PHOSPHORUS-RECOVERY FROM WASTE ACTIVATED SLUDGE (WAS) IN ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL (EBPR) PROCESSES

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ABSTRACT

Phosphorus is an essential element for every living organism, but when it exceeds certain limit in water bodies, it will cause serious environmental issues, such as eutrophication. P accumulation in water bodies can be caused by non-point sources, such as agriculture land runoff, as well as from point sources such as wastewater effluent discharges. In order to avoid eutrophication of water bodies the regulatory agency have traditionally pushed for the removal of phosphorus at wastewater treatment plants rather than regulating the non-point sources; this has been done imposing strict limits on wastewater effluents to natural watershed.

At the same time, phosphorus stock on the earth is a limited resource, and its quantity is decreasing steadily, due to its use in the fertilizers production. In order to support the population growth on the earth, phosphorus recovery, especially from wastewater streams has to be considered.

Biological processes that remove phosphorus from wastewater, are called Enhanced Biological Phosphorus Removal (EBPR in short) processes, and produce a P rich sludge that can be treated to recover phosphorus in forms that could be used as fertilizers. Several processes have been developed to recover P from EBPR plants, such as Waste Activated Sludge Stripping to Remove Internal Phosphorus (WASSTRIP) and the PhoStrip processes.

P recovery within EBPR plant often involved an anaerobic holding tank, where P is released due to PAO activity as well as bacteria decay. However, the impact of EBPR process operating conditions on P-release capacity and kinetics are not fully understood.
In addition, it is largely unclear how the anaerobic digestion process of the P-recovery process affects the microbial population, and therefore the EBPR activity in the mainstream, in system where the sludge is recirculated back to the mainstream.

In this study, P-release capacity and kinetics were studied by conducting day long endogenous anaerobic digestion tests on activated sludge withdrawn from lab scale sequencing batch reactors operating under different conditions (COD/P and SRT). P-release mechanisms during the digestion test were investigated by Live/Dead analysis, as well as soluble metal ion concentration measurements, which are usually associated with EBPR activity. In addition, PAO activities changes were explored by the microbial population quantification, combined with P-release rate in the present of VFA. Considering the microbial populations, in the acetate fed SBRs are different in quantity and possibly composition, from the population (especially PAOs) in full-scale samples, the same anaerobic test and measurements were performed on a full-scale EBPR WWTP WAS samples.

Under anaerobic starvation conditions, it was observed that low COD:P ratio with 10-20 days-SRT had better P recovery potential than other operation conditions involved in this study, in terms of higher amount ortho-P released and faster releasing rate. Among the released ortho-P during the digestion test, majority of it was found to be due by poly-P depletion. In addition, because of the reducing intercellular polymer storage, PAO activity also decreased significantly during the anaerobic digestion test. However, with different population and composition, PAO activity in full scale WAS samples actually increased after the test.
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1 Introduction

Phosphorus is a non-renewable key element in all living organisms as a component of cell membranes, nucleic acids and ATP (adenosine triphosphate), it is therefore critical to grow crops. The increasing world population is demanding an increase in crop productivity and phosphate rock deposits are being progressively depleted for the fertilizer industry to meet this demand. The current main phosphorus source is phosphorus-rich rock in the form of phosphate, which about 83% of the world’s easily exploitable is held by Morocco, China, South Africa and the U.S. The availability of high quality P can only support the U.S. for 40 years and 100 years globally (Sam J, 2011). The excessive application of phosphate fertilizers together with P from increasing human waste discharges has altered the natural P cycle (Figure 1). The inorganic cycle describes the cycle from erosion, transport into plant-available phosphates in soil, sediment, tectonic uplift and alteration of phosphate-containing rocks. Besides the inorganic phosphorus cycle, there are two organic cycles attached describing phosphorus as part of the food chain. One of the cycles takes place on land (soil-plant-humans/animals-organic waste-soil), the other is in water. These originally closed cycles are broken by the artificial fertilizer application (Cornel, 2009). Then, phosphates contained in wastewater effluent, as well as in the nonpoint runoff are transported to natural bodies of waters. The excessive accumulation of P in waters and sediments can cause serious negative impacts, especially presented by eutrophication (Cucarella Cabans, 2007). In order to reconnect the original cycle and meet the increasing needs of phosphorus in the future, there is a need to recovery phosphorus from the wastewater treatment processes.
At present, commercial phosphorus production is based almost exclusively on phosphate rock, primarily calcium phosphate in various forms, combined with a wide range of impurities. Because of the energy costs and environmental protection consideration, the current dominant production route uses the so-called ‘wet process’, where phosphate rock reacts with sulfuric acid to produce an impure phosphoric acid, often called ‘green’ acid, and waste Phospho-gypsum. The threat of limited resources and higher prices is pushing research towards new ways of recovering and recycling this vital material. An efficient P removal and recovery process is essential to the environmentally sound and cost effective repossession and reuse of P. The use of renewable sources moreover offers the immediate advantage of avoiding the environmental impacts associated with primary production from phosphate rock (Morse et al., 1998).

Enhanced Biological Phosphorus Removal (EBPR) is a phosphorous removal method widely used in municipal areas. EBPR is the removal of P by alternating aerobic and anaerobic stages of treatment, which favor the growth of Phosphorus Accumulating
Organisms (PAOs). PAOs, as the name entails, are able to accumulate P, thus removing it from the wastewater. In EBPR processes P can be removed up to 90% from the mainstream flow and reduce the eutrophication potential of the wastewater discharges (Oehmen et al., 2007).

Furthermore, the sludge generated from the EBPR process contains large amounts of P. The process therefore provides an effective method to concentrate phosphates in a reduced sludge volume, making this an ideal pretreatment of sludge for P recovery (Latimer et al., 2012).

P-Recovery avenues directly from WWTP and the potential use of the products can be divided into several categories (see Figure 2). The most common technology used for P recovery is where phosphorus rich sludge from EBPR is treated by anaerobic digestion to allow P release from the biomass into a liquid stream. Pelletized P is then precipitated from this side-stream flow in an up-flow fluidized bed reactor, as struvite (\(\text{NH}_4\text{MgPO}_4\cdot6\text{H}_2\text{O}\)) or hydroxyapatite (\(\text{Ca}_5\text{(PO}_4\text{)}_3\text{(OH)}\)). Feed and recycle stream fluidize the particles which are harvested at the bottom of the reactor. Effluent from this process can then be recycled or is returned to the head of the plant. During this crystallization process, approximately 80% of the P from the centrate can be recovered. The P enriched products can then be reused as slow release fertilizers (struvite) or feedstock (hydroxyapatite) for other industries (Latimer et al., 2012).

To date, studies have only been conducted on the quality and quantity of P-recovery production, since the processes involved the wasted activated sludge, and only decanted liquid were send back to the mainstream system (Le Corre et al., 2013, Bradford et al., 2012, Latimer et al., 2012, De Bashan et al., 2004). However, the anaerobic ‘digestion’
during P-recovery processes could have impacts on the microbial population, such as the population structure and activity. Better understanding of these impacts can provide useful information when recovering P from recycled activated sludge (RAS) is performed in combination with mainstream or side stream EBPR. Existing processes, where this information can advance the understanding and therefore the successful implementation of treatment/recovery strategies are the PhoStrip and the WASStrip processes.

Figure 2 Avenues of recovering phosphorus from wastewater and the potential use of the product (De-Bashan et al., 2004).
1.1 Outline of the thesis

Exploring the fundamentals of P recovery processes and their impact on the EBPR process is important for the development and widespread use of P recovery technology, and it was the main scope of this work. Below an outline of this thesis is given.

The process fundamentals, including aerobic/anaerobic P-Removal, microbiology, metabolism in Enhanced Biological Phosphorus Removal, Phosphorus cycle and storage situation, and P-Recovery methods from wastewater treatment plan will be presented in Chapter 2, with specific emphasis on Waste Activated Sludge Stripping to Remove Internal Phosphorous (WASSTRIP) process and the PhoStrip process. The different P-Recovery process categories are compared on the basis of their underlying principles and examples of full-scale application as well as pilot scale studies, are also given.

Hypothesis and scope are presented in Chapter 3, while a description of the experimental methodology, data acquisition and analysis is given in Chapter 4.

In Chapter 5 the results of the P-Recovery experimental work are presented; the first part of the chapter focuses on the lab scale SBR P-Recovery capacity and the effect of the endogenous digestion on the microorganisms, especially PAOs. Specifically we explore the effect of an important operational parameter, the sludge residence time, on the sludge P-recovery capacity and on the microbial population. The second part of the chapter focuses on full-scale WWTP samples.

Chapter 6 closes with general conclusions and perspectives for future research.
2 Literature review: EBPR and P-Recovery from WWTP

In EBPR process phosphorus removal is achieved by alternating anaerobic and aerobic stages, which favors the growth of PAOs, the bacteria responsible for the phosphorus removal. Excess sludge generated from EBPR processes contains large amounts of phosphorus (Esakki et al, 2012); indeed, comparing to the normal activated sludge system, phosphorus concentration in the EBPR sludge can reach about 5 times higher than in a normal activated sludge system (10% versus 2%) (Metcalf and Eddy, 2003). Therefore, EBPR processes provide an excellent method to concentrate phosphates in a reduced sludge volume. This bound phosphorus can be recovered to meet out its increase in demand (Raj et al, 2012).

This chapter describes the fundamental microbiology metabolism in EBPR, existing treatment process options to reduce the phosphorus content, as well as the kinetics and methodologies of P release and recovery from wastewater treatment.

2.1 EBPR Fundamental

The EBPR process relies on the selection and proliferation of a microbial population capable of storing orthophosphate in excess of its biological growth requirements. This functional group is referred to as polyphosphate-accumulating organisms (PAOs). With special design and operations conditions, which typically include alternative anaerobic and aerobic cycles, PAOs can thrive and dominate the biomass resulting in excessive accumulation of orthophosphate in the activated sludge. Phosphate can be then removed by wasting a certain amount of biomass (MOP, 2013).
2.1.1 Microbiology and Metabolism

When wastewater enters the anaerobic phase, the PAOs accumulate carbon sources (volatile fatty acids [VFAs]) as an internal polymer called polyhydroxyalkanoate (PHA). The main forms of these PHAs are poly-beta-hydroxybutyrate (PHB) and poly-beta-hydroxyvalerate (PHV). The energy to store this polymer is obtained from the breakdown of glycogen and hydrolysis of polyphosphate bonds. Polyphosphates are formed by a series of high-energy bonds; PAOs can subsequently obtain energy by breaking these bonds. Because polyphosphate is broken down to orthophosphate for energy supply, the phosphate concentration in the anaerobic phase increases. The ability of PAOs to accomplish anaerobic VFA uptake and to store PHA polymers is the main mechanism through which they gain a selective advantage in EBPR systems. During the experiment, this phenomenon is represented by observing an increase in ortho-P concentration in solution and the concomitant acetate uptake under anaerobic condition (See Figure 3).

![Figure 3 Typical P-release and uptake graph and acetate uptake results. (Lab scale batch tests results.) AN: Anaerobic phase; AE= Aerobic phase.](image)

The anaerobic phase needs to be followed by an oxygen rich phase (aerobic phase). During this phase, the stored PHB is consumed, generating energy for growth and uptake of orthophosphate from the liquid phase and generating energy and carbon for
replenishment of the glycogen and polyphosphate pools. Figure 4 presents the schematic diagrams of the anaerobic and aerobic PAO metabolism.

