%. It was observed that biochemical models proposed for PAOs associated *Candidatus Accumulibacter phosphatis* with with the PAO phenotype defined (Kong et al., 2004).

Identfying *Accumlibacter* as one of the PAOs is the major breakthrough. even though researchers are able to identify the *Accumlibacter*, they are still struggling to isolate them.

With the further studies, it was appered that all *Accumulibacter* species do not contain Poly-P granules and there were also other group of bacteria which contain Poly-P granules, one of those groups was *Actinobacter* species. Abundance of *Actinobacteria* (gram-positive bacteria with high GC content in DNA structure) are firstly observed in fullscale wastewater treatment plants in 1999 (Bond et al. 1999). While the volatile fatty acids (VFAs) uptake is major characteristic in many EBPR processes, Kong et al. 2005, identified *Actinobacter* species and found that they were not uptake VFAs conversely to *Accumulibacter.* Phosphate could taken up aerobically but not anoxically in the form polyphoshate by *Actinobacteria.* Therefore PHA was not stored intracellulary (Kong et al. 2005). Oehmen et al. 2007 suggested

that *Actinobacteria* appeare to grow up on different carbon sources, like amino acids (Oehmen et al. 2007). In full scale treatment plants in Denmark, 35% of all Bacteria are detected as *Actinobacteria* (Kong et al. 2005). In EBPR systems, the role of these microorganisms is still not explained.

In 1998, it was concluded that several different bacterial groups may play role rather than being a single dominant species of PAO (Mino et al. 1998). Liu et al. suggested that the microbial populations were diverse in phylogenetically even in under enriched and well controlled EBPR systems (Liu et al. 2000). *Candidatus Competibacter phosphatis* was identified as the phenotype of Glycogen Accumulating organisms (GAO) which are able to store PHA only anaerobically and can not store polyphosphate. GAOs compete with PAOs for for anaerobic substrate uptake and when the *Candidatus Competibacter phosphatis* is dominant in the anaerobic stage, phosphate release and ratio of VFA uptake which is a major feature observed in many EBPR processes are decrease (Oehmen et al.,2007).

All these studies and results show that multiple organisms are responsible for EBPR performance. In 2002, with using a deteriorated EBPR sludge, FISH probes are designed with generating a 16S rRNA gene clone library (Crocetti et al.,2002).

Characteristics and Concentrations of microorganisms involved to process are needed to investigate in different system to be able increase the performance and understand more of the mechanism of EBPR.

3.2.2 Identification of PAO

Despite of diffucult isolation of PAO in EBPR systems, with the molecular techniques (e.g 16S rRNA-based gene clone libraries, denaturing gradient gel electrophoresis (DGGE) or . fluorescence in situ hybridization (FISH) these microorganisms has been identified Seviour et al., 2003; Oehmen et al., 2007).

Acinetobacter (Wagner *et al*., 1994), subclass 2 *Betaproteobacteria* (which is closely related to *Rhodocyclus* and dominant in EBPR system exhibiting a good P-removal) (Bond *et al*.,1999) , *Candidatus Accumulibacter Phosphatis* (often abbreviated to *Accumulibacter*) (Hesselman *et al*., 1999) ; they are all identfied with applying the molecular techniques specially using FISH.

Beacuse of the *Accumulibacter* exhibite the characteristic PAO phenotype in different lab- and full-scale studies, researchers foccused on this group of microorganism.

For the identification and quantification of *Accumulibacter*, several FISH probes which target the *Accumulibacter* at the different areas of 16S rRNA are designed.

Several studies were suggested that various groups of PAO may exist in EBPR systems (Zilles *et al*., 2002; Wong *et al*., 2005). Two morphotypes of *Actinobacteria* (related to *Tetrasphera*), being aerobically able to remove phosphorus after consuming aminoacids under anaerobic conditions, were shown in different EBPR plants abundantly. But the association of these microorganisms to the EBPR process performance has not been defined because of the lack of data about their biomass activity and biochemical mechanisms (Wong *et al*., 2005) .

By the physiological and biomass activity tests it is supported that *Accumulibacter* is the only known PAO group appearing in research carried out at lab- and full-scale (Oehmen *et al*., 2007). But lots of microorganism are effected on the EBPR performance.

3.3 Denitrifying Phosphorus Removal

Early studies reported that PAOs have not the ability to use nitrate as electron acceptor and therefore PAOs could grow and accumulate phosphate only under aerobic conditions (Wentzel et al. 1986). Although there have been many studies claiming that under anoxic conditions phosphorus removal can be efficiently succeeded (Vlekke et al.,1988; Kerrn-Jespersen et al., 1993; Barker et al., 1996; Kong et al., 2005). When PHA-rich sludge and nitrate are present in the same (anoxic) reactor, anoxic phosphorus removal occurs and simultaneously denitrification and phosphorus removal perform. This process is known as denitrifying P removal. PAOs achieving this process are capable of to use nitrate and/or nitrite, instead of oxygen, as the final electron acceptor for P removal (DPAOs).

Denitrifying P removal process has advantages;

- Because of the aeration is not necessary for phosphorus uptake, this process is the energy savings.
- Also DPAOs use stored PHAs (the same substrate) for both denitrification and P removal.
- Beside of these, energy obtained from the respiratory PAO metabolism with nitrate is 40% lower compared to oxygen (Kuba et al., 1996), resulting in lower cell growth yield.

The comparison between the anoxic P removal process and a conventional anaerobic–aerobic process was showed that in the denitrifying P removal process savings can reach 50% in COD requirement, 30% oxygen consumption, and 50% sludge production (Kuba et al., 1996).

3.3.1 Anoxic Phosphorus Removal in EBPR Systems

When growth of DPAOs is enhanced in EBPR systems with specific operational conditions, anoxic phosphorus removal accrue. Predenitrification (Anaerobic-Anoxic-Oxic) and postdenitrification (Anaerobic-Oxic-Anoxic) configurations are favored designs.

These processes remove biological phosphorus along with simultaneous nitrification, denitrification. Ammonia is transformed into nitrite through nitrate (nitrification) in the aerobic tank. The returning supernatant in the aerobic tank is returned to the anoxic tank to proceed with denitrification. On the other hand, phosphate is released in the anaerobic tank. P is uptaken excessively in the later aerobic tank. Thus, phosphorus and nitrogen removal can be achieved simultaneously with those configurations. (Metcalf and Eddy, 1991)

In the pre denitrification configuration, in the anoxic reactor the internally stored organic carbon is used as electron donor for denitrification before entering the aerobic zone (Kuba et al., 1997). For denitrification, specially PHA plays a major role as a carbon source with parallel to phosphate uptake in the anoxic zone. With the numerous studies it was confimed that anoxic P uptake is directly related to the DPAOs growth within the system.

Predenitrification systems (such as UCT or A2O configurations) encourage the proliferation of DPAOs. the denitrifying activity of PAOs depends on the conditions experienced by the biomass. Denitrification activity is quite low with biomass between anaerobic/aerobic conditions (AO configuration), when the anoxic zones are included in the basic EBPR design denitrifying P removal is stimulated (Wachtmeister et al., 1997).

Furthermore, extension of the aerobic phase can lead the PAO cells to PHA depletion and, thus to affect their denitrifying potential. for the presence of DPAOs in EBPR systems, Hu et al. (2002) suggested that the extent of the anoxic P uptake appears to be associated to the nitrate load on the anoxic reactor.

When the main anoxic reactor is underloaded with nitrate, the P uptake tends to be limited to the aerobic reactor, when the nitrate load exceeds the denitrification

potential of OHOs (i.e., ordinary heterotrophic organisms), the anoxic reactor outflow contains nitrate and significant anoxic P uptake would be mostly observed.

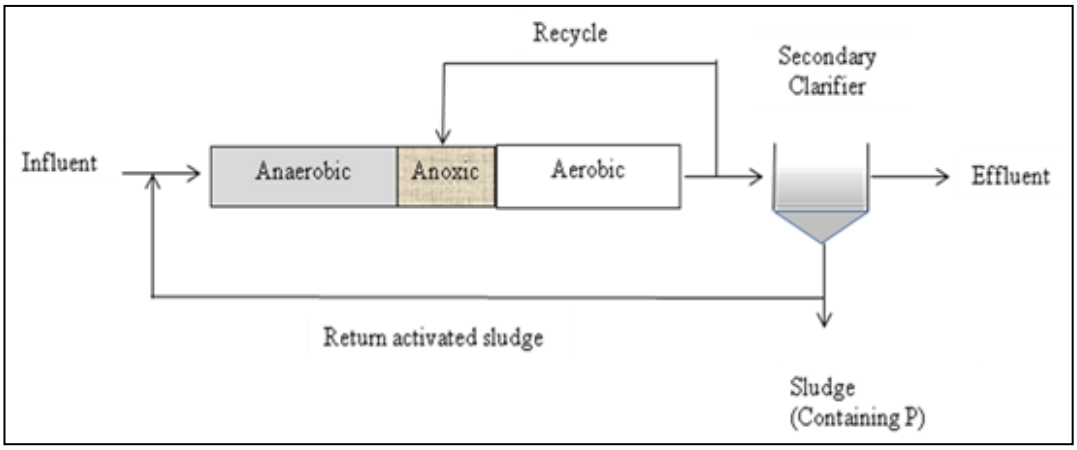

Figure 3.4 : Pre-denitrification configuration.

In conventional waste water treatment plants (WWTP), nitrogen removal is mostly achieved with pre-denitrification for two major reasons.

First one is improvement of denitrification rate (DNR) with biodegradable organic matter available in the anoxic zone, hence reducing the required volume of biological reactor. Second one is degradation of the part of the organic matter with the oxidation capacity of nitrate, hence reducing the oxygen demand and achieving savings in aeration requirement. Nevertheless, the nitrogen removal rate depends on the recirculation ratio that transfers the nitrate produced by nitrification in the aerated zone back to the anoxic zone and therefore consumes energy (Achary et al., 2006).

On the other hand, though conventional technologies of removing nitrogen have been playing an important role in disposing wastewater, there are many problems to be solved, such as the need of adding carbon source in treating the wastewater with low C/N rate, demanding more energy for oxidizing ammonia into nitrate than into nitrite, and occupying larger field and higher investment.

In postdenitrification, without a carbon source, the denitrification rate (DNR) is expected to be low and close to the endogenous DNR, the minimum DNR observed when no bacteria growth occurs (under maintenance and/or decay metabolism). However, as N-elimination occurs according to the natural nitrification/denitrification order, almost complete N-removal can be theoretically achieved, providing sufficient reactor volumes. In actual installed postdenitrification

WWTP a carbon source is always added to the anoxic reactor in order to minimize reactor volume.

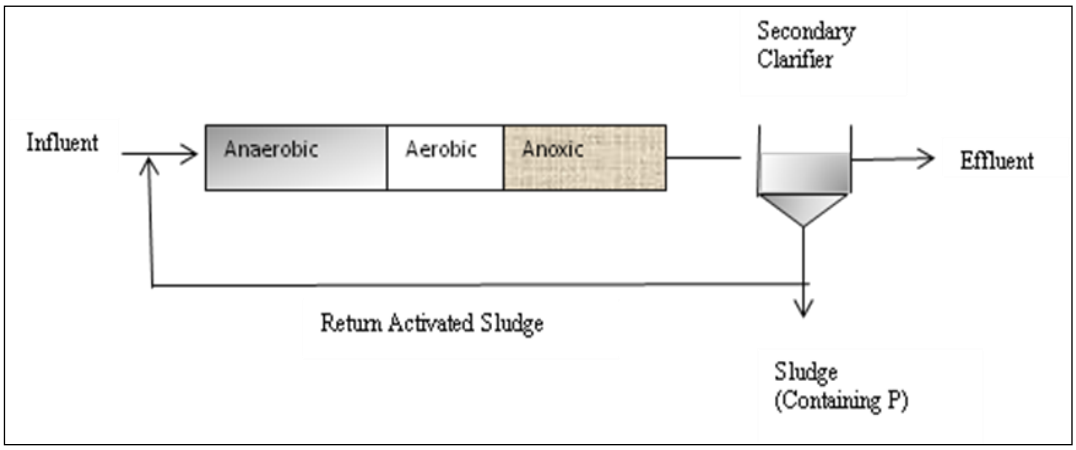

Figure 3.5 : Pre-denitrification configuration.