Figure 4 Schematic diagrams of the anaerobic and aerobic PAO metabolism. PHA: polyhydroxyalkanoate; Poly-P: polyphosphate (Yuan et al. 2012).

### 2.2 Environmental/Operation factors on EBPR

As a biological process, there are lots of environmental and operational factors that can affect EBPR performance, including dissolved oxygen, pH, temperature, COD:P ratio, carbon type, and sludge residence time (SRT) (Gu et al., 2008, Luz et al., 2004, Oehmen et al., 2004, Lemos et al., 1998). Some of these factors can consequently affect the potential P-Recovery capacity, and will be discussed in this section. The first factor to be considered is the type and amount (in terms of COD: P ratio) of the carbon source available during the anaerobic phase, which will affect the formation of PHAs and consequently the overall EBPR performance. The second main parameter, SRT is considered regarding its impact on EBPR microbial population and its P-removal performance, which also relates to biomass P content and, therefore, consequently could affect the P-recovery potential.
2.2.1 Carbon Source Type

Efficient phosphorus removal in aerobic phase can only be obtained based on the successful release during the anaerobic phase. In order to achieve the optimum release of phosphorus in the anaerobic phase, the system should be fed with an adequate amount of carbon. However, it has been found that is not only the availability of carbon that control the EBPR process, but also the carbon type; in other words, the form of COD that is available to microorganisms.

It is known that PAO under anaerobic conditions can uptake organic substrate (including short chain volatile fatty acid, amino acids and protein, etc.), thereby producing polyhydroxyalkanoates (PHA) with simultaneous phosphorus release to the external medium. Energy for this process comes from the breakdown of intracellular polyphosphate; the reducing equivalents needed are originated from glycogen consumption and substrate degradation in the tricarboxylic acid cycle. During the aerobic stage in the process, PHA previously accumulated in the anaerobiosis is metabolized for anabolic precursors and microorganism growth (Lemos et al., 1998, Maite et al., 2008).

It is known that the types of carbon source not only affect PAO and GAO performance, but also have impacts on polyhydroxyalkanoate (PHA) production. Oehmen et al (2004) presented that PAOs enriched using acetate as the sole carbon source are readily able to take up propionate, while GAOs enriched under similar conditions did not have the same ability. Hence, different carbon substrate could potentially be used to minimize GAOs and therefore provide a selective advantage for PAOs in EBPR systems. In addition, since PHA is both carbon and energy source of P-uptake, proliferation and glycogen replenishment, the differences in the biochemical pathways by which different VFAs are
converted to PHA, might explain the variations in P-removals observed in batch experiments (Arun et al., 1988, Pereira et al., 1996).

It was demonstrated in many laboratory-scale EBPR reactors that short VFA (mainly acetic acid) is more favorable than the long chain VFA (MOP, 2013). In actual treatment plants, the biodegradable VFA can be fermented in wastewater collection systems with proper retention time as long as there is adequate amount of organic matter.

2.2.2 **COD: P Ratio**

Randall et al. (1992) introduced the concept of the C/P ratio as a factor that affected the biological nutrient removal processes, in terms of SVI of microbial granules, P removal efficiency and stability, as well as microbial population structures. At higher COD/P ratio, a less compact microbial structure with lower SVI values was found (Lin et al., 2003). Moreover, the stability of EBPR processes was shown to be correlated with higher COD/P ratio, even though this higher ratio could significantly promoted the growth of GAO due to the phosphorus-limiting condition (Gu et al., 2008, Thongchai et al. 2007). Indeed, in EBPR system, PAOs and GAOs co-exist and compete for the carbon source. Both of these two types of bacteria are growing and reproducing in aerobic phase, which need carbon storage, accumulated during the anaerobic phase. It was reported that PAOs tend to dominate at a COD/P ratio of 10-20 mg COD mg/P, whereas GAOs tend to dominate at COD/P greater than 50 mg COD/mg P. Both populations coexist in the intermediate levels of COD/P ratio (Oehmen et al., 2007). As a result, there is the risk of EBPR failure when GAOs dominate in the system. Thus, an optimum COD:P to create a balance between PAOs and GAOs should be applied.
In addition, previous research has shown that the influent COD/P has close correlation with the EBPR biomass total phosphorus content and phosphorus removal, which is, furthermore, linked to advantages for P recovery process (Liu et al., 1997, Ma et al., 2005, MOP, 2013). Randall and Chapin (1995) stated that at lower influent C/P ratio, COD is limiting and the fraction of poly-P bacteria would be high, and this would lead to a high percentage of phosphorus in the wasted activated sludge (WAS). Figure 5 shows the effect of the influent COD:P on the sludge phosphorus content. It was found that, the P content in biomass increased with the reduction of COD:P ratio, which means the sludge fed with appropriately lower COD:P ratio feeding could release more phosphorus to be potentially recovered.

![Figure 5 Relation between the P/C feeding ratio and Px (P content [mg P/mg total suspended solids (TSS)] during the course of operation of reactors 1-3 (Liu et al., 1996).](image)

2.2.3 **Solids Retention Time**

Since the EBPR process relies on the selection and proliferation of PAO, the competition between PAOs and GAOs can directly impact the system performance. Among several factors, including COD/P ratio, pH, temperature and etc., solids retention time (SRT) is
probably the only parameter that can be easily adjusted for real practice. However the relationship between SRT and its impact on microbial population has not been sufficiently investigated in previously reported studies.

Rodrigo et al. (1999) concluded that shorter SRTs are beneficial for PAOs after observing the EBPR biomass activity degradation as SRT was extended, suggesting that GAOs could successfully compete with PAOs at longer SRTs.

According to Whang and Park (2006)’s study, with the operation of 30 °C and pH 7.5, the dominant species switched from GAO to PAO when SRT was changed from 10 to 3 days. Whang et al. (2007) inferred that, under the operating conditions applied by Whang and Park (2006), GAOs had a lower net biomass yield than PAOs, therefore, were outcompeted after SRT was shortened. However, because the lack of detail information about the effect of SRT on the population dynamics and microbial and biochemical mechanisms, and the specific operation condition, Whang and Park’s observation may not explain the scenario at ambient temperature, similar to those at full-scale facilities.

In addition, there are only a few studies that have explored the relationship between SRT and EBPR performance. It was illustrated that increasing SRT could lead to low biomass yield and extra waste sludge (USEPA, 1987). However, Randall et al (1995) reported that even though the phosphorus content in biomass increase, the overall phosphorus removal efficiency showed almost no change. Trembly et al (1999) and Wentzel et al (1991) also presented that longer SRT is more suitable for PAO proliferation, which indicate better performance of P-removal.
In a recent study, it was addressed that increasing SRT from 8 to 16 days can decrease P-removal efficiency and damage the stability of the sludge (Li et al. 2008). In summary, there are still a lot of contradictions about the effect of SRT on EBPR performances.

### 2.3 EBPR configurations

The general components of the EBPR process are anaerobic reactor, aeration tank, followed by a clarifier with sludge recycle. However, different configurations are possible, including those that include also concurrent nitrogen removal. There can be categorized as plug flow and completely mixed systems.


### 2.4 P recovery potential in EBPR system

Various wastewater streams with different P recovery potential can be identified in wastewater treatment plants, including the mainstream and the concentrated sidestreams. P recovery in the mainstream is usually not practiced due to the considerable high cost of chemicals for the precipitation (Ca, Mg) and for pH adjustments and tank size needed when utilizing existing proven technologies (Jeanmaire N. and Evans T., 2010). P recovery from concentrated sidestreams (processes operated on P-rich sidestreams within biological nutrient removal plants or sewage sludge processing lines) is considered economically and technologically feasible. As presented in Figure 6, based on an average phosphorus load of 9 mg/L, approximately 11% of incoming phosphorus load is removed with the primary sludge during primary sedimentation, 28% is incorporated into the biomass and removed with the surplus sludge.
If considering the permitted discharge concentrations of 1 or 2 mg/L, another 50% of the incoming phosphorus load has to be removed, either by biological or chemical-physical P-removal process or their combination. In summary, P removal from conventional WWTP systems is limited to approximately 30%, while from EBPR plant it can be achieved as high as to 90% from the mainstream flow, and the P is then incorporated into the sludge (Conel, 2009), providing considerable feasibility for P recovery.

For recovery processes, as explain in the next paragraph, it is useful to obtain a P-rich side stream. In order to obtain this sidestream, the sludge needs to go through a secondary phase of P release in an anaerobic unit. This unit can be already present in a WWTP (anaerobic digestion or non aerated holding tank) or may be specifically created for the P-recovery (Jeanmaire N. and Evans T., 2010).

During the anaerobic digestion of sludge, organically bound P is released in solution due to the degradation of biomass, the release of Poly-P as well as the reduction of iron phosphate. For EBPR sludge, about 62% of all P contained in the sludge is released from
the stored poly-P in PAOs. As a comparison only 40% of P is released from chemical-P sludge (Hester and Harrison, 2013).

Since P dissolved in sludge water is a starting point for P recovery from the liquid phase, by crystallization, precipitation or adsorption processes, EBPR can recovery P more effectively and economically than chemical P removal process.

However, due to release of poly-P along with Mg\(^{2+}\) and K\(^{+}\), struvite formation can be triggered with the present NH\(_4\)-N in digestion tank. This undesired struvite production can block pipe lines and reduce the recovery rate (Ronald et al., 2013). The controlled release of P under anaerobic condition is therefore essential to obtain the benefits of EBPR without the undesired consequences.

Hence, better understanding of the mechanisms of secondary P release can help to build up a more reliable and economical P recovery system.

### 2.5 P-Recovery from WWTP Fundamental

#### 2.5.1 Approaches to Recovery P from Wastewater

Phosphorus recovery technologies employ some variation of a process flow, whereby the nutrients are concentrated into a low volume stream from which nutrients can be extracted. Concentrating processes can be biological, chemical or electrochemical, while recovery methods can include physical as well as thermo-chemical processes (Latimer et al., 2012). According to the existing literature, and thorough reviews from previous studies, different P-recovery technologies are summarized in Table 1 (Sartorius et al 2011, Ronald et al., 2012). These technologies are divided into several categories based on the origin of the used matter (presented as wastewater and sludge, and sludge ash) and
the three major P-release processes, which are anaerobic digestion, hydrolysis, and thermal treatment.