Anoxic P Uptake with Nitrite

While performing a BNR system, nitrite appears between nitrification and denitrification steps. the accumulation of nitrite effects most of the biological processes, it causes severe problems in EBPR. Under anoxic conditions, NO_2^- could not used as a final electron acceptor for P uptake (Comeau et al., 1986; Kerrn-Jespersen et al., 1994). The recent studies have indicated that DPAOs are able to respire $NO2^-$ to perform EBPR, although only depend on a maximal $NO2^-$ concentration, above which, P uptake declines.

4. MATERIALS AND METHODS

4.1 Materials and equipment

4.1.1 Equipment

The laboratory equipment used during this study is listed in Appendix A.

4.1.2 Buffers, reagents and enzymes

The compounds and enzymes used during this study are listed in Appendix B; the recipes of buffers used in the experiments are given in Appendix C.

4.2 Methods

In this study two different Sequencing Batch Reactor (SBR) system (Figure 4.1) which have different configurations; predenitrification and postdenitrification, are performed EBPR. The SBRs were operated continuously in three cycles of 480 minutes (8 hr). Duration of the phases of both reactors were adjusted according to the concentration of ammonium, nitrate, nitrite and phosphorus in the effluent. The length (duration) of each phase are shown in Figure 4.1 and Figure 4.2, briefly. The synthetic wastewaters, shown in Table 4.1 and Table 4.2, are pumped into SBR reactors.

For the investigation of PAO occurrence in both predenitrification and postdenitrification reactor, Samples from postdenitrification and predenitrification SBRs were screened. In order to achieve the objectives of these studies various molecular biological methods were used (see below).

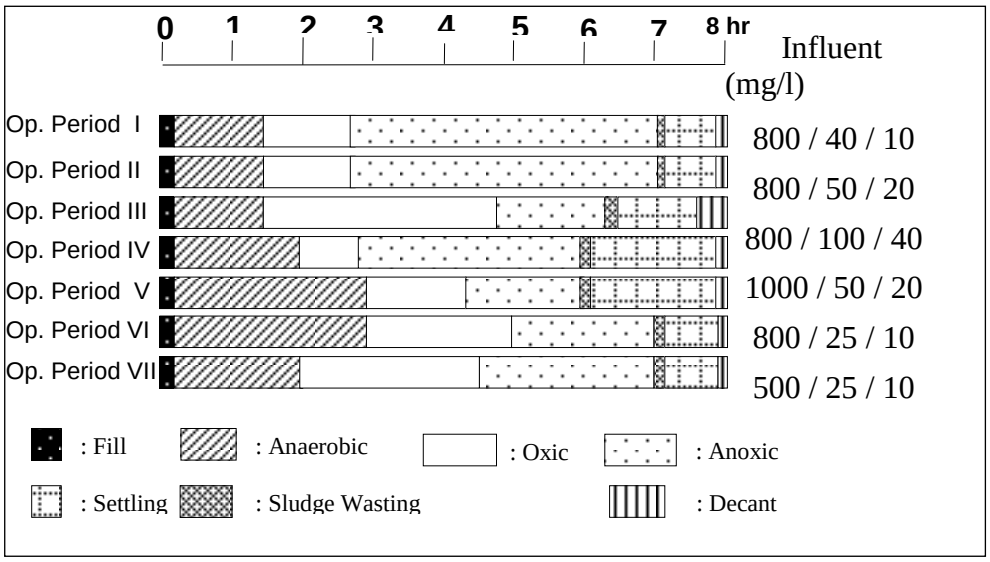

Figure 4.1 : Operational condition in the post denitrification SBR.

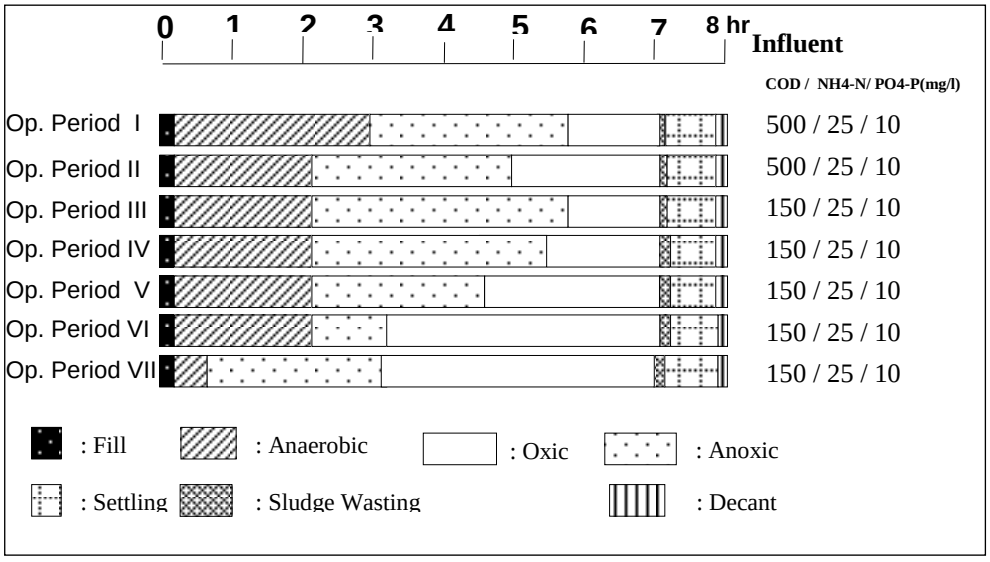

Figure 4.2 : Operational condition in the pre denitrification SBR.

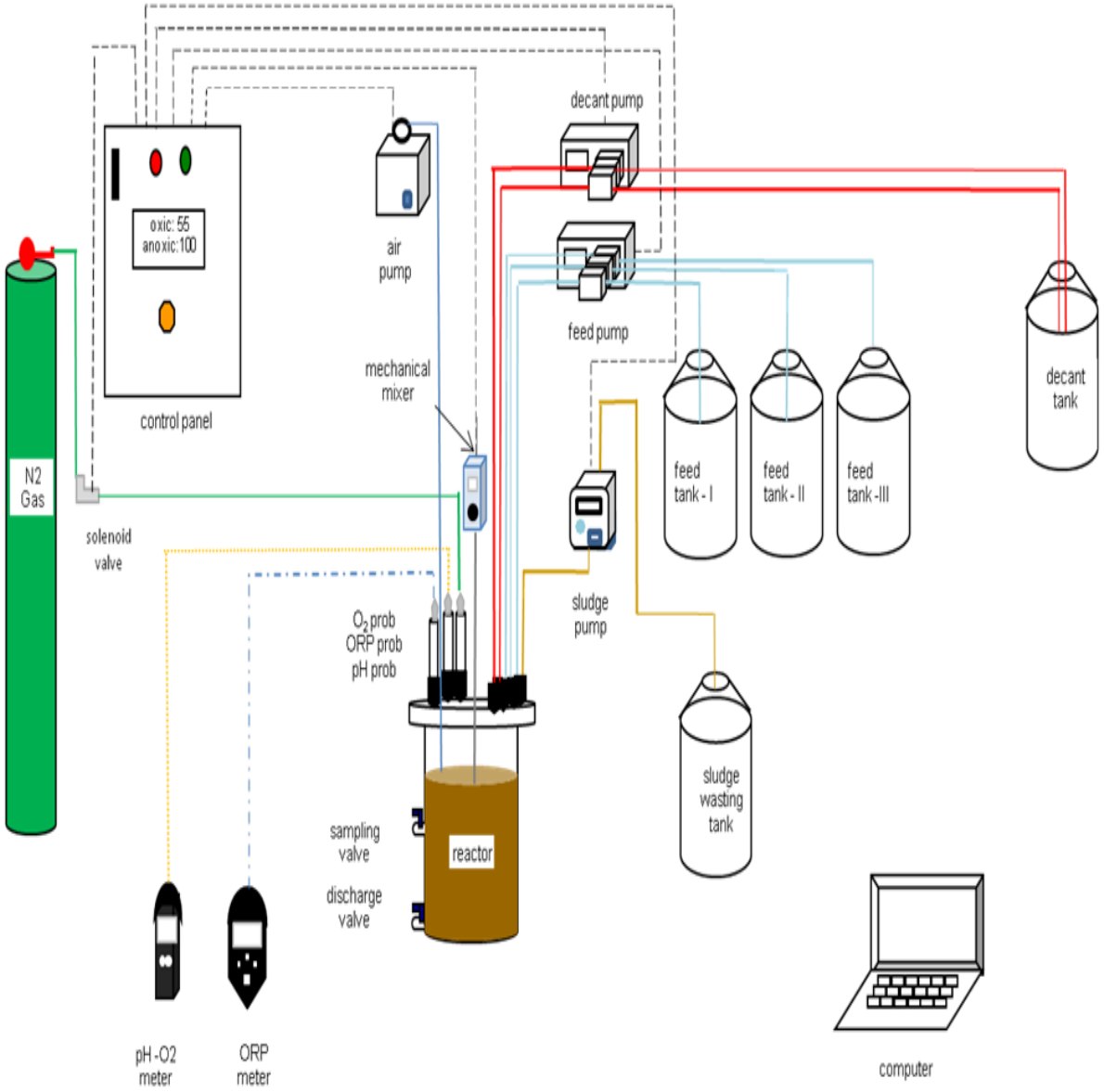

Figure 4.3 : Schematic presentation of SBR.

Table 4.1 : The synthetic wastewaters composition of postdenitrification system.

	Retention time							
Component	**I**	**II**	**III**	**IV**	**V**	**VI-VII**	**VIII**	**IX**
KOI, mg/L	800	800	800	1000	800	500	600	1800
NH_4-N, mg/L	40	50	100	50	25	25	80	80
PO_4-P, mg/L	10	20	40	20	10	10	30	30
$NaCH_3COOH$(146.43 g/L), mg/L	1171.4	1171.4	1171.4	1467.6	1171.4	733.8	733.8	733.8
KH_2PO_4 (197.33 g/L), mg/L	21.7	43.4	86.8	43.4	21.7	21.7	21.7	21.7
K_2HPO_4 (252.59 g/L), mg/L	27.78	55.56	111.12	55.56	27.78	27.78	27.78	27.78
$(NH_4)_2SO_4$, mg/L	189	236	472	236	118	118	118	118
$NaHCO_3$(mg/L $CaCO_3$)	300	300	300	375	300	150	150	150
$MgSO_4.7H_2O$, mg/L	180	180	180	180	180	180	180	180
$CaCl2.2H_2O$, mg/L	28.5	28.5	28.5	28.5	28.5	28.5	28.5	28.5
H_3BO_3	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
$CuSO_4.5H_2O$	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
$MnSO_4.H_2O$	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
$ZnSO_4.7H_2O$	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
$CoCl_2$	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
$FeSO_4.7H_2O$	3	3	3	3	3	3	3	3
$Na_2MoO_4.2H_2O$	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.1 2

Table 4.2 : The synthetic wastewaters composition of predenitrification system.

	Retention time						
Component	**I**	**II**	**III**	**IV**	**V**	**VI-VII**	**VII**
KOI, mg/L	1200	300	300	300	300	300	300
NH_4-N, mg/L	60	30	30	30	30	30	30
PO_4-P, mg/L	60	30	30	30	30	30	30
KH_2PO_4 (197.33 g/L), mg/L	52.4	21.7	21.7	21.7	21.7	21.7	21.7
K_2HPO_4 (252.59 g/L), mg/L	54	27.78	27.78	27.78	27.78	27.78	27.78
$(NH_4)_2SO_4$, mg/L	336	118	118	118	118	118	118
$NaHCO_3$(mg/L CaCO3)	150	150	150	150	150	150	150
$MgSO_4.7H_2O$, mg/L	180	180	180	180	180	180	180
$CaCl_2.2H_2O$, mg/L	28.5	28.5	28.5	28.5	28.5	28.5	28.5
H_3BO_3	0.30	0.30	0.30	0.30	0.30	0.30	0.30
$CuSO_4.5H_2O$	0.06	0.06	0.06	0.06	0.06	0.06	0.06
$MnSO_4.H_2O$	0.24	0.24	0.24	0.24	0.24	0.24	0.24
$ZnSO_4.7H_2O$	0.24	0.24	0.24	0.24	0.24	0.24	0.24
$CoCl_2$	0.3	0.3	0.3	0.3	0.3	0.3	0.3
$FeSO_4.7H_2O$	3	3	3	3	3	3	3
$Na_2MoO_4.2H_2O$	0.12	0.12	0.12	0.12	0.12	0.12	0.12

4.2.1 Sampling

4.2.1.1 Samples from lab-scale reactors

Samples were collected from predenitrification and postdenitrification SBRs to quantify the numbers of total bacteria and *Candidatus Accumulibacter phosphatis* (Table 4.1).

Three 20 ml samples were taken from each reactor, kept at cold chain (4^oC - 20^oC) until process and DNA extractions were carried out.

The detailed schematic structure of both predenitrification and postdenitrification SBRs is shown below.

Table 4.3 : Dates of all samples.

Sample	Samples Feature	Sampling date / Day
S1	Post-denitrification samples	29.06.2009 / 125
S2	Post-denitrification samples	04.08.2009 / 161
S3	Post-denitrification samples	15.10.2009 / 252
S4	Post-denitrification samples	02.12.2009 / 281
S5	Post-denitrification samples	01.02.2010 / 342
O1	Pre-denitrification samples	10.03.2010 / 1
O2	Pre-denitrification samples	10.06.2010 / 93
O3	Pre-denitrification samples	09.07.2010 / 122
O4	Pre-denitrification samples	05.08.2010 / 149
O5	Pre-denitrification samples	22.09.2010 / 197
O6	Pre-denitrification samples	03.01.2010 / 300
O7	Pre-denitrification samples	23.03.2011 / 379
O8	Pre-denitrification samples	27.06.2011 / 475
O9	Pre-denitrification samples	28.07.2011 / 506
O10	Pre-denitrification samples	12.08.2011 / 521

Figure 4.4 : SBR System.

4.2.2 DNA extractions

Genomic material contains all the information about the organism. Most of the molecular biological methods such as QPCR, DGGE, cloning and sequencing need a template from extracted nucleic acids (RNA and DNA). Isolation of nucleic acids even from uncultured environmental samples as template, made easier to collect information with further studies. [Wong DWS., 2006].

In this study, total genomic DNA of each sample was extracted using FastDNA Spin Kit for Soil (MP Bio, USA) according to the procedure supplied by the manufacturer. After the extraction of DNA each sample analyzed on agarose gels. The gels were prepared using 1% (w/v) agarose in 1XTAE buffer containing 0.5 μg/mL ethidium bromide. 4μl of DNA samples were mixed with 2x gel loading buffer. Electrophoresis was performed at 10V/cm and the gel was visualized under UV using gel imaging system (Gel Doc, BIORAD, US). DNA concentrations were measured using fluorimetric assay on Qubit fluorimeter (Invitrogen, US). Each sample was diluted in order to contain 10 ng/μL DNA. Samples were stored at 220 uC until used.

4.2.3 Polymerase Chain Reaction (PCR)

4.2.3.1 PCR Overview

The purpose of the PCR process is to amplify the template DNA enzymaticly using specific complimentary oligonucleotide sequences (Primers). Primers are usually around 20 nucleotides long and they are synthetically produced DNA sequences.

Polymerase Chain Reaction occurs in a simple buffer system contains thermostable DNA polymerase, two sequence-specific oligonucleotide primer pair and four deoxynucleoside triphosphates (dNTPs) (Table 4.4).

In this study, Bacterial PCRs targeting 16S rRNA gene fragments were performed.

4.2.3.2 Bacterial PCR

Amplification of 16S ribosomal ribonucleic acid (rRNA) fragments was performed on genomic DNA using *Candidatus Accumulibacter*-specific 518f (CCA GCA GCC GCG GTA AT) and PAO-846r (GTT AGC TAC GGC ACT AAA AGG) primers.

PCR is also carried out with a GC clamp added 518f (CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G – CCA GCA GCC GCG GTA AT) and PAO-846r primers.

Table 4.4 : Reagents used in PCR.

Reagents	**Total Volume**
ddH2O	17.875 µL
10X PCR Buffer (with 18mM MgCl2)	2.5 µL
Primer F	0.5 µL (0,2 pmol/µL)
Primer R	0.5 µL (0,2 pmol/µL)
dNTPs	2.5 µL (2,5 mM)
Taq Polimeraz	0,125 µL (5unit/µL)
Template	1 µL (10 ng)

Gradient PCR was performed to determine the optimum annealing temprature. (Table 4.5). PCR was performed with five minutes of initial denaturation at 94 ^{o}C and 30 cycles at 94 ^{o}C for one minute, 55 ^{o}C annealing for one minute, 72 ^{o}C extension for two minutes, and a 72 ^{o}C final extension for five minutes. Amplification reaction conditions were summarized in Table 4.6.

After the reaction, products were visualized by (1,5% w/v) gel electrophoresis following the staining with ethidium bromide in 1X TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM 0.5 M ethylenediaminetetraacetic acid, pH 8.0) for 30 minutes at 100 V cm^{-1} and a UV transilluminator using GelDocTM (BIORAD, USA).

Table 4.5 : PCR conditions for 16S rRNA gene.

Cycles	518 **F**-PAO-846 **R**
1	95^{o}C 5 min
34	95^{o}C 30 sec
	57^{o}C 35 sec
	72^{o}C 50 sec
1	72^{o}C 10 min
1	4^{o}C oo

Gradient PCR

Gradient PCR is a technique that allows the determination of an optimal annealing temperature using the least number of steps. Primers are usually supplied with

theoretical annealing temperatures, these can be calculated in various ways and are often specified for specific salt concentrations. An annealing temperature optimisation step is important when changing a sensitive assay from one thermal cycler to another. Block tolerances can vary and small differences in temperature may affect results, therefore using the gradient feature of a thermal cycler, the PCR can be optimised for each particular instrument.

In this study, gradient PCR was performed to optimize the annealling temperature depend on 518f and PAO-846r primers.

Table 4.6 : Gradient PCR conditions for 16S rDNA primers.

cycles	518 **F**-PAO-846 **R**
1	95°C 5 min
34	95°C 30 sec
	57 to 64°C 35 sec
	72°C 50 sec
1	72°C 10 min
1	4°C oo

4.2.4 Denaturing gradient gel electrophoresis (DGGE)

For separation of DNA fragments, Fischer et al. introduced denaturing gradient gel electrophoresis (DGGE) as a gel system about twenty five years ago [Fischer et al., 1979]. In conventional electrophoresis DNA molecules separate according to their sizes. The main principle of this methodology is using both temperature and chemical gradient across the gel, therefore DNA molecules separate according to base composition and sequence-related properties.

In DGGE, positive electrode forces to migrate through the pores of a polyacrylamide gel. When DNA molecule reaches the concentration of denaturing reagents at which it unwinds, it is chemically melted apart. Hydrogen bonding between the base pairs is broken by the temperature and the increasing gradient of denaturing chemicals (urea and formamide). Different sequences migrate at different positions in the gel. Fischer et al., 1983]. This separation is also supported considerably when a short sequence of

G's and C's (about 40 nucleotides), often called GC-clamp, is attached to one end of the amplified DNA products. [Sheffield et al., 1989]. Different taxonomic units (taxon) are refered by every separate bands. This provides to distinguish between mutated and wild type sequences without prior knowledge of sequences.

In this study, DGGE was performed on a D-Code apparatus (Bio-Rad, Hercules, USA). The equal amounts of PCR products were loaded onto 10% (wt/vol) polyacrylamide gels (37.5:1; acrylamide: bisacrylamide) with a denaturing gradient ranging from 35 % to 65% denaturant (100 % denaturant contains 40% [vol/vol] formamide and 7M urea in 1XTAE buffer). Electrophoresis was performed at constant 60^{o}C at 70V for 960 min in 1xTAE buffer. The gels were stained with SYBR Green I (1:5000) and visualized under UV using GelDoc imaging system (Bio-Rad, Dcode, USA). Hot start Taq polymerase is (Qiagen, Germany) used for producing PCR products that will be examined in DGGE. PCR reaction performed with Vf-GC and Vr primers. Final elongation time of PCR kept longer to ensure GC-clamped products are successfully elongated. The PCR condition has been listed in Table 4.7.

Table 4.7 : PCR conditions for 16S rDNA primers (PAO specific).

cycles	518 **F-GC**/PAO-846 **R**
1	95^{o}C 5 min
34	95^{O}C 30 sec
	55^{O}C 45 sec
	72^{O}C 55 sec
1	72^{O}C 15 min
1	4^{O}C oo

Preparation of DGGE Gel

10% (w/v) polyacrylamide [acrylamide-bisacrylamide (37.5:1)] solutions containing denaturing gradients of 0% and 100% were prepared (Table 4.8). Gel preparation steps are listed at Table 4.9. DGGE running and staining conditions are shown in Table 4.10.

Table 4.8 : Preparation of the % (w/v) acrylamide/bisacyrlamide (37.5:1) solutions containing denaturing gradients of 0% and 100%.

	40% Acrylamide/Bis	50X	Formamide	urea	dH2O	Total Volume
10% Gel 0% Denaturant	25 ml	2 ml	N	N	73 ml	100 ml
10% Gel 100% Denaturant	25 ml	2 ml%	40 ml	42 g	Final Vol.100	100 ml

Table 4.9 : Preparation of DGGE Gels.

Clean glass plates carefully with ethanol. Assemble the gel sandwich according to the supplier's instructions.
From the stock solutions of acrylamide and denaturants, prepare two solutions of 16 ml each (for a
Add 160 μL of 10% ammonium persulfate and 16 μL of TEMED to each solution and mix by gently
Pour the two gel solutions into the gradient maker
Insert the comb gently and at an angle and let the gel polymerize for 30–45 min.
Remove the comb and immediately flush the wells carefully with 1X TAE buffer to remove unpolymerized acrylamide

Table 4.10 : DGGE conditions.

	(518 F-GC/PAO-846 R)
Gel percentage	10%
Denaturing Gradient	35-65
Running Volt	180 volt
Running Temp.	60^{O}C
Running time	330 min
Staining	15 min.
De-staining	30 min.

4.2.5 Quantitative real time polymerase chain reaction (QPCR)

QPCR overview

Quantitative real-time PCR is based on detection of fluorescence emission from a reporter molecule at real time and detected signals from each reaction are converted into numerical value [Dorak, TM. 2006]. In this study, SYBR GreenI technology [iQ Sybr green supermix (Bio-Rad, Berkeley, CA)] is used for real-time quantitative PCR experiments. SYBR Green fluorescent dye has the ability to bind the double-stranded DNA. Fluorescence is emitted to the amount of double-stranded DNA.

QPCR experiments

In this study, 518f (5'-CCAGCAGCCGCGGTAAT-3') and PAO-846r (5'-GTTAGCTACGGCACTAAAAGG-3') primers are used to quantify total "*Candidatus Accumulibacter"* 16S rRNA genes. Q-PCR was carried out in triplicates for each sample (He et al.,2007). For the total bacterial quantification, an other reaction was performed using primer sets 341F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') targeting the V3 region of bacterial 16S rRNA genes (Muyzer et al., 1993). The PCR mixture contained 12.5 µl of the iQ Sybr green supermix (Bio-Rad, Berkeley, CA), 1 µl of each primer (0.4 µM), and 1 µl of each sample (Table 4.11). PCR condition was 95 °C for 10 min, followed by 45 cycles of 60 s at 95 °C, 60 s at 57 °C, 30 s at 72 °C, with data capture for each cycle at 78 °C for 15 s. Reactions were performed with iCycler iQ5 thermocycler and real-time detection system (Bio-Rad, Berkeley, CA).