Table 1 Overview of P recovery alternatives

<table>
<thead>
<tr>
<th>Recovery Source</th>
<th>Principle behind recovery</th>
<th>Harvest method</th>
<th>Chemical additions needed</th>
<th>Recovered element</th>
<th>Examples of technology</th>
<th>Reuse potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wastewater and Sludge</td>
<td>A concentration step (e.g., EBPR or adsorption onto selective media) acts to remove P from the mainstream flow. The P is then released into a smaller stream via anaerobic digestion, VFA stripping, or media regeneration. This stream is then subjected to chemical precipitation and crystallization under alkaline conditions.</td>
<td>Precipitation</td>
<td>Ca, NaOH</td>
<td>P, Ca</td>
<td>P-ROC, PhoStrip</td>
<td>replacement for P rock</td>
</tr>
<tr>
<td>Sludge ash</td>
<td>Acid addition to sludge ash re-dissolves nutrients. Selective precipitation of phosphate complexes is performed at pH 3.0</td>
<td>Leaching</td>
<td>H₂SO₄, Ca</td>
<td>Mg, P</td>
<td>AIRPREX</td>
<td>Fertilizer</td>
</tr>
<tr>
<td></td>
<td>Acid addition to sludge ash re-dissolves nutrients. The sludge is then dewatered to remove nutrient rich stream which is then subjected to chemical precipitation at alkaline pH.</td>
<td>Adsorption</td>
<td>Ca, NaOH</td>
<td>P, Ca</td>
<td>PIROSIDI</td>
<td>Fertilizer</td>
</tr>
<tr>
<td></td>
<td>Potassium or magnesium chlorides are added to the ash. This mixture is then heated to &gt;1000 °C to remove heavy metals chlorides. Potassium and magnesium phosphates can then be recovered directly from the residue.</td>
<td>Thermal treatment</td>
<td>Ca, NaOH</td>
<td>P, Ca</td>
<td>MEPHREC</td>
<td>Fertilizer</td>
</tr>
</tbody>
</table>

2.5.2 Commonly used P recovery process in EBPR plants

In one of the most common technologies used for P recovery, phosphorus rich sludge from EPBR is treated by anaerobic digestion to allow P release from the biomass into a liquid stream. Pelletized P is then precipitated from this side stream flow in an up-flow fluidized bed reactor (FBR), as struvite (NH₄MgPO₄·6H₂O) or hydroxyapatite (Ca₅(PO₄)₃(OH)). Feed and recycle streams fluids and particles are harvested at the bottom of the reactor (Figure 7).
A commercial application of this process is the Ostara Pearl reactor. The Ostara Pearl reactor adds magnesium to the phosphorus and ammonia rich centrate to generate struvite in a fluidized up flow reactor constructed with several different diameters. Even though the Ostara Pearl reactor has reduced centrate recycle phosphorus by an average, including during start up, of 82% along with a 14% reduction in ammonia, there was still a main problem, which is to collect concentrate and transport it to the reactors without generating struvite in the delivery system.

This challenge was solved by WASSTRIP (Figure 8), which divides the struvite synthesis into two patents pending systems. Waste Activated Sludge (WAS) is collected in an anaerobic release tank, combined with the supernatant from primary sludge partial fermentation tank, bringing VFA into the release tank to induce P and Mg release. This release tank is operated with short HRT, so there will not be ammonia release, which will cause struvite synthesis in undesirable places. Through thickened centrifuge process, the high P and Mg content liquid stream from the previous release tank is delivered to the FBR, while the solid phase is transported to further anaerobic digestion process along
with the primary sludge from partial fermentation tank. Since this anaerobic digestion tank is operated with long HRT, combined with the VFA from PS fermentation, ammonium can be produced as well as secondary P release. The liquid phase in the anaerobic digestion tank is separated by a dewatering centrifuge and conveyed to the FBR containing rich P and NH₄, combining with the P and Mg rich stream to synthesis struvite as final production. The bio-solids can be incinerated and disposed in a landfill.

![Diagram of wastewater treatment system](image)

**Figure 8 WASSTRIP™ + Ostara integrated into a wastewater treatment system (Baur et al., 2009).**

In the other commonly used P recovery technology called PhoStrip process, phosphorus is recovered by anaerobic treatment of a part of the return sludge flow in a stripper
(Figure 9). A pre-stripper can also be added, as a precaution against high nitrate levels in the sludge recirculating stream, as a denitrification reactor. Phosphate and short-chain organic acids concentrations accumulate and diminish with decreasing depth towards the surface in the stripper tank. In order to distribute the short-chain organic acids throughout the entire volume and to direct a portion of the re-dissolved phosphates into the stripper flow, the sludge from the stripper bed is recirculated and the phosphorus and short-chain organic acids thereby washed out (Kaschka et al., 1999). The dissolved phosphate is then precipitated with lime. Calcium phosphate produced from a side-stream in the treatment plant has a sufficient enough content of pollutants to be used as raw material in the phosphate industry (Levlin et al., 2003). In the PhoStrip process, because formation of supernatant is required, the total solids concentration in the P stripper is limited, so that, in practice, P concentrations range between 20 and 30 mg/L, which reduce the efficiency of crystallization or precipitation process. Furthermore, the hydraulic retention time in the stripper should be brief, because, otherwise, anaerobic processes will commence, leading to the formation of CO₂, CH₄, H₂S, or N₂O and may cause odor problems (Ronald et al., 2013).
2.5.3 **P-Recovery status around the world**

The Netherlands is among the pioneer countries in the field of phosphorus recovery from sludge with experiments performed in full-scale plants at municipal WWTPs. The West European phosphate industry, that as leaders such as, Thermphos International B.V from the Netherlands, has fixed an objective of replacing 20% of its current phosphate rock consumption by recovered phosphates, in order to reduce the consumption of phosphate rock and to close the phosphorus cycle (Stark, 2004).

Except economic driving force, regulation also can be a stimulating factor. Both Germany and Sweden have announced national objectives for phosphorus recovery for recycling from sewage. The Swedish EPA action (SEPA, 2002) has proposed an intermediate target for P-recycling that by 2015; at least 60% of the phosphorus in wastewater shall be restored to productive soil, of which half should be returned to arable land. Both the Swedish and German authorities recognize that phosphorus can be recovered for recycling by various processes, including recovery from wastewaters in
sewage works or from sewage sludge incineration ashes. The environmental authority of Aland, an autonomous province of Finland, has proposed a goal of 50% recovery of phosphorus from sewage as well (Miljobyran, 2002).

The Government of Canada has also started to take actions to reduce releases of pollutants to surface water, including from point sources like municipal wastewater treatment facilities, and at the same time recover useful product. Indeed, along with many other countries, Canada depends heavily on phosphate imports to meet its demands. Gilbert (2009) estimates that roughly 30% of Canada’s fertilizer consumption could be met by converting all of its wastewater treatment plants to biological treatment systems with struvite recovery technology. In the Manitoba Province, Canada, recent policy studies and legislative developments have identified nutrient reuse and specifically phosphorus recycling, as an important part of an overall strategy of reducing phosphate losses to surface waters and of upgrading wastewater and manure treatment. Specifically, the Manitoba “Water Quality Standards, Objectives and Guidelines Regulation”, 28th November 2011, acted in implementation of the Water Protection Act (2005), indicates that the “Best practical technology for beneficial use of valuable resources such as nutrients, organic matter and energy contained within municipal bio-solids and sludge” should be implemented for all new or expanding industrial and municipal wastewater treatment plants discharging into a water body. As a result of this type of regulation, Ostara has recently constructed the second full-scale WASSTRIP process for the city of Saskatoon, Saskatchewan in Canada.

In the U.S., there is also growing interest to produce “products” derived from sewage or sewage sludge. The processed sewage sludge materials (e.g., composted bio-solids,
alkaline stabilized bio-solids, and heat dried pellets) are starting to be used for nutrient recovery in several full-scale plants (Ulrich et al., 2009). There is also several large wastewater treatment facilities/utilities which have recently complete or embarked on projects to recover resources from wastewater. Examples of these are the Durham Advanced Wastewater Treatment Plant in Tigard, Oregon that recently upgraded the plant and installed the Pearl-OSTARA process, the plan on EBPR and struvite recovery in the city of Boise, ID and the long-term bio-solids beneficial use program in Hampton Roads Sanitation District, VA (WERF, 2010). Numerous studies have also been conducted or are underway in several facilities, including Nansemond in Virginia to verify the economic feasibility of P recovery technology.
3 Hypothesis and Objectives

P recovery within EBPR plant often involved an anaerobic holding tank, where P is released due to PAO activity as well as bacteria decay.

The factors that impact the rate and amount of P released within the anaerobic holding tank hold the key to successful P recovery as in WASSTRIP and PhoStrip processes. The microbial populations’ structure, particularly those functionally relevant to EBPR systems such as PAOs, GAOs, is expected to affect the P release process. Therefore, factors that impact the EBPR community structure and performance are consequently expected to influence the P release process. However, the impact of EBPR process operating conditions on P-release rates and kinetics are not fully understood. In addition, it is largely unclear how the anaerobic digestion process for the P-recovery process affects the microbial population.

It has been mentioned before, that both SRT and COD/P ratio can affect the microbial population in EBPR system, and therefore consequently change the P–removal performance. Indeed, different SRT conditions not only change the sludge-wasting load, but also the phosphorus content in the biomass (Ma et al., 2005). Previous research has also shown that the influent COD/P correlated well with the EBPR biomass total phosphorus content and phosphorus removal functions (Kisoglu et al., 2000; Liu et al., 1997; Schuler and Jenkins, 2003). Hence, it is expected that during the anaerobic digestion process, the P released amount and rate will also be different for WAS and RAS from systems operating at different SRT or different influent C/P ratio.

In addition, it has been reported that various plants in North America and Europe are experiencing improved performance by fermenting a small portion of the mixed liquor or
RAS in side-stream reactors, or by using unmixed in-line fermenters (Barnard et al., 2010, 2012, Carvanaugh et al., 2012, Vollertsen et al., 2006, Vale et al., 2008). Although the mechanisms involved in the side-stream EBPR process have not been fully understood yet, the review of literature related to EBPR biochemistry and RAS fermentation have indicated that the fermentation step can potentially allow the enrichment of PAOs over other organisms. If this hypothesis is true, P-recovery process can be not only applied on WAS, but also on part of the RAS, combining P-recovery potential to improved EBPR performance.

The overall objective of this study was to better understand the link between EBPR activity and the P-release process that takes place in the anaerobic digester and the stripping tank in P recovery schemes. The specific objectives are described as following:

1. Evaluate the operation condition (SRT and COD/P) impacts on P-release capacity and rate of EBPR sludge;

2. Investigate the mechanisms of P-release during the anaerobic digestion of EBPR sludge;

3. Explore the effect of the anaerobic digestion process on the PAOs in order to better understand what role this step plays for the mainstream EBPR process.

The specific experimental approach employed in this study is described in Chapter 4.
4 Materials and Methods

A schematic showing objectives, experimental approaches used and expected results is presented in Figure 10. As already highlighted in the previous chapter, this study aimed to evaluate the P release capacity of Bio-P sludge from systems operated at different SRT and C/P ratio, as well as to understand the effect of extended anaerobic condition on the microbial population. We also tried to identify the mechanisms of P release (e.g. bacteria decay or PAOs activity?) during the 24 hrs anaerobic times.