Six-point calibration curves for Q-PCR were produced by 10-fold serial dilution of positive controls at 10^3 to 10^8 target copies per reaction. Melting curve analyses are made at the end of each QPCR experiment.

Table 4.11 : Reagents used in QPCR.

Reactants	**Total Volume**
PCR water	10,5 µl
iQ Sybr green supermix	12,5 µl
F primer	1 µl
R primer	1 µl
Sample	1 µl

Table 4.12 : List of primers used in the study.

Primer	Sequence	Reference	Amplicon size (bp)	Primer specific annealing T (^{o}C)
518f	5'- CCAGCAGCCGCGGTAAT-3'	He et al., 2007	351	57
PAO-846r	5'-GTTAGCTACGGCACTAAAAGG -3'	He et al., 2007	372	57
518f-GC*	5'-CGCCCGCCGCGCCCCGCGCCC GTCCCGCCGCCCCCGCCCGCCAGCAGCCGCGGTAAT-3'	Baker et al.,2003 (GC-clamp)		
341f	5'- CCTACGGGAGGCAGCAG -3'	Muyzer et al., 1993	194	56
534r	5'- ATTACCGCGGCTGCTGG -3'	Muyzer et al., 1993		
Vf	5'-GGCCTACGGGAGGCAGCAG-3'	Muyzer et al., 1993	180	55
Vr	5'-ATTACCGCGGCTGCTGG-3'	Muyzer et al., 1993		

*used with PAO-846r

5. RESULTS

5.1 Sampling

In many cases, more than one group of microorganisms are responsible crucial steps in geochemical cycles. Microbial communities in wastewater treatment are very complex and conventional paramaters; disolved oxygen, alkalinity, salinity, pH, emperature, ; affect the diversity of microorganisms. Conventional parameters are collected from SBR reactors to have a understanding about the community structure and compare the results with EBPR system cultures.

In this study the sequencing anaerobic-aerobic-anoxic (post-denitrification) reactor was operated over 300 days and the sequencing anaerobic-anoxic-aerobic (pre-denitrification) reactor was operated over 400 days .

Measured value of P removal are given at Figure 5.1 and Figure 5.2.

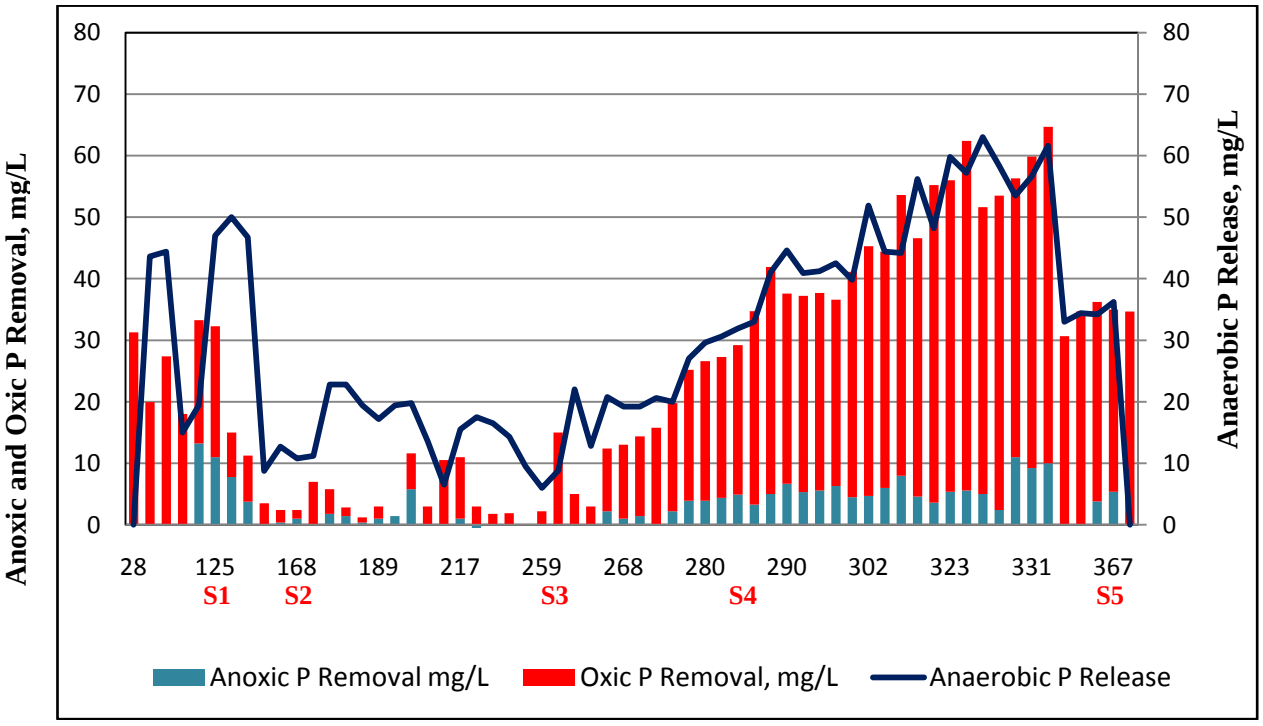

Figure 5.1 : Graphic presentation of P removal in postdenitrification.

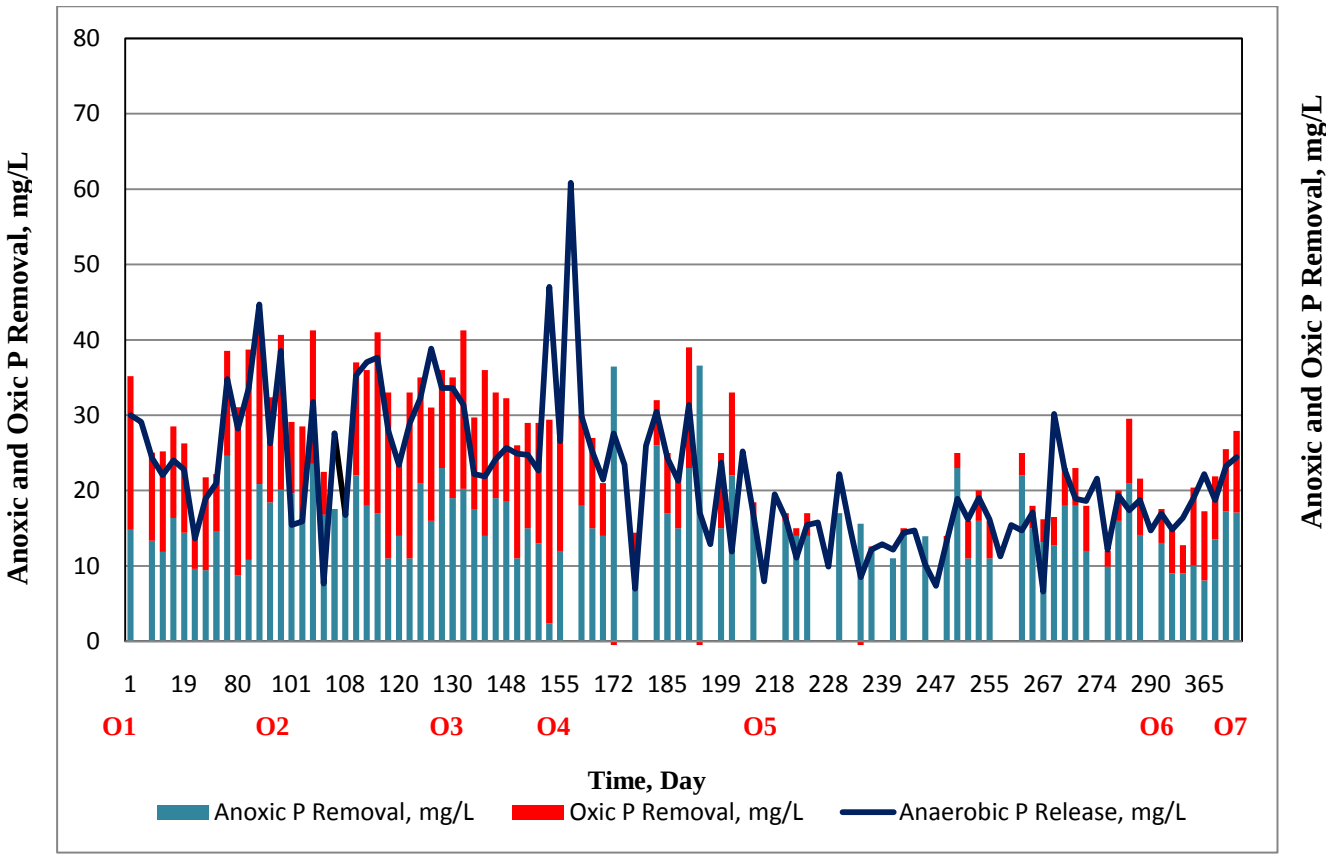

Figure 5.2 : Graphic presentation of P removal in predenitrification (Sampling).

5.1.1 DNA extraction results

DNA extraction results from predenitrification and postdenitrification sequencing batch reactors are given below. Prior to PCR-DGGE analysis of microbial diversity, nucleic acid concentrations were determined using fluorimetric assay on Qubit fluorimeter (Invitrogen, US) according to the procedure supplied by the manufacturer. Table 5.1 shows the DNA extraction results. Concentrations were measured above 10 µg/mL and diluted and stored at -20oC for subsequent PCR reactions. Agarose gel electrophoresis images of the genomic DNA showed that pure DNA were extracted (Figure 5.3).

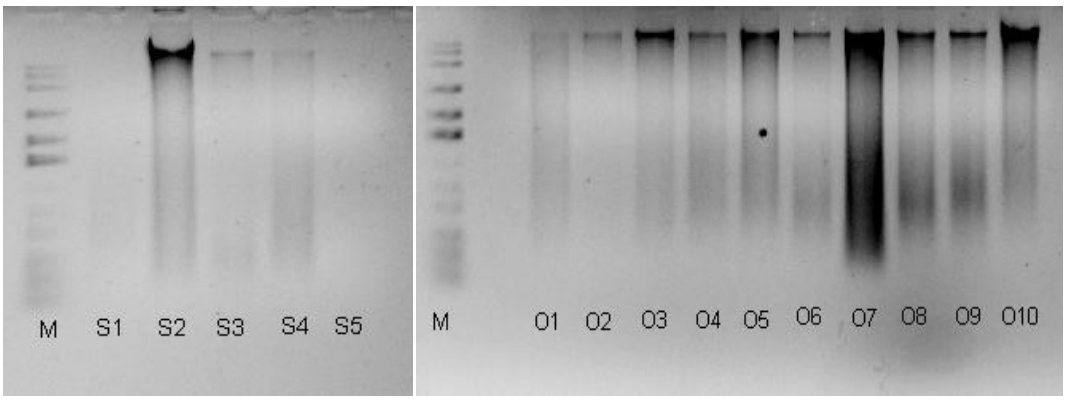

Figure 5.3 : Agarose gel electrophoresis (1% w/v) photos of DNA extraction results.

Table 5.1 : DNA concentrations of the samples.