![Figure 10 Objectives, Approaches and expected output for the study](image)

Four sequencing batch reactors were operated at different SRTs (5 to 30 days) and different influent C/P ratios (10 and 25) to provide bio-P sludge for testing. The different samples of sludge taken from the SBRs were exposed to anaerobic condition for a 24-26 hours period. P release rates and total amount of P released were evaluated and compared among the different sludge samples.
The P release during the anaerobic time could be due to the death of microorganisms with consequential release of phosphate, ammonia and other compounds in solution. The decay rate of PAO is significantly less than that of the other (heterotrophic) bacteria in activated sludge under anaerobic condition, and PAOs can release phosphorus from stored polyphosphate under anaerobic conditions even in the absence of VFAs for the microorganisms to derive sufficient energy for maintaining their metabolic activities. This is called secondary release of phosphorus (Barnard and Scruggs, 2003). Secondary release takes place at a much slower rate than primary release. There is also a possibility of P release associated with VFA uptake; where the VFA presence is due to the fermentation of the sludge. While primary sludge is commonly used as the source of VFAs, fermentation of return activated sludge (RAS) or WAS as an alternative source is not often applied. During the anaerobic digestion, fermentation of the WAS with consequently release of VFA (that can be taken up by PAOs) is expected to be a slow process, which might not have a big impact on the overall P released amount under the conditions of our experiments. In order to understand the mechanisms of P release the decay of bacteria, the release of metals (Mg, K), the production of VFA, as well as the EBPR activity were evaluated. It has been found that Mg\(^{2+}\) and K\(^+\) cations are bound as counter-ions in the poly-P chains, Me\(_{n+2}\)P\(_n\)O\(_{3n+1}\) (n indicate the chain length of poly-P and Me represents a metal cation). Hence the depletion of poly-P would raise soluble Mg\(^{2+}\) and K\(^+\) concentration in the sludge liquor (Jardin N. and Popel H.J., 2001, Choi et al., 2011).

In activated sludge, decrease in bacterial activity (biomass decay) can be caused by cell death (reduction in the amount of active bacteria) and activity decay (reduction in the
specific activity of active bacteria) (Hao et al., 2009; Manser et al., 2006; Mason et al., 1986; van Loosdrecht and Henze, 1999). The live/dead staining technique was therefore used to identify the ratio of viable cells to total cells by examining the integrity of the cell membrane.

Decrease in bacterial activity primarily takes place when the activated sludge is deprived of a growth substrate (Argrige and Chesbro, 1982; Hao et al., 2009; Lewis, 2000; Rice and Bayles, 2003; Yarmolinsky, 1995). In order to understand the impacts of the anaerobic ‘digestion’ process on microorganisms, especially the functional bacteria group PAOs, it is important to obtain the decay rate of PAOs. The bacteria decay could be indirectly determined by measuring the change in the phosphate release rate (PRR) of PAOs and the volatile fatty acid (VFA) uptake rate (VFAUR). Fluorescence in situ hybridization (FISH) (targeted at viable cells) was employed to determine the ratios of PAOs and GAOs to the total viable bacteria. The contribution of cell death and activity decay to the total bacterial activity could in this way be determined (Hao et al., 2010).

The following paragraphs describe in details the methodologies and material used in this study.

4.1 **Sequencing Batch Reactors (SBR)**

In order to assess the effect of solid residence time (SRT) on the P recovery potential of wasted activated sludge, four sequencing batch reactors were operated for over a year. Figure 11 shows the lab SBRs operated in the Environmental Engineering laboratory at Northeastern University. The reactors were seeded with activated sludge from a full-scale WWTP located in Las Vegas operating with an EBPR process. The seeded sludge was separated equally to four reactors and fed with synthetic wastewater (see next paragraph
for details) with no discharging for the first 2 weeks. Since the 3rd week, the wasted pump was activated, and the amount wasted was adjusted for each reactor to achieve the target sludge retention time. The reactors (working volume of 4 liters) were operated as SBRs with cycles consisting of feeding, anaerobic phase, aerobic phase, settling and decanting phase. The SBRs were operated for over 3 months at steady state condition before performing the endogenous digestion batch test.

Figure 11 Lab Scale SBRs.

4.1.1 Feeding

The influent to the SBRs is a synthetic wastewater with tap water, which has two components: Inorganic nutrient feed and concentrated organic feed. The quantity of the organic feed was adjusted during the operation of the reactors in order to achieve two COD: P ratios; for the first several months the reactor operated at a C/P ratio of 25, and in the second phase the C/P ratio was modified to 10. Detail feeding components are presented in Table 2 and Table 3.
Table 2 Inorganic and Concentrated Organic Feeding Components.

<table>
<thead>
<tr>
<th>FEED</th>
<th>COMPOUND</th>
<th>CONCENTRATION mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic nutrient feed</td>
<td>KCl (in)</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>NH₄Cl (in)</td>
<td>30.6 (8mg/L N)</td>
</tr>
<tr>
<td></td>
<td>MgCl₂ * 6H₂O (flour)</td>
<td>219</td>
</tr>
<tr>
<td></td>
<td>MgSO₄ * 7H₂O (in)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>CaCl₂ (flour)</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Yeast extract (in)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>NaH₂PO₄ *2H₂O (in)</td>
<td>40.28 (8 mg/l as P)</td>
</tr>
<tr>
<td>Trace mineral solution</td>
<td>1 ml/l</td>
<td></td>
</tr>
<tr>
<td>Nitrification inhibitor</td>
<td>Allylthiourea (in)</td>
<td>4</td>
</tr>
<tr>
<td>Concentrated organic feed</td>
<td>CH₃COONa*3H₂O (flour)</td>
<td>93668.4</td>
</tr>
<tr>
<td></td>
<td>Casamino acids (in)</td>
<td>9000</td>
</tr>
</tbody>
</table>

Table 3 Trace Mineral Solution Components.

<table>
<thead>
<tr>
<th>Trace mineral solution</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂BO₃ (II-3)</td>
<td>61</td>
</tr>
<tr>
<td>ZnSO₄*7H₂O (I-4)</td>
<td>300</td>
</tr>
<tr>
<td>KI (II-4)</td>
<td>15</td>
</tr>
<tr>
<td>CuSO₄*5H₂O (I-1)</td>
<td>61</td>
</tr>
<tr>
<td>Co(NO₃)₂*6H₂O (I-1)</td>
<td>75</td>
</tr>
<tr>
<td>Na₂MoO₄*2H₂O (II-1)</td>
<td>31</td>
</tr>
<tr>
<td>MnSO₄*H₂O (I-3)</td>
<td>340</td>
</tr>
<tr>
<td>FeSO₄*7H₂O (I-1)</td>
<td>300</td>
</tr>
</tbody>
</table>

4.1.2 SBRs Operation

The SBRs work through 4 six-hour cycles each day, as represented in the flow chart (Figure 12). The synthetic wastewater was feed into the reactors at the beginning of anaerobic phase, which last 110 minutes. Aerobic conditions are then reached by applying air bubble, using an aquarium pump. The dissolved oxygen (DO) concentration
was measured by a DO probe and meter (Thermo Scientific Orion). The temperature was maintained at a constant level of 20 °C performing the experiments in a thermostatic room (Filomena. et al. 2009).

Figure 12 Lab Scale SBRs operation.

The Hydraulic Retention Time (HRT) was maintained at 12 hours.

4.1.3 SBRs Maintenance and Monitoring

SBRs maintenance includes daily feeding and wasting, weekly TSS/VSS measurement, as well as monthly feeding tube and reactor container cleaning. pH, and dissolved oxygen (DO) concentrations were measured 3 times per week; on the same days effluent samples were taken and analyzed for ortho-P. In addition, the sludge thickness was
marked every two weeks at the end of settling period, as reference information of SBRs characteristics.

4.2 Batch Testing Methodologies

4.2.1 Endogenous Release test

The sludge taken from the SBRs operated with different SRTs and C/P ratios were exposed to 24 hrs anaerobic holding, following the procedure shown in Figure 13.

Figure 14 also show the set up used. Samples were collected at pre-set intervals, immediately filtered and stored for chemical analysis. At time zero and time 24 hrs two samples were collected: one sample was filtered and stored for chemical analysis and the second sludge sample was used for solids measurement as well as for the molecular work (FISH and live/dead testing). The live/dead assay was conducted within 30 minutes from sampling, whereas samples for FISH were fixed and stored at -20˚C until further analysis.

Aliquot of sludge from each SBR at the end of aerobic phase was collected in parallel to the sample used for the endogenous test and used to conduct the P-release test in presence of acetate (see next paragraph for procedure), which can help assessing the initial EBPR activity. The same type of test was performed on the remaining MLSS at the end of the endogenous release test, which can provide information of the holding time impacts on PAOs activity.
Figure 13: Endogenous Digestion Tests Procedure.

Figure 14: Picture of Batch Test set up.
4.2.2 **EBPR Activity Measurements**

EBPR activities are commonly measured via P release-uptake tests (Liu et al. (1997), Andrew et al. (2003) and Schuler et al. (2002), Filomena et al. (2005) Helmer et al., 1998), Liu et al., 1997)). The P release and uptake rates reflect indeed the overall activity of PAOs, which are the key agents in EBRP systems. Briefly, before the endogenous test, 2 liters of sludge was collected at the end of aerobic phase from each of the four reactors and concentrated to 1 liter by gravity to achieve required MLSS concentration range (2000-5000mg VSS/L), and the P-release test was performed on this sludge. At the end of endogenous test, the same type of test was performed on the sludge used during testing but without the concentration step. In order to create completely anaerobic condition, N₂ gas was pumped for the entire duration of the test. Dissolved oxygen (DO) concentration was measure by a DO probe and meter (Thermo Scientific Orion DO probe). The temperature was controlled at 20±2°C by a water bath. The temperature was measured by thermometers. pH was measured by a pH probe (Thermo Scientific Orion pH probe) and was controlled within 7-8, by the addition of 1M NaOH or 1 M HCl. After adding VFA (90 ppm as acetate) samples were taken at given intervals (5 to 15 min) and filtered through 0.45µm membranes immediately. All collected samples were analyzed for phosphate and acetate concentration, while a 50 ml mixed liquor sample was collected at the end of the test for TSS and VSS analysis.

4.2.3 **Chemical Analysis**

Both ortho-P samples for PAOs kinetic study and P-release capacity were measured by the colorimetric method according to Standard methods (APHA, 2005). Colorimetric method with stannous chloride (SM 4500 P D) has been considered much more sensitive
than the vanadomolybdophosphoric acid method and therefore was used in this study. Under acidic conditions, molybdophosphoric acid is formed in presence of phosphates and subsequently, reduced by stannous chloride to molybdenum blue. Absorbance is measured at 690 nm after 10 minutes with a UV-visible spectrophotometer (UVmini-1240, SHIMADZU).

Total Phosphorus was also measured to determine the TP content of the sludge; in this case the sludge sample is digested for 30 mins at 120°C before the colorimetric method. Mg\(^{2+}\), Ca\(^{2+}\), K\(^{+}\) were measured by ICP-MS (Aurora M90, Bruker).

### 4.2.4 Data Analysis

System performance and kinetics were evaluated by measuring total suspended solids and volatile suspended solids (TSS and VSS) by standard method, and phosphorus concentrations in filtered samples by ion chromatography (DIONEX DP ICS5000 with sampler ICS series AS-DV).

All data points were input to excel table, plotted and fitted with suitable trend line (Figure 15), which provided the slope during the anaerobic phase, which is used to determine the P-release rate accordingly to equation 1.

\[
P\text{-release rate (mg P/g VSS*hr)} = P\text{-release slope} \times 60/MLVSS \\
Acetate\text{-uptake rate (mg Ac/g VSS*hr)} = Acetate\text{-uptake slope} \times 60/MLVSS
\]

Specific P-release rates were also obtained using the MLTSS concentration or viable concentration respectively.