Sample	Samples Feature	Sampling date	Qubit Fluorometer Result (µg/mL)
S1	Post-denitrification samples	29.06.2009 / 125	10,6
S2	Post-denitrification samples	04.08.2009 / 161	49
S3	Post-denitrification samples	15.10.2009 / 252	16,5
S4	Post-denitrification samples	02.12.2009 / 281	29,5
S5	Post-denitrification samples	01.02.2010 / 342	13
O1	Pre-denitrification samples	10.03.2010 / 1	26,9
O2	Pre-denitrification samples	10.06.2010 / 93	12,2
O3	Pre-denitrification samples	09.07.2010 / 122	25,3
O4	Pre-denitrification samples	05.08.2010 / 149	34,1
O5	Pre-denitrification samples	22.09.2010 / 197	27
O6	Pre-denitrification samples	03.01.2010 / 300	23
O7	Pre-denitrification samples	23.03.2011 / 279	150
O8	Pre-denitrification samples	27.06.2011 / 475	64,5
O9	Pre-denitrification samples	28.07.2011 / 506	90,4
O10	Pre-denitrification samples	12.08.2011 / 521	122

5.2 Polymerase Chain Reaction (PCR)

DNA extracted from environmental and WWTP samples held at -20^{o}C until used for PCR screening. PCR analyses results are listed below.

5.2.1 Bacterial 16S rDNA genes

341f/534r primers

Presence of bacterial communities was detected using domain specific 16S rRNA gene primers. Amplicons obtained in PCR reactions served as a template for subsequent DGGE methodology to assess microbial diversity of each sample.

Briefly, 15 predenitrification and postdenitrification sequencing batch reactors samples were screened for Bacterial diversity.

After the amplification, 16S rDNA gene occurrence are shown and all of the PCR products were of the expected length (200bp).

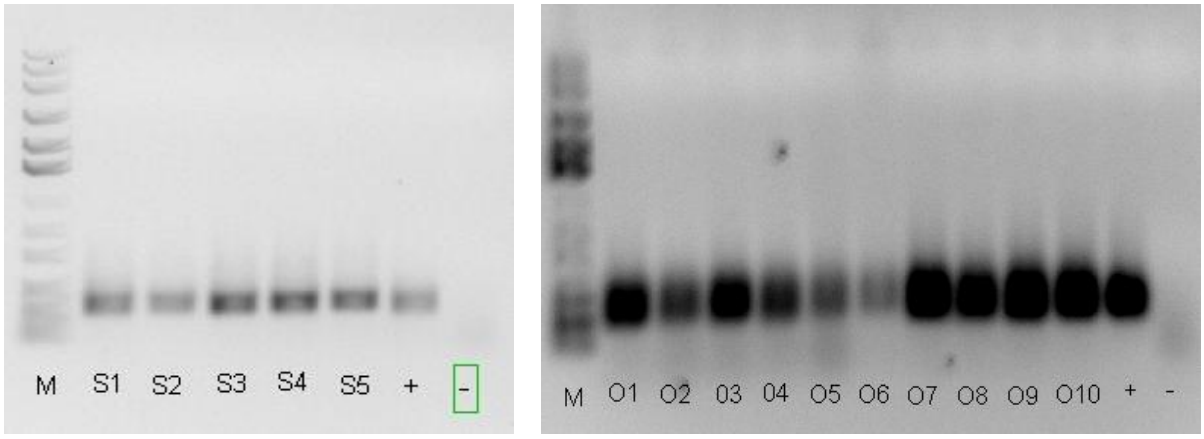

Figure 5.4 : PCR results of bacterial 16S rRNA gene amplification using VfGC/Vr primer.

518f/PAO846r Primers

Gradient PCR

Presence of "*Candidatus Accumulibacter"* was detected with 16S rRNA gene amplification using of "*Candidatus Accumulibacter"* specific 518f/PAO846r primers. For the determination of an optimal annealing temperature of 518f/PAO846r primers gradient PCR technique was performed (Figure 5.5). Optimum annealing temperature was determined as 57 ^{o}C.

After the amplification, "*Candidatus Accumulibacter"* specific 16S rDNA gene occurrence are shown and all of the PCR products were of the expected length (approximetely 300bp) (Figure 5.6).

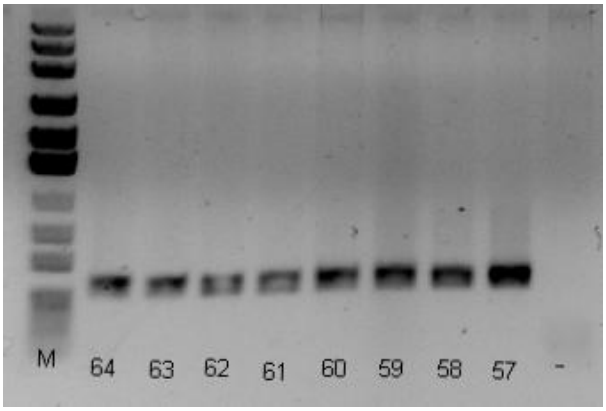

Figure 5.5 Results of Gradient PCR.

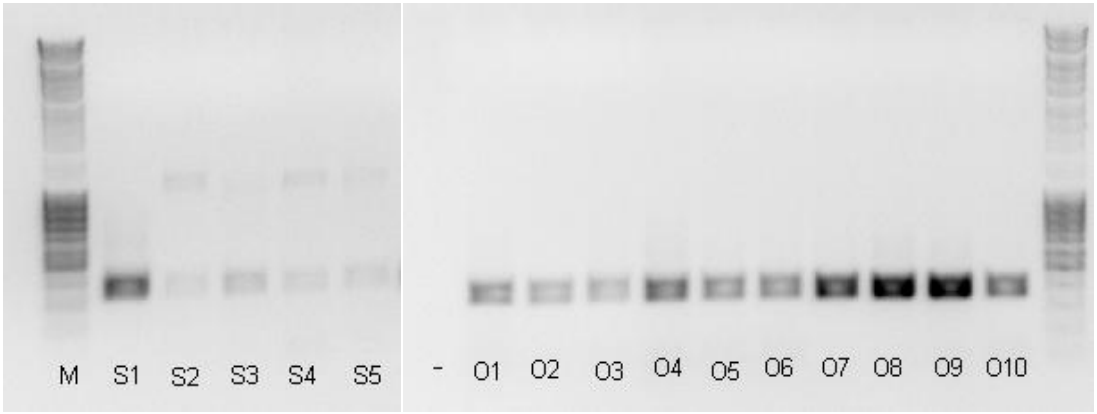

Figure 5.6 : PCR results of bacterial 16S rRNA gene amplification using PAO specific 518f/PAO846r primers.

5.3 Denaturing gradient gel electrophoresis (DGGE)

GC-clamped PCR products are used for DGGE analyses, for that purpose, GC-clamped primers are used. Bacterial 16S rDNA primers are used to screen diversity in WWTPs active sludge samples (see below).

PCR products that are amplified with GC-vf and vr are used in DGGE to examine bacterial diversity. DGGE band profiles indicate various bacterial species from both predenitrification and postdenitrification reactors (Figure 5.7).

Results indicate that predenitrification reactor profile much more diverse than postdenitrification's.

Occurrence of similar bands in bacterial postdenitrification SBR profiles demonstrated presence of similar taxonomic units though less diverse.

On the other hand, DGGE is not an exact quantitative method. But it can be suggested that microbial diversity increases in predenitrification configuration with reference to postdenitrification configuration.

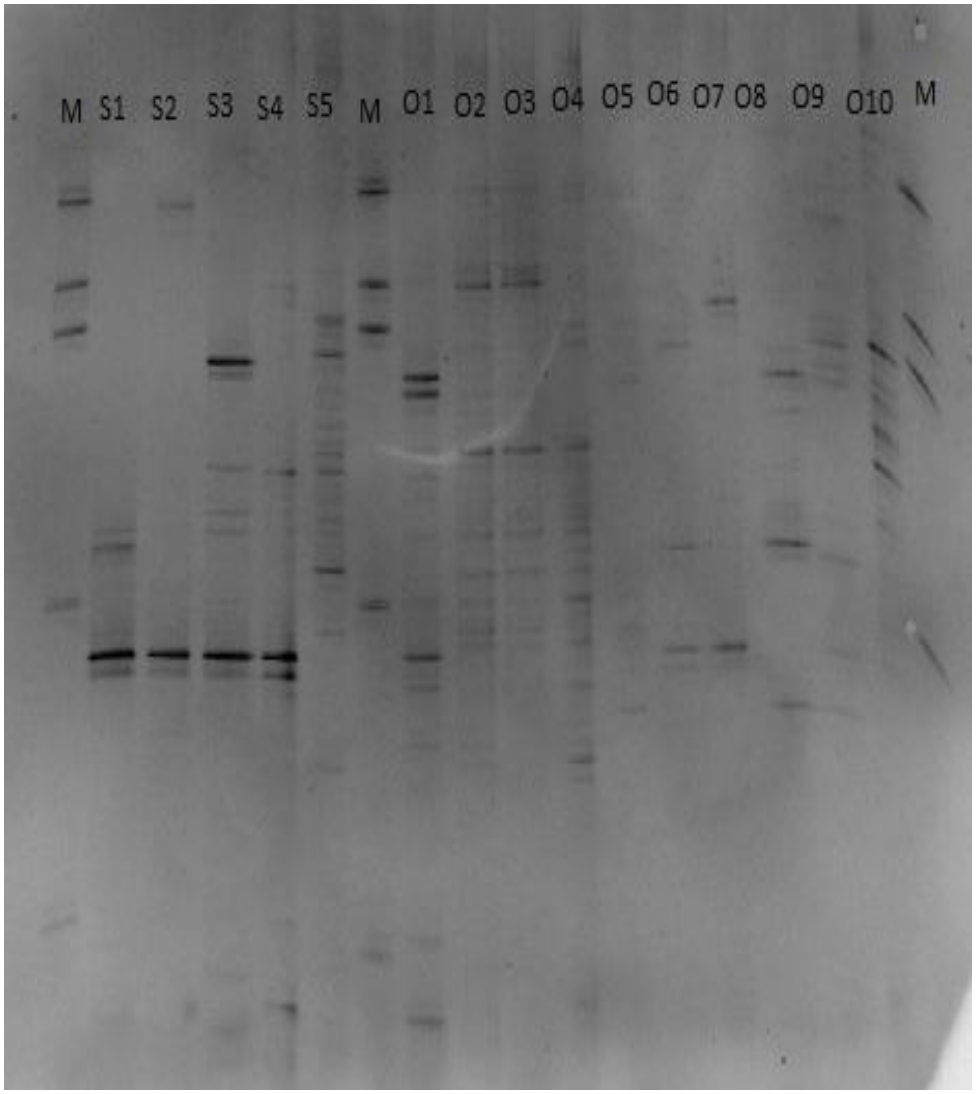

Figure 5.7 : Bacterial 16S rRNA DGGE profiles of cathode biofilm samples predenitrification and postdenitrification SBRs.

5.4 Quantitative polymerase chain reaction (QPCR)

In this study, SYBR GreenI technology [iQ Sybr green supermix (Bio-Rad, Berkeley, CA)] was used for real-time quantitative PCR experiments and 518f/PAO-846r primer set was performed to quantify total "*Candidatus Accumulibacter"* 16S

rRNA genes. Also 314f/534r universal primer set was performed to quantify total bacteria 16S rRNA genes. Q-PCR was carried out in triplicates for each sample (He et al.,2007). Six-point calibration curves for Q-PCR were produced by 10-fold serial dilution of positive controls at 10^3 to 10^8 target copies per reaction. Melting curve analysis for SYBR green assay was done after amplification for the determination of nonspecific amplification and primer dimers.

Quantitative polymerase chain reaction (QPCR) for "Candidatus Accumulibacter"

Candidatus Accumulibacter specific Q-RT PCR assay was very consistent. Figure 5.10 shows the strong inverse linear relationship between the threshold cycle numbers and the copy numbers of *Candidatus Accumulibacter*-specific 16S rRNA (R^2 = 0.996). The amplification efficiencies were 99,8% (slope= -3.326).

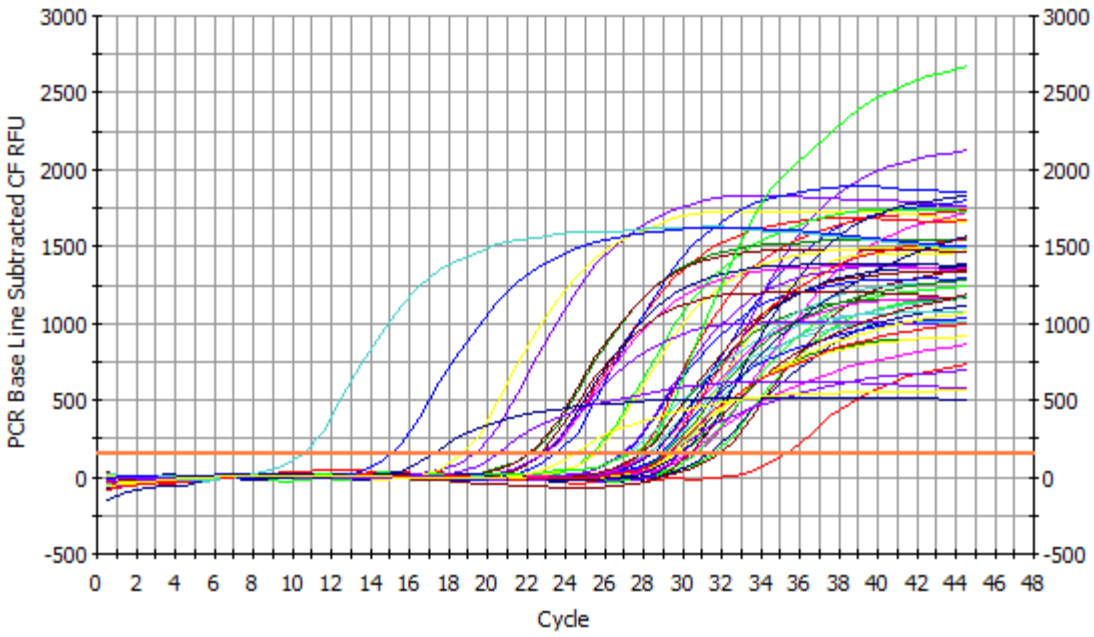

Figure 5.8 : PCR Amp/Cycle Graph for *Candidatus Accumulibacter* specific 16S rRNA fragment.

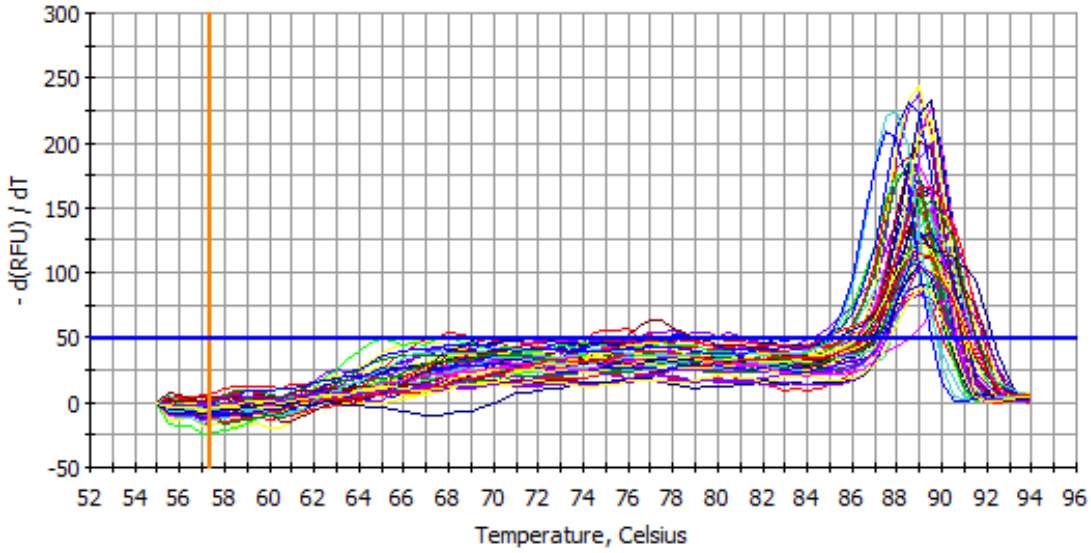

Figure 5.9 : Melting Curve Graph for *Candidatus Accumulibacter* specific 16S rRNA fragment.

Copy numbers (copies ml/culture) of sample groups of both predenitrification and postdenitrification SBRs are presented in Table 5.2. It was obtained that copy numbers of PAOs in the predenitrification SBR samples are statistically higher than postdenitrification SBR samples.

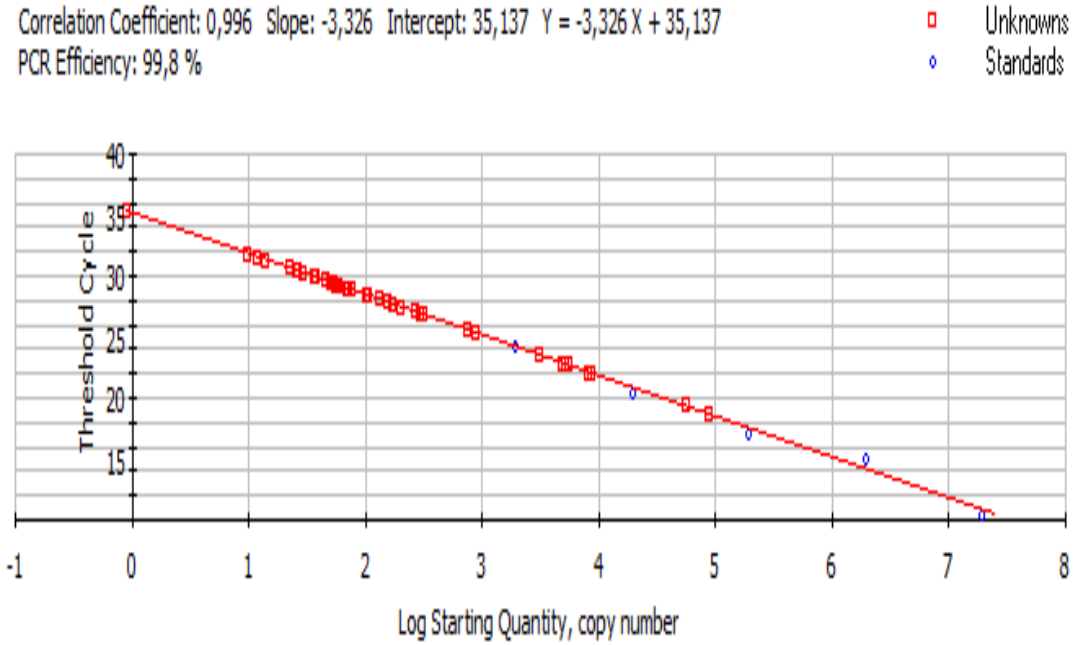

Figure 5.10 : Standard Curve Graph for *Candidatus Accumulibacter* specific 16S rRNA fragment.

According to conventional data, while released-P was high specially periods of S2 and S4 samples (samples of postdenitrification system), P removal was nominal and similarly copy number of *Candidatus Accumulibacter phosphatis* in these samples were quite low. On the other hand in samples of predenitrification system even if released-P was high, P removal was very effective and copy number of *Candidatus Accumulibacter phosphatis* in samples of predenitrification system supported that result.

Conventional data showed that predenitrification system is more appropriate for wastewaters with low carbon/nitrogen ratio. While a rising quantity of *Candidatus Accumulibacter phosphatis* was obtained in samples of predenitrification system, samples of postdenitrification system exhibited a fluctuated quantity.

Table 5.2 : *Candidatus Accumulibacter* specific 16S rRNA fragment copy numbers.

sample	PAO	CT
S1(125)	5,25E+03	22,77
S2(161)	1,06E+02	28,68
S3(252)	6,63E+01	29,09
S4(281)	2,97E+01	30,26
S5(342)	5,41E+01	29,37
O1(1)	1,63E+01	32,27
O2(93)	8,58E+01	29,06
O3(122)	2,00E+01	31,05
O4(149)	8,25E+01	29,23
O5(197)	1,74E+02	27,91
O6(300)	1,19E+02	28,26
O7(379)	4,89E+04	20,32
O8(475)	3,02E+03	24,87
O9(506)	5,47E+03	22,83
O10(521)	3,59E+02	27,58

Quantitative polymerase chain reaction for Quantification of the total number of copies of the bacterial 16S rRNA gene

The bacterial 16S rRNA gene specific Q-RT PCR assay was very consistent. Figure 5.12 shows the strong inverse linear relationship between the threshold cycle numbers and the copy numbers of the bacterial 16S rRNA (R^2 = 0.998). The amplification efficiencies were 101,6% (slope= -3.284).

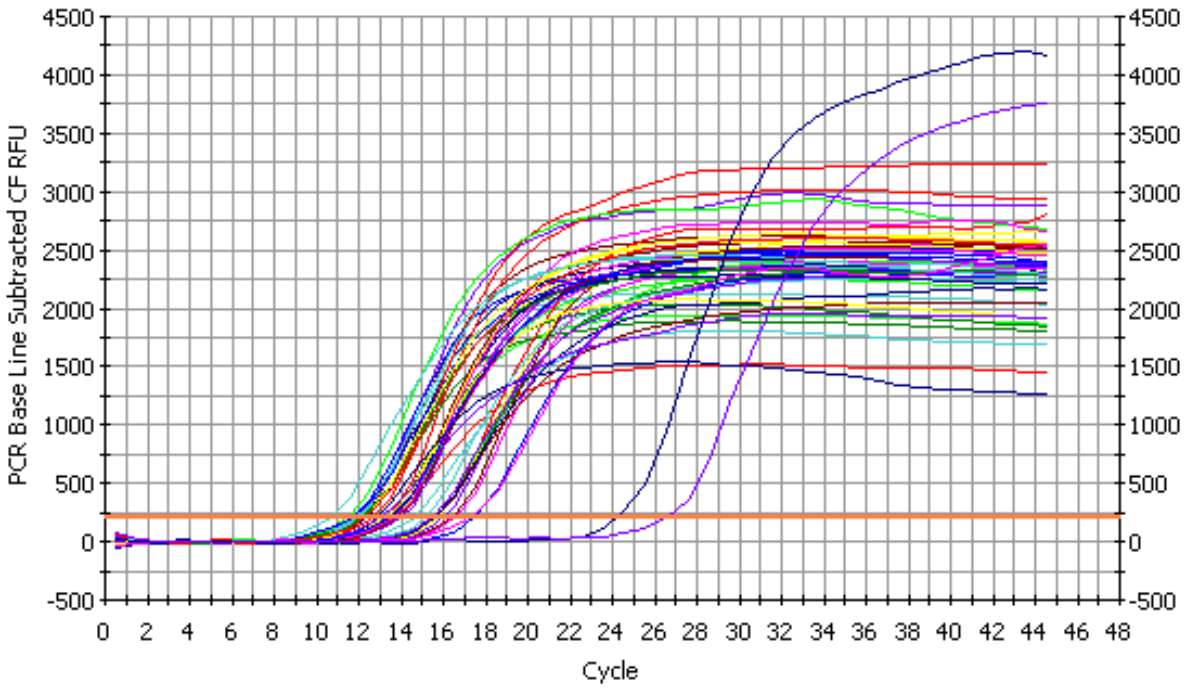

Figure 5.11 : PCR Amp/Cycle Graph for the total number of copies of the bacterial 16S rRNA gene.