The following equation was used to viable PAO concentration:

\[
\text{Viable PAO concentration} = VSS (t) \times \text{viable cell (}) (t) \times \text{total PAO (}} (t)
\]

Note: \( t \) = sampling time point (either before or after the digestion test)
Viable cell (%) = 1 - dead/ all cell (%)

All recorded temperatures during the test were averaged, and P release rates were then corrected (using an Arrhenius type equation (as shown below) and reported at a 20 °C.

\[
\text{Rate at 20°C} = \frac{\text{Rate at testing temperature}}{1.05^{\left(\frac{\text{Average testing temperature}-20°C}{4}\right)}}
\]  

(4)

Figure 15 Trend lines for P release and acetate-uptake rates calculations.

**4.2.5 Microbial Population Analysis**

Biomass abundance and microbial population structure were analyzed by TSS & VSS, Fluorescent In Situ Hybridization (FISH), DAPI and Live/Dead staining. DAPI and FISH were performed to determine abundance of candidate PAOs and GAOs population. Live/Dead analysis integrated with TSS & VSS results can represent the total population abundance change during the 24 hour endogenous digestion test. DAPI Staining

For the determination of PAO fraction, intracellular polyP was visualized by incubation with 50 ug/mL of 4',6-Diamidino-2-phenylindole (DAPI) for 1 min (Kawaharasaki et al., 1999). Under these conditions, cells containing a large amount of polyP are stained bright yellow while the rest of the cells are blue. The fractions of PAOs (yellow) were determined as the percentage of the total cells (blue+yellow). Stained cells were observed with an epifluorescent microscope (Zeiss Axioplan 2, Zeiss, Oberkochen, Germany).
Quantifications of population distributions were carried out using the software DAIME (Daime et al. 2006). Fluorescence In Situ Hybridization (FISH)

Observation and quantification of candidate PAOs and GAOs residing in the biomass from the aeration basins operated at different SRT were investigated by fluorescence in situ hybridization (FISH) targeting known PAOs and GAOs. FISH samples were fixed with a 4% paraformaldehyde (PFA) and stored in freshly prepared PBS/EtOH. Before staining, all samples were disrupted with 26 gauge syringes for 15-20 minutes in order to ensure uniform distribution of the cells on the slides. Then, each prepared sample was placed on gelatin-coated slide and air-dried. The slides were then soaked with ethanol of 50 %, 80 % and 96 % respectively for 3 min to further dehydrate. In situ hybridization of cells was performed with the labeled 16S rRNA-targeted oligonucleotide probes (see Table 4) then mixed with hybridization buffer (0.9M NaCl, 20mM Tris–HCl at pH 7.2, 0.01% sodium dodecyl sulfate (SDS), and 35% de-ionized formamide (DFA)). All in situ hybridizations were performed at 46 °C for at least 90 min and washed at 48 °C for 15 min with a washing buffer (20mM Tris–HCl at pH 7.2, 0.01% SDS, 5mM EDTA, and 70mM NaCl). After hybridization, the slides were counterstained with 1 µg/mL of DAPI solution for 3 min to quantify total cells to allow the estimation of the fraction of different PAOs and GAOs expressed as the percentage of the total cells.

Hybridized cells were observed with an epifluorescent microscope (Zeiss Axioplan 2, Zeiss, Oberkochen, Germany). Quantifications of population distributions were carried out using the software DAIME (Daime et al. 2006). Around 20-25 separate randomly chosen images were evaluated with final results reflecting the cumulative biovolumetric fractions of Accumulibacter, Actinobacter, Competibacter and Defluvicoccus present in
the corresponding samples. Microbial population fractions were expressed as percentage of DAPI stained cells.

Table 4 Oligonucleotide primer and probes used in this study and their respective target groups.

<table>
<thead>
<tr>
<th>Species</th>
<th>Probe</th>
<th>Target Group</th>
<th>Sequence (3’-5’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAOs</td>
<td>PAO462b</td>
<td>Most Accumulibacter</td>
<td>CCGTCATCTACWCAGGTTAACC</td>
<td>(Zilles et al. 2002)</td>
</tr>
<tr>
<td>PAOs</td>
<td>PAO651</td>
<td>Most Accumulibacter</td>
<td>CCGTCATCTACWCAGGTTAACC</td>
<td>(Crocetti et al. 2000)</td>
</tr>
<tr>
<td>PAOs</td>
<td>PAO846b</td>
<td>Most Accumulibacter</td>
<td>CCGTCATCTACWCAGGTTAACC</td>
<td>(Zilles et al. 2002)</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Actino-221a</td>
<td>Actinobacteria</td>
<td>CCGAGTTCATCCAGAAGAC</td>
<td>(Kong et al. 2005)</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Actino-658a</td>
<td>Actinobacteria</td>
<td>TCGGTCTCCCTACCCTACCAT</td>
<td>(Kong et al. 2005)</td>
</tr>
<tr>
<td>GAOs</td>
<td>GAOQ989</td>
<td>Some Competibacter</td>
<td>TTCCCCGGATGTCAGGC</td>
<td>(Crocetti et al. 2002)</td>
</tr>
<tr>
<td>GAOs</td>
<td>GAOQ431</td>
<td>Some Competibacter</td>
<td>TCCCCGGCTAAAGGCTT</td>
<td>(Crocetti et al. 2002)</td>
</tr>
<tr>
<td>GAOs</td>
<td>GB742</td>
<td>Some Competibacter</td>
<td>CGATCCTCTAGGCTAAGGCTACTCAG</td>
<td>(Kong et al. 2002)</td>
</tr>
<tr>
<td>GAOs</td>
<td>TFO_DF218</td>
<td>Defluvococcus Cluster 1</td>
<td>GAAGCCTTTTGCCCCCTACAG</td>
<td>(Wong et al. 2004)</td>
</tr>
<tr>
<td>GAOs</td>
<td>TFO_DF618</td>
<td>Defluvococcus Cluster 1</td>
<td>GCCTCCTCTCTCTCTCCCTACCCG</td>
<td>(Wong et al. 2004)</td>
</tr>
<tr>
<td>GAOs</td>
<td>DF988</td>
<td>Defluvococcus Cluster 2</td>
<td>GATACGACGCCCCATGTCAGGG</td>
<td>(Meyer, Saunders et al. 2006)</td>
</tr>
<tr>
<td>GAOs</td>
<td>DF1020</td>
<td>Defluvococcus Cluster 2</td>
<td>CCCGGCCGACCCGACTCCCT</td>
<td>(Meyer, Saunders et al. 2006)</td>
</tr>
</tbody>
</table>

4.2.6 LIVE/DEAD® Analysis

The LIVE/DEAD® BacLight TM bacterial viability kits (Type: L-7012) were used to discriminate between viable cells and dead cells. The BacLight® bacterial viability kits contain green fluorescent nucleic acid stain SYTO® 9 and red-fluorescent nucleic acid stain Propidium Iodide (PI). When used alone, the SYTO® 9 stain generally labels all bacteria that have both intact membranes and damaged membranes. In contrast, PI stain penetrates only those bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present. For this reason, bacteria with intact cell membranes (viable cells) stain green fluorescence, whereas bacteria with damaged membranes (dead cells) stain red fluorescence. Sludge samples (675µl from
each testing) were stained by 1 µl of SYTO® 9 and 1 µl of PI for 15 mins under dark condition and room temperature. Stained cells were observed with an epifluorescent microscope (Zeiss Axioplan 2, Zeiss, Oberkochen, Germany). Quantifications of the dead cell fractions were carried out using the software DAIME (Daims et al. 2006). Around 20-25 separate randomly chosen images were evaluated with final results reflecting the ratio of dead cells over viable cells; moreover the ratio of dead cell over all cell was determined.
5 P-Recovery Experimental Work Results

In this chapter the results of the P-release batch testing under endogenous condition are presented and discussed. The effect of endogenous digestion on microbial population and on PAOs is also examined.

5.1 Phosphorus Release under Endogenous Condition: Impact of SRT and COD/P Ratio

In the following paragraph the results of the 24 hours endogenous release tests for the WAS from the different SBRs reactor are presented and compared.

5.1.1 Wasted Activated Sludge from 5 day-SRT SBR

The 5-days-SRT SBR was operated with either an influent COD/P ratio of 10 or 25. When steady state conditions were obtained in the SBR, sludge samples were collected for testing. WAS samples were withdrawn at the end of the aerobic phase from the SBR operating at 5 days SRT and were concentrated from 2000 ml to 400ml; nitrogen gas was pumped in the sludge until anaerobic condition were reached. Solids and total phosphorus concentration were measured for both tests, which are reported in Table 5.

Table 5 Solid and total phosphorus concentration for 5-days-SRT.

<table>
<thead>
<tr>
<th></th>
<th>TSS</th>
<th>VSS</th>
<th>TP (mg P/L)</th>
<th>% of TP/TSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/P = 10</td>
<td>4332.8</td>
<td>3408.0</td>
<td>267.8</td>
<td>6.18 %</td>
</tr>
<tr>
<td>SRT = 5 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/P = 25</td>
<td>6222.3</td>
<td>4238.3</td>
<td>720.6</td>
<td>11.58 %</td>
</tr>
</tbody>
</table>

Figure 16 and Figure 17 show the phosphate concentration profiles during the tests with sludge from the SBR operated at 5-days-SRT, and COD/P at 10 and 25, respectively.
Figure 16 Ortho-P concentration during endogenous digestion test for WAS from 5-days-SRT reactor (COD:P=10).

Figure 17 Ortho-P concentration during endogenous digestion test for WAS from 5-days-SRT reactor (COD:P=25).

The error bars in the figure represent the errors in the two duplicate measurements performed, which is used in other SRT results as well. The results of these two measurements were consistent, despite the up to 400 dilutions required to be in the reading range of the colorimetric method used for the determination of the ortho-P concentration.

From Figure 16, it can be observed that the 5-days-SRT WAS released P at a fast rate for approximately 11 hours; it then seems to reach a plateau, and then increase slightly from 20 hour to 25 hour. The ortho-P concentration increased overall from 1.4 mg P/L to 106.5
mg P/L. This latest value represents about 39.8% of the total phosphorus initially present in the sludge. For the test with WAS from the SBR operated at highest COD/P ratio (COD:P=25), the phosphorus concentration in the liquid phase increased with a lower but constant rate going from 0.28 mg P/L to 87.27 mg P/L. At the end of the testing about 12.11% of the initial total phosphorus content was released.

5.1.2 Wasted Activated Sludge from 10 day-SRT SBR

The 10-days-SRT SBR was operated with an influent COD/P ratio of 10. When steady state conditions were obtained in the SBR, a sludge sample was collected for testing at the end of the aerobic phase and concentrated from 987 ml to 400ml. The concentrated TSS and VSS concentration were 7391.1 mg/L and 3979.2 mg/L respectively, and the TP content of the sludge was 1111.8 mg P/L, which represent 15.04% of TSS. Before the beginning of the test, nitrogen gas was pumped for about 40 minutes in order to obtain anaerobic conditions. Figure 18 shows the phosphate concentration profile during the testing.

![Figure 18 Ortho-P concentration during endogenous digestion test for WAS from 10 days-SRT reactor at COD:P=10.](image-url)
From this graph it can be observed that the 10 days-SRT WAS continuously released phosphorus, which increased from 0.6 mg P/L to 859.6 mg P/L. This latest value represents about 77.3% of the total phosphorus initially present in the sludge.

5.1.3 Wasted Activated Sludge from 20 day-SRT SBR

The WAS withdrawn at the end of the aerobic phase from the SBR operating at 20 days SRT, was concentrated from 519 ml to 400 ml before testing. The initial TSS and VSS concentrations were found to be of 6867.4 mg/L and 3146.4 mg/L respectively; the TP content of the concentrated sludge was 841.7 mg P/L, which represents 12.3% of TSS. Before the beginning of the test, nitrogen gas was pumped for about 40 minutes in order to obtain anaerobic conditions.

Figure 19 shows the phosphate concentration profile during the endogenous digestion test.

![Figure 19 Ortho-P concentration during endogenous digestion test for WAS from 20 days-SRT reactor at COD:P=10.](image)

From this graph it can be observed that the 20 days-SRT WAS continuously released phosphorus, which increased from 0.6 mg P/L to 621.4 mg P/L. A faster release rate was observed for the first 12 hours. After about 26 hours 73.8% of the total phosphorus initially present in the sludge was found in the liquid phase.
5.1.4 Wasted Activated Sludge from 30 day-SRT SBR

The 30-days-SRT SBR was operated with either an influent COD/P ratio of 10 or 25. When steady state conditions were obtained in the SBR, sludge samples were collected for testing. 400 ml of WAS sample was withdrawn at the end of the aerobic phase from the SBR operating at 30 days SRT and COD/P ratio of 10, and 350 ml of WAS was taken and concentrated to 300 ml from the SBR operated at 30 days SRT and COD/P ratio of 25. Nitrogen gas was pumped in the sludge until anaerobic condition was reached. Solids and total phosphorus concentration were measured for tests, which are reported in Table 6.

Table 6 Solid and total phosphorus concentration for 30-days-SRT.

<table>
<thead>
<tr>
<th></th>
<th>TSS (mg/L)</th>
<th>VSS (mg/L)</th>
<th>TP (mg P/L)</th>
<th>% of TP/TSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRT = 30 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/P = 10</td>
<td>7000.6</td>
<td>2777.2</td>
<td>779.9</td>
<td>11.14 %</td>
</tr>
<tr>
<td>SRT = 30 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/P = 25</td>
<td>3854.5</td>
<td>2544.7</td>
<td>305.3</td>
<td>7.92 %</td>
</tr>
</tbody>
</table>

Figure 20 and Figure 21 show the phosphate concentration profiles of 30-day-SRT SBR under COD:P=10 and 25 conditions during the endogenous digestion test, respectively.

![Phosphate concentration profile](image.png)

Figure 20 Ortho-P concentration during endogenous digestion test for WAS from 30 day-SRT reactor (COD:P = 10).
Figure 21 Ortho-P concentration during endogenous digestion test for WAS from 30 days-SRT reactor (COD:P=25).

From the first graph it can be observed that the 30 day-SRT WAS continuously released phosphorus, which increased from 0.6 mg P/L to 388.7 mg P/L. This latest value represents about 49.8% of the total phosphorus initially present in the sludge. While in the second graph (COD:P=25), the ortho P concentration increased from 0.45 mg P/L to 148.5 mg P/L. By the end of the test, about 48.7% of the initial total phosphorus content was released.

5.1.5 Summary of Phosphorus Release under Endogenous Condition

Table 7 presents the initial condition for all wasted activated sludge for different COD:P ratio, as well as a summary of the endogenous test results.
Table 7 Summary of Phosphorus released under endogenous condition.

<table>
<thead>
<tr>
<th>COD:P</th>
<th>25</th>
<th>30</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRT (days)</td>
<td>5</td>
<td>30</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>TSS (mg/L)</td>
<td>6222</td>
<td>3854</td>
<td>4332</td>
<td>7391</td>
<td>6867</td>
<td>7000</td>
</tr>
<tr>
<td>VSS (mg/L)</td>
<td>4238</td>
<td>2544</td>
<td>3408</td>
<td>3979</td>
<td>3146</td>
<td>2777</td>
</tr>
<tr>
<td>TP (mg P/L)*</td>
<td>720.55</td>
<td>305.28</td>
<td>267.82</td>
<td>1111.80</td>
<td>841.71</td>
<td>779.87</td>
</tr>
<tr>
<td>TP mg P/ mg TSS (%)</td>
<td>11.58</td>
<td>7.92</td>
<td>6.18</td>
<td>15.04</td>
<td>12.26</td>
<td>11.14</td>
</tr>
<tr>
<td>P released (mg P/L)</td>
<td>87.27</td>
<td>148.52</td>
<td>106.46</td>
<td>859.59</td>
<td>621.43</td>
<td>388.71</td>
</tr>
<tr>
<td>P released/ TP (%)</td>
<td>12.11</td>
<td>48.65</td>
<td>39.75</td>
<td>77.32</td>
<td>73.83</td>
<td>49.84</td>
</tr>
<tr>
<td>P release rate (mg P/ hr * VSS)</td>
<td>0.68</td>
<td>2.37</td>
<td>1.97</td>
<td>8.96</td>
<td>7.91</td>
<td>5.58</td>
</tr>
</tbody>
</table>

*The TP concentration here is at the beginning of the test; it is important to notice that the high value for the 10 and 20 days SRT are due to the fact that 2L and 1 L were concentrated down to 400 ml for the testing.

**SRT Effect on P Release Rate and Quantity**

The results of total P contents and P released amount from different SRT-SBR with same COD/P ratio are presented in Figure 22.

![Figure 22 Released ortho-P and total phosphorus content in different SRT WAS samples at COD:P=10.](image)

Comparing both overall quantity and percentages of released ortho-P over total phosphorus among different SRTs WAS samples but similar bio-mass concentration under same COD:P=10 condition, the 10 days-SRT WAS showed the highest P recovery potential (highest TP release and highest P released %), while the 5 days-SRT WAS had
the lowest one. Similar total phosphorus content (810 ± 30 mg/L) was found for WAS from reactors operating between 20 days to 30 days, but better P recovery potential was obtained for the lower SRT reactor. For SRT higher than 10 days, the TP content of the WAS as well as the amount of P released during the anaerobic holding time decrease. Similar trend was also found in their endogenous P release rates (see Figure 23). The 5 days-SRT-SBR had the lowest P release rate. Overall, these results combined show that WAS from the SRT operated between 10-20 had the best release rates, which translated in the highest amount of P release during the testing. Results of the weekly monitoring of the SBRs also indicated the 10-days and 20-days SRT reactors as the most successful, whereas the shortest SRT reactor had the worst performance (See monitoring results in appendix A).

![Figure 23](image-url) The rates of P-release for different SRT WAS samples at COD:P=10.

Figure 24 shows the initial VSS/TSS ratio for different SRT WAS samples at COD:P at 10. It was found the ratio consistently decreased as SRT increased, indicating the possible higher PAO fraction in longer SRT-SBRs (reference in PAOs abundance section 5.5) due to the high inorganic content of PAO biomass (Mogen et al., 2008). Despite this fact, the release rate and amount of P released were higher for the 10 and 20 days-SRT compared
to the 30 days SRT. It is possible that SRT affect the quantity but also the PAO diversity, as well as the structure of phosphorus accumulated in the cell. In a recent study Li et al (2014), try to link performances to poly-P chain length in PAOs, *Accumulibacter* diversity and operating SRT; and while no correlation was found between PAOs diversity and performance, the length of poly-P seems to be an important aspect. These results together with ours seem to indicate that SRT, could indeed affect the range of poly-P chain length in PAOs, which could in turn affect the rate of release of P (and overall amount) under endogenous condition.

![Figure 24 Initial VSS/TSS ratios for different SRT WAS samples at COD:P=10.](image)

**COD/P Ratio Effect on P Release Rate and Quantity**

Figure 25 and Figure 26 show the results for endogenous test performed with WAS from two SBRs operated with the same SRT but different influent COD:P ratios.

In Figure 25 and Figure 26, the blue bar presents the ortho P released amount under anaerobic condition as mg P/L, the red bar presents the total P content in WAS.
For 5 day-SRT WAS, the total P content was reduced with the decrease of the COD:P ratio, despite similar MLVSS content. This result is consistent with the reduced EBPR activity for this reactor when operating at the lowest COD/P ratio.

Conversely, reducing COD:P caused significantly increase of total P content in the 30 day-SRT WAS sample (Figure 26). However, in terms of % release, 30 days SRT WAS performed similarly (49 ± 1% of P released/TP) with both COD:P condition. The rate of P release was doubled in the WAS from the SBR operated at the lowest COD:P ratio.
The observation in 30 days-SRT confirm that the lower COD:P ratio can stimulate the growth of PAO, and therefore increasing the TP content, which translated in high P released amount at a significant higher release rate (about 390 mg/L vs 150 mg/L and 5.58 vs 2.37 mgP/gVSS*hr).

Despite the lower TP content in the WAS collected from the 5 days-SRT SBR, operated at the lower COD:P ratio, a larger amount of P was released at a faster rate compared to the WAS from the same system operated at the higher COD:P ratio (about 106 mg/L vs 87 mg/L and 1.97 vs 0.68 mgP/gVSS*hr).

The results seem to indicate that WAS from systems operated with lower influent COD:P ratio perform better in terms of quantity and rate of P release under anaerobic conditions, and might be preferable when operating P-recovery scheme.

### 5.2 Mechanism of P release under anaerobic condition

There are two possible pathways to release ortho-P during the anaerobic holding time, which are either PAO activity (secondary release) or PAO and other bacteria decay.

In order to understand the mechanisms of P release, and the contribution due to PAOs activity, we used the Live/Dead assay to quantify the bacteria decay during the endogenous test as well as the release of other ions in solution in addition to phosphate. When activated sludge from EBPR plants is anaerobically digested, the soluble concentrations of phosphate, magnesium, and potassium inside the digester increase because of polyphosphate degradation. Hence, the ratios of metal ions over P released will provide us the information about the source of P depletion.
5.2.1 Release of ions under Endogenous Condition

Figure 27 to Figure 30 show the metal ion concentration profiles during the anaerobic digestion tests with sludge from different SRT-SBRs operated at COD/P = 10. Table 8 summarized the concentration of various ions during the test for the different WAS samples as well as their ratios over unit of P released.

![Metal ions concentrations during endogenous digestion test for WAS from 5-days-SRT reactor (COD:P=10).](image)

From Figure 27, in 5-days-SRT-SBR samples, soluble Mg$^{2+}$ concentration followed a similar pattern as ortho-P, increasing for about 11 hours and then reaching a plateau. However, the ortho-P profile showed a second increase (after 20 hrs), which did not correspond with an increase of the Mg$^{2+}$ concentration. These results seem to indicate that the release in the first 11 hours is due to poly-P depletion, while the second P-release might have been associated with bacteria decay.

Differently from the ortho-P and Mg$^{2+}$, there were only slight shifts around 12th hour for soluble K$^+$ concentration, and it showed a decrease trend after 20 hour. This could be possibly explained by the appearance of small amount of acetate at the end of the test. Although with the detection limit of IC method, the accuracy of acetate was not ideal; the
presence of acetate may induce some bacteria uptake of K\(^+\) as well as other elements.