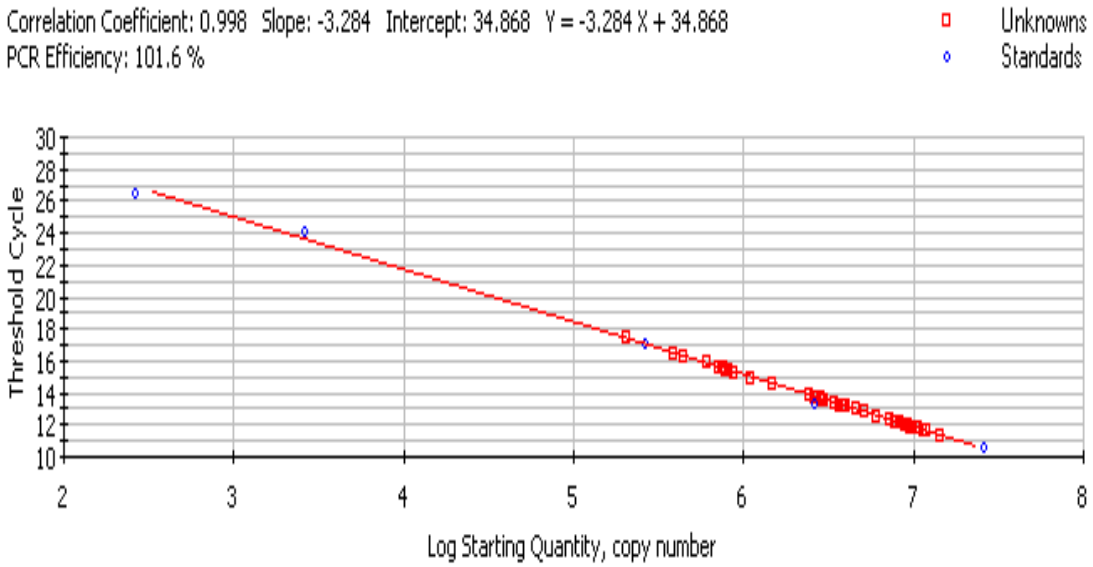

Figure 5.12 : Standard Curve Graph for the total number of copies of the bacterial 16S rRNA gene.

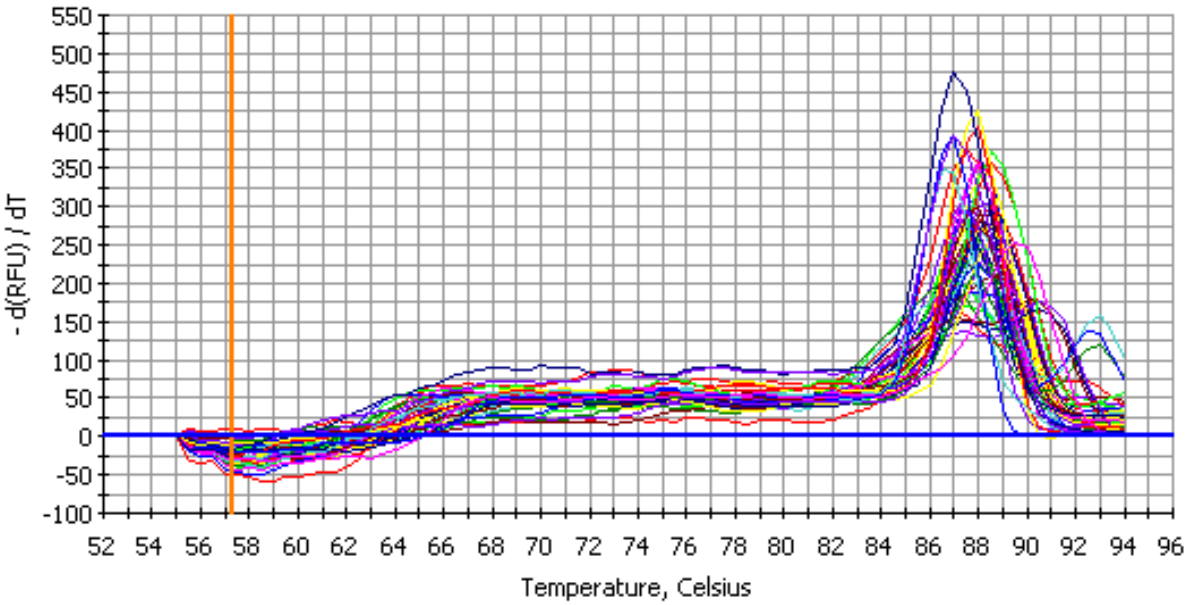

Figure 5.13 : Melting Curve Graph for the total number of copies of the bacterial 16S rRNA gene.

Copy numbers (copies ml/culture) of sample groups of both predenitrification and postdenitrification SBRs are presented in Table 5.3. It was obtained that both pre-and post-denitrification SBR samples contain similar numbers of bacteria approximately 4E+06.

Table 5.3 : *Total Bacteria* copy numbers.

Sample	Total Bacteria Number	Ct
O1	3,47E+06	13,41
O2	2,24E+06	14,06
O3	8,74E+06	12,08
O4	3,41E+06	13,43
O5	9,95E+06	11,90
O6	2,98E+06	13,61
O7	6,92E+05	15,69
O8	8,85E+05	15,35
O9	3,40E+05	16,77
O10	2,76E+06	13,72
S1	7,89E+05	15,50
S2	7,97E+06	12,41
S3	1,01E+07	11,88
S4	3,55E+06	13,42
S5	6,71E+06	12,49

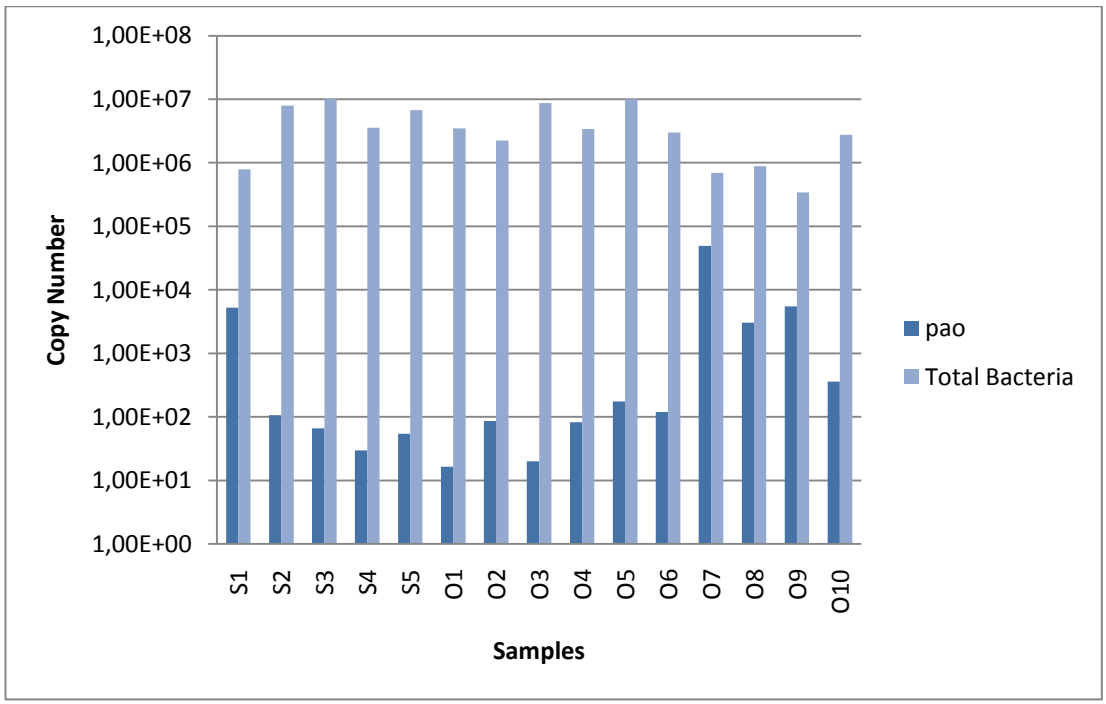

Figure 5.14 : Comparison between copy numbers of *Candidatus Accumulibacter phosphatis* and Total Bacteria.

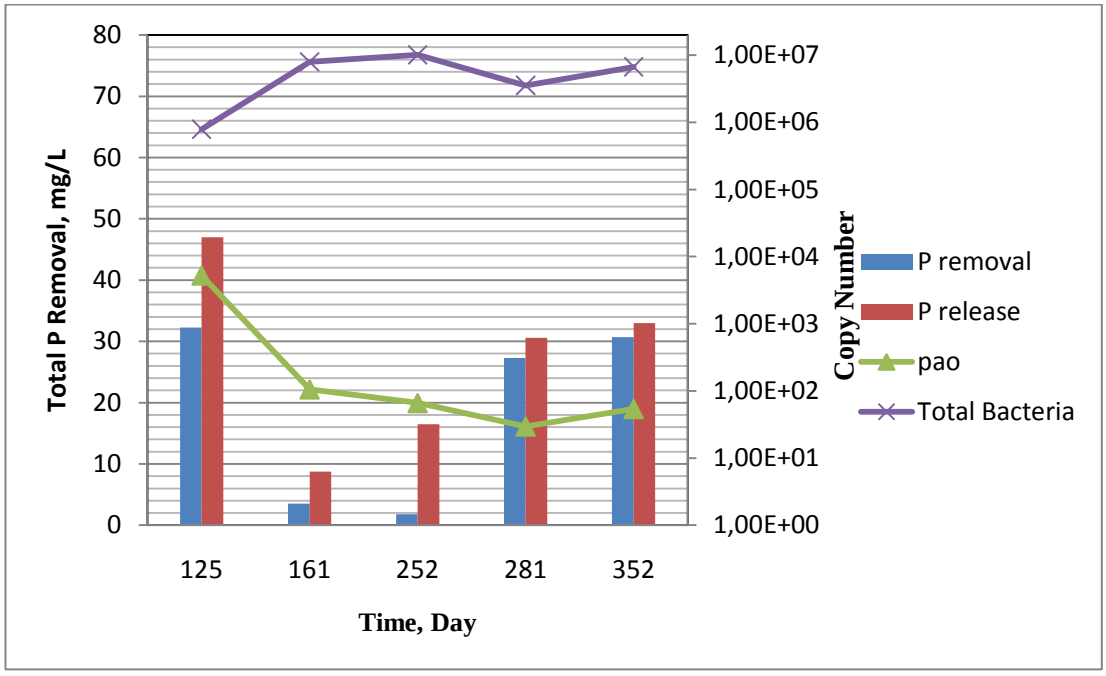

Figure 5.15 : Graphic presentation of comparison between total phosphorus release/removal rate and copy number of Candidatus Accumulibacter at sampling points in post denitrification reactor.

Influent/effluent PO_4-P concentration and P removal efficiency were shown in Figure 5.16. In spite of P removal efficiency was very high, there is no significant change in copy number of *Candidatus Accumulibacter* (Figure 5.15).

During post denitrification SBR operation, the phosphorus removal efficiency was affected by high acetic acid concentration adversely. While the post denitrification reactor has been operating, $NaHCO_3$ was added into the reactor for the adjustment of pH and supplementation of inorganic carbon for nitrification process. Due to break down of $NaHCO_3$ pump at day 139 for 24 hours which caused decrease of pH from 6.5 to 4.5. As a result; acetic acid concentration in the reactor at the end of day 139 was 500 mg/L. Randal and Chapin (1997) are suggested that when the concentration of acetic acid is 195 mg/L, acetic acid inhibition starts in the BPR system (Randal and Chapin (1997)) . As mentioned in the literature, our BPR system was seriously inhibited at 500 mg/L of acetic acid concentration. Approximately 4 months later, system was recovered and improved from the preceding operational periods.