For the 10-days SRT WAS sample, according to Figure 28, ortho P, Mg\(^{2+}\) and K\(^+\) all increased for the first 11 hour, while Mg\(^{2+}\) and K\(^+\) seemed to reach to a consistent value after 12 hour, ortho-P keep increasing at a fast rate. In addition, at 20-25 hours, Mg\(^{2+}\) increased slightly, while K\(^+\) concentration decreased, similarly to the change observed in the 5-days-SRT-SBR samples but at a slower rate. There was also a small amount of VFA present at the end of the digestion test, which could possibly induce further P release is association with VFA uptake.

Figure 28 Metal ions concentrations during endogenous digestion test for WAS from 10-days-SRT reactor (COD:P=10).
For the 20-days SRT WAS sample, Figure 29 shows increasing trends, similar as in 10-days-SRT SBR results, for the first 12, while slower rate of ortho-P release as well as small variation in the Mg$^{2+}$ concentration were observed during the remaining duration of the test.

In the 30-days-SRT SBR activated sludge, all of these three elements concentration increased continuously during the digestion test.
Table 8 Released metal ions and their ratios over P depletion under anaerobic conditions for different SRTs.

<table>
<thead>
<tr>
<th>SRT (days)</th>
<th>Final Metal Ion conc.</th>
<th>K/P</th>
<th>Mg/P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg (mg/L)</td>
<td>K (mg/L)</td>
<td>Average*</td>
</tr>
<tr>
<td>5</td>
<td>20.42</td>
<td>44.95</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>102.07</td>
<td>181.62</td>
<td>0.16</td>
</tr>
<tr>
<td>20</td>
<td>232.75</td>
<td>380.41</td>
<td>0.53</td>
</tr>
<tr>
<td>30</td>
<td>68.94</td>
<td>153.15</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* The average ratio was determined using the slope of the Mg/P curve as seen in Figure 31 below.

![Figure 31 Examples for the slopes of Mg/P rate calculations.](image)

According to the metal ion concentration profile during the digestion test, combined with the summarized ratios of K/P and Mg/P, the amount of Mg$^{2+}$ released in 5, 10 and 30-days-SRT SBR seemed to less than the typical value, 0.23 mass ratios in EBPR. The ratio was therefore also calculated for the first 12 hours of the test and the last 12 hours; the results show that higher ratio were found in the first 12 hours, while much lower ratio were found for the remaining 12 hours. This seems to indicate that majority of the poly-P depletion happens in the first 12 hours of the tests, especially for the 10 days SRT, for which the stoichiometric value of the Mg/P was found for the first 12 hours, while almost insignificant release of the Mg was observed for the remaining 12 hours. The lower amount of Mg/P ratio for the 5 days SRT samples, which is also parallel to no release of K could imply comparatively low poly-P in sludge or the P released is from cell degradation. The Mg/P ratio in 20 days-SRT-SBR samples was higher than typical value,
and additionally the K/P ratios were higher than the theoretical ratio, 0.29 mg K/mg P for EBPR sludge (Choi et al., 2011, Jardin N. and Popel H.J. 2001). These extra amount of metal ions released were probably because in activated sludge, metal ions can be adsorbed by some bacterial extracellular polymers for flocculation. Metals present in the ionic form may also be accumulated in the cytoplasm of a bacterial cell, or adsorbed on to the cell wall (Brown et al., 1979). Therefore, during the digestion process, when these bacteria decay, or the flocs break down, the concentrations of metal would also increase. For the 30 days-SRT WAS sample, the K/P ratio was found to be very close to the stoichiometry value, but lower in terms of Mg/P ratio; this could indicate different composition of the poly-P chain in samples from systems operated under different SRT.

5.3 Microorganism Population Change

Live/Dead analysis was performed before and after the endogenous digestion test on all SRTs WAS samples including different COD:P ratio. Table 9 summarized the live/dead results.

Table 9 Live/Dead analysis results for different SRTs summary.

<table>
<thead>
<tr>
<th>COD:P</th>
<th>25</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRT (days)</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>Dead/All Cell (%)</td>
<td>16.3 ± 4</td>
<td>26.6 ± 3</td>
</tr>
<tr>
<td>Before test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dead/All Cell (%)</td>
<td>34.4 ± 5</td>
<td>33.2 ± 4</td>
</tr>
<tr>
<td>After test</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results for the testing performed with WAS drawn from SBR operated with an influent COD/P ratio equal to 10 are reported also in Figure 32, in which blue bar presents the percentage of dead cells amounts over all cells before the endogenous digestions test, orange bar presents the same percentage but after the test. It is clear from
the results that, as expected, after the endogenous digestion test, the quantities of dead cells significantly increased among all samples. However, the amount of dead cells was different for different WAS samples. At COD:P = 10, the bacteria in 5 day-SRT were more affected by the endogenous condition, while the bacteria in SRTs larger than 10 days were more robust. The bacteria decay ratio increased while SRT extended when \( SRT \leq 20 \) days. However, the decay ratio increased when SRT became longer than 20 days.

![Figure 32 Dead/Live analysis results before and after the endogenous digestion tests of all SRT WAS samples at COD:P=10.](image)

Looking at the results of the dead/all cells, we can say that P release is in part due to cell decay; from a mass balance we could consider that the amount of P released due to bacteria decay is among 5-14 % of total P released. These fractions of P released by cell degradation could also explain the lower ratios of Mg/P found in the 5 and 30 days-SRT samples, as well as in the last 12 hours of the 10-SRT sample. Lower decay was observed for the 20-days SRT sample, which is also consistent with the metal ratio results.
5.4 Impact of Anaerobic condition on PAOs

As mentioned before, for process schemes where all or a fraction of RAS is treated in an anaerobic holding tank, to promote VFA production through fermentation, it is important to understand the impact of this holding time on PAOs. Indeed the anaerobic condition in the fermenter could potentially affect PAOs (positively or negatively), and therefore affect the mainstream process.

PAOs and competing GAOs abundance changes as well as PAOs activity change were measured; the next paragraphs show the results. During anaerobic condition in presence of VFA, only PAOs will release phosphorus (polyphosphate is broken down to orthophosphate for energy supply) when up-taking carbon source, therefore the P-release rate during the anaerobic phase can be used as an indication of the PAO activity.

5.4.1 Impact on PAOs abundance

In order to identify the bacteria species in the WAS and assess their abundance, quantitative fluorescence in situ hybridization (FISH) was applied using 10 different fluorescently labeled oligonucleotide probes targeting known PAOs and GAOs. DAPI stain was also used to determine total PAOs population. Figure 33 shows the PAO picture before and after the digestion test for different SRT-SBRs, in which the amount reduced obviously in 10, 20 and 30 days-SRT-SBRs. The detail data were summarized in Table 10, while Figure 34 and Figure 35 show respectively PAOs and GAOs relative abundance before and after the endogenous digestion test for different SRTs and influent COD:P equal to 10.
Figure 33 FISH pictures of PAO before and after the tests for different SRT-SBRs at COD:P=10.

Table 10 Abundance summary of known types of PAOs and GAOs for different SRTs at COD:P=10. Note: total PAOs % might be underestimated due to the amount of poly P depletion.

<table>
<thead>
<tr>
<th>SRT (days)</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accumulibacter (%) Before test</td>
<td>10.7 ± 5</td>
<td>52.6 ± 5</td>
<td>57.8 ± 5</td>
<td>79.4 ± 6</td>
</tr>
<tr>
<td>Accumulibacter (%) After test</td>
<td>12.7 ± 3</td>
<td>15.7 ± 2</td>
<td>27.5 ± 6</td>
<td>38.2 ± 6</td>
</tr>
<tr>
<td>Other PAOs (%) Before test</td>
<td>7.1 ± 8</td>
<td>8.2 ± 6</td>
<td>6.4 ± 5</td>
<td>7.4 ± 6</td>
</tr>
<tr>
<td>Other PAOs (%) After test</td>
<td>27.1 ± 4</td>
<td>13.3 ± 3</td>
<td>10.7 ± 7</td>
<td>22.9 ± 7</td>
</tr>
<tr>
<td>Total PAO (%) Before test</td>
<td>19.8 ± 4</td>
<td>61.1 ± 4</td>
<td>64.3 ± 3</td>
<td>86.8 ± 2</td>
</tr>
<tr>
<td>Total PAO (%) After test</td>
<td>39.8 ± 2</td>
<td>29.0 ± 2</td>
<td>38.2 ± 4</td>
<td>61.1 ± 3</td>
</tr>
<tr>
<td>Total PAO difference (%)</td>
<td>101.01</td>
<td>52.54</td>
<td>40.54</td>
<td>29.61</td>
</tr>
<tr>
<td>Competibacter GAO (%) Before test</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Competibacter GAO (%) After test</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Defluvicoccus GAO (%) Before test</td>
<td>ND</td>
<td>2.4 ± 0.4</td>
<td>3.5 ± 0.1</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Defluvicoccus GAO (%) After test</td>
<td>ND</td>
<td>8.9 ± 1.8</td>
<td>6.1 ± 2</td>
<td>4.7 ± 2.1</td>
</tr>
<tr>
<td>Defluvicoccus GAO difference (%)</td>
<td>--</td>
<td>270.83</td>
<td>74.29</td>
<td>23.68</td>
</tr>
</tbody>
</table>
In our SBRs, *Competibacter* type of GAO was not detected in all different SRT-SBRs, and *Defluvicoccus* GAO (presented as DF GAO below) was not found in the 5 days-SRT-SBR.

![Graph showing FISH analysis results for total PAOs before and after the endogenous digestion tests of all SRT WAS samples at COD:P=10.](image)

**Figure 34** FISH analysis results for total PAOs before and after the endogenous digestion tests of all SRT WAS samples at COD:P=10.

It was observed that before the endogenous digestion test SRT positively correlate to the total PAOs fractions (higher SRT, higher PAO fraction). However, after the 24 hrs anaerobic holding time, this fraction changed differently in each sample. In the 5 days-SRT-SBR sample, distinctively from the other samples, the total PAOs fraction increased over 2 times after the digestion process; this means that decay of non PAOs occurred during the anaerobic time, affecting the percentage abundance of PAOs. For all of the other WAS samples, an increase in the non-* Accumulibacter* PAOs was observed, however a significant decrease of *Accumulibacter* was observed for all WAS samples for SRT> 10 days. It should be noticed that total PAOs were identified by DAPI poly-P method, however, the poly-P in PAOs were most likely released in large amount during the digestion test, therefore the DAPI results might underestimate the actual amount of total PAOs. For WAS samples from SBR operated at an SRT longer than 10 days, the overall total PAOs fractions decreased, which indicates that PAOs decayed during the
digestion process. The reduction in PAOs was smaller for the 30 day-SRT sample; this could be in part due to the fact that less than 50% of the total P content was released in the 24 hrs, compared to over 70% for the 10 and 20 day-SRT samples, and therefore a better estimation of the total PAO was done. In addition, it is possible that a long SRT might select those bacteria having a better survival strategy by a lower decay rate, or induce a stronger survival capacity in the cells as already found by others (Hao et al., 2010, Salem et al., 2006).