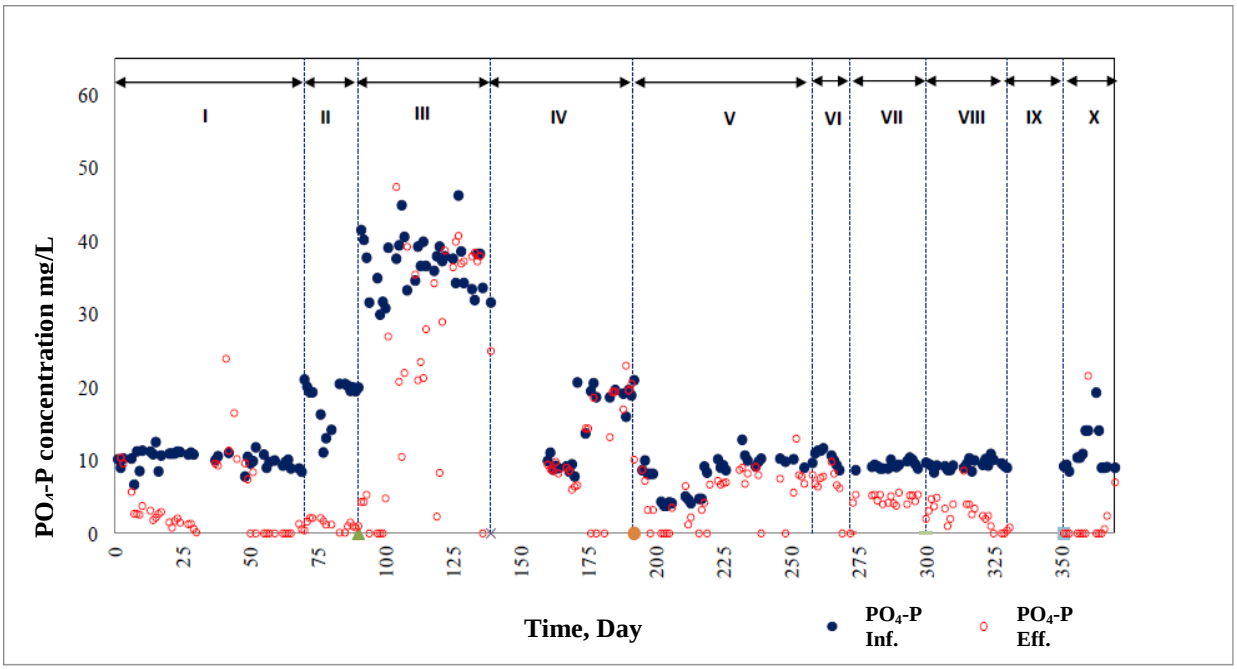

Figure 5.16 : Influent and effluent PO_4-P concentration in post-denitrification reactor.

Moreover, carbon sources (acetate to propionate ratios), temperature and pH levels have an andeniable impact on the growth of glycogen-accumulating organisms. The favorable condition of the existence of PAO (therefore, beneficial for the biological phosphorus removal process) is low temperature (10 ^{o}C). On the other hand, while HAc and HPr is supplied as C source at around 20 ^{o}C, pH has to be high as 7-7.5 for the enrichment of PAOs (Lopez et al. 2008).

Therefore activation and growth of *Candidatus Accumulibacter* was affected by low pH and a decreasing profile of copy number of *Candidatus Accumulibacter* was obtain after first sample (S1- day 125).

In post denitrification configurations, aerobic phase comes right after the anaerobic phase. Actinobacterial phosphate-accumulating organisms are able to take up Pi aerobicaly, after taking up organic substrates under anaerobic conditions (Okunuki et al. 2007). These results suggest that Actinobacterial phosphate-accumulating organisms might be dominant in post denitrification configurations.

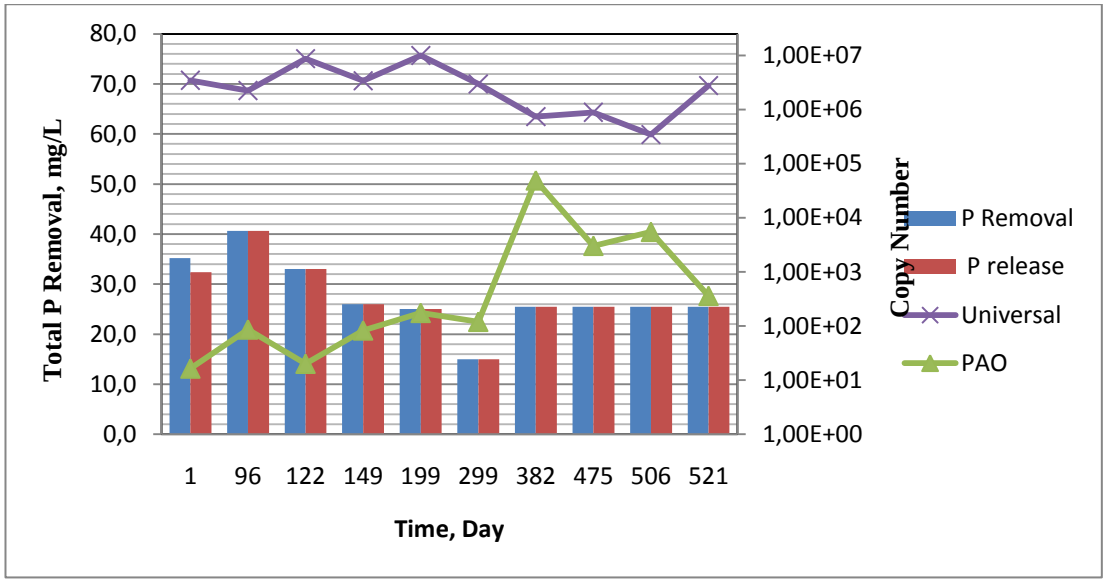

Figure 5.17 : Graphic presentation of comparison between total phosphorus release/removal rate and copy number of Candidatus Accumulibacter at sampling points in post denitrification reactor.

Influent/effluent PO_4-P concentration and P removal efficiency were shown in Figure 5.18. Copy number of *Candidatus Accumulibacter* was compatible with P removal efficiency (Figure 5.17).

In the last operation period, which include sample O7, of pre-denitrification reactor, while the length (duration) of anaerobic phase was reduced, the length (duration) of anoxic phase was induced (Figure 4.2). This arrangement caused the indication of the growth of *Candidatus Accumulibacter*.

In the pre-denitrification configurations anoxic phase comes right after the anaerobic one and in anoxic phase nitrate and nitrite are used as electron acceptors. Therefore pre-denitrification configurations is highly suitable for the seed with low C/N ratio.

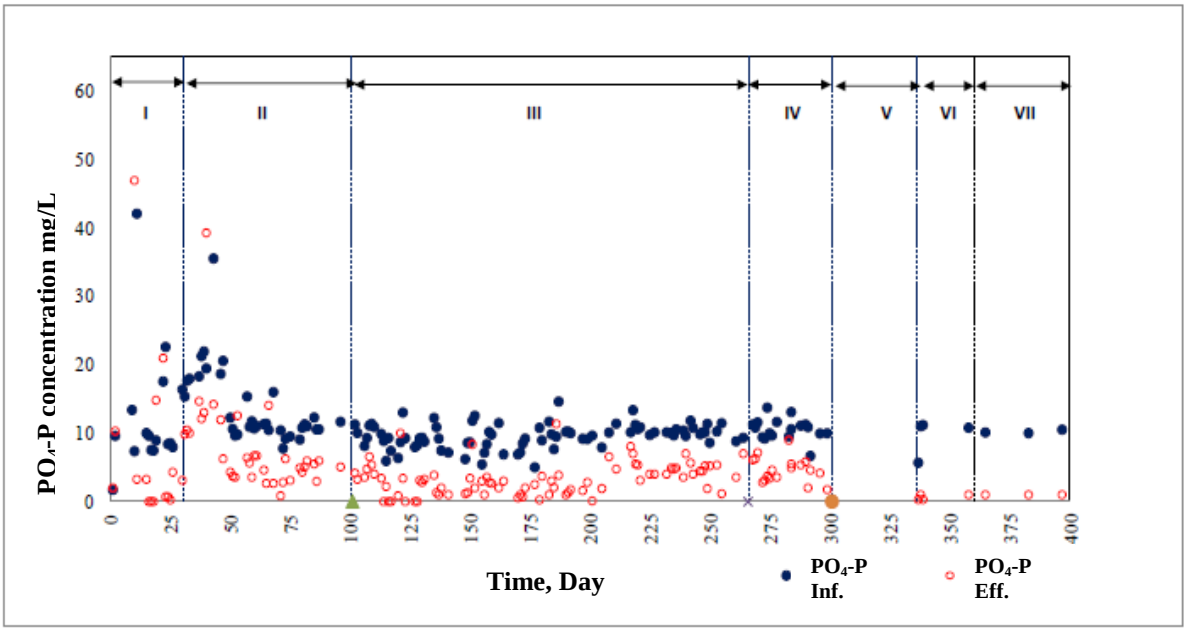

Figure 5.18 : Influent and effluent PO_4-P concentration in pre-denitrification reactor.

6. CONCLUSION

In this study investigation of the relation between the performance of two successfully operated denitrifying phosphorus (P) removal systems and their microbial community structure was aimed. For this purpose, two sequencing batch reactors (SBRs) were operated in oxic and anoxic conditions in post and predenitrification configurations.

Both conventional data in terms of P removal, Influent/Effluent PO_4-P concentration and quantitative microbial community analysis in terms of *Candidatus Accumulibacter phosphatis* with respect to total Eubacteria have been investigated.

In post denitrification BPR configurations, aerobic phase comes right after the anaerobic phase. Actinobacterial phosphate-accumulating organisms are able to take up Pi aerobicaly, after organic substrates utilized under anaerobic conditions (Okunuki et al. 2007). In this study, while released-P was high in postdenitrification system, P removal was unsteady and similarly copy number of *Candidatus Accumulibacter phosphatis* were quite low in the samples. In spite of the system's recovery, while the P removal efficiency increased, there was no significant change in copy number of *Candidatus Accumulibacter phosphatis.* These results suggest that Actinobacterial phosphate-accumulating organisms might be dominant in place of *Candidatus Accumulibacter* in post denitrification configurations.

On the other hand in samples of predenitrification system even if released-P was high, P removal was very effective and copy number of *Candidatus Accumulibacter phosphatis* in samples of predenitrification system supported that result. It could be suggested that due to longer duration of anaerobic phase and anoxic phase might enhanced the growth of *Candidatus Accumulibacter.*

Conventional data showed that predenitrification system is more appropriate for wastewaters with low C/N ratio. When compared in terms of *Candidatus Accumulibacter phosphatis* quantity; predenitrification system samples exhibited more steady profile rather than postdenitrification system's fluctuated quantity.

Results of such Denitrifying Biological Phosphourus Removal Systems might suggested that in terms of phosphorus removal, *Candidatus Accumulibacter phosphatis* found to be the dominant group in pre-denitrification configurations; however in post-denitrification configurations Actinobacter-related phosphate-accumulating organisms or other yet undefined groups of microorganisms may have important roles.

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APPENDICES

APPENDIX A: Laboratory Equipment

APPENDIX B: Chemicals

APPENDIX A

Laboratory Equipment

MOBGAM, Istanbul Technical University

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

APPENDIX B

Chemicals

40%ACRYLAMIDE/BIS SOLUTION 37.5:1	BIORAD
Formamide deionized solution	Sigma-Aldrich
UREA	Fluka
NaOH	Reidel-de Haen
Sodium Acetate Anhydrous	Sigma-Aldrich
Pottassium Hydrogen phosphate	J.T. Baker
Ethyl alcohol absolute	Sigma-Aldrich
EDTA molecular biology reagent	Sigma-Aldrich
TRIS-HCl	Sigma-Aldrich
Sodium Phosphate, Monobasic	Sigma-Aldrich
Ammonium Persulfate	Sigma-Aldrich
Acetic acid extra pure %99.5	Sigma-Aldrich
taq polymerase	INTRON
Hot start *taq* polymerase	Qiagen
primers	iontek
TEMED	Biorad
SYBR green Super mix	Biorad
NaHCO3	Fluka
KH2PO4	Fluka
NaCl J.T.	Baker
NaOH	Fluka

CURRICULUM VITAE

Name Surname: İpek ERGAL

Place and Date of Birth: Bilecik, 05.07.1988

E-Mail: ipekergal@gmail.com

Permanent Address: Ihlamurdere Cad. Ortak Ap.No:153 D:8 Beşiktaş/Istanbul

Universities and Colleges attended:
ITU, Molecular Biology, Biotechnology and Genetics 2011-2013, M. Sc.
Istanbul University, Molecular Biology and Genetics, 2006-2010, B. Sc.