![Figure 35 FISH analysis results for Defluvicoccus Cluster 2 GAOs before and after the endogenous digestion test of all SRT WAS samples at COD:P=10.](image)

With the exception of the 5 days-SRT-SBR, where DF GAO was not detected, the overall DF GAOs fraction increased after the anaerobic digestion test for all other samples. Differently from the positive relation with SRT at the initial condition, the changes of DF GAO fraction became smaller in longer SRT-SBR, which implies DF GAO in shorter SRT-SBR are even more endurable than in long SRT-SBR.

In summary, considering the population abundance results, the anaerobic digestion holding time affected *Accumulibacter* but did not have a large impact on GAOs, and other types of PAOs. These are preliminary results, and further testing are needed;
moreover we need to remark that the population in the acetate fed SBRs are quite different in quantity and possibly composition, from the one found in full-scale plant.

5.4.2 Impacts of Endogenous Digestion on PAOs Activity

Figure 36 shows the ortho-P concentrations during the 45 minutes P-release test for different SRTs at COD:P=10 in the presence of acetate as carbon source. The blue dots present the activity results before the digestion test, while the red dots show the results after the holding time. Trend lines were added to ortho-P concentration to determine the P-release rate according to equation 1 (see Chapter 4 for details). Table 11 summarized these PAO activity results.

![Figure 36 P release in presence of acetate, before and after digestion test for different SRT WAS samples at COD:P=10.](image)
<table>
<thead>
<tr>
<th>COD:P=10</th>
<th>SRT (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific P released ratio</td>
<td>Sample time point</td>
</tr>
<tr>
<td>Release rate (mg P/L* hr)</td>
<td>Before</td>
</tr>
<tr>
<td></td>
<td>After</td>
</tr>
<tr>
<td>mg P-re/ mg VSS*hr</td>
<td>Before</td>
</tr>
<tr>
<td></td>
<td>After</td>
</tr>
<tr>
<td>mg P released/ mg TSS*hr</td>
<td>Before</td>
</tr>
<tr>
<td></td>
<td>After</td>
</tr>
<tr>
<td>mg P released/ mg PAO*hr</td>
<td>Before</td>
</tr>
<tr>
<td></td>
<td>After</td>
</tr>
</tbody>
</table>

Note: PAOs amount were quantified by DAPI poly-P method. Since during the anaerobic digestion process, there's extraordinary amount of poly-P released, so it was possible that the amount of PAOs were underestimated.

When looking at the P release rates calculated in the unit of mg P/g VSS*hr, it was found that in different SRT-SBRs, the PAO activity was significantly reduced after the anaerobic holding time. These reductions could be attributed to PAOs decaying during the digestion process, as well limited availability of intracellular substrates and PAO decay (Lopez et al., 2006, Hao et al., 2010). Particularly, according to the mass balance, over 70% of total P in 10 and 20 days-SRT MLSS was released, where about 90% of released P was due to poly P depletion. Hence, the reductions of PAO activity during the digestion test were found to be in part due to low poly P content after the digestion test.
In addition, after the anaerobic digestion test, the VSS concentration was reduced not only because poly-P depletion as mentioned before, but also caused by cell degradation, which were not necessary all from PAOs. For these reasons, kinetics were also measured as mg P/g TSS*hr, and we also tried to estimate the specific rate to PAOs population (accordingly to equation (2) in Chapter 4). Correcting the results in terms of TSS and PAOs can indeed provide more information about the activity changes (See Table 12).

In the 5 days-SRT SBR, the release rate in terms of mg P/L*hr increased after the digestion test, while the rate decreased in other SRT-SBRs. This could be attributed to the increasing fractions of *Accumulibacter* and total PAO in 5 days-SRT-SBR after the test, which all decreased in other SRT-SBRs.

Similar with Lopez et al.’s results (2006), during the anaerobic time, the activity among 10 and 20 days-SRT-SPR dropped to approximately 30% of the initial rates in unit of mg P/mg TSS* hr, while the rate in 30 days-SRT-SBR only reduced to 59%. This could be explained by the fact that fewer total PAOs decayed in 30 days-SRT than in 10 and 20 days-SRT. In addition, it was found that the actual kinetics of remaining active PAOs
decreased in all WAS samples, and reduction percentage ranged from 6.33% (for the 30 days SRT sample) to 63.55% (for the 5 days SRT sample).

These results seems to confirm that at the low COD:P ratio and higher SRT, PAOs are more abundant and less affected in terms of kinetics by the anaerobic holding time.

5.5 Anaerobic Endogenous Digestion test for Full-Scale Activated Sludge Sample

The microbial populations, in the acetate fed SBRs are quite different from the population in full-scale samples, especially in terms of overall PAOs abundance, which might possibly affect the total amount of P released and the rate at which the P is released. The therefore conducted the endogenous P release test on a full scale WAS sample to quantify the amount of P released, the kinetic of the P release process as well as trying to determine the mechanisms. The WAS sample from a full-scale EBPR WWTP, which has influent bCOD/P mass ratio at 38.8, 7 days-SRT, and achieves over 90% of phosphorus removal.

5.5.1 Quantity and kinetic of P released

The collected WAS samples were settled in concealed bottle for about 3 hours before the testing, and the sludge was concentrated from 18L to 8 L. The concentrated WAS initially contained 1884 mg TSS/L and 1476 mg VSS/L, and TP of 172 mg/L, which represent 11.65% of TP/VSS. The low value for the solids was most likely due to the heavy rainfall that occurred the day of the sampling.
Table 12 summarized the information of full-scale samples, while Figure 38 shows the phosphate concentration profile during the endogenous digestion test.
Table 12 Summary of full-scale samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>bCOD/P (mg/mg)</td>
<td>38.8</td>
<td>TSS Before (mg/L)</td>
<td>1884</td>
</tr>
<tr>
<td>SRT (days)</td>
<td>7</td>
<td>TSS After (mg/L)</td>
<td>1768</td>
</tr>
<tr>
<td>TP content (mg P/gTSS)</td>
<td>0.09</td>
<td>VSS Before (mg/L)</td>
<td>1476</td>
</tr>
<tr>
<td>P released (mg/L)</td>
<td>20.6</td>
<td>VSS After (mg/L)</td>
<td>1450</td>
</tr>
<tr>
<td>Mg released (mg/L)</td>
<td>11.49</td>
<td>Activity Before (mg P/VSS*hr)</td>
<td>6.82</td>
</tr>
<tr>
<td>Average Mg/P *hr</td>
<td>0.38</td>
<td>Activity After (mg P/VSS*hr)</td>
<td>1.16</td>
</tr>
<tr>
<td>K released (mg/L)</td>
<td>15.15</td>
<td>Dead/All Before (%)</td>
<td>1.45</td>
</tr>
<tr>
<td>Average K/P *hr</td>
<td>0.57</td>
<td>Dead/All After (%)</td>
<td>15.25</td>
</tr>
</tbody>
</table>

Figure 38 Ortho-P concentration during the endogenous digestion test for WAS from full-scale WWTP.

It was found that the concentration of ortho-P in solution increased steadily from 1.1 mg/L to 20.6 with an average specific P-release rate of 1.22 mg P/g VSS*hr; this value is similar to the rate observed with the 5 days-SRT-SBR sample. After the digestions test, 12 % of the TP content was released.

Soluble magnesium concentration followed a similar trend to the ortho-P (See Figure 39). The average Mg/P mass ratio during the test was 0.38, which was similar to the ratio obtained for the 20 days-SRT-SBR. This value is higher than the stoichiometric value, however, the increasing concentration, associated with the ortho-P concentrations, is consistent with poly-P depletion. The concentration of K+ had an overall increasing trend,
and the average K/P ratio was found to be 0.57. Both metal/P ratios were higher than the stoichiometric values reported in literature, and significant concentration were present even before the digestion test. These metals in solution could be due to their presence in the flocs independently from poly-P depletion.

Figure 39 Metal ions concentrations during endogenous digestion test for WAS from full-scale WWTP.

Similar with the lab scale SBR samples, after the endogenous digestion test, the fraction of dead/all cell increased from 1.48% to 15.25%.

Figure 40 presents the PAO activity results for before and after the digestion test for the full-scale WAS sample. In terms of mg ortho-P released per unit of VSS*hr, the PAO activity significantly decrease from 6.82 to 1.16
Figure 40 P release in presence of acetate, before and after digestion test for full-scale WAS sample.
6 Conclusions

The overall objective of this study was to better understand the link between EBPR activity and the P-release process that takes place in the anaerobic digester and the stripping tank in P recovery schemes.

When evaluating the impact of SRT and influent COD/P on the P release capacity and rate our results seem to indicate that WAS from systems operated with lower influent COD:P ratio perform better in terms of quantity and rate of P release under anaerobic conditions, and might be preferable when operating P-recovery scheme. SRT in the range between 10-20 days showed also the highest P released, in terms of both quantity (about 70% of TP in the 24 hrs holding time) and rate. These higher rates will translate in lower footprint for the P release tank.

We also try to investigate the mechanism of P release during the digestion process. According to the mass balance on P as well as the amount of cell decay and the observed metal/P ratio it can be concluded that the majority of released P was due to poly-P depletion at all SRTs condition. The only type of VFA monitored during the test, was acetic acid; a small amount of this VFA was detected at the end of testing, which could indicate some fermentation of WAS occurring and possible P release associated with VFA uptake. However, with the limitation of the IC method, it was not clear whether there were other type of VFA produced and simultaneously up-taken.

The observed profiles of the P release as well as metal concentration seems to indicate that different mechanisms are responsible at different time intervals, in the future the effect of holding time should be assessed, measuring for example bacteria decay and PAOs activity for different holding time, shorter or longer than 24 hours.
The last objective was also to try to quantify the effect of the anaerobic digestion process on the PAOs. It was found that for all of the lab scale SBRs samples, the PAO activity was reduced after the digestion test; this was due in part of PAO decay (as shown by FISH results), and in part to limited availability of intracellular substrates. It was also found that the effect on the PAOs from the 30-days SRT WAS sample was less than for other samples. This result might be due in part of the fact that longer SRT might select PAOs having a better survival strategy by a lower decay rate, or induce a stronger survival capacity in the cells, and in part to higher availability of poly-P. This last aspect, which was confirmed both by P mass balance and P-release could be associated with different structure of the poly-P in this sample. The determination of the poly-P structure in our samples was beyond the scope of this work, but it should be evaluated in future studies.

A limit to our study was the use of DAPI to identify PAOs and FISH for known GA0s, in the future other methods; such as RAMAN spectroscopy could be used to follow the different polymers during the digestion process.

In order to better estimate the impact of the anaerobic digestion on the mainstream EBPR, P-uptake rate will also need to be measured in the future work. Indeed Pijuan et al. (2004) and Lopez et al. (2006) have found that during the digestion process, the total amount of PHA would be reduced and its type would also change from predominantly PHB to predominantly PHV, which could consequently affect the P-uptake rate during the aerobic condition, and therefore the mainstream P removal. In the future work, intercellular polymers measurements should be therefore performed.
In addition, in order to recover phosphorus more efficiently, VFA or fermented sludge can be added to the recovery process. Hence, it would be necessary to perform similar digestion test with different type of carbon source addition.

This study was one of the first attempts to understand the fundamentals of the P-release mechanism needed in P recovery scheme, we also investigate the potential impact of this process on the EBPR population. The author believe that both of these aspects are important for the development and widespread use of P recovery technology,
REFERENCES


